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# Antimicrobial susceptibility of milk bacteria from healthy and drug-treated cow udder

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**ABSTRACT**

The aim of this study was to evaluate the antimicrobial susceptibility of milk microbiota, considering udder health status and drug treatment history. Composite milk samples were taken aseptically from healthy cows without any signs of mastitis (n = 17) and drug-treated cows with cured mastitis (n = 19). Antimicrobial susceptibility testing was performed for 56 enterococci, 30 *Escherichia coli*, 24 enterobacteria and 94 staphylococci. Depending on the bacterial group or species, the following antibiotic disks were used: ampicillin, rifampin, chloramphenicol, linezolid, tetracycline, erythromycin, nitrofurantoin, vancomycin, penicillin, trimethoprim, cefoperazone, kanamycin, trimethoprim/sulfamethoxazole, nalidixic acid, ciprofloxacin, gentamicin, teicoplanin, sulfonamides, levofloxacin, clindamycin and amoxicillin/clavulanic acid. The occurrence of multiresistant *E. coli* and staphylococci was significantly higher ($P<0.05$) in the milk of drug-treated cows. The percentage ratio (drug-treated: healthy udders) of multiresistant *E. coli* was 56.3:0, enterobacteria 57.14:30 and staphylococci 56.5: 4.8. There was no significant difference ($P>0.05$) between the prevalence of multiresistant enterococci in the milk of healthy cows and cows with cured mastitis (87.2:73.7). The high prevalence of resistance in enterococci isolated from milk samples of healthy cows could be the result of animal cohabitation and cross-contamination.

**Key words:** antimicrobial resistance, milk, enterococci, udder health

## Introduction

Antimicrobial resistance is one of the leading public-health issues which is closely related to the interactions of farm animals, farmers, the environment and food of animal

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origin (GARIPCIN and SEKER, 2015). The connection between primary production at the farm level and food processing is particularly evident in the spread of antimicrobial resistance thorough the agri-food chain (ZDOLEC, 2016). The possibility of resistant organisms of animal origin becoming directly pathogenic to man, or transferring their resistance genes to pathogens of medical importance, is of particular concern (TEUBER, 2001). Due to the intensive use of antibiotics in public health and animal husbandry, antibiotic resistance in pathogens has been an increasing medical problem over the last decades. In addition to the spread of resistant zoonotic foodborne pathogens, there is also a possibility that food-related commensal bacteria or opportunistic pathogens are carriers of resistance genes, and therefore a potential hazard to consumers (SHARMA et al., 2014). With regard to dairy production, the most relevant are resistant mastitis-causing bacteria, such as staphylococci (PAJIĆ et al., 2014; ADEGOKE and OKOH, 2014), or resistant ubiquitous bacteria such as enterococci (GIMÉNEZ PEREIRA, 2005). Their presence in milk intended for human consumption or dairy products could be of public-health relevance. It is well known that coagulase-negative staphylococci (CoNS) are the most important bacteria involved in subclinical bovine mastitis, alongside *Staphylococcus aureus* (KALMUS et al., 2011). Their resistance to antimicrobial agents is common due to the high antibiotic pressure in conventional dairy farming. Usually different CoNS species from bovine milk differ significantly in their phenotypic and genotypic antimicrobial resistance profile, which is important for udder health management (SAMPIMON et al., 2011). Enterococci, on the other hand, have only limited clinical importance in dairy farming, but their ubiquitous nature and frequent carriage of resistance genes is a reason for concern. The results of many studies indicate the potential risk of acquired antimicrobial resistance in enterococci, and transfer of mobile genetic material to other bacteria, even in conditions of low antimicrobial pressure (COCCONCELLI et al., 2003).

Antimicrobial resistance surveys in dairy production are mostly focused on udder pathogens and milk samples from drug-treated animals. However, it is also important to evaluate the presence of resistant bacteria in regularly collected raw milk samples from clinically healthy animals, in order to assess the potential spread of resistant strains from raw material to dairy products. Hence, the aim of the present study was to determine the prevalence of resistant bacteria in milk samples from healthy, drug-untreated cows, which are farmed and milked in the same conditions together with drug-treated cows with cured bacterial mastitis.

## Materials and methods

*Milk sampling and microbiological analyses.* Composite milk samples (n = 36) were collected from 4 different dairy farms, including both healthy udder milk samples (n = 17) and drug-treated udder milk samples (n = 19), from each farm. Samples from

drug-treated cows were taken separately, i.e. on different sampling days than samples from healthy cows, in order to avoid potential cross-contamination. At the time of milk sampling the withdrawal period in the drug-treated group had expired. Before sampling, udder sanitation measures were implemented, and milk was taken aseptically in sterile microbiological tubes, stored at 4 °C and transported to the laboratory. One mL of 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 staphylococci, *Escherichia coli,* enterococci and enterobacteria enumeration. Staphylococci were grown on Manitol Salt Phenol-red Agar (Merck, Darmstadt, Germany) for 48 h at 37 °C, *E. coli* on Rapid *E. coli* 2 Agar (Bio-Rad, Marnes-la-Coquette, France) for 24 h at 44 °C, enterococci on Compass *Enterococcus* agar (Biokar Diagnostics, France) for 24 h at 44 °C, and enterobacteria on Crystal-Violet Neutral-Red Bile Glucose Agar (Merck, Germany) for 24 h at 37 °C. After incubation, colonies were selected for identification (Gram staining, oxidase, catalase, API STREP, API 20E and API Staph tests) and antimicrobial susceptibility testing.

*Antimicrobial susceptibility testing.* A total of 56 enterococci (37 from milk of drug-treated udders and 19 from healthy udders), 24 enterobacteria (14 from milk of drug-treated udders and 10 from healthy udders), 30 *E. coli* and 94 staphylococci isolates (53 milk from drug-treated udders and 41 from healthy udders) were collected for antimicrobial susceptibility testing by the disk diffusion method (ANONYMOUS, 2010). The number of tested bacterial isolates does not refer to the number of composite milk samples (n = 36), meaning that several strains of each bacterial group were randomly picked from the agar plates of the corresponding milk sample. Depending on the microbial group or species, the following antimicrobial disks (Bio-Rad, France) were used: ampicillin (10 µg), rifampin (5 µg), chloramphenicol (30 µg), tetracycline (30 µg), erythromycin (15 µg), nitrofurantoin (300 µg), vancomycin (30 µg), penicillin (10 IU), linezolid (30 µg), trimethoprim (5 µg), cefoperazone (75 µg), kanamycin (30 µg), trimethoprim/sulfamethoxazole (25 µg), nalidixic acid (30 µg), ciprofloxacin (5 µg), gentamicin (10/300 µg), teicoplanin (30 µg), sulfonamides (250 µg), levofloxacin (5 µg), clindamycin (2 µg) and amoxicillin/clavulanic acid (30 µg). The bacterial culture (0.5 McFarland) was streaked on Mueller-Hinton agar (Bio-Rad, France) and max. 6 disks were used per plate by means of a disk dispenser (Bio-Rad, France). After incubation (35 °C, 18-24 h), the zone diameter was measured and interpreted according to the CLSI document M100-S20. Vancomycin resistance in enterococci was tested by both the agar diffusion method and the E-test (AB BIODISK, bioMérieux).

*Statistical analysis.* Proportions of multiresistant bacterial groups and resistance to selected antimicrobial agents were compared using Chi-square test at $P<0.05$ probability level, by computer software (PREACHER, 2001).

**Results**

The occurrence of multiresistant *E. coli* and staphylococci was significantly higher (P<0.05) in the milk of drug-treated cows (Table 1). The percentage ratio (drug-treated: healthy udders) of multiresistant *E. coli* was 56.3:0, enterobacteria 57.14:30 and staphylococci 56.5:4.8. There was no significant difference (P>0.05) in the prevalence of multiresistant enterobacteria in milk from drug-treated udders and the milk of healthy cows, despite the evidently higher percentage in drug-treated samples. Interestingly, the percentage ratio of multiresistant enterococci in drug-treated udder milk samples and milk from healthy udders was quite similar, 87.2:73.7 % and the difference was not significant (P<0.05).

Table 1. Percentage of multiresistant strains related to udder health status and distribution of multiresistance toward antimicrobial agents

| Microorganisms | Multiresistant strains (%) | | Number of resistant strains (healthy udder/drug-treated udder) | Number of antimicrobial agents | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | Drug-treated udder | Healthy udder | | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 |
| Enterococci | 87.2[a] | 73.7[a] | | 2/3 | 1/1 | 1/4 | 6/7 | 4/10 | 0/6 | 0/3 | - | - | - |
| Enterobacteria | 57.14[a] | 30[a] | | 2/2 | 0/1 | 1/2 | 1/1 | 0/2 | 0/1 | 1/1 | - | - | - |
| *E. coli* | 56.3[a] | 0[b] | | 2/2 | - | 0/1 | 0/1 | 0/1 | - | - | - | 0/2 | 0/4 |
| Staphylococci | 56.6[a] | 4.8[b] | | 5/5 | 1/2 | 0/6 | 0/7 | 1/7 | 0/3 | 0/5 | - | - | - |

a, b - values with different letters in same row are significantly different (P<0.05)

In our study, staphylococci isolated from drug-treated udder milk samples were most frequently resistant to clindamycin, penicillin, ampicillin, linezolid and erythromycin. An evidently lower proportion of resistant staphylococci was found in milk samples from healthy udders (Fig. 1) where the most common resistance was found to penicillin (14.6 %), erythromycin and kanamycin (12.2 %). The occurrence of resistance in staphylococci to clindamycin, penicillin, ampicillin, linezolid, teicoplanin, rifampin, tetracycline and erythromycin was significantly higher (P<0.05) in milk samples from drug-treated udders. There was no difference (P>0.05) in the presence of resistance to chloramphenicol, kanamycin or sulphonamides in the staphylococci between the two groups.

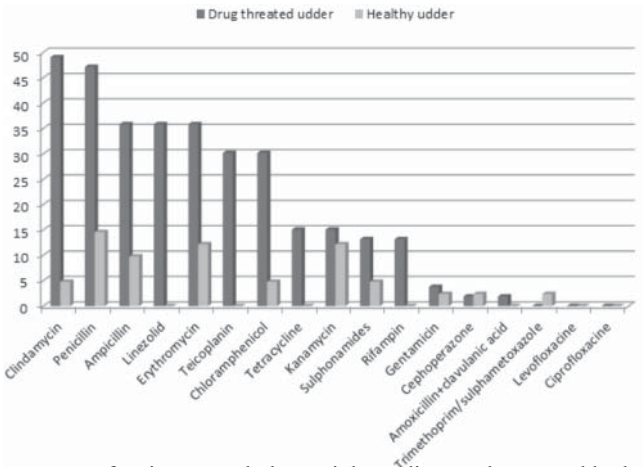


Fig. 1. Percentage of resistant staphylococci depending on the cow udder health status

In the present survey, more than 80 % of enterococci strains from drug-treated udders were resistant to penicillin, chloramphenicol and nitrofurantoin. However, the proportion of enterococci resistant to the same antimicrobials from healthy udders ranged from 50-60 % (Fig. 2). No difference in the occurrence of strains resistant to these antimicrobials was found ($P<0.05$) between healthy and drug-treated udders. Using the agar disk diffusion method, 19 enterococci with intermediate vancomycin zones were found. However, the E-test did not show any MIC above 32 µg/mL indicating the absence of phenotypically vancomycin-resistant enterococci (VRE).

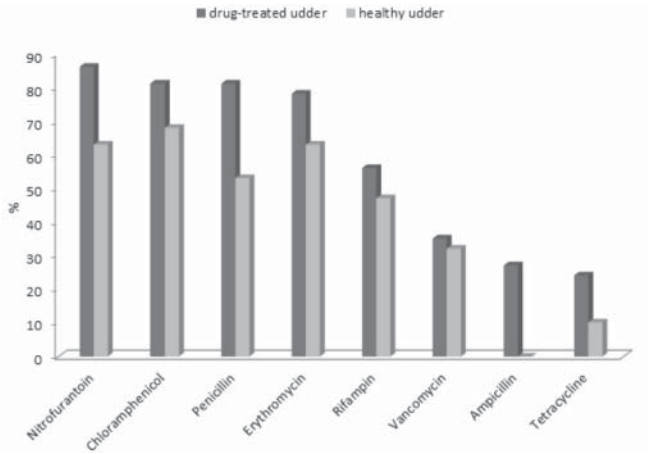


Fig. 2. Percentage of resistant enterococci depending on the cow udder health statuS

Four *E. coli* strains were resistant to 11 antimicrobials, and three enterococci and five staphylococci were resistant to 8 antimicrobial agents. *E. coli* strains from drug-treated udders were mostly (>50 %) resistant to trimethoprim, streptomycin, tetracycline, sulfonamides and chloramphenicol. Half of the tested *E. coli* strains from healthy udder milk samples were also resistant to trimethoprim. Enterobacteria isolated from drug-treated udders were mostly resistant to tetracycline, trimethoprim and streptomycin (>40 %), while in healthy-udder isolates the most common was tetracycline resistance (40 %). Gentamicin resistant strains were only isolated from untreated animals.

**Discussion**

The presence of milk bacteria resistant to antimicrobial agents is known as an important public-health issue and is mainly related to the treatment of mastitis. The aim of the present study was to gain insight into potential risks in terms of the potential occurrence of resistant bacteria in regularly collected milk originating from healthy cows cohabitating with drug-treated cows (with cured mastitis). There is a lack of similar studies performed in raw milk from healthy cows, whereas antimicrobial resistance has been surveyed in milk from mastitic cows with *Staphylococcus aureus* or CoNS (BENIĆ et al., 2012). JURMANOVIĆ et al. (2012) reported that *S. aureus* isolates from mastitic cows in Croatia are resistant to *β*-lactam antibiotics, aminoglycosides, lincosamides, oxytetracyclin, fluoroquinolones and sulfonamides. The results of their 9-year survey showed the increase in resistance of *S. aureus* (n = 2719) towards kanamycin, neomycin, enrofloxacin, lincomycin and penicillin. More recently, LESKOVEC et al. (2015) reported a significant increase in the resistance of *S. aureus* from mastitic cows to penicillin, ampicillin, neomycin, streptomycin and tetracycline, compared to previous reports in Croatia (BENIĆ et al., 2003). FREY et al. (2013) reported coagulase negative staphylococci (CoNS) from mastitic cows mostly resistant to oxacillin (47.0 % of the isolates), fusidic acid (33.8 %), tiamulin (31.9 %), penicillin (23.3 %), tetracycline (15.8 %), streptomycin (9.6 %), erythromycin (7.0 %), sulfonamides (5 %), trimethoprim (4.3 %), clindamycin (3.4 %), kanamycin (2.4 %), and gentamicin (2.4 %). In Croatia, resistance of CoNS was recently evaluated in pasteurized-milk cheeses, and most strains were resistant to erythromycin (38.46 %), penicillin (23.07 %) and tetracycline (5.12 %), with common multidrug resistance (ZDOLEC et al., 2013). Erythromycin and penicillin resistance were also found to be dominant in staphylococci from raw milk (this study). MIKULÁŠOVÁ et al. (2014) also found high multiresistance in CoNS from Bryndza, a traditional Slovak cheese. Multidrug resistant staphylococci are frequently found in ready-to-eat food, including cheeses (CHAJĘCKA -WIERZCHOWSKA et al., 2014). Considering *E. coli* resistance patterns, SRINIVASAN et al. (2007) reported that most strains from cow mastitis are resistant to ampicillin (98.4 %) and frequently resistant to streptomycin (40.3 %), sulfisoxazole (34.1 %), and tetracycline (24.8 %). Less than 20 % were resistant to carbenicillin, gentamicin, cephalothin, trimethoprim,

and amikacin. KALMUS et al. (2011) reported ampicillin, streptomycin and tetracycline resistance among *E. coli* isolates in 24.3 %, 15.6 % and 13.5 % of cases, respectively. The effect of antibiotic pressure is typically evident in *Escherichia,* which is presented in our study by the wide multiresistance patterns in strains from drug-treated animals only.

Enterococci are a quite controversial food-related microbial group, with both beneficial (food fermentation, production of enterocins) and potentially hazardous characteristics (biogenic amine producers, carriers of antimicrobial resistance genes). In our study, an equal distribution of resistant enterococci was present in both milk samples from healthy cow udders and drug-treated udders ($P>0.05$). Such a high proportion of resistant enterococci in both cow populations could be the result of animal cohabitation and cross contamination. Vancomycin resistant enterococci (VRE), as the clinically most relevant enterococci, were absent in both cow groups, based on E-test results. Similarly, JIMÉNEZ et al. (2013) reported the absence of VRE in ovine, feline, canine, porcine and human milk. The most isolates were resistant to chloramphenicol and tetracycline, and frequently resistant to erythromycin, which is partially presented in our study. Over the last few years, a low occurrence of VRE in raw milk has been reported by other authors (ÇETINKAYA et al., 2013; KROČKO et al., 2011). Dairy products, primarily cheeses, are more frequently positive to VRE strains (FURLANETO-MAIA, 2014). Enterococci of food origin are not a direct cause of resistant enterococci in humans, but they may transfer resistance determinants to human-adapted bacteria (ECONOMOU and GOUSIA 2015). Their frequent presence in raw cow milk collected for human consumption should be considered a potential risk, primarily when milk is consumed without any thermal treatment.

## Conclusion

The presence of bacteria resistant to antimicrobial agents in the primary production of milk is a significant public health issue due to the spread of resistance determinants to consumers. It is expected, in the conditions of higher antibiotic pressure, that more (multi) resistant bacteria will be found in animals, milk and the environment. Our study emphasizes another fact: the equal prevalence of resistance in enterococci isolated from milk samples of both healthy and drug-treated cows.

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**SAŽETAK**

Cilj je ovog rada bio istražiti osjetljivost na antimikrobne tvari mikroflore mlijeka s obzirom na zdravstveni status vimena i izloženost antimikrobnim tvarima. Uzorci mlijeka sterilno su uzeti od zdravih krava bez znakova mastitisa (n = 17) i liječenih krava s preboljelim mastitisom (n = 19). Istražena je osjetljivost 56 izolata enterokoka, 30 *Escherichia coli*, 24 enterobakterija i 94 stafilokoka. Sukladno bakterijskoj vrsti ili skupini, korišteni su diskovi ampicilina, rifampina, kloramfenikola, linezolida, tetraciklina, eritromicina, nitrofurantoina, vankomicina, penicilina, trimetoprima, cefoperazona, kanamicina, trimetoprim/sulfametoksazola, nalidiksične kiseline, ciprofloksacina, gentamicina, teikoplanina, sulfonamida, levofloksacina, klindamicina i amoksicilina s klavulanskom kiselinom. Pojavnost višestruko otpornih izolata *E. coli* i stafilokoka bila je značajno veća ($P<0,05$) u mlijeku liječenih krava. Postotni odnos (liječene:zdrave krave) višestruko otpornih izolata *E. coli* bio je 56,3:0, enterobakterija 57,14:30, te stafilokoka 56,5:4,8. Nije bilo značajne razlike ($P>0,05$) u pojavnosti rezistencije enterokoka u mlijeku između zdravih i liječenih krava. Visoka učestalost otpornosti na antimikrobne tvari enterokoka u mlijeku zdravih krava može biti posljedica kohabitacije životinja i križnog onečišćenja.

**Ključne riječi:** antimikrobna rezistencija, mlijeko, enterokoki, zdravlje vimena