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Antimicrobial resistance of coagulase-negative staphylococci and lactic acid bacteria from industrially produced dairy products

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Summary

In this research, the susceptibility to clindamycin, tetracycline, amikacin, amoxicillin + clavulanic acid, enrofloxacine, vancomycin, trimethoprim + sulphametoxazol, tobramycin, chloramphenicol, ciprofloxacin, erythromycin, penicillin and trimethoprim was tested in coagulase-negative staphylococci (n=78) and lactic acid bacteria (n=30) by means of disk diffusion test and E-test. The isolates were collected from soft and hard cheeses, butter and brine. All isolates of coagulase-negative staphylococci were susceptible to clindamycin, amikacin, amoxicillin + clavulanic acid, enrofloxacine, vancomycin, chloramphenicol and ciprofloxacin according to CLSI breakpoints. A total of 30 staphylococci isolates (38.46 %) were resistant to erythromycin, 18 to penicillin (23.07 %), 4 to tetracycline (5.12 %), and one isolate to trimethoprim, tobramycin and trimethoprim + sulfamethoxazole (1.28 %). Among 78 tested staphylococci, 35 of them were resistant to at least one antimicrobial substance (44.87 %). The rate of resistant isolates in different types of soft cheese ranged from 22 to 70 %, while resistant staphylococci were absent in hard cheese and brine. The growth of lactic acid bacteria was not influenced by trimethoprim + sulfamethoxazole (n=29), vancomycin (n=29), trimethoprim (n=28), amikacin (n=10) and tobramycin (n=10). The results show that significant part of apathogenic microbiota in different dairy products is phenotypically resistant to antimicrobial agents.

Key words: resistance, dairy products, apathogenic microbiota, coagulase-negative staphylococci, lactic acid bacteria

Introduction

Antimicrobial resistance is one of the most important issues of veterinary public health. Besides pathogenic bacteria, this resistance is common in apathogenic microbiota which could transfer resistance genes through a food chain. Coagulase-negative staphylococci (CNS) and lactic acid bacteria (LAB) are recognized as technologically/hygienically very important bacteria in food production and preservation (Hadžiosmanović et al., 2005; Šušković et al., 2010), however some health hazards could occur in the presence of strains that produce biogenic amines or enterotoxins and specially transfer antimicrobial resistance determinants (Dobranić et al., 2013; Zdolec et al., 2013a). In Croatia, only few studies were conducted in respect to prevalence of resistant food CNS and LAB, mainly autochthonous foodstuffs - fresh cow cheese and dry fermented sausages (Zdolec et al., 2011; Zdolec et al., 2012ab; Zdolec et al., 2013b). On the other hand, that kind of research was not performed in industrially produced dairy products. Thus, the aim of this study was to evaluate the antibiotic susceptibi-

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lity of CNS and LAB from fresh cheeses, brine, hard cheeses and butter produced in industrial facilities.

Material and methods

Isolates of CNS (n=78) and LAB (n=30) were collected from fresh cheeses produced from pasteurized milk, cheese brine, hard cheeses and butter. Samples were prepared for microbiological analyses following standard procedure (HRN ISO 7218:1999). Samples (0.1 mL) of selected decimal dilutions were plated on Manitol Salt Agar (MSA, bioMerieux, France) and de Man, Ragosa Sharpe agar (MRS, Merck, Germany) and incubated for 48 at 37 and 30 °C, respectively. MSA colonies were transferred to Brain Hearth Infusion broth (BHI, bioMerieux) and incubated for 24 hours at 37 °C. Bacterial culture was streaked again onto MSA, incubated (24 h, 37 °C), Gram stained and tested for coagulase activity (Bactident Coagulase, Merck). Gram positive, coagulase-negative cocci were selected for testing the antibiotic susceptibility. MRS colonies were grown in MRS broth (24-48 h at 30 °C), streaked onto MRS agar, Gram stained and tested for catalase. Gram positive, catalase-negative bacilli and coccobacilli were taken for antibiotic susceptibility testing.

Selected CNS and LAB isolates were tested for susceptibility towards following antimicrobial agents: clindamycin (2 μg), tetracycline (30 μg), amikacin (30 μg), amoxicillin + clavulanic acid (30 μg), enrofloxacine (5 μg), vancomycin (30 μg), trimethoprim + sulphametoxazol (25 μg), tobramycin (10 μg), chloramphenicol (30 μg), ciprofloxacin (5 μg), erythromycin (15 μg), penicillin (10 IU) and trimethoprim (5 μg) by means of disk diffusion method (antibiotic disks, Biorad, France) on Mueller-Hinton agar (bioMerieux, France). Additionally, minimal inhibitory concentration (MIC) were evaluated for 30 selected CNS using E-test (bioMerieux, France) for erythromycin, penicillin and tetracycline. Following incubation (24 h, 35±2 °C) inhibition zones were measured, as well as MICs (E-test), and results were interpreted according to CLSI criteria (Table 1; CLSI, 2008; 2010). Antibiotic susceptibility of LAB was tested only by disk diffusion method, and criterion of sensitivity was the occurrence of inhibition zone.

Table 1. Interpretative standards for resistance/sensitivity of staphylococci towards selected antimicrobial agents

Group	Antimicrobial agent	Zone of inhibition (mm)		MIC (μg/mL)	
		Resistant	Sensitive	Resistant	Sensitive
Lincosamines	Clindamycin	≤14	≥21	≥4	≤0.5
Tetracyclines	Tetracycline	≤14	≥19	≥16	≤4
Aminoglycosides	Amikacin	≤14	≥17	≥64	≤16
	Tobramycin	≤12	≥15	≥16	≤4
	Enrofloksacin	≤16	≥23	-	-
Glycopeptides	Vancomycin	-	-	≥16	≤2
Sulfonamides	Trimethoprim + sulphametoxazol	≤10	≥ 16	≥4	≤2
	Trimethoprim	≤10	≥16	≥16	≤8
Chloramphenicol	Chloramphenicol	≤12	≥18	≥32	≤8
2nd generation Quinolones	Ciprofloxacin	≤15	≥21	≥4	≤1
Macrolides	Erythromycin	≤13	≥23	≥8	≤0.5
Penicillins	Penicillin	≤28	≥29	≥0.25	≤0.12
Aminopenicillins + β-lactamase inhibitors	Amoxicillin + clavulanic acid	≤19	≥20	≥8	≤4

- Standard is not setted

Results and discussion

Results of antibiotic susceptibility testing of CNS are shown in tables 2-4. According to CLSI criteria related to disk diffusion method, all isolates were sensitive to clindamycin, amikacin, amoxicillin + clavulanic acid, enrofloxacin, vancomycin, chloramphenicol and ciprofloxacin. The most isolates were resistant to erythromycin, than penicillin and tetracycline. These isolates were additionally tested using E-test to determine MIC for mentioned antibiotics.

Results shown in table 2 indicate that MIC and inhibition zones correlated completely regarding interpretation of erythromycin, penicillin and tetracycline sensitivity/resistance. Results and interpretation of disk diffusion method could be influenced by several factors including bacterial species and strain, growth condition (time, temperature), pH of media and

Table 2. Number of resistant coagulase-negative staphylococci (CNS) according to testing method

Antimicrobial agent	Disk diffusion test		E-test	
	Number of resistants (n=78)	Range of inhibition zones (mm)	Number of resistants (n=78)	MIC* (μg/mL)
Erythromycin	30	0-10	30	8-32
Penicillin	18	14-20	18	0.5-1
Tetracycline	4	0-12	4	16-32
Trimethoprim	1	8	/	/
Trimethoprim + sulfamethoxazole	1	8	/	/
Tobramycin	1	0	/	/

*minimal inhibitory concentration
/not tested

Table 3. Number and percentage of resistant coagulase-negative staphylococci (CNS) toward specific antimicrobial agents

Antimicrobial agent	Number of isolates	Number of resistant isolates	Percentage (%) of resistant isolates
Erythromycin	78	30	38.46
Penicillin	78	18	23.07
Tetracycline	78	4	5.12
Trimethoprim	78	1	1.28
Trimethoprim + sulfamethoxazole	78	1	1.28
Tobramycin	78	1	1.28

Table 4. Number and percentage of resistant coagulase-negative staphylococci (CNS) in different dairy products

Dairy product	Number of isolates	Number of resistants	Percentage (%) of resistants
Fresh cheeses from pasteurized milk	58	32	55,2
Brine	9	0	0
Butter	3	3	100
Hard cheeses	8	0	0
Total	**78**	**35**	**44.87**

Table 5. Number and percentage of resistant lactic acid bacteria (LAB)

Antimicrobial agent	Number of isolates	Number of resistants	Inhibition zones (mm)	Percentage of resistance (%)
Amikacin	30	10	0	30.00
Tobramycin	30	10	0	30.00
Vancomycin	30	29	0	96.66
Trimethoprim	30	28	0	93.33
Trimethoprim + sulfametoxazole	30	29	0	96.66

recommended interpretative criteria (Bubonja et al., 2008). Despite E-test is not official CLSI method, our results showed its reliability compared to disk diffusion, as reported by others (Baker et al., 1991; Mayrhofer et al., 2008). Our results show a high number of resistant CNS from industrially produced dairy products, mainly fresh cheeses produced from pasteurized milk (44.87 %; n=78) which is potential hazard for consumers related to resistance genes transfer. The percentage of resistant strains in fresh cheeses ranged 22 to 70 % of tested staphylococci, while hard cheeses and brine were free of resistant staphylococci. Our results are in accordance with other studies that report the most frequent CNS resistance to erythromycin, tetracycline and penicillins (Simeoni et al., 2008; Resch et al., 2008; Even et al., 2011). The occurrence of penicillin-resistant staphylococci in dairy products is probably the consequence of their persistence in raw milk, as reported by Sampimon et al. (2011) and Kalmus et al. (2011). The presence of resistant staphylococci in milk and dairy products could be expected due to uncritical use of penicillins and tetracyclines in mastitis treatment or prevention (Zdolec et al., 2006; Vragović et al., 2012ab). However, our results show domination of erythromycin resistance, the antimicrobial agent that is not used intramammary. Thus, it could be assumed that high occurrence of erythromycin resistant strains is related to environmental factors (cross-contamination) or horizontal gene transfer.

The susceptibility of LAB to antimicrobials was tested only by disk diffusion method, and the absence of inhibition zones was used as criterion of resistance. All LAB isolates were sensitive to clindamycin, tetracycline, penicillin, erythromycin, amoxicillin + clavulanic acid, enrofloxacin, chloramphenicol, and ciprofloxacin. The growth of most isolates was not influenced by trimethoprim + sulfametoxsazole (n=29), vancomycin (n=29) and trimethoprim (n=28), and ten isolates (30 %) was resistant to amikacin and tobramycin (table 5). Our results are in agreement with other studies were the most frequently reported resistance of food-related LAB was to glycopeptides and aminoglycosiedes (Zhou et al., 2005; Klein, 2011; Zdolec et al., 2011). Džidić et al. (2008) emphasize that acquired antimicrobial resistance is not evaluated sistematicaly in LAB, which could be transfered to other bacteria.

Conclusion

Microbiologically acceptable milk for further processing must reach set criteria of total viable count and sommatic cells, while dairy product must fulfill the food safety and food hygiene criteria. These criteria recognize only coagulase-positive staphylococci and staphylococcal enterotoxins as hazardous in fresh cheeses. However, our results show that cheeses produced from pasteurized milk contain coagulase-negative staphylococci resistant to antibiotics. Despite the fact these cheeses are microbiologically safe products according to current legislation, we think that antimicrobial resistance of non pathogenic dairy microbiota should be evaluated more in terms of potential health risk. Risk assessment implies more data about the use of veterinary drugs in mastitis control, residues in milk and related occurrence of resistant CNS in raw milk, environment and dairy products.

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Otpornost na antimikrobne tvari koagulaza-negativnih stafilokoka i bakterija mliječne kiseline iz industrijskih mliječnih proizvoda

Sažetak

U ovom radu istražena je osjetljivost koagulaza-negativnih stafilokoka (n=78) i bakterija mliječne kiseline (n=30) na klindamicin, tetraciklin, amikacin, amoksicilin + klavulanska kiselina, enrofloksacin, vankomicin, trimetoprim + sulfametoksazol, tobramicin, kloramfenikol, ciprofloksacin, eritromicin, penicilin i trimetoprim primjenom disk-difuzijskog testa i/ili E-testa. Izolati su izdvojeni iz industrijski proizvedenih mekih i tvrdih sireva, maslaca i salamure. Svi izolati stafilokoka bili su osjetljivi na klindamicin, amikacin, amoksicilin + klavulansku kiselinu, enrofloksacin, vankomicin, kloramfenikol i ciprofloksacin prema CLSI (Clinical and Laboratory Standards Institute) kriterijima. Ukupno 30 izolata (38,46 %) stafilokoka bilo je otporno na eritromicin, 18 na penicilin (23,07 %), 4 na tetraciklin (5,12 %), te po jedan izolat na trimetoprim, tobramicin i trimetoprim + sulfametoksazol (1,28 %). Od ukupno 78 testiranih izolata stafilokoka, njih 35 bilo je rezistentno na najmanje jednu antimikrobnu tvar (44,87 % izolata). Udio rezistentnih izolata u mekim sirevima kretao se od 22 do 70 % testiranih stafilokoka, dok u tvrdom siru i salamuri rezistentnih izolata nije bilo. Na rast bakterija mliječne kiseline nisu utjecali trimetoprim + sulfametoksazol (n=29), vankomicin (n=29), trimetoprim (n=28), amikacin (n=10) i tobramicin (n=10). Dobiveni rezultati ukazuju na to da značajan udio nepatogene mikroflore različitih mliječnih proizvoda s hrvatskog tržišta pokazuje fenotipsku rezistenciju na antimikrobne tvari.

Ključne riječi: rezistencija, mliječni proizvodi, nepatogena mikroflora, koagulaza-negativni stafilokoki, bakterije mliječne kiseline

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