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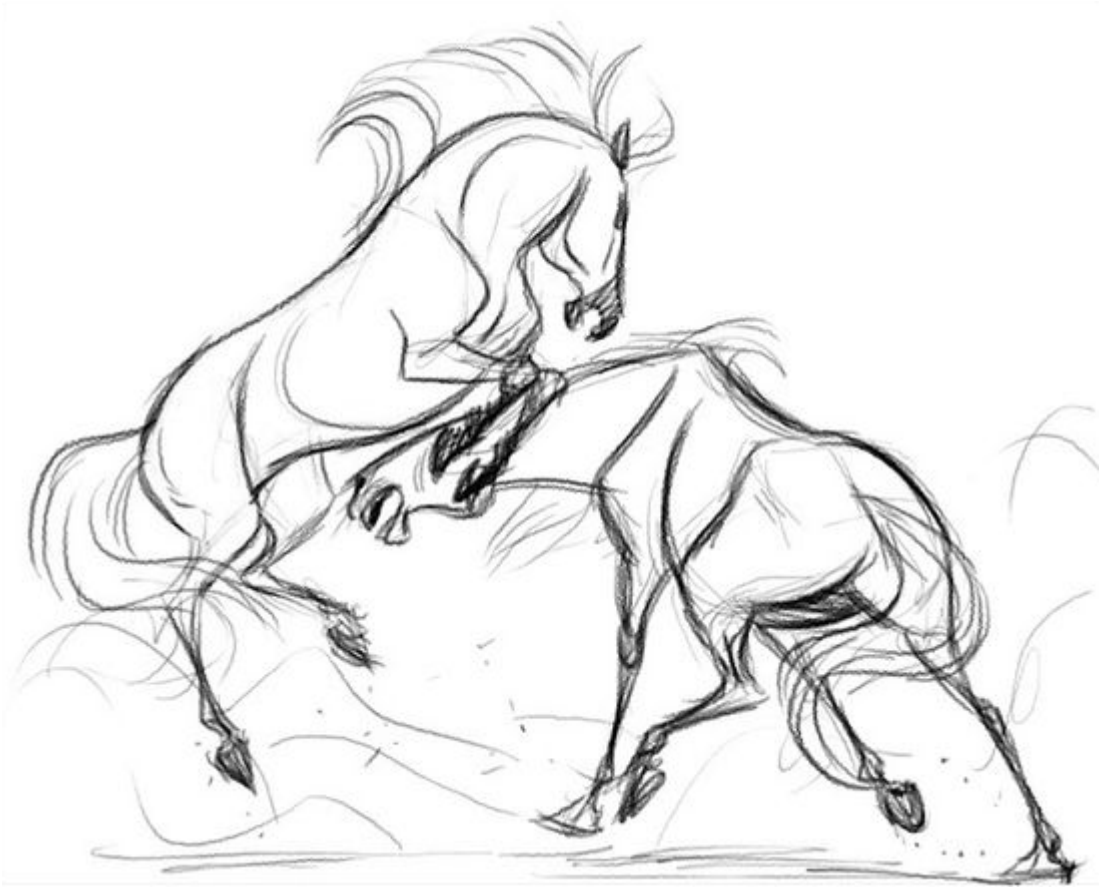

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21000 Novi Sad, Trg D. Obradovića 8
Tel.:++(021) 6350-711; 4853-308;
Fax:++(021) 6350-019
web:http://www.polj.uns.ac.rs
e-mail: stocarstvo@polj.uns.ac.rs

UNIVERSITY OF BELGRADE
FACULTY OF AGRICULTURE
INSTITUTE OF ANIMAL SCIENCE

11080 Zemun-Belgrade, Nemanjina 6
Tel.:++(011) 2615-315; 2197-425;
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web: www.agrif.bg.ac.rs
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ANTIBIOTIC RESISTANCE IN LEUCONOSTOC ISOLATES OF CHEESE ORIGIN

Ledina T.[1], Djordjevic J.[1], Bulajic S.[1]

Abstract: *Leuconostoc* spp. are widely distributed in dairy environments, mostly as adjunct cultures and non-starter lactic acid bacteria. In order to gain Qualified Presumption of Safety status, bacteria must not carry transferable resistance to antibiotics. Although members of lactic acid bacteria are recognized as safe, constant monitoring of antibiotic resistance is recommended by European Food Safety Authority. The aim of this study was to evaluate antibiotic resistance in *Leuconostoc* isolates of cheese origin.

This study was conducted on 20 *Leuconostoc* isolates belonging to two species: *Leuconostoc mesenteroides* (n=18) and *Leuconostoc pseudomesenteroides* (n=2). All investigated isolates originated from Serbian traditional raw milk cheeses – Zlatar, Sjenica and Homolje cheese. Minimum inhibitory concentrations were determined by broth microdilution method for 8 antibiotics and results were interpreted according to European Food Safety Authority guidance. Most prevalent resistance phenotype was resistance to kanamycin (n=15), following with resistance to chloramphenicol (n=6), and streptomycin (n=5). One *Ln. mesenteroides* isolate showed multiresistance, with resistance to 7 out of 8 investigated antibiotics. Genetic basis of tetracycline resistance (*tet*(A), *tet*(B), *tet*(C), *tet*(K), *tet*(L), *tet*(M), *tet*(O) and *tet*(W) genes) was analyzed by PCR method in all isolates. None of the analyzed genes was found in any of the isolates. According to the results of this study *Leuconostoc* isolates are not a reservoir of tetracycline resistance. However, determining genetic basis of antibiotic resistance is necessary for the complete risk assessment regarding transferable antibiotic resistance in the isolates.

Keywords: *Leuconostoc*, antibiotic resistance, lactic acid bacteria

Introduction

Although resistance to antibiotics is probably as old as bacterial populations, overuse and misuse of antibiotics in human and veterinary medicine have put huge selective pressure on bacteria, enabling survival only of the resistant cells (Allen et al., 2010). Bacterial resistance to antibiotics can be intrinsic characteristic of a genus or species, but can also be acquired through horizontal gene transfer and mutations (Mathur and Singh, 2005). While intrinsic and mutational resistance are threat to human health only in pathogenic bacteria, acquired resistance in commensal bacteria is indirect health hazard, as it can be transferred

[1] Ledina Tijana, PhD, teaching assistant; Djordjevic Jasna, PhD, teaching assistant; Bulajic Snezana, PhD, associate professor, University of Belgrade, Faculty of Veterinary Medicine, Belgrade, Serbia.
Corresponding author: Ledina Tijana; email: tijana.ledina@vet.bg.ac.rs

from commensal to pathogenic bacteria (Devirgiliis et al., 2011). In order to fully comprehend antibiotic resistance phenomenon, it is necessary to acknowledge ecological dimension of antibiotic resistance – sources of resistant bacteria and the main routes for the dissemination through different environments (Wang et al., 2006). Food chain is one of the possible routes for the exchange of antibiotic resistant bacteria between human and animal populations, as gene transfer from resistant to susceptible bacteria can happen during processing and production, as well as in gastrointestinal tract of consumers (Rossi et al., 2014). Having in mind their diversity and abundance, commensal bacteria originating from food may play an important role in antibiotic resistance genes transfer (Marshall et al., 2009). *Leuconostoc* species are heterofermentative lactic acid bacteria, which participate in the formation of aroma in many dairy products. Strains of *Leuconostoc* metabolize citrate in milk to produce dyacetil and are associated with the formation of other flavor compounds, such as lactic acid, acetic acid end ethanol (Hemme and Foucaud-Scheunemann, 2004). Together with lactobacilli they constitute non-starter lactic acid bacteria (NSLAB), which are crucial for the characteristic sensory properties of many traditional cheeses (Nieto-Arribas et al., 2010). *Leuconostoc* species are commonly isolated from Serbian traditional cheeses, where they constitute a substantial part of NSLAB (Begovic et al. 2011; Golić et al., 2013).

The aim of this study was to evaluate antibiotic resistance in *Leuconostoc* isolates originating from traditional Serbian cheeses, as part of the isolates' safety assessment.

Materials and methods

Material for this research were 20 *Leuconostoc* spp. isolates belonging to two species: *Leuconostoc mesenteroides* (n=18) and *Leuconostoc pseudomesenteroides* (n=2). All investigated isolates originated from Serbian traditional raw milk cheeses (Homolje, Zlatar and Sjenica cheese) and were previously identified by MALDI (Matrix-Assisted Laser Desorption/Ionization) TOF (Time of Flight) mass spectrometry, as described in our previous study (Ledina et al., 2018).

Antibiotic susceptibility testing

Susceptibility testing was performed following EFSA recommendations (European Food Safety Authority, EFSA, 2012), by determining MIC (Minimum Inhibitory Concentrations) values for following antibiotics: gentamicin (gentamicin sulphate; Fluka™, Honeywell, Germany), kanamycin (kanamycin sulphate; Dr. Ehrenstorfer, Augsburg, Germany), streptomycin (streptomycin sesquisulphate hydrate; Sigma-Aldrich, Germany), tetracycline (tetracycline hydrochloride; HiMedia, India), erythromycin (erythromycin dihydrate; Sigma-Aldrich, Germany), clindamycin (clindamycin hydrochloride, Sigma-Aldrich, Germany), chloramphenicol (chloramphenicol; Sigma-Aldrich, Germany) and ampicillin (ampicillin trihydrate; Dr. Ehrenstorfer, Augsburg, Germany). MIC values were defined as the lowest concentration of antibiotic that completely inhibited growth in the microtiter well after 48h of incubation. Susceptibility testing was performed using broth microdilution method, following procedure described in ISO standard 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)" (International Organization for Standardization,

ISO, 2010). Susceptibility to antibiotics was assessed according to the EFSA guidelines (EFSA, 2012).

DNA extraction and detection of *tet* genes

The extraction of total bacterial DNA was performed using commercial kit (Wizard Genomic DNA purification kit, Promega, WI, USA), following manufacturer's instructions. Prior to the DNA extraction, 1 ml of overnight MRS (de Man, Rogosa Sharpe) broth (Merck, Germany) culture was centrifuged at 12,000 rpm/2 min. The pellet was suspended in 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 in order to improve cell lysis. The presence of following genes associated to tetracycline resistance was investigated in all isolates: *tet*(A), *tet*(B), *tet*(C), *tet*(K), *tet*(L), *tet*(M), *tet*(O) and *tet*(W). Primer sequences were designed and PCR amplification conditions were set as described in Hansen et al. (1996), for *tet*(A) gene; Roe et al. (1995) for *tet*(B) gene, Frech and Scwarz (2000) for *tet*(C) gene, Zycka-Krzesinska et al. (2015) for *tet*(K) and *tet*(L) genes; Nawaz et al. (2011) for *tet*(M) and *tet*(O) genes and Aminov and Mackie (2001) for *tet* (W) gene.

Results and discussion

Lactic acid bacteria have long history of safe use, which granted them QPS (Qualified Pressumption of Safety) status. However, it is highly recommended to test phenotypic resistance in bacteria that are added on purpose to food and, if the phenotypic resistance is present, to analyze genetic basis of the resistance (EFSA, 2012). In this research, following EFSA guidelines, resistance/susceptibility patterns of *Leuconostoc* isolates was determined by broth microdilution method. Distribution of MIC values for *Leuconostoc mesenteroides* isolates is shown in Table 1.

Table 1. Distribution of MIC values for *Leuconostoc* spp.

Ab	Number of isolates with the following MIC value (µg/ml)																	Ecoff (µg/ml)
	0.016	**0.032**	**0.064**	**0.125**	**0.25**	**0.5**	**1**	**2**	**4**	**8**	**16**	**32**	**64**	**128**	**256**	**512**	**1024**	
GEN							1	5	10	2	1		**1**					**16**
KAN									1		4	**7**	**4**	**3**			**1**	**16**
STR											1	8	6	**4**	**1**			**64**
TET						1	12	2	1		1	1	**2**					**8**
ERY				2	13	4				**1**								**1**
CLI		3	13	3							**1**							**1**
CHL									14	**5**			**1**					**4**
AMP				3	13	4												**2**

White area represents concentrations of antibiotic distributed in microtiter plate wells for each antibiotic. Ecoff values (µg/ml), are ecological cut off values (breakpoints) for the

phenotypic resistance, proposed by EFSA (2012). If the isolate had MIC value greater than Ecoff, it was considered resistant to antibiotic. Ab – antibiotic; GEN – gentamicin, KAN – Kanamycin, STR – Streptomycin, TET – Tetracycline, ERY – Erythromycin, CLI – Clindamycin, CHL – Chloramphenicol, AMP – Ampicillin.

Most prevalent phenotypic resistance in *Leuconostoc mesenteroides* was resistance to kanamycin (n=14), following with resistance to chloramphenicol (n=6) and resistance to streptomycin (n=5). Regarding two *Leuconostoc pseudomesenteroides* isolates, one isolate was susceptible to all investigated antibiotics and one isolate was resistant to kanamycin and tetracycline. Resistance to aminoglycoside antibiotics and chloramphenicol were as well reported as most prevalent in dairy *Leuconostoc* strains from the previous studies (Alegría et al., 2015; Flórez et al., 2016). Although resistance to aminoglycosides and chloramphenicol was common in the *Leuconostoc* isolates, MIC values for kanamycin, streptomycin and chloramphenicol were only one to two-fold higher than proposed breakpoints for most isolates. In the research by Flórez et al. (2016) it is suggested that breakpoint values for kanamycin and streptomycin should be reevaluated. However, in order to revise breakpoint values, it is necessary to evaluate MICs in considerable number of strains; and, to our knowledge, antibiotic resistance in dairy *Leuconostoc* has not been extensively investigated to date.

Distribution of MIC values among strains in a bacterial population is one of the indicators if the resistance is intrinsic or acquired. There is a general scientific consensus that bacteria with intrinsic resistance have unimodal (Gaussian) distribution of MIC values, as opposed to bacteria with acquired resistance which have bimodal distribution if MIC values. (Murray et al., 2003). Since the resistant isolates from this research show unimodal distribution of MIC values for kanamycin, streptomycin and chloramphenicol, it can be assumed that the resistance to these antibiotics is intrinsic, and, as such do not pose a risk for the antibiotic resistance genes transfer. However, molecular characterization of antibiotic resistance is necessary in order to conclude if the resistance is intrinsic or acquired.

Resistance to gentamicin, tetracycline, erythromycin and clindamycin was a characteristic of one isolate, which showed multiresistance (resistance to three or more classes of antibiotics), being susceptible only to ampicillin. Multi drug resistant *Leuconostoc* isolates of dairy origin were also reported in other studies (Flórez et al., 2016; Morandi et al., 2013). Tetracycline resistance genes are widely spread in lactic acid bacteria, and can be detected even in phenotypically susceptible strains. Genetic basis of tetracycline resistance (*tet*(A), *tet*(B), *tet*(C), *tet*(K), *tet*(L), *tet*(M), *tet*(O) and *tet*(W) genes) was analyzed by PCR method in all isolates. None of the analyzed genes was found in any of the isolates. Having in mind that more than 40 *tet* genes is described to date (Roberts and Schwarz, 2016), tetracycline resistance in two phenotypically resistant isolates could be coded by the genes that were not analyzed in this study, or could be chromosomally encoded.

Conclusions

Resistance to aminoglycoside antibiotics kanamycin and streptomycin, as well to chloramphenicol was frequent among isolates originating from traditional Serbian cheeses. These resistances can be intrinsic and not transferable, but genetic basis of the resistance

should be further analyzed, in order to exclude risk of gene transfer. According to the results from this study, *Leuconostoc* isolates could not serve as a reservoir for tetracycline resistance.

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References

1. Alegría Á., Delgado S., Flórez A. and Mayo B. 2013. Identification, typing, and functional characterization of *Leuconostoc* spp. strains from traditional, starter-free cheeses. Dairy Science and Technology 93(6), 657-673.
2. Allen H., K. 2014. Antibiotic resistance gene discovery in food-producing animals. Current Opinion in Microbiology 19, 25–29.
3. Aminov R. I., Mackie R. I. 2007. Evolution and ecology of antibiotic resistance genes. FEMS Microbiology Letters 271,147–161.
4. Begovic J., Brandsma J. B., Jovcic B., Tolinacki M., Veljovic K., Meijer W. C. and Topisirovic L. 2011. Analysis of dominant lactic acid bacteria from artisanal raw milk cheeses produced on the mountain Stara planina, Serbia. Archives of Biological Sciences 63(1), 11-20.
5. Devirgiliis C., Barile S. and Perozzi G. 2011. Antibiotic resistance determinants in the interplay between food and gut microbiota. Genes and Nutrition 6, 275–284.
6. European Food Safety Authority, EFSA. 2012. Guidance on the assessment of bacterial susceptibility to antimicrobials of human and veterinary importance. EFSA Journal 10, 1–10.
7. Flórez A.B., Campedelli I., Delgado S., Alegría Á., Salvetti E., Felis G.E., Mayo B. and Torriani S. 2016. Antibiotic susceptibility profiles of dairy *Leuconostoc*, analysis of the genetic basis of atypical resistances and transfer of genes in vitro and in a food matrix. PLoS ONE 11(1), e0145203.
8. 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. Journal of Applied Microbiology 89, 633–641.
9. Golić N., Čadež N., Terzić-Vidojević A., Šuranská H., Beganović J., Lozo J., Kos B., Šušković J., Raspor P. and 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. International Journal of Food Microbiology 166, 294–300.
10. Hansen L., Blanchard P. and Hirsh D. 1996. Distribution of *tet*(H) among *Pasteurella* isolates from the United States and Canada. Antimicrobial Agents Chemotherapy 40, 1558–1560.

11. Hemme D. and Foucaud-Scheunemann C. 2004. *Leuconostoc*, characteristics, use in dairy technology and prospects in functional foods. International Dairy Journal 14, 467–494.
12. Ledina T., Golob M., Djordjević J., Magas V., Čolović S. And Bulajic S. 2018. MALDI-TOF mass spectrometry for the identification of Serbian artisanal cheeses microbiota. Journal of Consumer Protection and Food Safety 13(3), 309-314.
13. Marshall B. M., Ochieng D. J. and Levy S. B. 2009. Commensals: Underappreciated reservoir of antibiotic resistance. Microbe 4(5), 231-238.
14. Mathur S. and Singh R. 2005. Antibiotic resistance in food lactic acid bacteria - A review. International Journal of Food Microbiology 105, 281–295.
15. Morandi S., Cremonesi P., Silvetti T. and Brasca M. 2013. Technological characterisation, antibiotic susceptibility and antimicrobial activity of wild-type *Leuconostoc* strains isolated from North Italian traditional cheeses. Journal of Dairy Research 80, 457-466.
16. Murray P.R., Baron E.J., Jorgensen J.H., Pfaller M.A. and Yolken R.H. 2003. Manual of Clinical Microbiology. Washington, American Society for Microbiology.
17. Nawaz M., Wang J., Zhou A., Ma C., Wu X., Moore J. E., Cherie Millar B. and Xu J. 2011. Characterization and transfer of antibiotic resistance in lactic acid bacteria from fermented food products. Current Microbiology 62, 1081–1089.
18. Nieto-Arribas P., Seseña S., Poveda J. M., Chicón R., Cabezas L. and Palop L. 2011. Enterococcus populations in artisanal Manchego cheese: Biodiversity, technological and safety aspects. Food Microbiology 28, 891–899.
19. nternational Organization for Standardization, ISO. 2010. ISO 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).
20. Roberts M. C. and Schwarz S. 2016. Tetracycline and phenicol resistance genes and mechanisms: importance for agriculture, the environment, and humans. Journal of Environment Quality 45(2), 576-592.
21. Roe D. E., Braham P. H., Weinberg A. and Roberts M. C. 1995. Characterization of tetracycline resistance in *Actinobacillus actinomycetemcomitans*. Oral Microbiology and Immunology 10, 227–232.
22. Rossi F., Rizzotti L., Felis G. E. and Torriani S. 2014. Horizontal gene transfer among microorganisms in food: Current knowledge and future perspectives. Food Microbiology 42, 232–243.
23. Wang H. H., Manuzon M., Lehman M., Wan K., Luo H., Wittum T. E., Yousef A. and Bakaletz L. O. 2006. Food commensal microbes as a potentially important avenue in transmitting antibiotic resistance genes. FEMS Microbiology Letters 254, 226–231.
24. Zycka-Krzesinska J., Boguslawska J., Aleksandrzak-Piekarczyk T., Jopek J. and Bardowski J. K. 2015. Identification and characterization of tetracycline resistance in *Lactococcus lactis* isolated from Polish raw milk and fermented artisanal products. International Journal of Food Microbiology 211, 134–141.