ANTIBIOTIC RESISTANCE OF AUTOCHTHONOUS POTENTIAL PROBIOTIC BACTERIA

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ABSTRACT

Traditional cheeses represent great source for isolation and selection of lactic acid bacteria and potential application as starter cultures or potential probiotic bacteria. Nowadays, probiotic criteria (ability to survive gastrointestinal tract, antimicrobial activity, antibiotic resistance) and clinical trials, became very important for selection of autochthonous potential probiotic bacteria. Some potential probiotic strains may carry transmissible plasmid-encoded antibiotic resistance genes. The aim of this work was to evaluate the antibiotic resistance of 85 autochthonous potential probiotic strains isolated from traditional Serbian cheeses. Antibiotic resistance was determined for ampicillin, vankomycin, oxacillin, neomycin, chloramphenicol, gentamycin, tetracycline, erythromycin, kanamycin, penicillin, streptomycin using disk diffusion test and E-test. All the strains showed resistance to kanamycin, while most of the LAB strains showed resistance to streptomycin (94%) and vancomycin (82%). Slightly less number of strains showed resistance to gentamicin (65%), neomycin (56%) and oxacillin (53%). All strains showed sensitivity to penicillin, while several strains showed resistance to ampicillin, erythromycin, tetracycline and chloramphenicol. Strains showed minimal inhibitory concentracion (MIC) for streptomycin ≥24 μg mL^{-1}, gentamicin ≥48 μg mL^{-1}, oxacillin ≥1.5 μg mL^{-1}, tetracycline ≥3 μg $mL^{-1}$and erythromycin ≥1 μg mL^{-1}. The results obtained in this study indicate that resistance of autochthonous potential probiotic lactic acid bacteria isolated from traditional Serbian cheeses to antibiotics, is very important feature in characterisation and selection of strains for application as potential probiotic bacteria in production of functional food.

INTRODUCTION

Lactic acid bacteria (LAB) represent heterogeneous group of bacteria which are widely spread in the nature: gastrointestinal and urogenital tract of human and animals, dairy products, numerous fermented food, etc. These bacteria traditionally present natural microflora of different fermented dairy product, such as cheeses, as well as different cereals, fermented sausages, vegetables, etc.

According to the definition given by FAO/WHO (2002) probiotics are „live microorganisms which when administrated in adequate amounts, confer a health benefits on the host". Many LAB are used as probiotic bacteria due to their potential health benefits to the consumer health. Regarding this, LAB represent important part of food industry.

Lactic acid bacteria strains isolated from traditional made cheeses constitute a reservoir of unexplored potential in biotechnology. Considering the fast growing interest for application of probiotic strains in food production, it could be presumed that it is possible to isolate some strains, with potential probiotic ability, among the autochthonous strains (Radulovic *et al.*, 2010). In order to be used as probiotics LAB should fulfill essential characteristics which include the following: (1) recognition as safe (GRAS; generally recognized as safe); (2) viability during processing and storage; (3) antagonistic effect against pathogens; (4) capability to survive in the intestinal ecosystem and (5) adherence to the intestinal epithelium

of the host among others (McFarland *et al.*, 1997; Begley *et al.*, 2005; Vesterlund *et al.*, 2005; Lin *et al.*, 2006), as well as sensitivity to the antibiotic. Considering the importance of antibiotic resistance of LAB in food chain, antibiotic susceptibility of autochthonous potential probiotic bacteria is a very important criteria for their selection (Radulovic *et al.*, 2012).
The extensive use of antibiotic in animal husbandry for therapeutic and prophylactic use as well as growth promoters (Barton, 2000, Singer *et al.*, 2003), lead to the use of antibiotic as integral part of feed. Autochthonous LAB isolated from dairy products obtained from animals that have been treated with antibiotics, could carry antibiotic resistant determinates (Teuber *et al.*, 1999). This resistance could be transmitted to the human population through food chain as one of the main pathways for the transmission of antibiotic resistant bacteria from animals to humans (Singer *et al.*, 2003). Although many strains are not pathogenic, autochthonous LAB could constitute a reservoir of genes conferring resistance to antibiotics which might be transferred to pathogenic strains (Lukasova and Sustackova, 2003). The circulation of genes coding for antibiotic resistance from beneficial LAB in the food chain via animals to humans is a complex problem (Radulovic *et al.*, 2012). Therefore, there is a need to evaluate the safety of autochtonous potential probitic lactic acid bacteria regarding their ability to acquire and disseminate antibiotic resistance determinants in selection of LAB.
The aim of this work was to examine resistance of 85 autochthonous lactic acid bacteria to different antibiotic and to determine minimal inhibitory concentration of antibiotics to the LAB.

MATERIAL AND METHODS

Traditional cheeses

Autochthonous lactic acid bacteria were isolated from traditional made white brined Sjenicki cheese. Samples of cheeses were collected from different household in region of Sjenica in Serbia. The collected samples of cheeses were kept in sterile bags at 4°C and analyzed within the following 24 h.

Isolation and selection of lactic acid bacteria

From each cheese samples, 10 g were weighed aseptically and transfered into a sterile Stomacher bag under unseptic conditions and homogenized in 90 mL of sterile sodium citrate 20% (w/v) for 5 minutes using Lab Blender 400 stomacher (Seward, London, UK). Appropriate decimal dilutions of the samples were prepared using the same diluents and plated on different growth media. Lactobacilli were isolated from plates which were incubated in anaerobic conditions (Gas Pak, BBL, Germany) on MRS agar (Oxoid, CM 361) for 48 h on 30°C and 45°C. Lactococci were accumulated on M17 agar (Oxoid, CM 785) under aerobic conditions on 30°C and 45°C for 48 h.
After incubation, few single colonies were randomly picked from MRS and M17 agar plates and streaked on new agar plates for purification. Pure Gram-positive and catalase-negative isolates were cultivated in MRS and M17 broth overnight and stored under -80°C in MRS and M17 broth supplemented with 20% glycerol. Isolates were revitalized by two consecutive transfers on MRS broth for lactobacilli on 37°C and M17 broth for lactococci on 30°C.
Overall, 161 pure cultures were isolated form Sjenicki cheese and 85 strains were selected based on their technological characterisation and identification with API system (BioMerieux, France). The following 85 strains of LAB were examined for antibiotic resistance: *Lactococcus lactis* (twenty-one strains), *Lactococcus cremoris* (seven strains), *Leuconostoc* (five strains), *Lactobacillus plantarum* (twenty-eight strains), *Lactobacillus paracasei* (nineteen strains), *Lactobacillus brevis* (three strains) and *Lactobacillus pentosus* (two strains).

Testing for antibiotic resistance

Selected autochthonous LAB were tested for their resistance to 11 antibiotic: penicillin (10U), ampicillin (10 µg), vancomycin (30 µg), streptomycin (10 µg), kanamycin (30 µg), erythromycin 15 µg), tetracycline (30 µg), gentamicin (10 µg), chloramphenicol (30 µg), neomycin (30 µg) and oxacillin (1 µg). Antibiotics were produced by Becton, Dickinson and

Company, USA. Testing was performed using the standard disc diffusion test (National Committee for Clinical Laboratory Standards, 1993).

Determination of antibiotic minimal inhibitory concentracion

E-test strips (BioMerieux, France) for determination of minimal inhibitory concentration of antibiotic susceptibility for gentamycin, oxacillin, tetracycline, streptomycin and erythromycin were used according to the manufacturer's instruction. Briefly, a bacterial suspension was made by picking a few colonies of LAB from MRS or M17 agar plates using sterile loop and transferred to sterile sodium chloride solution (0.9%) to reach density corresponding to the McFarland 0.5 value. Using sterile swab, the suspension of isolates was swabbed on appropriate agar plates in three directions, rotating the plates for 60 degrees each time to evenly distribute the suspension of isolate. The E-test strips were placed on air dried surface of plates. For lactobacilli and lactococci isolates were used MRS and M17 agar plates which were incubated anaerobically at 37°C for 48 h and aerobically at 30°C for 48 h, respectively. The minimal inhibitory concentration (MIC) for each antibiotic was read as the lowest antibiotic concentration in which the growth was inhibited.

RESULTS AND DISCUSSION

The results obtained for antibiotic resistance of selected autochthonous strains using the disk diffusion method are shown in table 1.

Among 85 strains of autochthonous potential probiotic lactic acid bacteria, none of the strains was totally susceptible to the tested antibiotics and some of the strains showed multiple resistances. Results showed that all strains were susceptible to penicillin, while one strain of *Lb. plantarum* and *Lb. brevis* showed resistance to amplicilin. Also, resistance to erythromycin, tetracycline and chloramphenicol, regardless the species strains, was present in only several strains which is in accordance to the results of Herros et al., 2005. Autochthonous potential probiotic lactic acid bacteria showed resistance to kanamycin, while almost all strains were resistant to streptomycin and vancomycin. These results are in accordance to the several papers (Herros *et al.*, 2005, Zhou *et al.*, 2005, Kastner *et al.*, 2006) which indicate that LAB are normally resistant to the principal types of antibiotics.

Table 1. Resistance of 85 strains of autochthonous potential probiotic lactic acid bacteria determined by disk diffusion test

	Lc. lactis	*Lc. cremoris*	*Leuconostoc*	*Lb. plantarum*	*Lb. paracasei*	*Lb. brevis*	*Lb. pentosus*
Number of strains	21	7	5	28	19	3	2
Ampicillin (10 µg)	0	0	0	1 RS	0	1 RS	0
Chloramphen. (30 µg)	1 RS	0	0	0	1 RS	0	0
Erythromycin (15 µg)	0	0	0	1 RS	0	0	0
Gentamicin (10 µg)	15 RS	4 RS	4 RS	20 RS	11 RS	1 RS	0
Kanamycin (30 µg)	21 RS	7 RS	5 RS	28 RS	19 RS	3 RS	2 RS
Neomycin (30 µg)	12 RS	4 RS	2 RS	15 RS	11 RS	2 RS	1 RS
Oxacillin (1 µg)	11 RS	2 RS	1 RS	17 RS	10 RS	2 RS	2 RS
Penicillin (10U)	0	0	0	0	0	0	0
Streptomycin (10 µg)	21 RS	6 RS	5 RS	26 RS	18 RS	2 RS	2 RS
Tetracycline (30 µg)	1 RS	1 RS	0	3 RS	0	0	0
Vancomycin (30 µg)	18 RS	6 RS	3 RS	24 RS	15 RS	2 RS	2 RS

RS-resistant strains

The incidence of antibiotic resistance of potential probiotic bacteria varied notably depending on the minimal inhibitory concentration (MIC). Among the strains which were tested for streptomycin, one *Lb. plantarum* strain showed ≥24 µg mL^{-1} for MIC, while the rest of the tested strains showed significant higher concentration (192-1024 µg mL^{-1}). A broad range o

MIC distributions of potential probiotic bacteria was detected for erythromycin, 0.19 μg mL
for one *Lb. paracasei* strain, 0.50 μg mL^{-1} for *Lb. plantarum* and *Leuconostoc*, 1 μg mL^{-1} fo
Lb. plantarum, 4 μg mL^{-1} for two *Lc. lactis* strains and 6 μg mL^{-1} for one *Lb. plantarum* strain
Tetracycline MIC distributions was detected for *Lc. lactis* 0.064 μg mL^{-1}, 0.094 μg mL^{-1} and
μg mL^{-1}, 3 μg mL^{-1} for *Lb. planatrum* strain and 4 μg mL^{-1} for *Lc. cremoris* strain. A wid
range of gentamycin MIC distributions was determined, 48 μg mL^{-1} for *Lb. plantarum* and *Lb*
paracasei, 64 μg mL^{-1} for *Lb. paracasei* strain, 96 μg mL^{-1} for three *Lb. plantarum* strains an
one *Lb. paracasei* strain, 128 μg mL^{-1} for *Lc. cremoris* and *Lb. paracasei* and 256 μg mL^{-1} fo
one *Lc. lactis* and *Lb. paracasei* strain. For oxacillin, MIC range of antibiotic susceptibilit
was clearly narrow, 1.5 μg mL^{-1} for *Lb. paracasei*, 2 μg mL^{-1} was detected for six *Lb*
paracasei strains and one *Lc. lactis* strain, and 3 μg mL^{-1} for one *Lb. plantarum* strain.
These results agree with other studied on probiotic bacteria (Hummel *et al.*, 2007, Matto e
al., 2007, Korhonen, 2010).
Knowledge on the antibiotic resistance of autochthonous potential probiotic bacteria is sti
limited. However, resistance to certain antibiotic is not unusual for autochthonous LAB whic
includes potential probiotic and starter cultures (Florez *et al.*, 2005, Katla et al., 2001).
Results of many studies, including this one, indicate that genera *Lactobacillus*, *Leuconosto*
and *Lactococcus* are generally quite sensitive to clinically relevant antibiotic such a
penicillin, ampicillin, erythromycin and tetracycline. In contrast, some resistance appear t
be intrinsic for LAB, like vancomycin, streptomycin, kanamycin and gentamycin (Kastner e
al., 2006, Korhonen, 2011).
Determination of MIC breakpoint values for LAB is very important, considering the fact that
may affect on the decision on whether resistance could be considered as intrinsi
Furthermore, there is a not standard and the National Committee for Clinical Laborator
Standards does not establish MIC breakpoints for LAB, except the *Enterococcus* spp.

CONCLUSIONS

The food chain was considered as the main route of transmission of antibiotic resistant lacti
acid bacteria between the animals and human population. Fermented dairy products an
fermented meats, which are not heat-treated before consumption, provide a vehicle fo
antibiotic resistant LAB with a direct link between the animal indigenous microflora and th
human gastrointestinal tract. Results from the present study suggest that autochthonou
lactic acid bacteria used as potential probiotic bacteria isolated from traditional cheese
might carry and possibly spread antibiotic resistance determinants. Since there has been
significant rise in the consumption of probiotic products, it is important that probiotics shoul
be tested for the presence of transferable resistance genes before being used as probiot
bacteria. In the future research, continuous attention should be paid to the selection
probiotic strains free of transferable antibiotic-resistance determinants.

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