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ORIGINAL ARTICLE

Tetracycline resistance in lactobacilli isolated from Serbian traditional raw milk cheeses

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Abstract The aim of this study was to investigate the presence of tetracycline resistance in lactobacilli isolated from traditional Serbian white brined raw milk cheeses (Homolje, Sjenica, Zlatar). Isolation of presumptive lactobacilli was initially performed using MRS-S agar without tetracycline, or supplemented with 16 and 64 μg/mL of tetracycline. Rep-PCR $(GTG)_5$ genotyping showed a high diversity of the isolates obtained, as examination of 233 isolates resulted in 156 different Rep-PCR fingerprints. Ninety out of 156 (57.69%) of the strains, representatives with different $(GTG)_5$ fingerprints, were identified by MALDI-TOF MS as lactobacilli, while 66 out of 156 (42.31%) strains were identified as members of other LAB genera. All except one out of 90 *Lactobacillus* isolates further tested by microdilution method, demonstrated unimodal distribution of tetracycline MIC values which were equal to or lower from the breakpoint MIC values (EFSA in EFSA J 10: 1–10, 2012. https://doi.org/10.2903/j.efsa.2012.2740). Only one *Lb. paracasei* isolate showed the presence of *tet*(M) gene, while the other analyzed *tet* genes [*tet*(A), *tet*(B), *tet*(C) *tet*(K), *tet*(L), *tet*(O) and *tet*(W)] were not detected in any of the isolates. The results of this study indicates that lactobacilli from traditional Serbian raw milk cheeses do not present considerable tetracycline resistance reservoirs. For final conclusions about the safety of these autochthonous cheeses regarding the possible tetracycline resistance transferability, the assessment of the entire cheese microbiota is needed.

Keywords Antibiotic resistance · Tetracycline · Lactobacilli · Raw milk cheese · MALDI TOF mass spectrometry · Rep-PCR

Introduction

Antimicrobial resistance represents an increasing worldwide problem that the World Health Organization (WHO) recognized as one of the greatest threats to human health (Clementi and Aquilanti 2011). The food chain is considered to be one of the main routes for the transmission of antibiotic resistant bacteria between human and animal population (Witte 1997).

Resistance to antibiotics may be intrinsic; e.g. inherent to bacterial genus or species, or acquired, either through mutations or through transfer of antibiotic resistance genes from other bacteria. Intrinsic and mutational antibiotic resistances have low potential for horizontal spreading in bacterial populations. On the contrary, the risk of horizontal transfer is significantly higher in the case of acquired genes (Levy and Marshall 2004). Commensal microorganisms, including lactic acid bacteria (LAB), may serve as a potential reservoir for the antibiotic resistance genes (Teuber et al. 1999). Although the resistant commensal microorganisms do not present a threat to human health, a horizontal dissemination of resistance genes is more likely

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to occur among commensal and pathogenic microorganisms, than directly from one pathogenic microorganism to another (Andremont et al. 2003).

Bacterial resistance to tetracyclines is very well documented, with more than 40 *tet* genes described, and is often used as a model for the monitoring of antibiotic resistance ecology (Thaker et al. 2010; Roberts and Schwarz 2016). Resistance to tetracyclines is associated with the mobile genetic elements, such as plasmids, transposons, and conjugative transposons, which facilitate transfer of the tetracycline resistance genes among bacteria belonging to a large number of species and genera. Moreover, the prevalence of certain *tet* genes in many Gram-negative and Gram-positive species suggests that the *tet* genes are being exchanged among microorganisms from different environments, including human and animal microbiota (Roberts and Schwarz 2016; Gevers et al. 2003).

White brined cheeses, such as Sjenica, Homolje and Zlatar cheese, traditionally made from raw milk in the mountain regions, are consumed in high amounts in Serbia. The artisanal cheese-making in Serbia is quite simple and similar among the cheese varieties. Immediately after milking, the rennet is added and formation of a curd takes approximately 1–2 h. The curd is afterwards cut into smaller cubes to facilitate the whey separation, or it is transferred into a linen strainer without cutting. The strainer is tied into a knot and hung on wooden hooks to enable the drainage. In the next phase of curd pressing, a wooden board loaded with stone is put at the top of the curd. The curd is then cut, salted, laid in layers into a barrel, and completely overlaid with brine. The cheese ripening usually takes 1–2 months.

Artisanal cheeses contain distinct and typical microbial populations, owing to the use of unpasteurized milk, addition of homemade rennet and brine, and the absence of deliberately added starter cultures (Devirgiliis et al. 2013; Golic et al. 2013). The production process is not standardized and it is open to contamination which also contributes to the wide microbial diversity (Veljovic et al. 2007). Strains belonging to *Lactobacillus* spp. are among the most abundant non-starter lactic acid bacteria (NSLAB) isolated from Serbian traditional raw milk cheeses, where they play an important role (Veljovic et al. 2007; Golic et al. 2013).

The aim of this study was to determine the prevalence and genetic profiles of tetracycline resistance in *Lactobacillus* strains isolated from traditional Serbian cheeses, as a part of safety assessment regarding the potential for dissemination of antibiotic resistance trough the food chain.

Materials and methods

Isolation of bacterial strains from cheese samples

Presumptive *Lactobacillus* spp. were isolated from Serbian traditional raw milk cheeses Homolje (n = 3), Sjenica (n = 12) and Zlatar (n = 16).

Approximately 10 g of cheese samples were diluted in 2% sodium-citrate, homogenized, and plated on three versions of de Man, Rogosa, Sharpe (MRS) agar (Merck, Germany) with 0.14% sorbic acid (MRS-S agar): agar plates without tetracycline, plates supplemented with 16 μg/mL, and plates supplemented with 64 μg/mL of tetracycline (HiMedia, India). Plates were incubated under anaerobic conditions (GenBox, bioMerieux, France) at 30 °C/72 h. Up to five discrete colonies of presumptive lactobacilli were randomly picked from all three versions of countable plates. All Gram positive, catalase negative bacteria were chosen for further investigation.

Rep-PCR analysis

All isolates were genotyped by Rep-PCR fingerprinting method using $(GTG)_5$ primer (Versalovic et al. 1994).The amplification program was 95 °C for 3 min, 35 cycles of 95 °C for 1 min, 40 °C for 1 min and 72 °C for 2 min and 72 °C for 5 min. Gel images were analyzed with software package BioNumerics™ Version 5.1 (Applied Maths, Sint-Martens-Latem, Belgium). Calculation of similarity was based on Dice correlation coefficient, and an average linkage (UPGMA or unweighted pair group method with arithmetic averages).

Species identification

Bacterial isolates were identified using the matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF) mass spectrometry (MS). Tested isolates (n = 156) were inoculated onto blood agar plates and incubated for 24 h at 37 °C under aerobic conditions. One colony per isolate was subjected to MALDI-TOF MS identification (Microflex LT system; Bruker Daltonics, Germany), employing MALDI Biotyper 3.1 software (Bruker Daltonics, Germany). A direct-transfer method was used, i.e. a small amount of bacterial colony was transferred as a thin film directly onto a MSP 96 polished steel BC target, incubated at room temperature for approximately 1 min to dry, overlaid with 1 μl of HCCA matrix solution (α-cyano-4-hydroxycinnamic acid; Bruker Daltonics, Germany) and subjected to MALDI-TOF MS analysis. As proposed by the manufacturer, the criteria for successful identification were confidence scores ≥ 2.0 for species and ≥ 1.7 for genus level.

In the case of unsatisfactory results, generated for some isolates with score cut-off values below 1.7, the extended direct transfer method with 70% formic acid was used.

Tetracycline susceptibility testing and MIC determination

The minimum inhibitory concentration (MIC) of tetracycline was determined by microdilution method according to ISO standard (ISO 10932 2010). Briefly, colonies were picked from the solid media, and suspended in the sterile saline solution to achieve cell density corresponding to McFarland value 1 (corresponding app. 3×10^8 log CFU/mL). Bacterial suspension was diluted 500-times in LAB susceptibility test medium (LSM broth), consisting of 90% ISO Sensitest broth (Oxoid) and 10% MRS broth (Merck, Germany). 55 µL of LSM broth with bacterial suspension was added in the previously prepared microtiter plates, containing 0.125–64 µg/mL tetracycline (HiMedia, India). Microtiter plates were incubated anaerobically at 28 °C/48 h in case of *Lactobacillus brevis* and *Lactobacillus plantarum* and anaerobically at 37 °C/48 h for all the other *Lactobacillus* species.

Minimum inhibitory concentration (MIC) value was defined as the lowest concentration of antibiotic with no visible growth in the microtiter well. Results of the tetracycline susceptibility assessment were interpreted according to the guidelines of European Food Safety Authority (EFSA 2012). Strains were considered resistant if MIC value was > 32 µg/mL for *Lactobacillus plantarum*, > 4 µg/mL for *Lactobacillus paracasei*, and > 8 µg/mL for obligate (*Lactobacillus brevis, Lactobacillus kefiri, Lactobacillus parakefiri, Lactobacillus diolivorans* and *Lactobacillus buchneri*) and facultatively heterofermentative (*Lactobacillus curvatus, Lactobacillus paraplantarum* and *Lactobacillus coryniformis*) lactobacilli.

DNA extraction and PCR detection of the tetracycline resistance genes

Initially, 1 mL of overnight culture was centrifuged at 12,000 rpm/2 min. To improve bacterial lysis, the pellet was suspended in 600 µl of 0.5 mM EDTA solution (pH 8.0) containing lysozyme (1 mg/mL) and mutanolysine (25 U/mL) (Sigma-Aldrich, Germany) and incubated at 37 °C/1 h. The total DNA was further extracted using Wizard Genomic DNA purification kit following manufacturer's instructions (Promega, WI, USA).

The presence of the following *tet* genes was investigated: *tet*(A), *tet*(B), *tet*(C) *tet*(K), *tet*(L), *tet*(M), *tet*(O) and *tet*(W). PCR reaction mixes (total volume 20 µl) contained 0.5 µM of each primer (Invitrogen, Thermo Fisher Scientific, MA, USA), 1 × PCR buffer, 1.5 mM × $MgCl_2$, dNTPs at concentration 200 µM, and 1 U of Taq DNA polymerase (Kappa Biosystems, MA, USA). All amplifications were performed in thermal cycler (Mastercycler gradient 5331, Eppendorf AG, Hamburg, Germany, and Surecycler G8800, Agilent Technologies, Santa Clara, Ca, USA) with following programs: initial denaturation at 95 °C/1 min, 30 cycles of 95 °C for 30 s, T_{an} for 30 s, and elongation at 72 °C for 30 s, with final elongation at 72 °C for 5 min. All primer sequences, annealing temperatures, amplicon sizes and positive controls are indicated in Table 1.

PCR products were separated by electrophoresis on 1% agarose gel and were visualized with Midori green advanced stain (Nippon genetics, Germany).

Results and discussion

In recent years, raw milk cheeses have received increasing attention for their microbial diversity and a source of promising strains with the potential to be used as starter or protective cultures in cheese production. On the other hand, raw milk cheese could also serve as a vehicle for dissemination of antimicrobial resistance and introduction of antibiotic resistance bacteria to humans. In this study, we focused to tetracycline resistance since, according to recent available data, about 15 tons of tetracyclines, as one of the most used antibiotic groups, was sold per year in the period from 2011 to 2014 in Serbia, for veterinary use (Medicines and Medical Devices Agency of Serbia 2014). Extensive use of antibiotics including tetracyclines can generate selective pressure in bacteria, including LAB, leading to acquisition of antimicrobial resistance determinants (Teuber 2001). Furthermore, the correlation between antimicrobial usage and development of resistance has been well documented (Bronzwaer et al. 2002).

Therefore, the first aim of our work was to assess the prevalence of phenotypic tetracycline resistance in lactobacilli as one of the most abundant LAB group in Serbian raw milk cheeses. All cheese samples from this study were initially plated on both MRS-S agar as a selective culture medium designed to favor the growth of lactobacilli, and MRS-S agar supplemented with tetracycline. Plates supplemented with tetracycline in concentrations of 16 µg/mL were used for enumeration and isolation of tetracycline resistant (Tc^r) subpopulations of lactobacilli, since MIC breakpoints for most members of *Lactobacillus* genus are in the range 4–8 µg/mL, as proposed by EFSA (European Food Safety Authority 2012). Since MIC value of 32 µg/mL is proposed as a tetracycline breakpoint for *Lb. plantarum*, the plates supplemented with 64 µg/mL tetracycline were used for the enrichment of the plates with presumptive Tc^r *Lb. plantarum* subpopulation, as well as for

Table 1 PCR reactions conditions for the *tet* genes detection

Gene	Primer sequence	T_{an} (°C)	Amplicon size (bp)	Positive control	Literature source
tet(A)	F: GTAATTCTGAGCACTGT R: CCTGGACAACATTGCTT	55	670	*Escherichia coli* DSM 3876	(Hansen et al. 1996)
tet(B)	F: CAGTGCTGTTGTTGTCATTAA R: GCTTGGAATACTGAGTGTAA	52	500	*E. coli* IM 648	(Roe et al. 1995)
tet(C)	F: AACAATGCGCTCATCGT R: GGAGGCAGACAAGGTAT	58	1138	Plasmid pbR322	(Frech and Schwarz 2000)
tet(K)	F: TTATGGTGGTTGTAGCTAGAAA R: AAAGGGTTAGAAACTCTTGAAA	55	348	*Staphylococcus aureus* subsp. *aureus* DSM 4911	(Zycka-Krzesinska et al. 2015)
tet(L)	F: GTMGTTGCGCGCTATATTCC R: GTGAAMGRWAGCCCACCTAA	55	696	*Enterococcus mundtii* IM 613	(Zycka-Krzesinska et al. 2015)
tet(M)	F: GTTAAATAGTGTTCTTGGAG R: CTAAGATATGGCTCTAACAA	50	750	*Enterococcus faecium* IM 338	(Nawaz et al. 2011)
tet(O)	F: AACTTAGGCATTCTGGCTCAC R: TCCCACTGTTCCATATCGTCA	52	515	*Bifidobacterium adolescentis* IM 677	(Nawaz et al. 2011)
tet(W)	F: GAGAGCCTGCTATATGCCAGC R: GGGCGTATCCACAATGTTAAC	64	168	*Bifidobacterium lactis* subsp. *lactis* LMG 18314	(Aminov and Mackie 2001)

enumeration of lactobacilli subpopulations with higher resistance levels.

The results of the plate counting of bacteria in Zlatar, Sjenica and Homolje cheeses on MRS-S agar without tetracycline or on the same medium supplemented with tetracycline in concentrations of 16 and 64 μg/mL are presented in Figs. 1, 2 and 3. The number of bacteria detected on MRS-S agar without added tetracycline ranged between 6.12 and 8.45 log CFU/g.

Concentration of tetracycline of 16 μg/mL had a moderate influence on the number of CFU in the case of examined Homolje cheese samples. For Zlatar and Sjenica cheese samples, generally, 16 μg/mL of added tetracycline reduced the number of CFU for 1–3 log units. In other words, 0.1–10% of the colonies grown on MRS-S agar (10^4–10^7 CFU/g) were able to grow in the presence of 16 μg/mL of tetracycline. In one sample of Zlatar cheese and two samples of Sjenica cheese, no visible growth (< 100 CFU/g) was observed on tetracycline plates supplemented with 16 μg/mL. The addition of 64 μg/mL of tetracycline into the agar medium resulted in complete inhibition of the growth in 8 samples of cheese (4 Zlatar, 3 Sjenica, 1 Homolje) and partial inhibition in 23 samples. There was, however, up to about 5 log CFU/g resistant bacteria detected in Zlatar and Homolje cheese, and up to 6 log CFU/g in Sjenica cheese.

The colonies (n = 233) were isolated from MRS-S agar plates with 16 μg/mL tetracycline (n = 32), with 64 μg/mL tetracycline (n = 26) or without tetracycline (n = 175) and genotyped by Rep-PCR fingerprinting using $(GTG)_5$ primer. As identification to the species level is of utmost importance in order to accomplish a correct interpretation of the susceptibility testing results, all isolates with unique fingerprints were further identified using MALDI-TOF mass spectrometry. The results of $(GTG)_5$ genotyping showed a high diversity of the isolates obtained, as out of 233 isolates, 156 different $(GTG)_5$ fingerprints were observed. Although the MRS medium is considered as a lactobacilli selective medium, only 90 out of 156 (57.69%) of the strains, representatives with different Rep-PCR fingerprints were actually identified as lactobacilli, while 66 out of 156 (42.31%) strains were identified as members of other LAB genera (*Lactococcus* spp, *Leuconostoc* spp, *Enterococcus* spp, *Pediococcus* spp). Therefore, it should be considered that the results of plate counting of presumptive lactobacilli on three different agar media presented in Figs. 1, 2 and 3 also comprises a certain portion of other LAB. The low selectivity of MRS agar, however, was observed also in several other studies (Devirgiliis et al. 2008; Flórez et al. 2017; Tušar et al. 2014).

Among *Lactobacillus* isolates, the following species were identified: *Lb. plantarum* (n = 37), *Lb. paracasei* (n = 29), *Lb. brevis* (n = 13), *Lb. kefiri* (n = 4), *Lb. curvatus* (n = 3), *Lb. parakefiri* (n = 1), *Lb. paraplantarum* (n = 1), *Lb. coryniformis* (n = 1), *Lb. diolivorans* (n = 1) and *Lb. buchneri* (n = 1). Eighty-seven isolates were identified with confidence score ≥ 2, while 2 *Lb. plantarum* strains were identified with confidence scores 1994 and 1996, and one *Lb. kefiri* isolate with confidence score 1839, implying that the isolation was successful to a genus

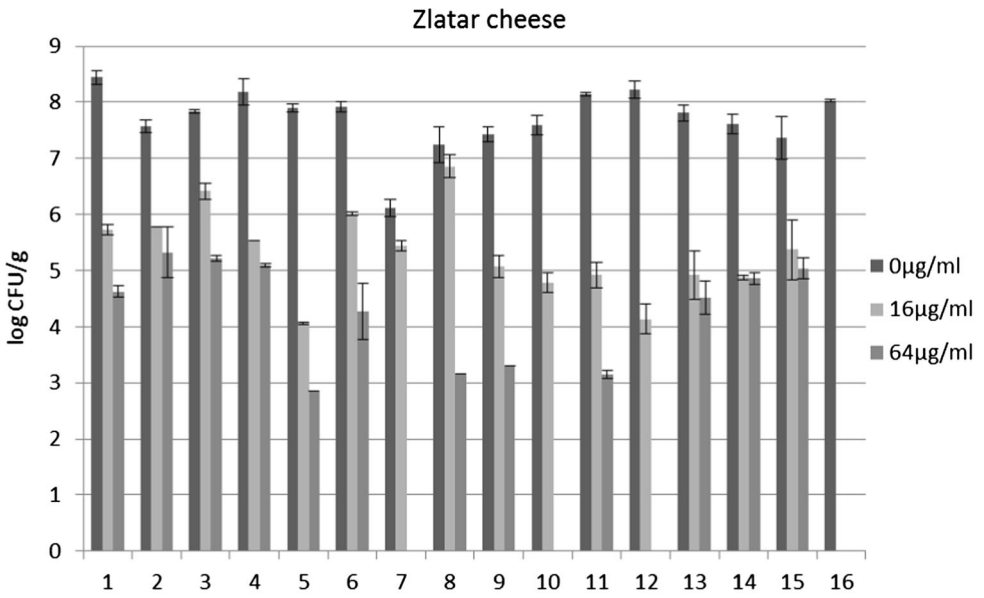

Fig. 1 Zlatar cheese—number of colonies on MRS-S agar without tetracycline and MRS-S agar supplemented with 16 and 64 µg/mL tetracycline

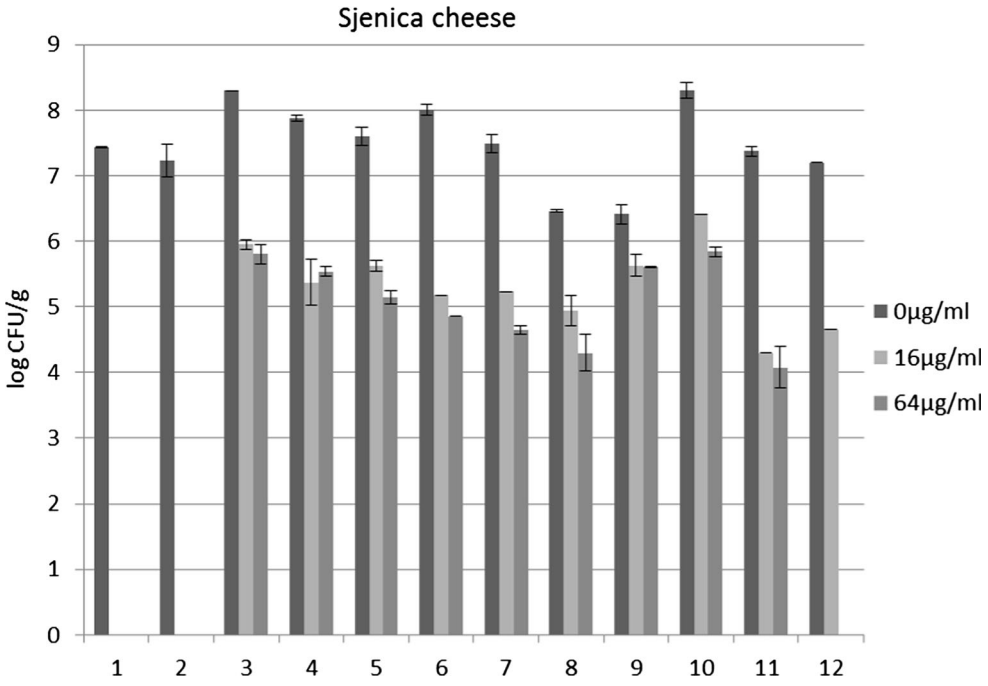

Fig. 2 Sjenica cheese—number of colonies on MRS-S agar without tetracycline and MRS-S agar supplemented with 16 and 64 µg/mL tetracycline

level. Besides PCR based methods and 16S rDNA sequencing which are widely used for the identification of microorganisms isolated from food, MALDI TOF mass spectrometry is also gaining importance in the species identification as a simpler and highly efficient method. Accuracy of MALDI-TOF mass spectrometry for

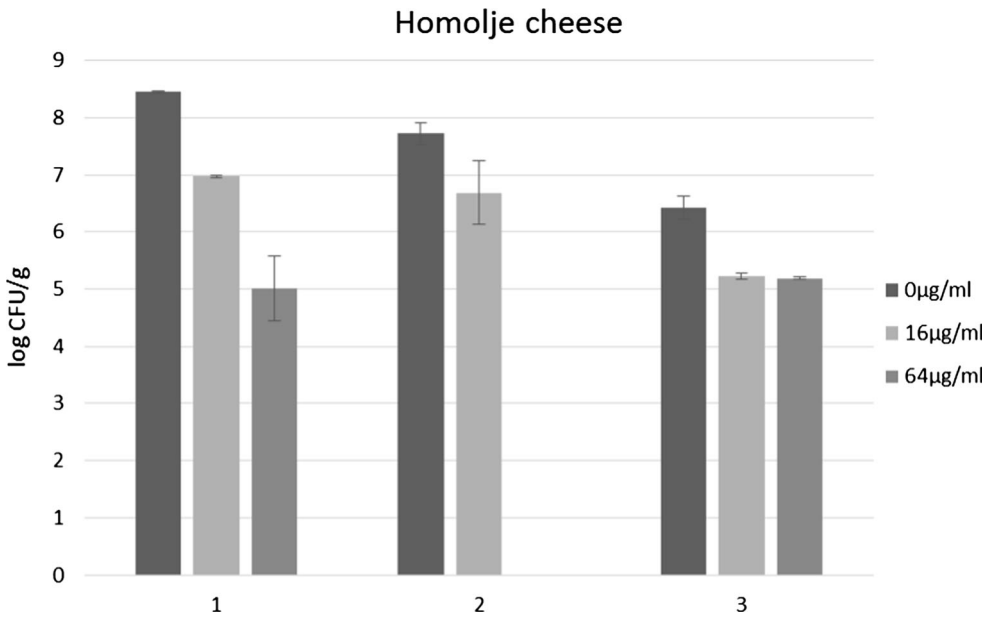

Fig. 3 Homolje cheese—number of colonies on MRS-S agar without tetracycline and MRS-S agar supplemented with 16 and 64 μg/mL tetracycline

identification of food isolates has significantly improved with the upgrade of databases (Pavlovic et al. 2013). Still few literature data is available regarding identification of lactobacilli from food by MALDI-TOF mass spectrometry. The results of the study of Dušková et al. (2012) and Nacef et al. (2017) demonstrated high accuracy of the species identification of *Lactobacillus* strains.

The lactobacilli isolates were subjected to susceptibility testing using the microdilution method.

Minimal inhibitory concentrations (MICs) range and distribution for *Lactobacillus* isolates are shown in the Table 2.

All except one of 90 *Lactobacillus* isolates differing in their Rep-PCR fingerprints demonstrated unimodal distribution of tetracycline MIC values which were equal or lower from the breakpoint MIC values (EFSA 2012). The only strain belonging to *Lb. diolivorans* showed moderate phenotypic resistance to tetracycline, with MIC value of 16 μg/mL.

Fifteen out of these 90 strains were isolated from MRS-S supplemented with tetracycline. Surprisingly, in none of the strains isolated from agar supplemented with tetracycline, the MIC of tetracycline measured by microdilution assay (1-8 μg/mL) was as high as the concentration of tetracycline in the agar used for the primary selection of resistant strains (16 or 64 μg/mL). One *Lb. paracasei* strain that was found to be phenotypically susceptible (MIC 1 μg/mL) was derived from the plate supplemented with 64 μg/mL tetracycline.

Such discrepancy could be assigned to the lower antibacterial activity of tetracycline in the MRS based agar medium (pH = 5.5–5.9) compared to the activity in the liquid medium used in the microdilution assay, i.e. standard ISO Sensitest broth with 10% MRS (pH = 6.7). The differences between phenotypical resistance exhibited by the strains in antibiotic supplemented agar and the real MIC values, were also reported by Zonenschain et al. (2009). The inoculum size, incubation temperature, gaseous atmosphere and incubation period can also affect the results of antimicrobial susceptibility testing (Ammor et al. 2007). Moreover, already Danielsen and Wind (2003) pointed out that little is known about the interactions between MRS agar and antimicrobial agents, on the contrary to Mueller–Hinton and Iso-Sensitest medium.

Resistance to tetracycline in most bacterial species is horizontally acquired, and *tet* genes coding for the resistance are highly mobile, due to their localization on the conjugative transposons (Robert and Schwartz 2016). The most abundant *tet* gene in LAB is *tet*(M) probably due to the association of *tet*(M) with integrative and conjugative transposons, facilitating horizontal transfer (Burrus et al. 2002). Genes *tet*(K), *tet*(L), *tet*(O), *tet*(Q), *tet*(S), *tet*(W), *tet*(Z) and *tet*(36), were also found in lactobacilli (Devirgiliis et al. 2013, Roberts and Schwarz 2016).

In addition to phenotypic determination of tetracycline resistance, all *Lactobacillus* isolates with unique $(GTG)_5$ fingerprints were subjected to detection of tetracycline resistance genes. Only one *Lb. paracasei* isolate showed the presence of *tet*(M) gene, while all the other analyzed *tet* genes, *tet*(A), *tet*(B), *tet*(C) *tet*(K), *tet*(L), *tet*(O) and *tet*(W) were not detected in any of the isolates. *Lb. paracasei* species originates from the gastrointestinal tract of humans and animals, and it can enter the environment and/or contaminate raw material (Comunian et al. 2010).

Table 2 MICs range and distribution in lactobacilli isolates

Lactobacillus spp. species	MIC values distribution (μg/mL)									
	0.125	0.25	0.5	1	2	4	8	16	32	64
Lactobacillus plantarum (32)*					1	5	31			
Lactobacillus paracasei (4)		2	11	14		2				
Lactobacillus brevis (8)						5	8			
Lactobacillus kefiri (8)						3	1			
Lactobacillus curvatus (8)		1	1	1						
Lactobacillus parakefiri (8)							1			
Lactobacillus paraplantarum (8)							1			
Lactobacillus coryniformis (8)							1			
Lactobacillus diolivorans (8)								1		

*EFSA breakpoint MIC values (μg/mL) are indicated in the parenthesis after the species names

Having in mind its origin, it can be presumed that strains of *Lb. paracasei* can easily acquire *tet* genes, since horizontal gene transfer is most likely to take place in the gut environment where microorganisms are abundant and in close contact. Furthermore, *tet*(M) gene is usually located on Tn916 conjugative transposon, which is highly mobile and has many hosts in different environments, either G+ or G− bacterial genera (Clewell et al. 1995). Interestingly, the *Lb. paracasei* strain carrying the *tet*(M) gene, showed no phenotypic resistance and could be classified as phenotypically susceptible (MIC 1 μg/mL). The association of *tet*(M) gene with the variability of measured MIC values was reported also in other studies (Gevers et al. 2003; Flórez et al. 2008). In contrary with these findings, Comunian et al. (2010) showed that the transcription levels of *tet*(M) gene were correlated with the concentration of tetracycline.

None of the analyzed *tet* genes was detected in *Lb. diolivorans* isolate that showed phenotypic resistance. Nevertheless, tetracycline resistance is coded with many more genes (Roberts and Schwarz 2016; Thaker et al. 2010), suggesting that the resistance observed in our isolate is probably associated with *tet* gene other than those analyzed in this study or with chromosomal mutation.

Low prevalence of tetracycline resistance in lactobacilli isolated from Serbian raw milk cheeses is in accordance with the results on lactobacilli of fermented food origin demonstrated in other studies (Ammor et al. 2007; Comunian et al. 2010; Ma et al. 2017). Comunian et al. (2010), however, reported that the prevalence of tetracycline resistance in *Lb. paracasei* isolates from food is tightly connected with the breeding practice. In the areas where the livestock is traditionally pastured and no systematic use of antibiotics is present, the prevalence of antibiotic resistance is relatively low. On the contrary, where an intensive livestock farming takes place, antibiotics are systematically applied, for therapeutic or for growth promoting purposes where this is still allowed, and the prevalence of resistant bacterial strains increases. Furthermore, Flórez and Mayo (2015) pointed out that the wild type LAB strains that don't carry acquired resistance genes reach higher numbers and grow faster in cheese environment than the strains carrying acquired resistance determinants. Serbian raw milk cheeses are produced in mountain regions where livestock is mainly pastured, no intensive farming techniques are applied, and antibiotics are rarely, if ever, used. Therefore, low prevalence of antibiotic resistance that was demonstrated in this study was expected.

However, when the presence of the *tet* genes in the cheese matrix, rather than in bacterial isolates was investigated, the presence of *tet* genes was significantly higher (Flórez et al. 2014; Flórez and Mayo 2015). In the study by Flórez et al. (2014) genes *tet*(S) and *tet*(W) had the highest prevalence, while *tet*(K), *tet*(L), *tet*(M), *tet*(O) were also detected. Only one cheese out of 20 was free from *tet* genes. High prevalence of *tet* genes in cheese matrix indicated that cheeses are burdened with *tet* genes, but these genes could originate from any bacteria, therefore it can't be concluded that the high prevalence of *tet* genes was due to the presence of resistant lactic acid bacteria and that any of the LAB isolates carried the resistance genes.

Conclusion

This is to our knowledge the first study concerning Serbian autochthonous cheeses as a potential vehicle for the spread of tetracycline resistance.

The results of phenotypic testing using microdilution method and genotypic testing based on PCR suggest low prevalence of tetracycline resistance in lactobacilli isolated

from traditional Serbian white brined raw milk cheeses Sjenica, Homolje and Zlatar. Significant number of lactobacilli and other LAB, was able to grow in the MRS agar medium supplemented with tetracycline (16 or 64 μg/mL) in concentrations higher from the breakpoint MICs set for lactobacilli (32 μg/mL for *Lb. plantarum* and 4–8 for other species). The lactobacilli strains derived from the MRS agar with tetracycline, however, were found susceptible by microdilution assay, which can be explained by the effects of media composition and growth parameters on the activity of tetracycline against lactobacilli. The results of the present study indicate that viable lactobacilli from the examined traditional Serbian cheeses do not present considerable reservoir of antibiotic resistance genes. These results can contribute to a growing body of knowledge about antibiotic resistance through the food chain, and may be a part of more in-depth safety evaluation of Serbian raw milk cheeses. For further conclusions about the safety of these autochthonous cheeses regarding possible risks in terms of antibiotic resistance transferability, the entire cheese microbiota should be examined.

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References

Aminov RI, Mackie RI (2001) Molecular ecology of tetracycline resistance: development and validation of primers for detection of tetracycline resistance genes encoding ribosomal protection proteins. Appl Environ Microbiol 67:22–23. https://doi.org/10.1128/AEM.67.1.22

Ammor MS, Flórez AB, Van Hoek AHAM, de Los Reyes-Gavilán CG, Aarts HJ, Margolles A, Mayo B (2007) Molecular characterization of intrinsic and acquired antibiotic resistance in lactic acid bacteria and bifidobacteria. J Mol Microbiol Biotechnol 14:6–15. https://doi.org/10.1159/000106077

Andremont A, How A, Flora C (2003) Commensal flora may play key role in spreading antibiotic resistance. ASM News 69:601–607

Bronzwaer SLAM, Cars O, Buchholz U, Mölstad S, Goettsch W, Veldhuijzen IK, Kool JL, Sprenger MJW, Degener JE (2002) The relationship between antimicrobial use and antimicrobial resistance in Europe. Emerg Infect Dis 8:278–282. https://doi.org/10.3201/eid0803.010192

Burrus V, Pavlovic G, Decaris B, Guédon G (2002) Conjugative transposons: the tip of the iceberg. Mol Microbiol 46:601–610. https://doi.org/10.1046/j.1365-2958.2002.03191.x

Clementi F, Aquilanti L (2011) Recent investigations and updated criteria for the assessment of antibiotic resistance in food lactic acid bacteria. Anaerobe 17:394–398. https://doi.org/10.1016/j.anaerobe.2011.03.021

Clewell DB, Flannagan SE, Jaworski DD, Clewell DB (1995) Unconstrained bacterial promiscuity: the Tn916–Tn1545 family of conjugative transposons. Trends Microbiol 3:229–236. https://doi.org/10.1016/S0966-842X(00)88930-1

Comunian R, Daga E, Dupré I, Paba A, Devirgiliis C, Piccioni V, Perozzi G, Zonenschain D, Rebecchi A, Morelli L, De Lorentiis A, Giraffa G (2010) Susceptibility to tetracycline and erythromycin of *Lactobacillus paracasei* strains isolated from traditional Italian fermented foods. Int J Food Microbiol 138(1–2):151–156. https://doi.org/10.1016/j.ijfoodmicro.2009.11.018

Danielsen M, Wind A (2003) Susceptibility of Lactobacillus spp. to antimicrobial agents. Int J Food Microbiol 82:1–11. https://doi.org/10.1016/S0168-1605(02)00254-4

Devirgiliis C, Caravelli A, Coppola D, Barile S, Perozzi G (2008) Antibiotic resistance and microbial composition along the manufacturing process of Mozzarella di Bufala Campana. Int J Food Microbiol 128:378–384. https://doi.org/10.1016/j.ijfoodmicro.2008.09.021

Devirgiliis C, Zinno P, Perozzi G (2013) Update on antibiotic resistance in foodborne Lactobacillus and Lactococcus species. Front Microbiol 4:1–13. https://doi.org/10.3389/fmicb.2013.00301

Dušková M, Šedo O, Kšicová K, Zdráhal Z, Karpíšková R (2012) Identification of lactobacilli isolated from food by genotypic methods and MALDI-TOF MS. Int J Food Microbiol 159:107–114. https://doi.org/10.1016/j.ijfoodmicro.2012.07.029

European Food Safety Authority (2012) Guidance on the assessment of bacterial susceptibility to antimicrobials of human and veterinary importance. EFSA J 10:1–10. https://doi.org/10.2903/j.efsa.2012.2740

Flórez AB, Mayo B (2015) Diversity and dynamics of antibiotic-resistant bacteria in cheese as determined by PCR denaturing gradient gel electrophoresis. Int J Food Microbiol 214:63–69. https://doi.org/10.1016/j.ijfoodmicro.2015.07.027

Flórez AB, Ammor MS, Mayo B (2008) Identification of tet(M) in two Lactococcus lactis strains isolated from a Spanish traditional starter-free cheese made of raw milk and conjugative transfer of tetracycline resistance to lactococci and enterococci. Int J Food Microbiol 121:189–194. https://doi.org/10.1016/j.ijfoodmicro.2007.11.029

Flórez AB, Alegría A, Rossi F, Delgado S, Felis GE, Torriani S, Mayo B (2014) Molecular identification and quantification of tetracycline and erythromycin resistance genes in Spanish and Italian retail cheeses. Biomed Res Int. https://doi.org/10.1155/2014/746859

Flórez AB, Vázquez L, Mayo B (2017) A functional metagenomic analysis of tetracycline resistance in cheese bacteria. Front Microbiol. https://doi.org/10.3389/fmicb.2017.00907

Frech G, Schwarz S (2000) Molecular analysis of tetracycline resistance in Salmonella enterica subsp. enterica serovars Typhimurium, Enteritidis, Dublin, Choleraesuis, Hadar and Saintpaul: construction and application of specific gene probes. J Appl Microbiol 89:633–641. https://doi.org/10.1046/j.1365-2672.2000.01160.x

Gevers D, Masco L, Baert L, Huys G, Debevere J, Swings J (2003) Prevalence and diversity of tetracycline resistant lactic acid bacteria and their tet genes along the process line of fermented dry sausages. Syst Appl Microbiol 26:277–283. https://doi.org/10.1078/072320203322346137

Golić N, Cadež N, Terzić-Vidojević A, Suranská H, Beganović J, Lozo J, Kos B, Sušković J, Raspor P, Topisirović L (2013) Evaluation of lactic acid bacteria and yeast diversity in traditional white pickled and fresh soft cheeses from the mountain regions of Serbia and lowland regions of Croatia. Int J Food Microbiol 166:294–300. https://doi.org/10.1016/j.ijfoodmicro.2013.05.032

Hansen L, Blanchard P, Hirsh D (1996) Distribution of tet(H) among Pasteurella isolates from the United States and Canada. Antimicrob Agents Chemother 40:1558–1560

International Standardization Organization ISO (2010) 10932: 2010 Milk and milk products determination of the minimal inhibitory concentration (MIC) of antibiotics applicable to bifidobacteria and non-enterococcal lactic acid bacteria (LAB)

Levy SB, Marshall B (2004) Antibacterial resistance worldwide: causes, challenges and responses. Nat Med 10:S122–S129. https://doi.org/10.1038/nm1145

Ma Q, Fu Y, Sun H, Huang Y, Li L, Yu Q, Dinnyes A, Sun Q (2017) Antimicrobial resistance of *Lactobacillus* spp. from fermented foods and human gut. LWT–Food Sci Technol 86:201–208

Medical Devices Agency of Serbia (2014) http://www.alims.gov.rs/ciril/files/2015/12/vet-promet2014.pdf/accessed. Accessed on 03 May 2017

Nacef M, Chevalier M, Chollet S, Drider D, Flahaut C (2017) MALDI-TOF mass spectrometry for the identification of lactic acid bacteria isolated from a French cheese: the maroilles. Int J Food Microbiol 247:2–8

Nawaz M, Wang J, Zhou A, Ma C, Wu X, Moore JE, Millar BC, Xu J (2011) Characterization and transfer of antibiotic resistance in lactic acid bacteria from fermented food products. Curr Microbiol 62:1081–1089. https://doi.org/10.1007/s00284-010-9856-2

Pavlovic M, Huber I, Konrad R, Busch U (2013) Application of MALDI-TOF MS for the Identification of Food Borne Bacteria. Open Microbiol J 7:135–141. https://doi.org/10.2174/1874285801307010135

Roberts MC, Schwarz S (2016) Tetracycline and phenicol resistance genes and mechanisms: importance for agriculture, the environment, and humans. J Environ Qual 45(2):576–592

Roe DE, Braham PH, Weinberg A, Roberts MC (1995) Characterization of tetracycline resistance in *Actinobacillus actinomycetemcomitans*. Oral Microbiol Immunol 10:227–232

Teuber M (2001) Veterinary use and antibiotic resistance. Curr Opin Microbiol 4:493–499. https://doi.org/10.1016/S1369-5274(00)00241-1

Teuber M, Meile L, Schwarz F (1999) Acquired antibiotic resistance in lactic acid bacteria from food. Antonie van Leeuwenhoek, Int J Gen Mol Microbiol 76:115–137. https://doi.org/10.1023/A:1002035622988

Thaker M, Spanogiannopoulos P, Wright GD (2010) The tetracycline resistome. Cell Mol Life Sci 67:419–431. https://doi.org/10.1007/s00018-009-0172-6

Tušar T, Žerdoner K, Bogovič Matijašić B, Paveljšek D, Benedik E, Bratanič B, Fidler N, Rogelj I (2014) Cultivable bacteria from milk from slovenian breastfeeding mothers. Food Technol Biotechnol 52(2):242–247

Veljovic K, Terzic-Vidojevic A, Vukasinovic M, Strahinic I, Begovic J, Lozo J, Ostojic M, Topisirovic L (2007) Preliminary characterization of lactic acid bacteria isolated from Zlatar cheese. J Appl Microbiol 103:2142–2152. https://doi.org/10.1111/j.1365-2672.2007.03450.x

Versalovic J, Schneider M, de Bruijn FJ, Lupski JR (1994) Genomic fingerprint of bacteria using repetitive sequence-based polymerase chain reaction. Methods Mol. Cell. Biol. 5:25–40

Witte W (1997) Impact of antibiotic use in animal feeding on resistance of bacterial pathogens in humans. Cyba Found Symp 207:61–71

Zonenschain D, Rebecchi A, Morelli L (2009) Erythromycin- and tetracycline-resistant lactobacilli in Italian fermented dry sausages. J Appl Microbiol 107:1559–1568. https://doi.org/10.1111/j.1365-2672.2009.04338.x

Zycka-Krzesinska J, Boguslawska J, Aleksandrzak-Piekarczyk T, Jopek J, Bardowski JK (2015) Identification and characterization of tetracycline resistance in Lactococcus lactis isolated from Polish raw milk and fermented artisanal products. Int J Food Microbiol 211:134–141. https://doi.org/10.1016/j.ijfoodmicro.2015.07.009