Antibiotic susceptibility and antimicrobial activity of autochthonous starter cultures as safety parameters for fresh cheese production

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Summary

The antibiotic susceptibility and antimicrobial activity, as food safety parameters important for application of autochthonous lactic acid bacteria (LAB), that previously satisfied technological criteria for functional starter cultures in fresh cheese production were examined. Soluble whole cell protein patterns of autochthonous LAB strains from fresh cheese, obtained by SDS-PAGE, revealed the presence of two predominant strains, which were identified as *Lactobacillus fermentum* A8 and *Enterococcus faecium* A7. These strains were not resistant and shown susceptibility to antibiotics: ampicillin, bacitracin, penicillin G, azithromycin, chloramphenicol, clarithromycin, clindamycin, spiramycin, tetracycline, streptomycin, neomycin, gentamicin, erythromycin, rifampicin and novobiocin. *Lb. fermentum* A8 strain displayed phenotypic resistance to vancomycin, but this resistance is intrinsic, not transferable and it is acceptable from the safety aspect. The capacity of *Lb. fermentum* A8 and *Ec. faecium* A7 to inhibit growth of test-microorganisms *Listeria monocytogenes* ATCC 11911, *Escherichia coli* 3014, *Salmonella enterica* serovar Typhimurium FP1 and *Staphylococcus aureus* 3048, was also analysed. According to obtained results, *Lb. fermentum* A8 and *Ec. faecium* A7 are safe from the aspect of spreading antibiotic resistance and could be useful as bioprotective cultures that inhibit common bacterial food contaminants, including *L. monocytogenes*.

Key words: antimicrobial activity, antibiotic susceptibility, autochthonous starter cultures

Introduction

The emergence of antibiotic-resistant bacteria is a worldwide problem primarily caused by the excessive and inappropriate use of antibiotics in human a veterinary medicine and as growth promoters of farm animals. The food chain has been recognized as one of the main routes for the transmission of antibiotic-resistant bacteria between animal and human populations. There are very few systematic studies that investigate the acquired antibiotic resistance in lactic acid bacteria (LAB) of food origin (Šušković, 1996; Džidić et al., 2008; Zdolec et al., 2013). LAB are widely used as starter cultures or as probiotics in fermented dairy products and as such en-

ter animal or human gastrointestinal tract in a high viable cell numbers and there interact with the intestinal microbiota (Bernardeau et al., 2008; Kos et al., 2008). This could be an issue concerning their safety as some of LAB are resistant to antibiotics, but these resistance is often intrinsic and nontransmissible (Salminen et al., 1998). However, some LAB may carry potentially transmissible plasmid-encoded antibiotic resistance genes, as shown for example in certain *Lactobacillus fermentum*, *Lb. plantarum* and *Lb. reuteri* strains (Fons et al., 1997). The transmission of antibiotic resistance genes to unrelated pathogenic or potentially pathogenic bacteria in the gut microbiota is a major health concern, hence should be considered during the selection and characterisation of

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both probiotic and functional starter culture strains (Džidić et al., 2008). In general, strains harbouring antibiotic resistance plasmids are considered unsuitable for application as functional starter cultures and probiotics (Salminen et al., 1998; Saarela et al., 2000). On the other hand, intrinsically antibiotic-resistant probiotic strains may benefit patients whose normal intestinal microbiota has become unbalanced or greatly reduced in numbers due to the administration of antibiotics (Salminen et al., 1998).

The production of many traditional cheeses in different European countries as well as in Croatia relies on spontaneous fermentation by LAB (Rosetti et al., 2009; Leboš Pavunc et al., 2012; Beganović et al., 2013). Fresh cheese, produced from raw milk by activity of autochthonous starter cultures, represents an environment in which LAB originate mainly from milk. The antibiotic resistance of these microorganisms can be affected by antibiotic treatment of the milk-producing animals. Therefore, the objective of the present study was to investigate the antibiotic susceptibility and antibacterial activity against food pathogens, of autochthonous starter cultures *Lactobacillus fermentum* A8 and *Enterococcus faecium* A7, which are important traits from safety aspects. These cultures were previously defined by Leboš Pavunc et al. (2012), from functional and technological aspects, as starter cultures for fresh cheese production. Since dairy products have been frequently reported as contaminated with *Listeria monocytogenes*, antilisterial activity of *Lb. fermentum* A8 and *Ec. faecium* A7, but also antimicrobial activity towards *Salmonella enterica* serovar Typhimurium FP1, *Escherichia coli* 3014 and *Staphylococcus aureus* 3048 in order to assess the potential of these LAB strains to inhibit the growth of the most common pathogens detected in fermented dairy products, was investigated.

Materials and methods

Bacterial strains

All LAB strains (n=15) were isolated and characterised in the Laboratory of Antibiotics, Enzymes, Probiotics and Starter Cultures Technology, Faculty of Food Technology and Biotechnology University of Zagreb. Examined strains were stored at -80 °C in the De Mann Rogosa Sharpe (MRS) broth (Difco, Darmstadt, Germany) with 15 % (v/v) glycerol. Test-microorganisms *L. monocytogenes* ATCC 11911, *S. aureus* 3048, *E. coli* 3014 and *S. enterica* serovar Typhimurium FP1 were stored on nutrient broth (Biolife, Milano, Italy) plus 20 % (v/v) glycerol at -80 °C. All cultures were subcultured twice before use in appropriate medium.

SDS-PAGE of soluble cell proteins and computer-aided comparison of the electrophoretic protein patterns

SDS-PAGE of soluble cell proteins was performed according to Beganović (2008). Protein SDS-PAGE gels were scanned (Scanjet 3800; Hewlett Packard, CA, USA) and stored as TIFF files. Recording of the electrophoretic protein patterns and grouping of the bacterial strains by Unweighted Pair Group Method Using Average Linkage (UPGMA) cluster analysis were performed by using Gel-Compar II (Applied Maths, St-Materns-Latem, Belgium).

Plasmid DNA isolation

The minipreparation of plasmid DNA was performed from 50 ml of the overnight grown *Lb. fermentum* A8 and *Ec. faecium* A7 cultures. Plasmid DNA was extracted using Midi-prep kits (Promega, San Luis Obispo, CA) according to the manufacturer's instructions. Extracted plasmids were separated by horizontal agarose gel electrophoresis (0.8 % agarose gel in 1 Tris-Phosphate-EDTA buffer, 100 V, 2 h). After ethidium bromide staining, DNA was visualised and digitalized images captured under UV light transillumination (MiniBIS Pro, DNR Bio-Imaging Systems Ltd.). For the estimation of the molecular weight of each plasmid, Gene Ruler™ 1kb DNA Ladder Fermentas marker was used to estimate the molecular mass of the plasmids.

Antibiotic disk diffusion tests

For antibiotic disk diffusion tests, overnight grown culture of *Lb. fermentum* A8 and *Ec. faecium* A7, diluted to give viable cell count of approximately 10^8 CFU/mL, was added into 12 mL of melted MRS agar cooled to 50 °C, and then was poured into petri dishes. After the MRS agar plates had been surface dried, susceptibility test disks of each of the

antibiotics were placed aseptically on the MRS agar. The 16 different antibiotics were tested in order to cover all the known chemical and functional groups of antibiotics: ampicillin, bacitracin, penicillin G, vancomycin, azithromycin, clindamycin, chloramphenicol, clarithomycin, erythromycin, gentamicin, streptomycin, spiramycin, tetracycline, neomycin, rifampicin and novobiocin. All antibiotic discs were purchased from Oxoid (Basingstoke, United Kingdom). Agar plates with antibiotic disks were then incubated for 24 h. The diameters of the inhibition zones were measured using a ruler and an average of 3 readings was calculated.

E test

Bacterial suspensions with a turbidity equivalent to McFarland standard 1 were inoculated into MRS agar plates. After drying the surfaces of the plates, the E test strips, (M. I. C. E. Evaluators™, Oxoid Ltd, Baksingstoke, UK) of the antibiotic tested (ampicillin, bacitracin, benzyl penicillin, clindamycin, chloramphenicol, erythromycin, tetracycline, rifampicin and vancomycin) were applied. The plates were incubated under the same condition as for the antibiotic disk diffusion tests. The minimal inhibitory concentration (MIC) is the lowest antibiotic concentration that inhibits the visible bacterial growth after overnight incubation at optimal growth temperature of the certain strain. MICs were read directly from the test strip according to the manufacturer´s instructions and were expressed as micrograms per millilitre.

Antibacterial activity of functional starter cultures

Three different methods were used in order to investigate an antibacterial activity of *Lb. fermentum* A8 and *Ec. faecium* A7. Overnight cultures (5 µL) of *Lb. fermentum* A8 and *Ec. faecium* A7 were spotted onto the surface of MRS agar (1.2 % w/v) plates, which were then incubated at 37 °C for 24 h. Test-microorganisms were inoculated into soft agar medium (nutrient broth containing 0.7 % w/v agar) to a final concentration of approximately 10^7 CFU/mL. The soft media were poured on the plates which were incubated for 24 h at the optimal growth for indicator strains. Inhibition was scored positive in the presence of a detectable clear zone around the colony of the producer strain. The ratio of the inhibition diameter (ID) to the spot culture diameter (CD) was calculated for each assay, to determine the effective inhibition ratio (EIR): ((ID-CD)/CD). *Lb. fermentum* A8 and *Ec. faecium* A7 were further tested for their antimicrobial activity against *L. monocytogenes* ATCC 19111, *S.* Typhimurium FP1, *E. coli* 3014 and *S. aureus* 3048 using the well-diffusion method described by Kos et al. (2008) and by turbidimetric method described by Beganović et al. (2011).

Results and discussion

Autochthonous LAB strains SDS-PAGE protein analysis and plasmid DNA isolation

In order to define which LAB genus is predominant in the autochthonous fresh cheese bacterial population, SDS-PAGE analysis of the soluble cell proteins was performed from the selected MRS colonies and their protein profiles were compared. Results obtained by SDS-PAGE of soluble bacterial cell proteins, indicated that these were actually only two different bacterial strains, i.e. eleven cocci-shaped isolates showed identical whole-cell protein profiles and four rod-shaped isolates showed identical whole-cell protein profiles, respectively (Figure 1). This implies that lactobacilli, but also enterococci represent a significant proportion of the Croatian fresh cheese microbiota, being in accordance with Giraffa (2003) and Bernadeau et al. (2008) who reviewed that traditionally produced cheeses originating from Southern and Eastern European regions contain high percentage of *Lactobacillus* and *Enterococcus* strains among total autochthonous cheese microbiota. Accurate taxonomic identification of the microorganism intended for application as functional starter culture is essential for its safety assessment. Hence two representatives of autochthonous strains were identified by DNA fingerprinting using AFLP™ as *Lactobacillus fermentum* A8 and *Enterococcus faecium* A7, respectively (Leboš Pavunc et al., 2012).

Before the application of functional starter culture, especially those from *Enterococcus* genera, in the food or feed, the presence of transferable resistances genes must be excluded, especially if these genes are present on the plasmids which often serve as vehicles to shuttle DNA between bacteria (D´Aimmo et al., 2007; Bernardeau et al., 2008). Hence, in this study *Lb. fermentum* A8 and *Ec.*
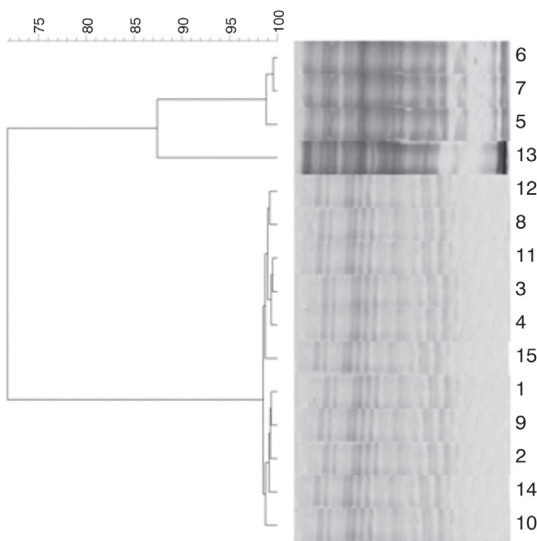
Figure 1. Dendrogram based upon fingerprints obtained by SDS-PAGE of soluble bacterial cell proteins of 15 autochthonous lactic acid bacteria isolates (lines indicate autochthonous LAB isolates: 6,7,5,13-*Lb. fermentum*; the other lines- *Ec. faecium*). Clustering was performed by UPGMA
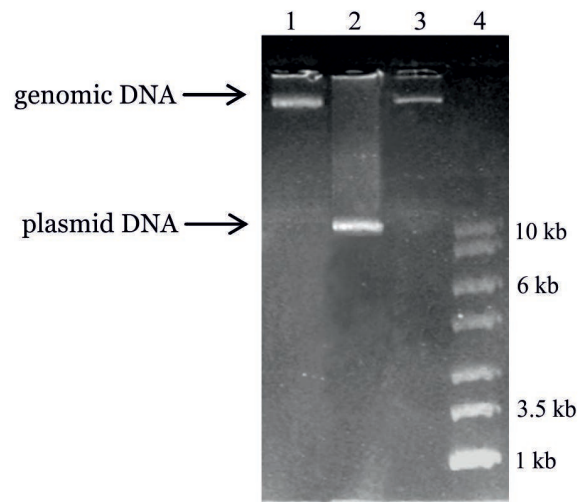

Figure 2. Agarose gel electrophoresis showing plasmid profiles 1 *Ec. faecium* A7; 2 *Lb. fermentum* A8; 3 *Lactococcus lactis* LMG 9450; 4 Gene Ruler™ DNA Ladder Fermentas marker

faecium A7 were tested for the presence of plasmids to determine whether these strains might carry any plasmid-encoded antibiotic resistance genes (Figure 2). Plasmid DNA analysis showed that strain *Lb. fermentum* A8 possess a plasmid of approximately 10.0 kb, while no plasmid was detected in case of *Ec. faecium* A7 (Figure 2). Gfeller et al. (2003) reported that *Lb. fermentum* ROT1, isolated from a raw milk cheese, has 19.3-kb plasmid pLME300 with two antibiotic-resistance determinants *vat*(E) for streptogramin A and *erm*(LF) for erythromycin. Therefore, further antibiotic susceptibility was investigated to examine whether these strains maybe have potential plasmids which may carry antibiotic resistance genes.

Antibiotic susceptibility of Lb. fermentum A8 and Ec. faecium A7

Antibiotic susceptibilities of *Lb. fermentum* A8 and *Ec. faecium* A7 were tested against antibiotics chosen in order to provide a diverse representation of antimicrobial agent classes. According EFSA (2008), as a basic requirement, the minimal inhibitory concentration (MIC) of the antibiotics expressed as mg/L or µg/mL should be determined.

The antibiotics used in this study were chosen in order to maximise the probability to identify possibly present resistance genotypes to the most commonly used antibiotics by assessing the resistance phenotypes of *Lb. fermentum* A8 and *Ec. faecium* A7 in order to cover main chemical and functional groups of antibiotics: ampicillin, bacitracin, penicillin G, vancomycin, azithromycin, chloramphenicol, clarithromycin, clindamycin, erythromycin, gentamicin, streptomycin, spiramycin, tetracycline, neomycin, rifampicin and novobiocin, with concentrations indicated at Table 1. The appearance of inhibition zone is taken as criterion for the antibiotic susceptibility. *Lb. fermentum* A8 and *Ec. faecium* A7 were susceptible to all tested antibiotics, with exception of the absence of *Lb. fermentum* A8 susceptibility to vancomycin (Table 1). Examined strains were shown to be less sensitive to aminoglycoside antibiotics gentamicin, streptomycin and neomycin, what could be due to the MRS medium's low pH because of lactic acid production. Namely, the activity of aminoglycosides is markedly reduced at acidic pH values (Klare et al., 2007; Leboš Pavunc et al., 2011).

Table 1. Antibiotic susceptibility of functional starter cultures *Ec. faecium* A7 and *Lb. fermentum* A8 for fresh cheese production

Antibiotics	μg/disk	*Ec. faecium* A7	*Lb. fermentum* A8
		Inhibition zones (mm)	
Ampicillin(AMP)	2	18±4	26±1
Bacitracin(B)	10	13±0	13±1
Penicillin G(P)	1	21±1	27±3
Vancomycin(VA)	30	11±1	0
Azithromycin(AZM)	15	11±2	17±1
Chloramphenicol(C)	30	19±1	25±1
Clarithromycin(CLR)	5	18±1	23±2
Clindamycin(DA)	2	17±2	25±0
Erythromycin (E)	15	16±1	21±1
Gentamicin (CN)	10	8±0.5	10±0.5
Spiramycin(SP)	100	17±1	19±1
Streptomycin (S)	10	11±1	10±0.5
Tetracycline(TE)	10	12±1	18±2
Neomycin (N)	10	9±0	10±3
Rifampicin (RD)	5	16±1	25±2
Novobiocin(NV)	5	13±1	13±2

Lb. fermentum A8 strain displayed phenotypic resistance to vancomycin assayed by antibiotic disk diffusion tests and E-test. Among antibiotic resistances, vancomycin resistance is of the major concern because vancomycin is one of the last antibiotics broadly efficacious against clinical infections caused by multidrug-resistant pathogens (Zhou et al., 2005). Coming back to detected vancomycin resistance of *Lb. fermentum* A8, it should be stated that some LAB however, including strains of *Lactobacillus* sp., *Pediococcus* sp. and *Leuconostoc* sp., are resistant to vancomycin. Such resistance is intrinsic, meaning that is chromosomally encoded and not transmissible (Zhou et al., 2005). This intrinsic resistance to vancomycin is explained by the presence of D-alanine:D-alanine ligase related enzymes (Bernardeau et al., 2008). Vancomycin inhibits the peptidoglycan biosynthesis by binding to muramylpentapeptides that terminate in D-alanyl-D-alanine (D-Ala-D-Ala) and blocks the addition of these precursors into the peptidoglycan backbone. Many *Lactobacillus* strains incorporate D-alanyl-D-lactate (D-Ala-D-Lac) into their muramylpentapeptides by aid of D-Ala-D-Ala ligase (Ddl). This is the basis for their vancomycin resistance as peptides that terminate in D-Ala-D-Lac

are bound 1000-fold less efficiently by vancomycin compared to peptides terminating in D-Ala-D-Ala (van Pijkeren and Britton, 2012).

While the transfer of vancomycin resistance from *Lactobacillus* sp. to other bacteria has not been observed, in many *Enterococcus* sp. vancomycin resistance is often plasmid encoded and transmissible. Several investigations showed the occurrence of vancomycin resistant enterococci (VRE) isolated from food or of animal origin, hence the most important factor during the selection of enterococci, as potential functional starter cultures, is to test their resistance against glycopeptides like vancomycin. Antibiotic susceptibility results obtained for strain *Ec. faecium* A7 are in accordance with Lopes et al. (2006) who demonstrated that bacteria from *Enterococcus* genus, isolated from autochthonous dairy products, were intrinsically susceptible to the majority of the antibiotics used to cope with common infections, namely, penicillins, aminoglycosides, macrolides and vancomycin. Moreover, literature concerning antibiotic resistance in enterococci is limited to clinical enterococci and confuses general characteristics of the genus *Enterococcus* including strains from fermented food origin, with characteristics of clinical enterococcal isolates.

Table 2. Minimum inhibitory concentration (MIC) of different antibiotics determined for *Ec. faecium* A7 and *Lb. fermentum* A8, respectively

Antibiotics	*Ec. faecium* A7	*Lb. fermentum* A8
	MIC (µg/mL)	
Ampicillin	0.03	0.06
Bacitracin	1.50	3.00
Benzyl penicillin	0.032	0.064
Vancomycin	8.00	0
Chloramphenicol	1.00	1.00
Clindamycin	0.064	0.094
Erythromycin	1.00	0.75
Tetracycline	2.00	1.50
Rifampicin	0.064	0.094

Table 2 summarises the distribution of MICs to several antibiotics which mode of action is based on inhibiting the protein synthesis, cell wall synthesis or DNA synthesis, determined by E-test assay. According to the results both strains were susceptible to the nine different antibiotics applied in E-test. Although MIC determined for vancomycin in case of *Ec. faecium* A7 is slightly higher than demanded according to EFSA (2008) guidelines, it should be emphasised that this value is much lower than in *Enterococcus* strains which possess vanA or vanB transferable vancomycin resistance with MIC values >64 µg/mL and up to 1000 µg/mL, respectively. Some enterococci may possess intrinsic, but not transferable, resistance against vancomycin, coded by vanC (MIC 2-32 µg/L) (Sahlström et al., 2009). Obtained results are in agreement with investigations of Rossetti et al. (2009) who studied the antibiotic susceptibility of dominant LAB associated with Grana Padano cheese whey starters. As stated by EFSA, microbiological breakpoint values for vancomycin are not required for *Lb. fermentum* since obligate and facultatively heterofermentative lactobacilli are known to be intrinsically resistant to glycopeptide antibiotics such as vancomycin (Danielsen and Wind, 2003; EFSA, 2008; Rossetti et al., 2009).

Antibacterial activity of Lb. fermentum A8 and Ec. faecium A7

When proved safe from the aspect of antibiotic resistance, another important feature of autochthonous LAB, isolated from artisanal fermented dairy products, is a potential to improve the microbiological safety of food, because these strains are well adapted to the environmental conditions of the milk as substrate and their antimicrobial activity is resulting at least from the lactic acid production (Šušković et al., 2010; Kos et al., 2011). Due to the availability of nutrients in milk, fresh raw milk cheeses may host different pathogenic microorganisms. Levels of *L. monocytogenes*, as high as 10^7 CFU/g, have been found in some naturally contaminated cheeses (Settani et al., 2011). Besides, *L. monocytogenes*, *E. coli* is one of the foodborne pathogens of concern for the dairy industry. Raw milk is often recognised as matrix for the transmission of this pathogen, and milk and different dairy products, including cheeses, have been implicated in *E. coli* O157:H7 outbreaks (Rodrigues et al., 2005). Settani et al. (2011) reported that besides these two mentioned pathogens, according to European Commission Regulation, the presence of the two main other pathogens *Staphylococcus aureus* and *Salmonella* must be checked to assess microbiological safety of the cheese. Hence, in this study antimicrobial activity of *Lb. fermentum* A8 and *Ec. faecium* A7 against these four food pathogens was investigated. The cell-free supernatants from these strains were sterilized by filtration, and tested by well-diffusion assay against *S.* Typhimurium FP1, *E. coli* 3014, *L. monocytogenes* ATCC 11911 and *S. aureus* 3048 (Table 3).

Apart from the best inhibitory activity against *L. monocytogenes* ATCC 11911, culture supernatants also inhibited *S.* Typhimurium FP1, *E. coli* 3014 and *S. aureus* 3048. These results are in accordance with the results of Kos et al. (2008) who demonstrated the antagonistic activity of probiotic strain *Lb. helveticus* M92, isolated from dairy product, against *S.* Typhimurium FP1, *E. coli* 3014, *L. monocytogenes* and *S. aureus* 3048 by *in vitro* competition test. The growth inhibition of four different pathogens by *Lb. fermentum* A8 and *Ec. faecium* A7 cell free supernatants, analysed by turbidimetric method, is presented in Figure 3.

Table 3. Antagonistic activity of *Ec. faecium* A7 i *Lb. fermentum* A8 against *E. coli* 3014, *S.* Typhimurium FP1, *L. monocytogenes* ATCC 11911 and *S. aureus* 3048, tested by a) agar-well diffusion assay and b) agar spot test

a)

Test-microorganisms	Inhibition zones obtained with culture supernatants (mm)	
	Ec. faecium A7	*Lb. fermentum* A8
E. coli 3014	17.3	12.0
S. Typhimurium FP1	20.7	15.0
L. monocytogenes ATCC 11911	25.0	18.0
S. aureus 3048	19.0	16.3

b)

| Test-microorganisms	EIR*	
	Ec. faecium A7	*Lb. fermentum* A8
E. coli 3014	0.384	0.333
S. Typhimurium FP1	0.600	0.466
L. monocytogenes ATCC 11911	0.787	0.680
S. aureus 3048	0.555	0.504

*Effective inhibition ratio achieved by culture supernatants

Figure 3. Antibacterial activity of cell-free supernatants (25 %, 50 %, 75 %, 100 % dilution in nutrient broth) from strains: a) *Ec. faecium* A7, b) *Lb. fermentum* A8, against *E. coli* 3014, *S.* Typhimurium FP1, *L. monocytogenes* ATCC 11911 and *S. aureus* 3048 assayed by turbidimetric method, after 24 h of inhibition
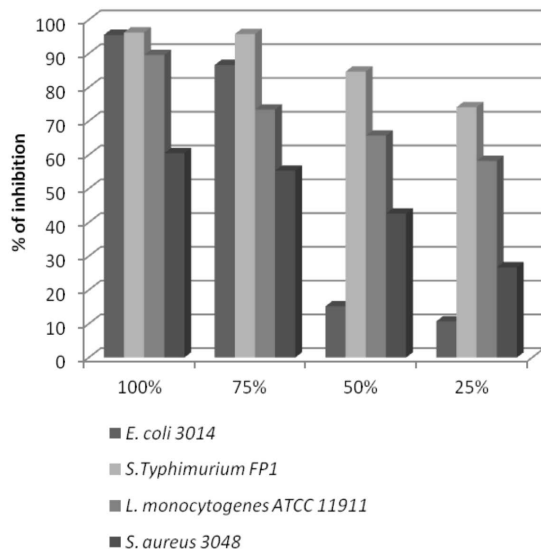
a)   b)
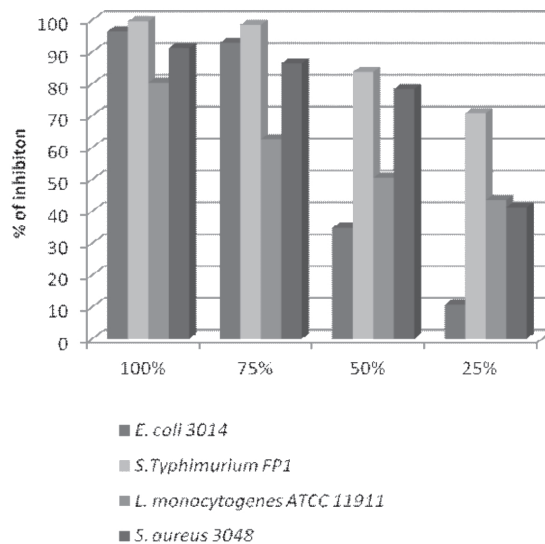
According to the results, *Lb. fermentum* A8 and *Ec. faecium* A7 reduced growth of the 4 examined strains within 24 h, although differences in the degree of inhibition were observed among the strains. Cell-free supernatants (100 %) significantly reduced pathogen levels. However, at lower dilutions of *Lb. fermentum* A8 and *Ec. faecium* A7 cell-free supernatants (75 %; 50 % and 25 %) inhibitory effects decreased although were still strong (Figure 3 a-b). The antimicrobial ability attributed to many LAB against food and clinical pathogens is primarily due to the production of organic acids, but also to other antimicrobial metabolites such as bacteriocins. Previously Leboš Pavunc et al. (2012) reported that *Lb. fermentum* A8 and *Ec. faecium* A7 produce high concentrations of lactic acid after overnight growth, 8.74 gL^{-1} and 7.94 gL^{-1}, respectively. Alakomi et al., (2000) studied the antibacterial effect of lactic acid against *Escherichia coli* O157:H7, *Pseudomonas aeruginosa*, and *Salmonella enterica* serovar Typhimurium and reported that is largely, but not totally, assigned to its ability in the undissociated form to penetrate the cytoplasmic membrane, resulting in reduced intracellular pH and disruption of the transmembrane proton motive force. Additionally high concentrations of lactic acid may act as a potentiator of the effects of other antimicrobial substances produced by LAB such as bacteriocins. Our results suggest that these strains, previously developed as functional starter culture, could offer an advantage due to their antimicrobial activity towards common food pathogens, during fresh cheese production.

Conclusions

Strains *Lb. fermentum* A8 and *Ec. faecium* A7, originally isolated from autochthonous Croatian fresh cheeses, were sensitive to all clinically effective antibiotics applied in this study, with exception of the absence of *Lb. fermentum* A8 susceptibility to vancomycin, which is referred to the specific structure of cell wall peptidoglycan, which is intrinsic and not transferable resistance. Instability of aminoglycoside antibiotics, gentamicin, streptomycin and neomycin at lower pH values is a reason of obtained lower sensitivity of examined strains to those antibiotics. *Lb. fermentum* A8 reveals a presence of the plasmid which is approximately of 10.0 kb in size, but its role is yet to be determined. As *Lb. fermentum* A8 and

Ec. faecium A7 are previously defined as functional starter cultures from the technological point of view, exhibited a strong inhibitory activity against food pathogens and especially against *L. monocytogenes*, they may be useful in controlling the growth of these pathogens during production of fresh cheese.

Osjetljivost na antibiotike i antimikrobna aktivnost autohtonih starter kultura kao parametri sigurnosti njihove primjene u proizvodnji svježeg sira

Sažetak

U ovom radu istražena je osjetljivost na antibiotike i antimikrobna aktivnost odabranih autohtonih sojeva bakterija mliječne kiseline, koji su u prethodnim istraživanjima zadovoljili tehnološke kriterije za funkcionalne starter kulture namijenjene proizvodnji svježeg sira. Prema profilu topljivih staničnih proteina, dobivenom SDS-PAGE metodom, ustanovljeno je da u tradicionalno proizvedenom svježem siru prevladavaju dvije vrste bakterija mliječne kiseline, identificirane kao *Lactobacillus fermentum* A8 i *Enterococcus faecium* A7. Ovi bakterijski sojevi nisu bili rezistentni, nego su pokazali osjetljivost na testirane antibiotike: ampicilin, bacitracin, penicillin G, azitromicin, kloramfenikol, klaritromicin, klindamicin, spiramicin, tetraciklin, streptomicin, neomicin, gentamicin, eritromicin, rifampicin i novobiocin. Bakterijski soj *Lb. fermentum* A8 posjeduje fenotipsku rezistenciju na vankomicin, ali ta je rezistencija uređena, nije prenosiva na druge bakterije i stoga je prihvatljiva s aspekta sigurnosti njegove primjene kao starter kulture. Ispitano je i antimikrobno djelovanje kultura *Lb. fermentum* A8 and *Ec. faecium* A7 prema test-mikroorganizmima: *Listeria monocytogenes* ATCC 11911, *Escherichia coli* 3014, *Salmonella enterica* serovar Typhimurium FP1 i *Staphylococcus aureus* 3048. Prema dobivenim rezultatima, *Lb. fermentum* A8 i *Ec. faecium* A7 su sigurni s aspekta ši-

renja antibiotičke rezistencije i mogu biti korisne kao zaštitne kulture koje inhibiraju bakterije, najčešće kontaminante hrane, uključujući i *L. monocytogenes.*

Ključne riječi: antimikrobna aktivnost, antibiotička rezistencija, autohtone starter kulture

References

1. Alakomi, H.L., Skyttä, E., Saarela, M., Mattila-Sandholm, T., Latva-Kala, K., Helander, I.M. (2000): Lactic Acid Permeabilizes Gram-Negative Bacteria by Disrupting the Outer Membrane. *Applied Environmental Microbiology 66 (5)*, 2001-2005.

2. Beganović, J., Pavunc, A.L., Gjuračić, K., Špoljarec, M., Šušković, J., Kos, B. (2011): Improved sauerkraut production with probiotic strain *Lactobacillus plantarum* L4 and *Leuconostoc mesenteroides* LMG 7954. *Journal of Food Science 76 (2)*, M124-M129.

3. Beganović, J., Kos, B., Leboš Pavunc, A., Uroić, K., Džidara, P., Šušković J. (2013): Proteolytic activity of probiotic strain *Lactobacillus helveticus* M92. *Anaerobe, section Molecular biology, genetics and biotechnology 20*, 58-64.

4. Beganović, J. (2008): Dissertation, Application of proteomics and other molecular methods in characterisation of functionality of the probiotic bacteria, Faculty of Food Technology and Biotechnology, University of Zagreb.

5. Bernardeau, M., Vernoux, J., Henri-Dubernet, P., Guéguen, S.M. (2008): Safety assessment of dairy microorganisms: The *Lactobacillus* genus. *International Journal of Food Microbiology 126*, 278-285.

6. D`Aimmo, M.R., Modesto, M., Biavatto, B. (2007): Antibiotic resistance of lactic acid bacteria and *Bifidobacterium* spp. isolated from dairy and pharmaceutical products. *International Journal of Food Microbiology 115*, 35-42.

7. Danielsen, M., Wind, A. (2003): Susceptibility of *Lactobacillus* spp. to antimicrobial agents. *International Journal of Food Microbiology 82(1)*, 1-11.

8. Džidić, S., Šušković, J., Kos, B. (2008): Antibiotic Resistance Mechanisms in Bacteria: Biochemical and Genetic Aspects. *Food Technology and Biotechnology 46 (1)*, 11-21.

9. European Food Safety Authority (2008): Update of the criteria used in the assessment of bacterial resistance to antibiotics of human or veterinary importance. *The EFSA Journal. 732*, 1-15.

10. Fons, M., Hege, T., Ladire, M., Raibaud, P., Ducluzeau, R., Maguin, E. (1997): Isolation and characterization of a plasmid from *Lactobacillus fermentum* conferring erythromycin resistance. *Plasmid 37*, 199-203.

11. Gfeller, K.Y., Roth, M., Meile, L., Teuber, M. (2003): Sequence and genetic organization of the 19.3-kb erythromycin- and dalfopristin-resistance plasmid pLME300 from *Lactobacillus fermentum* ROT1. *Plasmid 50*, 190-201.

12. Giraffa, G. (2003): Functionality of enterococci in dairy products. *International Journal of Food Microbiology 88*, 215-222.

13. Kos, B., Šušković, J., Beganović, J., Gjuračić, K., Frece, J., Iannaccone, C., Canganella, F. (2008): Characterization of the three selected probiotic strains for the application in food industry. *World Journal of Microbiology and Biotechnology 24*, 699-707.

14. Kos, B., Beganović, J., Jurašić, L., Švađumović, M., Leboš Pavunc, A., Habjanič, K., Šušković, J. (2011): Coculture-inducible bacteriocin biosynthesis of different probiotic strains by dairy starter culture *Lactococcus lactis*. *Mljekarstvo 61 (4)*, 273-282.

15. Klare, I., Konstabel, C., Werner, G., Huys, G., Vankerckhoven, V., Gunnar K., Hildebrandt, B., Müller-Bertling, S., Witte, W., Goossens, H. (2007): Antimicrobial susceptibilities of *Lactobacillus*, *Pediococcus* and *Lactococcus* human isolates and cultures intended for probiotic or nutritional use. *Journal of Antimicrobial Chemotherapy 59*, 900-912.

16. Leboš Pavunc, A., Beganović, J., Kos, B., Uroić, K., Blažić, M., Šušković, J. (2012): Characterization and application of autochthonous starter cultures for fresh cheese production. *Food Technology and Biotechnology 50 (2)*, 141-151.

17. Lopes, DeF., M., Simões, A.P., Tenreiro, R., Figueiredo Marques, J.J., Barreto Crespo, M.T. (2006): Activity and expression of a virulence factor, gelatinase, in dairy enterococci. *International Journal of Food Microbiology 112*, 208-214.

18. Rossetti, D., Carminati, M., Zago, G., Giraffa, A. (2009): Qualified Presumption of Safety approach for the safety assessment of Grana Padano whey starters *International Journal of Food Microbiology 130*, 70-73.

19. Rodriguez, E., Arques, J.L., Nuñez, M., Gaya, P., Medina, M. (2005): Combined effect of high-pressure treatments and bacteriocin-producing lactic acid bacteria on inactivation of *Escherichia coli* O157:H7 in raw-milk cheese. *Applied Environmental Microbiology 71* (7), 3399-3404.

20. Saarela, M., Mogensen, G., Fonden, R., Matto, J., Mattila-Sandholm, T. (2000): Probiotic bacteria: safety, functional and technological properties. *Journal of Biotechnology 84*, 197-215.

21. Sahlström, L., Rehbinder, V., Albihn, A., Aspan, A., Bengtsson, B. (2009): Vancomycin resistant enterococci (VRE) in Swedish sewage sludge. *Acta Veterinaria Scandinavica 51*, 24.1-9.

22. Salminen, S., von Wright, A., Morelli, L., Marteau, P., Brassart, D., Vos de, W.M., Fonde'n, R., Saxelin, M., Collins, K., Mogensen, G., Birkeland, S.E., Sandholm, T.M. (1998): Demonstration of safety of probiotics-a review. *International Journal of Food Microbiology 44*, 93-106.

23. Settanni, L., Franciosi, E., Cavazza, A., Cocconcelli, P.S., Poznanski, E. (2011): Extension of Tosèla cheese shelf-life using non-starter lactic acid bacteria. *Food Microbiology 28 (5*), 883-890.

24. Šušković, J. (1996): Dissertation, Influence of growth medium composition on the kinetics of production of lactic acid for food purposes, Faculty of Food Technology and Biotechnology, University of Zagreb.

25. Šušković, J., Kos, B., Beganović J., Leboš Pavunc, A., Habjanič, K., Matošić, S. (2010): Antimicrobial Activity - The Most Important Property of Probiotic and Starter Lactic Acid Bacteria. *Food Technology and Biotechnology 48* (3), 296-307.

26. van Pijkeren, J.P, Britton, R.A. (2012): High efficiency recombineering in lactic acid bacteria. *Nucleic Acids Research 40 (10)*, 1-13.

27. Zdolec, N., Dobranić, V., Zdolec, G., Đuričić, D. (2013): Otpornost na antimikrobne tvari koagulaza-negativnih stafilokoka i bakterija mliječne kiseline iz industrijskih mliječnih proizvoda. *Mljekarstvo 63 (1)*, 30-35.

28. Zhou, J.S., Pillidge, C.J., Gopal, P.K., Gill, H.S. (2005): Antibiotic susceptibility profiles of new probiotic *Lactobacillus* and *Bifidobacterium* strains. *International Journal of Food Microbiology 98*, 211-217.