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Antimicrobial susceptibility of lactic acid bacteria isolated from fermented sausages and raw cheese

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ABSTRACT

The aim of this study was to determine lactic acid bacteria from Croatian traditional sausages and raw cow's cheese and to investigate their susceptibility/resistance to 23 antimicrobials. All isolates of *Lactobacillus* spp. and *Leuconostoc* spp. (n = 12) were susceptible to tetracycline, penicillin, bacitracin, chloramphenicol, cefalexin, linkomycin, erythromycin, ampicillin, spiramycin and amoxicillin, but resistant to vancomycin, nalidixic acid and metronidazole. *Lactobacillus curvatus* and *Lactobacillus brevis* 2 were resistant to nitrofurantoin and trimethoprim, *Lactobacillus curvatus* and *Lactobacillus fermentum* to oxacillin, while *Lactobacillus brevis* 1*, Lactobacillus fermentum, Lactobacillus paracesei* ssp. *paracasei* 1 and 2 to neomycin. *Leuconostoc* spp. showed resistance to streptomycin, enrofloxacine, nitrofurantoin, sulphonamides, polymyxin B and oxacillin. These preliminary results indicate the need for further investigations of LAB populations to obtain relevant data in risk assessment related to traditional food products.

Key words: *Lactobacillus* spp., *Leuconostoc* spp., antimicrobial resistance

Introduction

The prescription of antimicrobial drugs in prophylaxis, ignorance of real causative agents, improper dosage, inapposite therapy periods and other irregularities have caused the appearance of antimicrobial resistance in bacteria which is nowadays an emerging (veterinary) public health issue (TEUBER, 2001; HUSSEIN et al., 2005; ZDOLEC et al., 2006;

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MARCINČÁK et al., 2006; ROASTO et al., 2009). The most important problem is the increase in resistance in clinically significant bacteria, but today it is clear that comensal bacteria could also contribute to spreading genes resistant to pathogens (MATHUR and SINGH, 2005; RUZAUSKAS et al., 2009). In this respect, the transfer of antimicrobial resistance is the only relevant reason for caution in lactic acid bacteria (LAB). However, LAB possess a significant natural resistance, especially to vancomycin, which is not transmissible (BERNARDEAU et al., 2008).

The aim of this study was to isolate and determine LAB from traditional Croatian fermented sausages and raw cow's cheese, and to investigate their susceptibility and resistance towards selected antimicrobials. Based on the results obtained, we were able to estimate the presence of resistant or multi-resistant strains in traditional foods and the level of potential risk for consumer health regarding the spread of antimicrobial resistance in the food chain.

Materials and methods

Lactic acid bacteria isolates. In this study we tested previously isolated strains (n = 7) from home-made fermented sausages (ZDOLEC et al., 2007), and isolates from raw cow's cheese produced in a local Istrian dairy (n = 5). Isolates were kept in a Microbank system at -20 ºC.

Antimicrobials. The antimicrobial susceptibility of lactic acid bacteria was tested using selected antimicrobials listed in Table 1 (antimicrobial disks, BIO-RAD, France).

Table 1. Antimicrobials used in susceptibility assay

Antimicrobials	Concentration	Antimicrobials	Concentration
Streptomycin	500 µg	Cephalexin	30 µg
Enrofloxacin	5 µg	Oxacillin	1 µg
Nitrofurantoin	300 µg	Lincomycin	15 µg
Neomycin	30 UI	Vancomycin	30 µg
Trimethoprim	5 µg	Gentamycin	10 µg
Sulphonamides	300 µg	Metronidazol	4 µg
Tetracycline	30 µg	Erythromycin	15 µg
Polymyxin	300 µg	Kanamycin	30 µg
Penicillin	6 µg	Ampicillin	10 µg
Nalidixic acid	30 µg	Spiramycin	100 µg
Bacitracin	10 UI	Amoxicillin	25 µg
Chloramphenicol	30 µg		

Determination of isolates. Isolates (n = 12) were grown in MRS broth (Merck, Germany) for 24-48 hours at 30 ºC. The culture was spread onto MRS agar and incubated for 48-72 hours at 30 ºC. A well isolated colony was taken by swab and spread at high density on MRS and incubated for 24 hours at 30 ºC. The further procedure with API 50 CHL was performed according to the manufacturer's instructions (bioMérieux, France). Interpretation of results was performed with Api*web*™ V 1.2.1.

Susceptibility assay. The agar diffusion disk method was performed according to MAYRHOFER et al. (2008) with modifications: isolates were grown in MRS broth for 24 hours at 30 ºC, re-suspended and re-incubated twice. The active culture (0.1 mL) was spread onto MRS agar. After 20 minutes at room temperature, the discs (6 per plate) were put onto the inoculated media. The plates were incubated for 24 hours at 30 ºC and examined for inhibition zones, which were then measured (mm). We included the disk diameter (6 mm) in the width of zone.

Results

Biochemical identification showed the profiles of the species:

a) In fermented sausages: *Leuconostoc mesenteroides* subsp. *mesenteroides, Lactobacillus curvatus, Lactobacillus brevis* (n = 2)*, Lactobacillus fermentum, Lactobacillus paracasei* subsp. *paracasei* (n = 2).

b) In raw cheese: *Leuconostoc mesenteroides* subsp. *cremoris* (n = 2) and *Leuconostoc mesenteroides* subsp. *dextranicum* (n = 3).

Results of the susceptibility test for LAB originating from the fermented sausages and raw cheese are presented in Tables 2 and 3, respectively. The results showed that all isolates are sensitive to tetracycline, penicillin, bacitracin, chloramphenicol, cefalexin, linkomicin, erythromycin, ampicillin, spiramycin and amoxicillin. The growth of all LABs was not influenced by vancomycin, nalidixic acid and metronidazole, while sulphonamides inhibited the growth of the strain *Ln. mesenteroides* subsp. *cremoris* 2.

Analysing only the results obtained for lactobacilli, besides the antimicrobials listed above, their growth was also disabled in the presence of streptomycin. Two isolates were resistant to nitrofurantoin, trimethoprim (*Lb. curvatus* and *Lb. brevis* 2) and oxacillin (*Lb. curvatus* and *Lb. fermentum*), and four of them to neomycin (*Lb. brevis* 1*, Lb. fermentum, Lb. paracesei* subsp. *paracasei* 1 and 2). Regarding the *Leuconostoc* species, resistance was found towards streptomycin, enrofloxacin, nitrofurantoin, sulphonamides, polymyxin and oxacillin.

Table 2. Results of evaluation of sausage-originated LAB sensitivity to antimicrobials

Antimicrobials	Zone of inhibition (mm)						
	Leuconostoc mesenteroides subsp. *mesenteroides*	*Lactobacillus curvatus*	*Lactobacillus brevis* 1	*Lactobacillus brevis* 2	*Lactobacillus fermentum*	*Lactobacillus paracasei* subsp. *paracasei* 1	*Lactobacillus paracasei* subsp. *paracasei* 2
Streptomycin	-	8	10	12	8	8	8
Enrofloxacin	-	-	-	10	-	-	-
Nitrofurantoin	30	-	24	-	22	20	20
Neomycin	-	8	-	10	-	-	-
Trimethoprim	-	-	14	-	13	14	15
Sulphonamides	-	-	-	-	-	-	-
Tetracycline	10	12	12	12	14	10	12
Polymyxin	-	-	-	-	-	-	-
Penicillin	30	26	26	24	30	26	25
Nalidixic acid	-	-	-	-	-	-	-
Bacitracin	13	12	12	16	13	10	13
Chloramphen.	16	24	20	20	24	20	25
Cephalexin	15	11	14	12	16	12	12
Oxacillin	10	-	8	8	-	8	8
Lincomycin	13	16	15	18	15	15	15
Vancomycin	-	-	-	-	-	-	-
Gentamycin	-	-	-	-	-	-	-
Metronidazol	-	-	-	-	-	-	-
Erythromycin	24	20	20	25	24	20	20
Kanamycin	-	-	-	-	-	-	-
Ampicillin	20	26	20	26	20	24	25
Spiramycin	18	18	18	20	18	16	20
Amoxicillin	18	25	24	32	24	24	20

Table 3. Results of evaluation of cheese-originated LAB sensitivity to antimicrobials

Antimicrobials	Zone of inhibition (mm)				
	Leuconostoc mesenteroides subsp. *cremoris* 1	*Leuconostoc mesenteroides* subsp. *cremoris* 2	*Leuconostoc mesenteroides* subsp. *cremoris* 3	*Leuconostoc mesenteroides* subsp. *dextranicum* 1	*Leuconostoc mesenteroides* subsp. *dextranicum* 2
Streptomycin	22	25	20	24	20
Enrofloxacin	10	12	12	12	-
Nitrofurantoin	-	12	-	-	-
Neomycin	16	20	18	18	15
Trimethoprim	-	-	-	-	-
Sulphonamides	-	12	-	-	-
Tetracycline	30	30	25	25	22
Polymyxin	-	10	10	12	8
Penicillin	25	25	30	25	25
Nalidixic acid	-	-	-	-	-
Bacitracin	26	30	24	28	28
Chloramphenicol	22	25	25	28	25
Cephalexin	16	20	18	20	16
Oxacillin	8	8	10	12	-
Lincomycin	28	30	25	25	15
Vancomycin	-	-	-	-	-
Gentamycin	14	20	14	15	-
Metronidazol	-	-	-	-	-
Erythromycin	20	30	22	20	20
Kanamycin	12	18	12	10	12
Ampicillin	22	30	25	26	20
Spiramycin	14	20	20	20	18
Amoxicillin	22	25	25	24	22

Discussion

Lactobacilli are usually sensitive to penicillin and β-lactamase inhibitors, but more resistant to oxacillin and cephalosporins (COPPOLA et al., 2005; DANIELSEN and WIND, 2003). Our results are in agreement with these findings, but other studies have reported the appearance of penicillin resistance. Thus, YUKSEKDAG and BEYATLI (2008) isolated penicillin-resistant strains of *Lb. casei* and *Lb. helveticus* from milk products. In that study among 24 LAB isolates (*Lactobacillus* spp., *Leuconostoc* spp., *Streptococcus* spp., *Lactococcus* spp.), as many as 44% were resistant to penicillin. Similar results were reported by ZARAZAGA et al. (1999). According to CONDON (1983) non-permeability

of the lactobacilli cell wall is the main reason for their resistance, but several non-specific mechanisms could cause differences among strains within the same species (PUTMAN et al., 2001). Most lactobacilli are resistant to glycopeptides (vancomycin), while susceptibility to bacitracin is variable (COPPOLA et al., 2005; KATLA et al., 2001). Our study confirms the well-known resistance of lactobacilli to vancomycin which is considered as intrinsic and untransferable (KLEIN et al., 1998), but all our strains were sensitive to bacitracin. Lactobacilli are usually sensitive to inhibitors of protein synthesis such as chloramphenicol, erythromycin, clindamycin and tetracycline and more resistant to aminoglycosides (neomycin, kanamycin, streptomycin and gentamycin) (CHARTERIS et al., 1998; COPPOLA et al., 2005; ZHOU et al., 2005). This is partially presented in our results, where all lactobacilli were inhibited by chloramphenicol, erythromycin, tetracycline and streptomycin. On the other hand, some lactobacilli were resistant to neomycin, and especially to kanamycin and gentamycin. Antimicrobial resistance is known to be present in this group of antibiotics, in particular for chloramphenicol (LIN et al., 1996), erythromycin (CATALOLUK and GOGEBAKAN, 2004) and tetracycline (KASTNER et al., 2006). Furthermore, our results showed the insensitivity of lactobacilli to antimicrobials which inhibit the synthesis of nucleic acids, as reported by others (CHARTERIS et al., 1998; KATLA et al., 2001; COPPOLA et al., 2005). Leuconostocs are usually resistant to glycopeptides, cefoxitin, metronidazole, nalidixic acid, gentamycin, kanamycin, streptomycin, nitrofurantoin, sulphadiazin and trimethoprim (SWENSON et al., 1990; HERRERO et al., 1996; ZARAZAGA et al., 1999; KATLA et al., 2001; FLÓREZ et al., 2005). Most Leuconostocs are sensitive to rifampicin, chloramphenicol, erythromycin, clindamycin and tetracycline (SWENSON et al., 1990; FLÓREZ et al., 2005). Our results showed that isolated *Leuconostoc* spp. were resistant to vancomycin, nalidixic acid and metronidazol, but some strains additionally to streptomycin, enrofloxacin, nitrofurantoin, sulphonamides, polymyxin and oxacillin.

In conclusion, antimicrobial resistance of food-borne lactic acid bacteria is real today and emerging due to potential gene transfer to pathogenic bacteria. The goal of our preliminary study was to evaluate the presence of resistant LABs in local traditional foods such as fermented sausages and raw cheese. We only characterized some strains, so further investigations of LAB populations are necessary to obtain relevant data to be included in risk assessment related to traditional food products.

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SAŽETAK

Cilj ovoga rada bio je ustanoviti bakterije mliječne kiseline u tradicionalnim fermentiranim kobasicama i svježem kravljem siru te istražiti njihovu osjetljivost na 23 antimikrobne tvari. Svi izolati *Lactobacillus* spp. i *Leuconostoc* spp. (n=12) bili su osjetljivi na tetraciklin, penicilin, bacitracin, kloramfenikol, cefaleksin, linkomicin, eritromicin, ampicilin, spiramicin i amoksicilin, a otporni na vankomicin, nalidiksičnu kiselinu i metronidazol. *Lactobacillus curvatus* i *Lactobacillus brevis* 2 bili su otporni na nitrofurantoin i trimetoprim, *Lactobacillus curvatus* i *Lactobacillus fermentum* na oksacilin, a *Lactobacillus brevis* 1*, Lactobacillus fermentum, Lactobacillus paracesei* subsp. *paracasei* 1 i 2 na neomicin. *Leuconostoc* spp. pokazali su otpornost na streptomicin, enrofloksacin, nitrofurantoin, sulfonamide, polimiksin B i oksacilin. Ovi preliminarni rezultati upućuju na potrebu daljnjih istraživanja antimikrobne osjetljivosti bakterija mliječne kiseline radi prikupljanja relevantnih podataka u procjeni rizika povezanog s autohtonom hranom.

Ključne riječi: *Lactobacillus* spp., *Leuconostoc* spp., otpornost na antimikrobne tvari