Antibacterial activity and enterocin genes in enterococci isolated from Bryndza cheese

ANNA BELICOVÁ – MÁRIA MIKULÁŠOVÁ – JURAJ KRAJČOVIČ – ROMAN DUŠINSKÝ

Summary

Antimicrobial activity of 112 *Enterococcus faecium* and 33 *E. faecalis* isolates from Slovakian bryndza cheese was determined against fifteen strains of lactic acid bacteria (LAB) and nine potential bacterial pathogens by the well diffusion agar assay. The observed inhibitory activity was strain-specific. The cell-free supernatants of *E. faecium* isolates obtained from MRS broth cultures showed higher antimicrobial activity against LAB than supernatants obtained from reconstituted milk cultures, while the opposite was observed for *E. faecalis* isolates. All supernatants showed a direct inhibitory activity against *Listeria innocua*, *Staphylococcus lentus*, *E. faecalis* V583, *Acinetobacter calcoaceticus*, *Sphingomonas paucimobilis*, *Escherichia coli*, *Edwardsiella tarda*, *Serratia marcescens* and *Salmonella enterica*. Supernatants from enterococcal isolates with pH adjusted to 6.5 possessed bacteriocinogenic activity against *L. innocua*, *Staph. lentus, S. enterica* and *E. faecalis*. In MRS medium, enterococci produced from 5.0 $g \cdot l^{-1}$ to 9.9 $g \cdot l^{-1}$ of titratable organic acids. Presence of at least one enterocin structural gene was demonstrated in 63 isolates by PCR. Structural genes for enterocin A, B, P, L50A/L50B and bac31 were detected, *ent*A+*ent*B and *ent*A+*ent*P being the most frequent combinations of the genes. None of the isolates carried the enterocin AS-48 structural gene.

Keywords

Enterococcus sp.; bryndza cheese; antibacterial activity; lactic acid; enterocin genes

Slovakian bryndza cheese is a natural, spreadable cheese, manufactured according to the traditional method, by milling a lump of matured ewes' cheese or by milling a mixture of lump ewes' cheese and lump cows' cheese. It has been granted the status of a Protected Geographical Indication (PGI) by the European Commission in 2008. Bryndza cheese represents a variable pool of indigenous lactic acid bacteria (LAB) such as lactobacilli, enterococci, lactococci, streptococci and pediococci [1]. Enterococci belong to dominant LAB species in bryndza cheese, which most probably play a key role in the cheese-making process by providing aroma, affecting texture and/or permitting the growth of other microorganisms [2]. It is known that the *Enterococcus* genus is ubiquitously distributed in different ecological niches. In particular, enterococci are natural inhabitants of the gastrointestinal tract of mammals and are also isolated from a wide range of foodstuffs such as meat, cheese and milk and from environmental sources such as soil, sand, air and water. The antagonistic activities of LAB against food pathogens, potential pathogenic microorganisms and spoilage microorganisms *in vitro* and *in vivo* were described [3, 4]. They proposed mechanisms including production of lactic and other organic acids, production of hydrogen peroxide, competition and nutrient depletion, alteration of oxidation-reduction potential, and production of bacteriocins. Bacteriocins are small, extracellularly released antibacterial peptides or proteins that display inhibitory activity against closely related Gram-positive bacteria [5]. A majority of bacteriocin-producing enterococci have been isolated from food, such as cheese, meat, fish and vegetables [4]. Bacteriocins from LAB were classified into four distinct classes based on their biochemical and functional properties [6]. A wide variety of enterocins produced by enterococci belong to class II bacteriocins containing enterocin A, B, P, L50, Q, mundticin, enterocin 1071 [7]. The bacteriocinogenic enterococci

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acting either as starter/co-cultures or as producers of bacteriocins may play important role in new and improved natural food preservation technologies [8]. Moreover, enterococci are used as probiotics to refine the microbial balance of the intestinal flora or as a treatment for gastroenteritis in humans and animals [4].

Detailed enterococcal profile of bryndza cheese obtained from various producers in different seasons was described, for review see [9]. Enterococci were found in bryndza cheese at high levels (10^7–10^8 CFU·g^{-1}), the dominant species being *Enterococcus faecium*, followed by *E. durans* and *E. faecalis*. The antibiotic susceptibility of these isolates was investigated [10]. To date, no data have been published on the antimicrobial activity of enterococci from bryndza cheese against other LAB or against potential bacterial pathogens. No data have been neither published on the production of organic acids and the presence of structural genes for bacteriocins.

Our goals were (a) to evaluate antimicrobial effects of enterococcal isolates from bryndza cheese against selected lactic acid bacteria and potential pathogens by the well diffusion method, (b) to determine the production of titratable organic acids by enterococci and (c) to detect the presence of genes encoding for bacteriocins.

MATERIALS AND METHODS

Bacterial strains

Enterococcal isolates were isolated from bryndza cheese from five different producers from middle and south-west Slovakia, namely, Liptovský Mikuláš (LM), Ružomberok (R), Červený Kameň (CK), Tisovec (T) and Zvolenská Slatina (ZS). Samples were produced in January (I), June (II) and October (III). The isolates were characterized in our previous study [2]. *Enterococcus faecium* (112) and *E. faecalis* (33) isolates were randomly selected for screening of their antimicrobial activity. The identification of the species was confirmed by PCR using species-specific primers targeting *ddl* genes. None of selected enterococcal isolates was proven to carry either vancomycin resistance *vanA* or *vanB* genes, or virulence factors (e.g. gelatinase, aggregation substance or cytolysin) [2].

Indicator strains *Streptococcus thermophilus* CCM 4757; *Lactococcus lactis* subsp. *lactis* CCM 1881; *Lactobacillus plantarum* CCM 4281; *L. brevis* CCM 1815; *L. paracasei* subsp. *paracasei* CCM 4649; *L. rhamnosus* CCM 1828; *L. acidophilus* CCM 4833; *L. sakei* subsp. *carnosus* CCM 3728; *L. helveticus* strains CCM 3806 and CCM 4289; *L. delbrueckii* subsp. *lactis* strains 2344 and CCM 2772; *L. delbrueckii* subsp. *bulgaricus* strains CCM 4288, CCM 4290 and CCM 4755; *Listeria innocua* CCM 4030; *Staphylococcus lentus* CCM 3472; *Acinetobacter calcoaceticus* CCM 4503; *Sphingomonas paucimobilis* CCM 3293; *Escherichia coli* CCM 2260; *Edwardsiella tarda* CCM 2233; *Serratia marcescens* CCM 303, and *Salmonella enterica* subsp. *enterica* serovar Typhimurium strain TA100 CCM 3812 were obtained from the Czech Collection of Microorganisms, Brno, Czech Republic. *E. faecalis* V583 ATCC 700802 was obtained from the University of Oklahoma, Norman, Oklahoma, USA. For long-term storage, bacteria cultures were kept at –80 °C in their respective broths supplemented with 20% glycerol (Merck, Darmstadt, Germany). All strains were subcultured twice prior to the experiments.

Preparation of natural and pH-adjusted cell-free supernatants of enterococci

Enterococci were grown in broth according to De Man, Rogosa and Sharpe (MRS broth; Merck) or in 10% reconstituted skim milk at 37 °C for 24 h. Anaerobic incubation was used to rule out any inhibition due to hydrogen peroxide production. Natural cell-free supernatants (NCFS) and adjusted cell-free supernatants (ACFS) were obtained by centrifugation (3300 × *g*, 20 min, 5 °C) of overnight cultures and sterilized by filtration using a 0.45 μm pore filter (Millipore, Billerica, Massachusetts, USA). Before sterilization, ACFS were adjusted to pH 6.5. Both cell-free supernatants were stored frozen (–80 °C).

Well diffusion agar assay of enterococcal cell-free supernatants with LAB

Twenty milliliters of MRS or Elliker agar (Merck) melted and tempered at 45 °C were vigorously mixed with 200 μl of an overnight culture of LAB and poured into Petri dishes. Wells of 9 mm in diameter were made in the agar layer and 180 μl of NCFS were pipetted into each well. The plates were incubated aerobically overnight at 37 °C, Elliker plates for lactococci were incubated at 25 °C). Growth inhibition was detected by visual observation of either clear (complete inhibition) or partial inhibition halos (weak inhibition) around the wells. The experiments were repeated three times.

Well diffusion agar assay of enterococcal cell-free supernatants with potential pathogenic bacteria

Twenty milliliters of a melted agar medium containing 1 ml of the bacterial indicator were poured into a Petri dish. Six wells of 9 mm in diameter were then aseptically excavated into the

agar and filled with 180 μl of NCFS or ACFS obtained from the cultures of enterococci. Plates were incubated at 37 °C for 24 h and the diameter (in mm) of halos was measured. Enterococci were qualified as bacteriocinogenic (Bac^+) when their ACFS inhibited growth of indicator strains with halos of $d > 10$ mm. The experiments were repeated three times. All data were analysed using paired Student's *t*-test and variance analysis ANOVA.

Determination of production of organic acids

Enterococci were cultivated in MRS broth at 37 °C for 24 h. Supernatants were obtained by centrifugation at 8800 × *g* for 15 min at 5 °C. The quantity of produced acids was determined by titration with 0.1 $mol \cdot l^{-1}$ NaOH and expressed as concentration of titratable organic acids in $g \cdot l^{-1}$. Data were expressed as means of at least three separate experiments.

PCR detection of enterocin genes

The presence of structural genes *ent*A, *ent*B, *ent*P, *ent*L50 A/B, *ent*AS-48 and *bac*31 for enterocins A, B, P, AS-48, L50 A/B and bacteriocin 31, respectively, were evaluated. PCR amplification was performed for all enterococci using the rapid alkaline lysis method [11]. The specific primers and conditions were utilised as described previously [12–17]. Control strains from our collection were included for PCR detection of six enterocin structural genes. Amplified fragments were analysed by electrophoresis in 1.5% agarose gels and visualized with an UV transilluminator after staining with ethidium bromide. A 100-bp ladder was used as the molecular weight standard. Presence of bands at expected sizes for each primer pair was considered as a positive result in each experiment.

RESULTS AND DISSCUSION

Interaction of enterococcal cell-free supernatants with selected LAB by well diffusion agar assay

The antimicrobial screening of NCFS from 145 enterococcal isolates confirmed the different potential of enterococci isolated from bryndza cheese to inhibit the growth of 15 LAB strains (Tab. 1). The NCFS from ZSIII 26 and ZSIII 11 *E. faecium* isolates were the most active causing inhibition (complete or partial) of 12 and 10 LAB indicator strains, respectively. The NCFS from other *E. faecium* isolates inhibited variable numbers of tested LAB strains, 46 isolates inhibited 1–5, and 62 isolates 6–10 indicator strains, respectively. In comparison, no inhibition was observed by NCFS from LMI 27 and CKI 2 isolates of *E. faecium*.

The most effective isolate of *E. faecalis* was ZSII 11 inhibiting the growth of all 15 LAB strains. Inhibition of 6 to 14 LAB strains was observed with supernatants from 16 *E. faecalis* isolates. Seven isolates inhibited 1–5 indicator strains. Other nine *E. faecalis* supernatants did not show any effect on the growth of LAB strains.

Generally, antimicrobial potential of enterococci was heterogeneous and strain-specific, depending on the culture medium used. Similarly, the observations of various interactions between lactic acid starters and the probiotic bacteria were reported by VINDEROLA et al. [18]. The cell-free supernatants of *E. faecium* isolates obtained from MRS broth culture showed higher antimicrobial activity against LAB than supernatants obtained from reconstituted milk cultures, while the opposite was observed for NCFS of *E. faecalis* isolates. It seems that this phenomenon results from a better ability of *E. faecium* isolates to utilize glucose from MRS than lactose from milk, which influences the production of organic acids and other antimicrobials. Higher antimicrobial activity of *E. faecalis* supernatants obtained from milk could be explained by the fact that *E. faecalis* expresses highest acidifying potential among enterococci cultivated in milk as demonstrated and described in several papers, e. g. [19]. The source and different seasonal intervals of collected specimens did not significantly influence the effect of enterococci on tested LAB.

Antimicrobial activity of natural and pH-adjusted cell-free supernatants of enterococcal isolates against selected potential pathogens

The antimicrobial activity of 145 enterococcal isolates against nine potential pathogens was tested using NCFS and ACFS obtained from MRS broth. All NCFS showed an inhibitory effect on the growth of all indicator strains as presented in Fig. 1. Detected inhibition zones measured from 12 to 19 mm in diameter. The highest inhibitory effect of enterococci was observed against *E. faecalis* V583, *S. lentus* and *A. calcoaceticus*. It is known, that LAB including enterococci produce several agents with antimicrobial activity [5]. Generally, strength of antimicrobial effect of NCFS was partially dependent on the effect of lactic acid and other antimicrobials. The substantial role of lactic acid in the growth inhibition of different microorganisms was also described by others [18]. In our work, no relationship could be established between the amount of titratable organic acids produced by enterococci and their antimicrobial activity against indicator strains.

Antimicrobial activity ACFS of enterococci

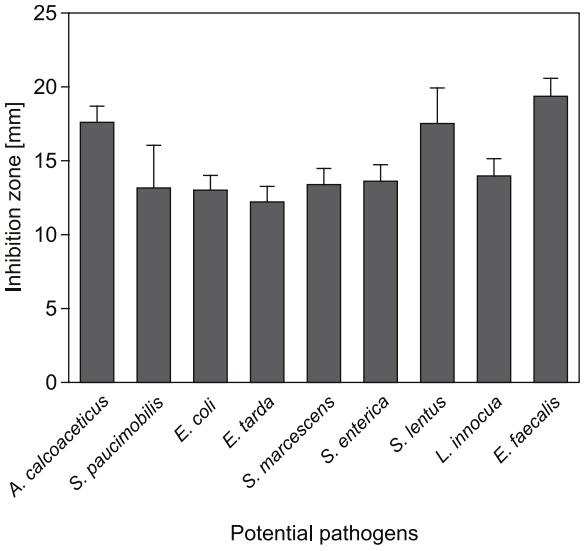

Fig. 1. Antimicrobial activity of natural cell-free supernatants of enterococcal isolates from bryndza cheese against nine potential pathogens.
Mean values ± standard deviation of three experiments are presented.

was also screened. After neutralization to pH 6.5, several ACFS from enterococcal strains retained their inhibitory activity against potential pathogens. This finding suggests that not only the organic acids were responsible for antibacterial activity of the screened enterococci. The results are summarized in Tab. 1. Sixty-eight isolates (47%) were bacteriocinogenic (Bac$^+$, 54.5% of *E. faecium* isolates and 21.2% of *E. faecalis* isolates). *L. innocua*, *S. lentus* and *S. enterica* were inhibited by 34.5%, 33.8% and 16.6% of the Bac$^+$ isolates, respectively. The taxonomically related *E. faecalis* V583 was inhibited by 34.5% Bac$^+$ isolates, while *A. calcoaceticus*, *S. paucimobilis, E. coli, E. tarda* and *S. marcescens* were not inhibited. The antimicrobial effect of ACFS is based on production of bacteriocins or bacteriocin-like substances [20]. Bacteriocinogenic enterococci from bryndza cheese inhibited the growth of both, the closely-related G$^+$ bacteria such as *L. innocua*, *S. lentus* and *E. faecalis* V583, as well as the G$^-$ bacterium *S. enterica*. The Bac$^+$ enterococcal isolates from bryndza cheese showed similar inhibitory spectra, most of them inhibited the growth of three or four of the tested species. The Bac$^+$ *E. faecium* and *E. faecalis* strains of different origin studied by De Vuyst et al. [3] also did not show significantly different inhibitory spectra. The inhibition zones observed did not exceed a diameter of 11–13 mm (including the 9 mm of the well), which is comparable with the results obtained by other authors [21, 22]. ACFS and concentrated ACFS of LAB from dairy, meat and vegetable products, and isolates from various environments, showed antimicrobial activity against a broad spectrum of microorganisms, which included pathogens and spoilage or breakdown organisms [3, 8]. Number of bacteriocinogenic species, character of inhibition zones and activity range of tested collections of LAB varied. No correlation between enterococci from different sources of bryndza cheese and their antagonistic activity was observed. Similar results have been obtained by other researchers [3, 23]. These results can be summarized in a statement that the antimicrobial effect of LAB is a strain-specific property influenced by the culture medium and the experimental conditions [24].

Determination of the organic acids production

Some differences in the production of the organic acids were observed in enterococcal isolates (Tab 1). In MRS medium, enterococci produced from 5.0 $g \cdot l^{-1}$ to 9.9 $g \cdot l^{-1}$ of titratable organic acids. The majority of isolates produced 5.8–7.8 $g \cdot l^{-1}$ of organic acids. The major metabolic end-product of enterococci fermentation is lactic acid, which decreases pH in the culture media. In our experiments, we regularly observed that after 24 h of fermentation, the pH of MRS broth dropped from 6.5 to 4.1–5.0 (data not shown). It is known that enterococci are weak acidifiers in comparison with other LAB, but production of lactic acid by enterococcal isolates from bryndza cheese was comparable to that determined for LAB and bifidobacteria in the standard media following 24 h of fermentation [25]. Strains of lactobacilli and *Pediococcus* sp. isolated from bryndza produced 8–15 $g \cdot l^{-1}$ of lactic acid after 72 h of aerobic cultivation in MRS medium [26].

Detection of structural genes for enterocins by PCR

Detection of structural genes for enterocins by PCR was performed with 145 isolates of *E. faecium* and *E. faecalis* obtained from bryndza cheese. The results are summarized in Tab. 1. Sixty-three enterococcal isolates carried at least one of the tested structural genes for enterocins (51% of *E. faecium* and 18% of *E. faecalis* isolates). The enterocin genes were rarely presented as singletons except for enterocin A. The chromosomally located enterocin A gene was found in 17 *E. faecium* isolates. At least one enterocin gene was observed in the majority of strains from different sources (animal isolates, food and feed) in other studies [27, 28]. Similar to our results,

Tab. 1. Properties of enterococcal isolates from bryndza cheese: antimicrobial activity against LAB strains and potential pathogens, occurrence of enterocin-encoding structural genes and organic acids production

E. faecium strains	Natural CFS from MRS or skim milk cultures																										pH-adjusted CFS				Genes for enterocins	Organic acids production [g·l^{-1}]
	ST		LLI		LPl		LB		LPa		LR		LA		LS		LH1		LH2		LDl1		LDl2		LDb1		LDb2		LDb3		LI	SL	EF	SE		
	M	S	M	S	M	S	M	S	M	S	M	S	M	S	M	S	M	S	M	S	M	S	M	S	M	S	M	S	M	S						
LMI 5	c		c		c								c		c						c		c		c						I	I	I	I	A B	7.9
CKI 13	c		c		c								c						c		c		c		c				c		I	I	I	I	A B	7.9
ZSII 15	c		c										p												c	c					I	I	I	I	A B	7.3
TII 6	c												p		c	c									c						I	I	I	I	B P	7.9
TII 14	c		p	c																					c	p					I	I	I	I	B bac31 L50	7.9
TIII 30	p		c										p		c								c		p				p		I	I	I	I	A B P bac31 L50	8.5
CKIII 20	p		p										c		c	p	p				p		p		c				p		I	I	I	I	A B	8.1
LMI 4	c		c						c	c													p						p	p	I	I	I		A P	7.9
LMI 7	p		p	c	p										c	c							p		c	c					I	I	I		A	8.0
LMI 26	c		c		c								p								p		p		c						I	I	I		A B	7.9
LMI 29	c		c						c	c			p																c	c	I	I	I		A	7.4
RI 4	c		c		p												p	p			p		c		p						I	I	I		A L50	7.9
RI 10	c	c	p	c							p	p					p	p					p	c			c	c			I	I	I		A	8.3
RI 13	c																								p						I	I	I		A P	6.9
RI 15	c	p	c																						c				c	p	I	I	I		A B	8.1
RI 23	c		c		c						p						p		p		p		p		c						I	I	I		A	8.3
RI 24	c				c								p										c		c	c			p		I	I	I		A	7.9
RI 25	c				c								c										p		c	c					I	I	I		A	8.8
CKI 26	c	p	c		c								c						c		c		c		c				c		I	I	I	I	A B	7.6
RII 29	c		c				p								c	c									c						I	I	I		A	7.9
ZSII 6			p		c										p						p				p						I	I	I		A B P bac31 L50	7.9
ZSII 17			p		p								c								c		c		c						I	I	I		A P	7.3
ZSII 30			p		p								c								c		p		c						I	I	I	I	A B bac31	7.9
TII 4	c		p				p								c						c		p		c				c		I	I	I		A P	8.0
TII 9																									c						I	I	I		A P	7.9
TII 20	c												p												c	c					I	I	I		A P	7.9
TII 24	c																								c	c					I	I	I		A P	7.9
RIII 7	c		c										p		c				p		c		c		c						I	I	I		A P	8.1
ZSIII 11	c	c	c	p	p								c	p	c	c	c	p			c	p	c	p	c	c			c	p	I	I	I		A P	8.5
ZSIII 26			c		p	p	c		p				c	c	c	c	c	c	c	c	c	c	c	c	c				c	c	I	I	I		A	7.6
TIII 16	c		c										p		p		p				p		c		c						I	I	I		A P	7.9
TIII 18	c		c										p		p		p						c		p				p		I	I	I		A P	8.2

Tab. 1. continued

E. faecium strains	Natural CFS from MRS or skim milk cultures																												pH-adjusted CFS				Genes for enterocins	Organic acids production [g·l^{-1}]
	ST		LLl		LPl		LB		LPa		LR		LA		LS		LH1		LH2		LDl1		LDl2		LDb1		LDb2		LDb3		Ll	SL	EF	SE		
	M	S	M	S	M	S	M	S	M	S	M	S	M	S	M	S	M	S	M	S	M	S	M	S	M	S	M	S	M	S						
TIII 26	p	p	p		c						p	p	c		c	p	c	c					c	c	p				c	p	l	l	l	l	A B	7.2
TIII 27			c		c								c		c	p	c						p		p				c		l	l	l		A B	8.4
CKIII 5	p		c										c		p		c						c	c	c						l	l	l		A L50	8.2
CKIII 10	c		c										c		p		c						c		c				c		l	l	l		A	7.2
CKIII 21	c		p										c	p	c		c						p	p	c				c	p	l	l	l		A L50	9.0
CKIII 24	c		p										c	p	p	p	p		p				p	p	c				p	p	l	l	l		A P	8.1
CKIII 25			c										c		c								p		c				c		l	l	l	l	A B	7.9
CKIII 27	c		c										c		c		p		p				c		c				p		l	l	l		A	7.4
CKIII 30	c		p										p		c		c						c		c				c		l	l	l		A B	7.9
CKIII 23			c		c								c	c	c	c		p					p	p	c				p	p		l	l	l	B P	8.4
LMI 17	c		p		c								p										c		c						l	l			A bac31	7.7
ZSI 13	c		c																				p		c	c			p		l	l			A bac31	7.9
LMI 2	c		p		p								p										p		c	c						l		l	A bac31	7.9
LMI 23	c	c	p		c				c	c			p			c					c		p		c	c					l				A	7.5
RII 23	p		c		c								p						c				c		c						l				A	7.3
LMI 24	c		c		c								p		c						p		p		c											9.7
RII 20	p												p										c		c	c						l	l	l	A B bac31	7.8
LMI 9	c		c		c								p		p						c		c		c									l		7.5
LMI 18	c		p		c								p								c		p		c									l		7.2
LMI 27																																		l		5.7
LMI 30			c																						p	p								l		5.8
RI 2	p													c											c	c								l		7.1
RI 27	c		c																						c	p			p					l		7.2
TI 14			c		c																													l		7.2
ZSII 12			c		c								p								c		c		p									l		7.1
ZSII 18	c		c		p		p						p								p		c		c	c			p					l		7.2
RIII 10	c		c										p		c	p			c	p			p		c				p							8.8
RIII 16	c		c										c		c	c							p		c				p							7.6
RIII 22	c		c										c		c	c							c		c											7.5
CKIII 14	c		c										c		c	p	p		p				c		c											8.9
LMI 1			c																																	5.5
LMI 8	p				p								p										p		p	c									A	5.9

Tab. 1. continued

E. faecium strains	Natural CFS from MRS or skim milk cultures																												pH-adjusted CFS				Genes for enterocins	Organic acids production [g·l^{-1}]
	ST		LLI		LPI		LB		LPa		LR		LA		LS		LH1		LH2		LDl1		LDl2		LDb1		LDb2		LDb3		LI	SL	EF	SE		
	M	S	M	S	M	S	M	S	M	S	M	S	M	S	M	S	M	S	M	S	M	S	M	S	M	S	M	S	M	S						
LMI 13	c		p		c								p								p		c		c										A	7.3
LMI 15	c		c																				c		c	c										7.7
LMI 19	p		c																																	6.8
LMI 28	c																										p									6.8
RI 3	c																				c				c	c										6.6
RI 6			c	c																					c	c			p							6.8
RI 8				c																					c	c			p	p						7.1
RI 14			c																						p	p			p	p						6.2
RI 19	c				p																		p						c							7.2
RI 20			c		p																				p											6.7
RI 22			c																						c	c										7.0
RI 29	c		p																						p	p										5.6
ZSI 1	p		c		p																		p		c	c										7.2
ZSI 2			c																		c		c		p		c									7.2
ZSI 3	c		c		p												p						c		c		p		c							7.5
ZSI 5	c		c																				c		p	p										7.7
ZSI 14	c		c		p										p								p		c	c			p							7.3
ZSI 15			c										p										c		c		c		c							7.2
ZSI 29			c																				p		c											7.1
CKI 2																																				5.6
CKI 10	c		c		p								c						c		p		c		c											9.2
RII 21	c																								c	c			p	p					A	7.8
ZSII 1			c		c										p						c		p		p											6.8
ZSII 9			c		c								p								c		c		p											7.5
TII 5													p												c											5.9
TII 11															c						c	c			c	c			c	c						7.3
TII 17			p																						c											5.9
TII 22	p												p												c	c										6.8
TII 23	p																								c	c										5.8
TII 25													p												c	c										6.8
RIII 1	p		c																		c		c		c	c			p							7.2
RIII 9	c		c										c		c				p	p	p		c		c											8.9

Tab. 1. continued

E. faecium strains	Natural CFS from MRS or skim milk cultures																												pH-adjusted CFS				Genes for enterocins	Organic acids production [g·l^{-1}]
	ST		LLI		LPI		LB		LPa		LR		LA		LS		LH1		LH2		LDI1		LDI2		LDb1		LDb2		LDb3		LI	SL	EF	SE		
	M	S	M	S	M	S	M	S	M	S	M	S	M	S	M	S	M	S	M	S	M	S	M	S	M	S	M	S	M	S						
RIII 11	c		c										c		c	p			p	p	p		c		c											8.6
RIII 15	c		c										c		c	c	p						c		c				p						A	7.2
RIII 19	p		c										c		c	p		p					p		c											7.3
RIII 21	c		c												c	c			p		c		p		c											9.5
RIII 30	c		c		p								p		c	c	p		c		p		c		c				c							9.4
ZSIII 24	c		c				p						c		c		c						c		c	p			p							9.5
ZSIII 30	c		c										c				c				p		c		c	p			p							7.2
TIII 1	p		p		p								p																							5.5
CKIII 1	c		c										p		p		c								c											6.6
CKIII 3	c		c										c		c	p	c								c											7.3
CKIII 4			c		p								c				p	p							c											7.5
CKIII 7	p		c										c		p		p						c		c				c							7.5
CKIII 12	c		c										c		c	p	c				p		c		c				p							9.6
CKIII 13	c		c										c		c		c						p		c				c						A	7.5
CKIII 16			c		p								c		c	p	p				p	p	p		c											7.2
CKIII 17			c										p		c		p						p		c				p							6.9
number of c	64	3	70	5	22	0	1	0	3	3	0	0	34	3	35	10	14	2	7	1	20	2	42	4	84	27	3	1	20	3	45	46	43	23		
number of p	17	3	20	1	19	1	4	0	1	0	3	2	33	3	11	11	16	6	8	3	16	2	32	4	17	8	2	0	23	11						

E. faecalis strains	Natural CFS from MRS or skim milk cultures																												pH-adjusted CFS				Genes for enterocins	Organic acids production [g l^{-1}]
	ST		LLI		LPI		LB		LPa		LR		LA		LS		LH1		LH2		LDI1		LDI2		LDb1		LDb2		LDb3		LI	SL	EF	SE		
	M	S	M	S	M	S	M	S	M	S	M	S	M	S	M	S	M	S	M	S	M	S	M	S	M	S	M	S	M	S						
ZSI 21	c	c		p		p	c	c		p	p	c	p	c				c						c					c	I	I	I		B P	8.1
ZSII 11	c	c	c	c	c	c	c	c	c	c	c	c	c	c	p	c	c	c	c	c	c	c	c	c	c	c	c	c	c	c	I	I	I		B P bac31 L50	8.5
ZSII 14	c	c	p	c	p	c	c	c	p	c	c	c	c	c	p	c	c	c	c	c	c	c	c	c			p	p	c	c	I	I	I		B P bac31 L50	8.3
ZSI 24	c	c		p		p	c	c		p	c	c	c	c			c	c					p	c	p					c	I		I		B bac31 L50	8.1
RI 18	p	p	p	p			p	c			c	c	c	p			p	c					c	c						p	I		I		B P	8.0
RI 7		p	p	p		p		c			p	c		c				c					p	c						c						7.2
ZSI 12	p	p	p	p			c	c			c	c	p	c			c	c					c	c						c			I		B bac31	7.8
CKII 22	c	p	c		p	p	c	c			c	p				c	c			c			p		p								I	I		7.4
RI 17	p	p	p	p			c	c			c			p		p	c			c		c	c					p		c						9.1

Tab. 1. continued

E. faecalis strains	Natural CFS from MRS or skim milk cultures																												pH-adjusted CFS				Genes for enterocins	Organic acids production [g l^{-1}]
	ST		LLI		LPl		LB		LPa		LR		LA		LS		LH1		LH2		LDl1		LDl2		LDb1		LDb2		LDb3		LI	SL	EF	SE		
	M	S	M	S	M	S	M	S	M	S	M	S	M	S	M	S	M	S	M	S	M	S	M	S	M	S	M	S	M	S						
RI 1		p	p	p			p	c			p	c		c				p					p	c						c						7.9
RI 21	p		p								p		c										p		c				p							5.9
RI 27			p	p	p						p		p																							6.4
ZSI 10	p		p				c	p			c		c			p	c			c		c	c						p							7.2
ZSI 17	p	c		p		p	p	c		p	p	c	p	c				c						c						c							7.5
ZSI 25	c	c					c	c			c	c	c	c			c	c					p	c	p					c							5.7
TI 6											p		p												p												7.0
TI 19																																					5.8
LMII 14											p																										7.2
LMII 19																									p												7.2
RII 3										p										c		c								c							6.9
ZSII 7																																					6.5
ZSII 21																																					6.8
ZSII 22	p	p			c				p	p	c		c			p		p	p	p			p		c												6.3
ZSII 28		c		c		p		c		c		c		c		c		c		c		c		c				p			c						9.7
TII 19																														p	p						6.0
CKII 3	p																																				6.9
CKII 14	c	p	p						p	p	p		p			c		p	p		c	p	c		c												6.4
RIII 25																																					6.3
ZSIII 23																																					7.4
ZSIII 25																																					7.1
CKIII 18																																					5.5
CKIII 19																																					9.1
CKIII 29																																					5.5
number of c	7	7	2	3	2	2	9	13	1	3	10	11	8	10	0	5	8	10	2	7	3	6	7	11	4	1	1	1	2	12	5	3	7	1		
number of p	8	8	10	9	3	6	3	1	3	6	9	1	6	2	2	3	1	3	2	1	0	1	7	0	5	0	1	4	3	1						

CFS – cell-free supernatants, M – MRS culture, S – skim milk culture.
ST – *Streptococcus thermophilus* CCM 4757, LLI – *Lactococcus lactis* subsp. *lactis* CCM 1881, LPl – *Lactobacillus plantarum* CCM 4281, LB – *L. brevis* CCM 1815, LPa – *L. paracasei* subsp. *paracasei* CCM 4649, LR – *L. rhamnosus* CCM 1828, LA – *L. acidophilus* CCM 4833, LS – *L. sakei* subsp. *carnosus* CCM 3728, LH1 – *L. helveticus* CCM 3806, LH2 – *L. helveticus* CCM 4289, LDl1 – *L. delbruecki* subsp. *lactis* CCM 2344, LDl2 – *L. delbruecki* subsp. *lactis* CCM 2772, LD1 – *L. delbruecki* subsp. *bulgaricus* CCM 4288, LD2 – *L. delbruecki* subsp. *bulgaricus* CCM 4290, LD3 – *L. delbruecki* subsp. *bulgaricus* CCM 4755.
c – complete inhibition, p – partial inhibition; blank cell – no inhibition.
I - inhibition of potential pathogens: LI – *Listeria innocua* CCM 4030, SL – *Staphylococcus lentus* CCM 3472, EF – *Enterococcus faecalis* V583 ATCC 700802, SE – *Salmonella enterica* subsp. *enterica*, serovar Typhimurium strain TA100 CCM 3812.
Genes for enterocins: A – *ent*A, B – *ent*B, P – *ent*P, L50 – *ent*L50 A/B, AS-48 – *ent*AS-48, bac31 – *bac*31, blank cells – no genes detected.

other authors found the structural gene of enterocin B to be always associated with the presence of the gene encoding for enterocin A [3, 23, 28]. Combinations of enterocin genes *ent*A+*ent*B and *ent*A+*ent*P were the most frequent. A higher incidence of the combination of genes encoding for enterocin A with P, and a low occurrence of more than two enterocin genes in one isolate, were reported by De Vuyst et al. [3]. Enterocin A and P are antilisterial bacteriocins of class II.1 bacteriocins of the pediocin family. Enterocin B, a broad-spectrum acting bacteriocin, belongs to subgroup II.3 [6]. The plasmid-encoded enterocin L50A and L50B, as well as enterocin bac31, were detected in both *E. faecium* and *E. faecalis* isolates. Interestingly, no plasmid-encoded gene for enterocin AS-48 was detected in contrast to other screening studies, where the presence of AS-48 gene in enterococci was observed [3, 29]. Our results indicate that the antimicrobial activity against G^+ potential pathogens of most of ACFS from enterococcal isolates correlated with the detection of the enterocin genes. However, we observed that pH-adjusted cell-free supernatants of 5 isolates harbouring *ent*A gene (LMII 8, 13, RII 21, RIII 15 and CKIII 13) inhibited no indicator strain. The occurrence of PCR^+ among Bac^- isolates showed the lack of the phenotypic expression also in the presence of enterocin gene. This phenomenon may be explained by down-regulation or low levels of gene expression or by an inactive gene product [30]. The existence of silent genes in enterococcal strains was referenced by several works previously [3, 30]. None of enterocin genes screened for were found in 9 of *E. faecium* and 1 of *E. faecalis* isolates with Bac^+ phenotype (exclusively against *S. enterica*). Therefore, different structural bacteriocin genes, not tested in this study, could be responsible for the antimicrobial activity of 10 PCR^- isolates. The antimicrobial potential of enterococci against *S. enterica* can be explained by production of some other compound(s) with antimicrobial effect [31, 32].

CONCLUSIONS

Natural cell-free supernatants obtained from MRS or from skim milk of enterococci isolated from bryndza cheese showed the antagonistic activity only against certain LAB strains but this activity was sufficient for inhibition of the potential pathogens studied. From the results of our work it is apparent that *E. faecium* isolates TII 14, TIII 30, ZSII 6 and *E. faecalis* isolate ZSI 24 produce enterocins that inhibit tested potential pathogens (*L. innocua, Staph. lentus, E. faecalis* V583 and *S. enterica*) without any strong antagonistic effect against tested LAB. These enterococci do not bear virulence factors and therefore this characteristic qualifies them as suitable for use as effective bacteriocin producers or as protective cultures in bryndza cheese manufacture.

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