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CHARACTERIZATION OF ANTIBIOTIC RESISTANCE PHENOTYPES AND RESISTANCE GENES IN *ENTEROCOCCUS* SPP. ISOLATED FROM CHEESES

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Abstract - Strains of *Enterococcus* spp. isolated from a collection of 123 artisanal and industrial cheese samples were studied for the phenotypic and genotypic assessment of antibiotic resistance. A total of 226 isolates included 119 *E. faecium* (52.65%), 40 *E. durans* (17.7%), 37 *E. hirae* (16.37%), 29 *E. faecalis* (12.83%) and 1 *E. gallinarum* (0.44%). Out of 61 tested strains, 15 (24.59%) strains exhibited resistance to one or more tested antibiotics, as determined by the disc diffusion method. The resistance phenotypes were as follows: gentamicin (45.45%), tetracycline (31.82%), erythromycin (9.09%), vancomycin (9.09%) and penicillin (4.55%). The presence of tetracycline and erythromycin resistance genes [*tet*(M), *tet*(L) and *erm*(B), respectively] and integrase gene (*int*), associated with Tn*916-1545* transposon family, was detected by PCR procedures. The *tet*(M) gene was determined in all 7 tested strains, but none of the analyzed strains harbored *tet*(L) determinant. The *erm*(B) gene was not detected in 9 strains characterized by phenotypic resistance to erythromycin. All 16 strains were positive for the presence of the *int* gene. The presented results show the presence of antibiotic resistance genes and the transposon integrase gene associated with transferable resistance in enterococci, indicating a potential for gene transfer through the food chain.

Key words: cheese; enterococci; antibiotic resistance; potential of antibiotic-resistant gene transfer

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INTRODUCTION

Enterococci represent a major component of the microflora of artisanal cheeses produced in southern Europe. Because of their metabolic activities, it has been suggested that enterococci could be evaluated as starter or adjunct cultures, playing an important role in ripening and aroma development (Franz et al., 2001; Schirru et al., 2012). Recent studies suggest that cheeses may serve as a reservoir of antibiotic-resistant enterococci with intrinsic characteristics that allow them to persist and spread in the community (Bertrand et al., 2000). Previous studies on European cheeses (Teuber et al., 1999) detected strains of enterococci (mainly *E. faecalis* and *E. faecium*) resistant to penicillin, tetracycline,

chloramphenicol, erythromycin, gentamicin, lincomycin, rifampicin, fusidic acid and vancomycin (Giraffa et al., 2000). Prevalence of multiple drug resistance was also observed (Bertrand et al., 2000). Resistance to tetracycline is conferred by genes [*tet*(K) and *tet*(L)] and [*tet*(M), *tet*(O) and *tet*(S)] encoding efflux mechanisms and ribosomal protection proteins, respectively. The *tet*(M) gene is associated with the Tn*916-1545* family of conjugative transposons that have a broad host range. In addition, tetracycline resistance can be conferred by an unknown mechanism encoded by *tet*(U) (Chopra et al., 2001; Haack et al., 2000). Erythromycin resistance in enterococci is determined by genes encoding erythromycin methylases (*erm*), efflux pumps (*msr*C, *mef*A, *mef*E) and inactivating enzymes (*mph*A) (Singh et al., 2001). The aim of this investigation was to examine the prevalence of antibiotic-resistant phenotypes and resistance genes among enterococcal strains isolated from artisanal and industrial cheeses originating from Serbia.

MATERIALS AND METHODS

Cheese samples

The material under investigation consisted of 123 cheese samples, including fresh, rennet coagulated cheeses made from raw (n=13) and cooked milk (n=18); fresh, sour coagulated cheeses made from raw (n=14) and cooked milk (n=4); cheeses in brine made from raw (n=21) and cooked milk (n=15); samples of kashkaval (n=12) originated from Stara Planina; Sombor (soft) cheese (n=16) and industry-made cheeses (n=10). Production and consumption of white cheese in brine is dominant in central Serbia, although its homemade production occurs in all parts of Serbia. Sombor cheese (made without the addition of starter cultures) and cheese from Stara Planina are cheeses whose traditional production persists over time and is part of the cultural identity of the people of the region concerned. Cow, sheep and goat milk used for kashkaval production originates from Stara Planina.

Microbiological analysis

Ten grams of each cheese sample were homogenized with a sterile solution of sodium citrate (20 g l^{-1}), adequately diluted in sterile Ringer solution and spread on Kanamycin Aesculine Azide (KAA; Oxoid) plates. After 24 h incubation at 37°C in aerobic conditions, colonies that displayed the typical enterococcal growth and cell morphology were picked up and purified twice on KAA plates. The phenotypization of isolated strains was performed according to the following tests: catalase activity, growth in MRS broth at 10°C and 45°C, growth at pH 9.6, growth in broth containing 6.5% NaCl, growth in 0.1% methylene blue milk, resistance at 60°C/15 and 30 min, Voges/Proskauer reaction and fermentation of ribose. The identification of *Enterococcus* spp. was accomplished by API system (api® 20 Strep, bioMeriux, France).

Antibiotic susceptibility testing

Antibiotic resistance was tested by the agar diffusion method on Muller-Hinton agar plates according to the recommendations of *NCCLS* (2001), with the commercial BBL Sensi-Disc Antimicrobial Susceptibility Test Discs (BBL™ Sensi-Disc™ Antimicrobial Susceptibility Test Discs) (Becton, Dickinson and Company, Le Pont de Claix, France). The inocula were prepared by suspending a few isolated colonies in Brain Heart Infusion (BHI) broth, giving a density of 0.5 McFarland standard and swabbed for confluent growth onto Mueller Hinton agar. Discs of lincomycin (2 μg), gentamicin (10 μg), streptomycin (10 μg), erythromycin (15 μg), tetracycline (30 μg), vancomycin (30 μg), sulfamethoxazole+trimethoprim (23.75+1.25 μg) and penicillin (10 IU) were added onto inoculated Muller-Hinton agar plates. Plates were incubated for 24 h at 37°C.

Measurements of zone diameter and interpretative categories (susceptible, intermediate and resistant) were calculated according to the manufacturer's recommendations.

Detection of antibiotic resistance and integrase genes

Isolation of bacterial DNA was performed according to the manufacturer's recommendation (Dneasy Blood and Tissue Kit, Qiagen). For all detection assays, a common PCR core mixture (total volume, 25 µl) was used that consisted of 1 x PCR buffer, deoxynucleoside triphosphates (GeneAmp DNTPs, (Applied Biosystem)) at a concentration of 200 µM each, 2.5 U of Taq polymerase (Qiagen) and 0.4 µM of each primer. A 10 ng portion of intact total DNA was used as the PCR template. All PCR amplifications were performed in a GeneAmp 9700 PCR system (Applied Biosystem, Warrington, United Kingdom). The following conditions for multiplex PCR were used: an initial denaturation step at 94°C/ 3 min, followed by 30 cycles at 94°C/1 min, 55°C/1 min (50°C for *int* gene), 72°C/2 min and a final extension step at 72°C for 10 min. The presence of genes was analyzed in the PCR reaction (Doherty et al., 2000; Sutcliffe et al., 1996) with primers specific for *tet*(M), *tet*(L), integrase (*int*) and *erm*(B) genes. The nucleotide sequences of the primers used for detection were: 5'- AGTTTTAGCTCATGTTGATG and 5'- TCCGACTATTTGGACGACGG for *tet*(M) (product size 1 862 bp); 5'-GTMGTTGCGCGCTATATTCC and 5'-GTGAAMGRWAGCCCACCTAA for *tet*(L) (product size 696 bp); 5'-GCGTGATTGTATCTCACT and 5'- GACGCTCCTGTTGCTTCT for Tn*916-1545*(product size 1 046 bp); 5'-GAAAAGGTACTCAACCAAATA and 5'-AGTAACGGTACTTAAATTGTTTAC for *erm*(B) (product size 639 bp). PCR amplicons were analyzed electrophoretically on 1% agarose gels and visualized by ethidium bromide fluorescence.

RESULTS

Among the 226 *Enterococcus* isolates tested, 119 (52.65%) were *E. faecium*, 40 (17.7%) *E. durans*, 37 (16.37%) *E. hirae*, 29 (12.83%) *E. faecalis* and 1 (0.44%) *E. gallinarum*. In the present study 15 of 61 (24.59%) tested enterococci strains exhibited resistance to one or more tested antibiotics. According to the disc diffusion method, the prevalence of resistance phenotypes was as follows: gentamicin (45.45%), tetracycline (31.82%), erythromycin (9.09%), vancomycin (9.09%) and penicillin (4.55%). No resistance was observed towards lincomycin and sulfamethoxazole-trimethoprim (Table 1). Only one strain of *E. faecalis* exhibited resistance to three antibiotics: tetracycline-gentamicin-vancomycin. Resistance to gentamicin was detected in one and three strains of *E. faecalis* and *E. faecium*, respectively. One strain of *E. faecalis*, two *E. hirae* strains and one strain of *E. faecium* showed resistance only to tetracycline. Resistance to gentamicin-penicillin and gentamicin-tetracycline was detected in one *E. faecium* and two *E. faecalis* strains, respectively. One strain of *E. faecalis* and *E. faecium* each exhibited resistance to gentamicin. Only one strain of *E. faecalis* was resistant to vancomycin. For the 10 µg streptomycin disc, all strains displayed (intrinsic) resistance to this antibiotic. The determined resistance profiles of enterococci strains are shown in Table 2.

The genetic basis of the observed tetracycline and erythromycin resistance was investigated by PCR amplification of the genes *tet*(L), *tet*(M) and *erm*(B), respectively. By the same method, potential conjugative transfer of resistance determinants was estimated based on integrase (*int*) gene detection. The presence of the *tet*(M) gene was investigated in seven enterococci strains characterized by phenotypic resistance to tetracycline. The amplification product of the expected size was obtained in all seven tested strains. The gene for *tet*(L) could not be detected (Fig. 1). In none of the erythromycin resistant strains (n=9), could the gene for *erm*(B) be amplified. The integrase (*int*) gene was detected in all investigated enterococci strains: 7 strains with tetracycline-resistant phenotype that carried *tet*(M) and 9 strains with erythromycin-resistant phenotype (Fig. 2). The presence of the *int* gene in resistant enterococci indicates that they contain a member of the broad-host range Tn*916-1545* conjugative transposon family.

Table 1. The prevalence of resistance phenotypes in *Enterococcus* spp.

Antimicrobial agents	Resistance phenotypes (n)	Resistance phenotypes (%)
Gentamycin	10	45.45
Tetracycline	7	31.82
Erythromycin	2	9.09
Vancomycin	2	9.09
Lincomycin	1	4.55
Penicillin	0	0
Sulfamethoxazole-Trimethoprim	0	0
Streptomycin*	0	0

Table 2. Resistance profiles in *Enterococcus* spp.

Antimicrobial agents (n)	Antimicrobial agents that strain expressed resistance to	*Enterococcus* spp.
3	Tetracycline, Gentamicin, Vancomycin	*E. faecalis* 342
2	Tetracycline, Gentamicin	*E. faecalis* 513 *E. faecalis* 512
2	Gentamicin, Erythromycin	*E. faecium* 593 *E. faecalis* 163
2	Gentamicin, Penicillin	*E. faecium* 982
1	Tetracycline	*E. faecium* 802 *E. hirae* 713 *E. hirae* 61 *E. faecalis* 102
1	Gentamicin	*E. faecium* 701 *E. faecium* 343 *E. faecium* 352 *E. faecalis* 192
1	Vancomycin	*E. faecalis* 702

DISCUSSION

Enterococci are ubiquitous cocci that occur and grow in a variety of dairy and other food products. These organisms may enter the milk either directly from human or animal feces, indirectly from contaminated water sources, and from the milking equipment and the bulk-milk storage tank. In addition to different species of enterococci found in cheese,

E. faecalis and *E. faecium* are the predominant species in raw milk, pasteurized milk and cheese and in other milk products from different countries (Suzzi et al., 2000). Among the 101 isolates of *Enterococcus* spp. found in Turkish white cheese samples (Çitak et al., 2004), *E. faecalis* (61.3%) and *E. faecium* (24.7%) were the most commonly isolated species, followed by *E. durans* (6.9%), *E. mundtii* (4.9%) and *E. hirae* (1.9%). *E. faecium*, *E. durans* and *E. faecalis* in Bryndza cheese at frequencies of 57%, 22% and 16%, respectively, have been found (Belicová et al., 2007). Similar frequencies of *E. faecium* (52.65%), *E. durans* (17.7%) and *E. faecalis* (12.83%) were found in the present investigation. In a study conducted by Ortigosa et al. (2008), *E. faecium* was the predominant species in cheeses made from pasteurized cow and goat milk, while these species with *E. faecalis* were found at similar levels in pasteurized ewe milk

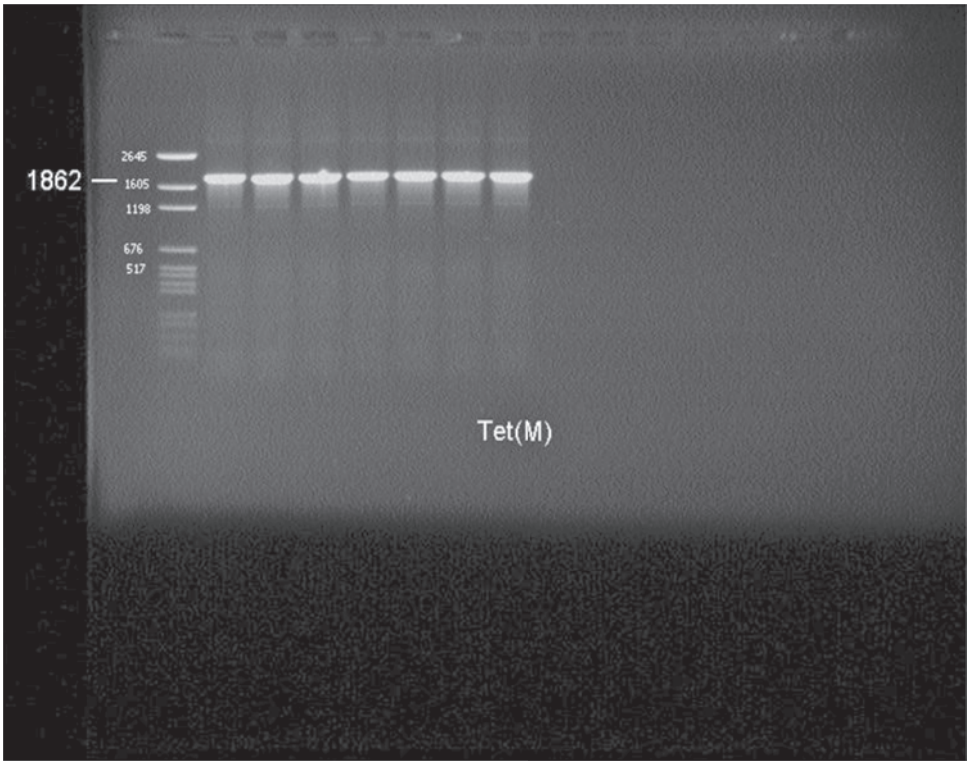

Fig. 1. Agarose gel showing PCR products of the *tet*(M) gene in *enterococci* isolates with tetracycline resistance phenotype (lanes 1-7). The molecular weight marker (MW) was a DNA ladder (BenchTop pGEM DNA; Promega)

cheeses. The results of Kročko et al. (2011) confirmed equal numbers of *E. faecalis* and *E. faecium* in traditional Slovak Bryndza cheese.

According to Teuber et al. (1999), *E. faecalis* and *E. faecium* resistant to one or more antibiotics, including penicillin, tetracycline, chloramphenicol, erythromycin, gentamicin, lincomycin, rifampicin, fusidic acid and vancomycin, have been isolated from European cheeses. The resistance of enterococci to cephalosporins, lincosamides, many β-lactams and aminoglycosides is defined as intrinsic, while acquired resistance refers to resistance to chloramphenicol, erythromycin, tetracycline and glycopeptides (Pavia et al., 2000). Lopes et al. (2003) do not agree with the generalized idea that enterococci are intrinsically resistant to gentamicin, which is explained by the possible gene transfer from clinical or commensal bacteria to dairy enterococci. In this work, for the 10 μg gentamicin disc, 83.6% of dairy isolates were susceptible, which is in accordance with 42% of susceptible isolates represented by Lopes et al. (2003). Previous studies (Teuber et al., 1999) showed a much higher incidence of resistance to gentamicin (80%) of enterococci isolated from cheeses. Contrary to this, Belicová et al. (2007) reported the absence of resistance to gentamicin in *E. faecium*, *E. durans* and *E. faecalis* from Bryndza cheese. Finding susceptibility of enterococcal isolates from cheese to vancomycin is advantageous as it is used as a therapeutic alternative (Franz et al., 2001; Belicová et al., 2007). According to the results of the current study, resistance to vancomycin was detected in only two *E. faecalis* strains. This indicates that strains isolated from the Serbian cheeses did not acquire resistance determinants towards vancomycin. Similar results are also reported by Pesavento et al. (2014), who detected a low number of strains (all from fresh soft cheese) resistant to vancomycin. However, Çitak et al. (2004) have found considerably higher values of resistance

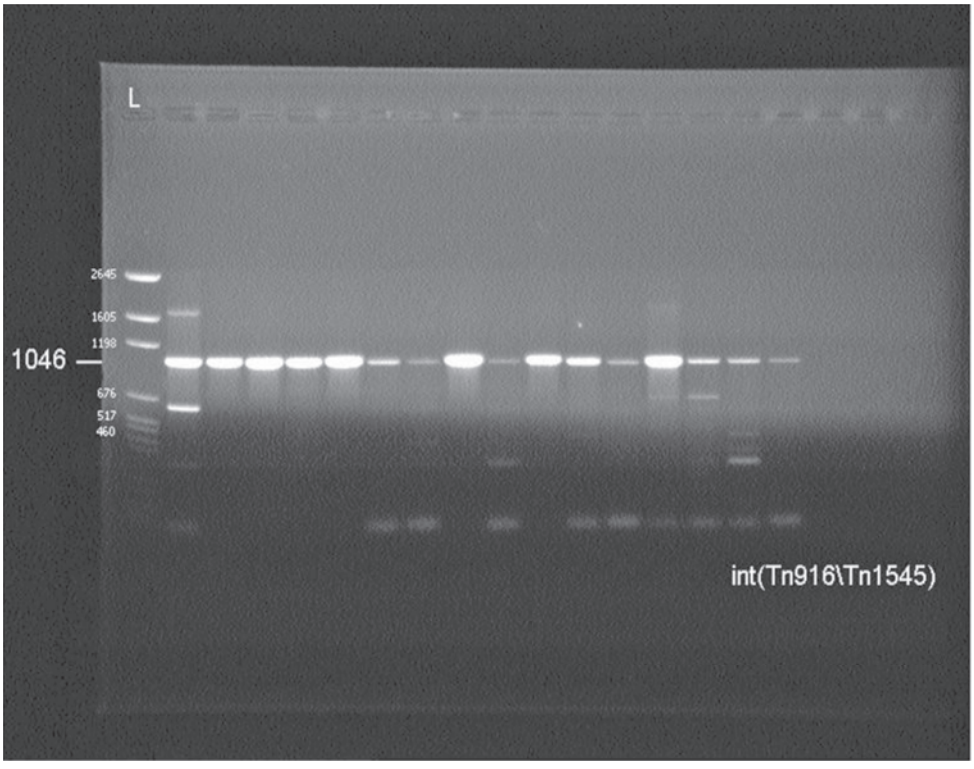

Fig. 2. Agarose gel showing PCR products of the *int* gene in *enterococci* isolates with tetracycline and erythromycin resistance phenotypes (lanes 1-16). The molecular weight marker (MW) was a DNA ladder (BenchTop pGEM DNA; Promega) (lane L).

to vancomycin in strains of *E. faecalis* and *E. faecium* (96.7% and 76%, respectively) isolated from Turkish white cheeses. According to some authors, tetracycline-resistant isolates exhibiting co-resistance to erythromycin is indicative of administration of antibiotics to farm animals

(Šustáčková et al., 2004) and of transferable antibiotic resistance determinants. Considerably high levels of enterococci resistance to tetracycline (55.4%) and erythromycin (93%) were reported by Çitak et al. (2004). On the contrary, only 6% of isolates from Bryndza cheese were resistant to erythromycin, while resistance to tetracycline was not detected (Kročko et al., 2011). In the current investigation, the prevalence of resistance phenotypes to tetracycline and erythromycin was 31.82% and 9.09%, respectively. Resistance only to tetracycline was expressed by two strains of *E. hirae*, one strain of *E. faecium* and one strain of *E. faecalis*.

The results presented in previous studies (Huys et al., 2004) demonstrate that tetracycline resistance in enterococci, mainly originating from European cheeses, is conferred by the *tet* genes [*tet*(M), *tet*(L) and *tet*(S)]. The *tet*(M) gene is often associated with the *Tn*916-*Tn*1545 family of conjugative transposons (Clewell et al., 1995). In this work, the presence of *tet*(M) gene was determined in all seven strains with tetracycline-resistant phenotype (Fig. 1), but none of the analyzed strains harbored *tet*(L). Similar was the case in a study on European cheeses (Huys et al., 2004) where *tet*(M) was the predominant genotype, followed by *tet*(L). In contrast to these results, *tet*(L) was the most commonly detected gene among the tetracycline-resistant enterococci strains (94%), followed by *tet*(M), which occurred in 63% of the strains (Hummel et al., 2007). Determinants of erythromycin resistance include methylases, efflux pumps and inactivating enzymes (Singh et al., 2001). The *erm*(B) gene is considered the most wide-

spread macrolide-resistant gene among enterococci from food (Teuber et al., 1999). It is well known to occur either on conjugative plasmids or on transposons such as Tn*916-1545*, often associated with other antibiotic resistance determinants (Hummel et al., 2007). Results of the current study showed that the *erm*(B) gene was not detected in enterococci characterized by phenotypic resistance to erythromycin, but amplification was achieved in PCR reaction with primers specific for integrase (*int*). In contrast to the present study, the *erm*(B) gene could be detected in 18 of erythromycin-resistant strains (Hummel et al., 2007) and at an incidence of 100% (Khan et al., 2002). Since the DNA of

E. faecium strains reacted with the *msr*A/B gene primers, the *msr*A/B efflux pump was probably responsible for the observed erythromycin resistance in strains that did not contain a detectable *erm*(B) gene (Hummel et al., 2007). All 16 examined strains in the current study were positive for the *int* gene (Fig. 2). The presence of the *int* gene in all tested strains of enterococci indicates that they contain a member of the broad-host range *Tn*916-*Tn*1545 conjugative transposon family. According to Hummel et al. (2007), the integrase gene was observed in 13 of 16 tetracycline-resistant enterococci, nine of which carried both the *tet*(M) and *int* genes, indicating that tetracycline resistance is possibly transferred by transposon. The association of tetracycline resistance and *erm*(B) genes in eight strains containing the Tn*916-1545* integrase gene, indicates that these strains might harbor transposon, by which antibiotic resistance is transferred (Hummel et al., 2007). Another previous study showed that all *erm*(B)-containing strains were positive for the detection of a Tn*916-1545* element, as well as a majority of *tet*(M) containing isolates (Huys et al., 2004).

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