BIOACTIVE ENTEROCOCCI ISOLATED FROM SLOVAK EWES' LUMP CHEESE*

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Enterococci are widespread organisms; some of their properties are desired in dairy industry. They can produce antimicrobial proteinaceous substances (enterocins) linked to food biopreservation. This study focused on bioactive *Enterococcus faecium* and *Enterococcus faecalis* strains from Slovak ewes' lump cheeses to check genes encoding enterocins production and inhibition activity. The total counts of enterococci in ewes' lump cheeses reached 5.95 ± 2.44 log CFU/g on average. Genotypization by PCR and identification by MALDI-TOF mass spectrometry alloted 12 strains to the species *Enterococcus faecium* and 18 strains to the species *E. faecalis.* Enterococci were hemolytic phenotype free. Gelatinase negative strains were tested for the presence of enterocins genes. *E. faecium* and *E. faecalis* strains from Slovak ewes' lump cheeses possessed mostly genes for enterocins P and A. Enterocin gene free *E. faecalis* EE29E3 inhibited indicator *Enterococcus avium* EA5 (inhibition zone > 10 mm); EE36E1inhibited *Listeria innocua* LMG 13568 (inhibiton zone 12 mm). Among *E. faecium* possessing enerocins genes, inhibition activity was only noted in EF27E4 strain (against *E. avium* EA5, *Listeria monocytogenes* CCM4699; inhibiton zone 10–22 mm). *E. faecium* EF27E4 was selected for more detailed studies *in vitro* aimed at its potential use in dairy industry.

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INTRODUCTION

Enterococci are ubiquitous microorganisms. They belong to obligatory gastrointestinal microbiota and represent lactic acid producing Firmicutes. Many positive properties of enterococci (lactic acid bacteria) are desired in dairy industry. Compared to other lactic acid bacteria, they can reach higher proteolytic activity important e.g. for cheese ripening. In cheese making, enterococci beneficially contribute to hydrolysis of milk fat by esterases and production of flavour components acting in the development of cheese aroma. Owing to their properties some enterococci have been used as starter cultures and starter adjuncts (Franz et al., 1999; Lauková, Czikková, 2001). In 1996, the British Advisory Committee on Novel Foods and Processess even accepted the use of *Enterococcus faecium* strain K77D as a starter culture in fermented dairy products. The contribution of enterococci to food is not limited only to the final taste development through their primary and secondary metabolisms; they also produce bacteriocins, mostly enterocins, that are linked to food biopreservation (Giraffa, 2003). In general, enterocins inhibit growth of a broad spectrum of microbes including spoilage or pathogenic bacteria (Lauková et al., 1993; Giraffa, 1995; Franz et al., 2007), which substantiates their use in dairy industry.

Slovak lump cheeses are produced in agrofarms and belong to the traditional products made from ewes' milk. Ewes' milk is used for manufacturing traditional and European Union protected dairy specialities in

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Slovakia, including ewes' lump cheeses (OA EC- Other Acts European Commission 2010/C20/9) (K o l o š t a et al., 2014). These products are ideal for the isolation of 'wild' lactic acid bacteria, in this case enterococci, to check their beneficial properties to protect dairy products from contaminant bacteria. This study was focused on bioactive *E. faecium* and *E. faecalis* strains isolated from traditional Slovak ewes' lump cheeses, to detect enterocins-encoding genes and inhibition activity. Besides the basic research, we aimed at selecting a candidate strain suitable for future studies and potential use in dairy industry. Novelty of the study is also in detecting enterocins genes in enterococci from Slovak ewes' lump cheeses.

MATERIAL AND METHODS

Isolation of enterococci

Thirty eight ewes' lump cheeses (made from raw milk) were supplied by 34 different agrofarms in central Slovakia. Samples of cheeses were treated by the standard microbiological method (ISO 15214:1998) using appropriate dilutions; 10 g of cheese were inserted into 90 ml of peptone water (Merck, Darmstadt, Germany); samples were stirred using a stomacher Masticator (IUL Instruments, Barcelona, Spain) and diluted. The appropriate dilutions were plated on Slanetz-Bartley agar (Merck, Darmstadt, Germany) and incubated at 37°C for 24–48 h. Bacterial richness was calculated as an average count of colonies grown in the highest dilution per sample and expressed as logarithm of colony-forming units (CFU) per gram of sample ± standard deviation.

To check the purity of colonies, 59 presumed colonies were inoculated on Brain heart agar (Difco, Baltimore, USA) enriched with 5% of defibrinated sheep blood.

Identification of enterococci

First, pure colonies were identified using Matrix-assisted Laser Desorption/Ionisation Time-of-Flight Mass Spectrometry (MALDI-TOF MS) based on protein 'fingerprints' (MALDI-TOF mass spectrometer; Bruker Daltonics, Billerica, USA) (A l a t o o m et al., 2011), performed using a Microflex MALDI-TOF mass spectrophotometer (Microflex, Leipzig, Germany). This method is proposed especially for research microbiology. Briefly, a single colony from Slanetz-Bartley agar (Merck) was mixed with matrix (α-cyano-4-hydroxycinnamic acid and trifluoroacetic acid) and the suspension was spotted onto a MALDI plate and ionized with a nitrogen laser (wave length 337 nm, frequency 20 Hz). Lysates of bacterial cells were prepared according to the producer's instructions (Bruker Daltonics). The results were evaluated using the MALDI Biotyper 3.0 (Bruker Daltonics) identification database. Taxonomic allotment was evaluated on the basis of highly probable species identification (score value 2.300–3.000) and secure probable species identification/probable species identification (2.000–2.299). Positive controls were *Enterococcus faecium* CCM4231 (L a u k o v á et al., 1993) (Czech Culture Collection of Microorganisms, Brno, Czech Republic) and *Enterococcus faecalis* DSM 20478 (Deutche Sammlung von Mikroorganismen GmbH Braunschweig, Germany) (Bruker Daltonics database, 2008). Identical colonies evaluated by MALDI-TOF score value were excluded. For the other testing, identified strains were maintained on M-Enterococcus agar (Difco) (ISO 7899-2-2000) and stored with the Microbank system (Pro-Lab Diagnostic, Richmond, Canada).

Genotypization by PCR method

Genotypization using the polymerase chain reaction (PCR) method (Techgene KRD thermocycler; Techne, Cambridge, UK) was also used to confirm taxonomic allotment of the strains followed by agarose electrophoresis in 0.8% agasose gel (Sigma-Aldrich, St. Louis, USA) buffered with Tris-Acetate-Ethylenediaminetetraacetic acid (TAE) (Merck) containing 1 µg/ml of ethidium bromide (Sigma-Aldrich). The molecular mass standard (Promega, Madison, USA) was used according to the manufacturers' instructions. DNA (template) from each strain was isolated by the rapid alkaline lysis method (B a e l a e et al., 2001). The sequences of the primer pairs used for PCR amplification of *Enterococcus faecium* were 5′-GCAAGCTTCTTAGAGA-3′ and 5′-CATCGTGTAAGCTAACTTC-3′ (550 bp; Invitrogen, Carlsbad, USA) and of *Enterococcus faecalis* 5′-ATCAAGTACAGTTAGTCTT-3′ and 5′-ACGATTCAAAGCTAACTG-3′ (941 bp; Invitrogen) (W o o d f o r d et al., 1997). *Enterococcus faecium* EK13-CCM7419 (M a r e k o v á et al., 2003) and *Enterococcus faecalis* CCM 4224 (Czech Culture Collection of Microorganisms, Brno, Czech Republic) were positive controls.

Hemolytic and gelatinase activity

Hemolysis was detected by streaking the cultures on de Man-Sharpe-Rogosa (MRS) agar (Difco) supplemented with 5% of defibrinated sheep blood. Plates were incubated at 37°C for 24–48 h under semi-anaerobic conditions. Presence or absence of clearing zones around the colonies was interpreted as β-hemolysis and negative gamma-hemolysis, respectively (S e m e d o - L e m s a d d e k et al., 2003, 2013).

Gelatinase activity was detected with a 3% gelatin medium (Todd-Hewitt agar; Becton and Dickinson, Cockeysville, USA). After growth of tested strains (48

Table 1. Sequences of the primer pairs (F – forward; R – reverse) used for PCR amplification of the structural genes of enterocins (Ent) A, P, B, and L50B

	Primers	References
Ent A	F5'R5'-CCC TGG AAT TGC TCC ACC TAA-3' R5'-CCC TGG AAT TGC TCC ACC TAA-3'	Aymerich et al. (1996)
Ent P	F5'-GCT ACG, CGT TCA TAT GGT AAT-3' R5'-TCC TGC AAT ATT CTC TTT AGC-3'	Cintas et al. (1997)
Ent B	F5'-CAA AAT GTA AAA GAA TTA AGA TCG-3' R5'-AGA GTA TAC ATT TGC TAA CCC-3'	Casaus et al. (1997)
Ent L50B	F5'-ATG GGA GCA ATC GCA AAA TTA-3' R5'-TAG CCA TTTT TTC AAT TTG ATC-3'	Cintas et al. (1998)

h at 37°C), plates were flooded with a 15% solution ($HgCl_2$ in 20% HCl). Loss of turbidity halos around colonies was then checked at 4°C (Kanemitsu et al., 2001). Gelatinase positive strains were excluded from the next test.

Detection of the structural genes for enterocin production

The enterocins (Ents) genes that have been tested were Ents A, P, B, and L50B (Table 1). They were selected because of their most frequent detection in different enterococci (Strompfová et al., 2008). Primers sequences for PCR amplification of Ents genes were used according to Aymerich et al. (1996), Casaus et al. (1997), and Cintas et al. (1997, 1998): 5 min denaturation at 95°C, 30 cycles of 30 s at 95°C, 30 s at 58°C, 30 s at 72°C; 5 min at 72°C, 94°C. Annealing temperature for Ent P, L50B, and B was 56°C instead of 58°C. The PCR product was visualized by 2% agarose electrophoresis (1 µg ethidium bromide). Positive controls were: *E. faecium* EK13-CCM7419 (Marková et al., 2003) for Ent A, P; *E. faecium* L50 (Cintas et al., 1997, 1998) for Ent L50B and B. The method was in greater detail described previously by Szabóová et al. (2012). Briefly, template (2 µl) was added to 8.75 µl of reagent mixture which contained 0.5 µl of each primer, 1 µl (10 mmol/l) of nucleotide triphosphates containing deoxyribose called dNTPs (Invitrogen), and water to a total volume of 50 µl. The sequences of the primer pairs used for PCR amplification of the Ents structural genes are summarized in Table 1. DNA (template) was extracted by the rapid alkaline lysis method (Baele et al., 2001).

Bioactivity/inhibition activity testing

Bacteriocin activity of the strains possessing Ents genes was tested using qualitative agar diffusion method according to Skalka et al. (1983) against the four principal indicator strains such as *E. avium* EA5 (isolate from piglet, our laboratory), *L. monocytogenes* CCM 4699 (CCM), *Listeria innocua* LMG13568 (kindly supplied by prof. De Vuyst, University Brussel, Belgium), and *Staphylococcus aureus* SA5 (our strain isolated from mastitis milk). Bacteriocin activity was expressed as clearing inhibition zones around producing strain. *E. avium* EA5 is our principal indicator which means the most sensitive strain used in bacteriocin activity testing (Lauková et al., 1993). The rest of indicators were used as the representatives of frequent milk contaminants. Briefly, Brain heart agar plates (Difco) inoculated with tested enterococci were incubated at 37°C overnight. Then, the plates were overlaid with 2.5 ml of soft agar (0.7%) seeded with 200 µl of overnight cultures of indicator organisms (optical density measured at 600 nm – OD600; 0.4–0.6). The plates were incubated overnight and widths of the clear inhibition zones were measured (in mm). *E. faecium* CCM4231 (our isolate deponed to CCM) was used as a bacteriocin-producing positive control (Lauková et al., 1993).

RESULTS

The total counts of enterococci in ewes' lump cheeses reached 5.95 ± 2.44 log CFU/g on average. In this study, 12 strains of *E. faecium* and 18 strains of *E. faecalis* were identified (Tables 2, 3). The majority of *E. faecium* strains were evaluated with the score value in the range 2.086–2.171; it corresponds with secure probable species identification/probable species identification (2.000–2.299). Only *E. faecium* strains EF11E9, EF25E8, EF29E4 reached the score for a probable genus identification (1.700–1.999); however, their taxonomic allotment to the species *E. faecium* was confirmed by PCR. Similarly, two *E. faecalis* strains EE1E1 and EE25E1 were evaluated with the score in the range 1.700–1.999, but PCR confirmed their allotment to the species *E. faecalis*. The other *E. faecalis* strains showed highly probable species identification

Table 2. Enterocins genes and bacteriocin activity of hemolysis and gelatinase negative *Enterococcus faecium* strains

Strain	Ents genes	Bc activity	Score value
EF1E4	A, P	ng	2.096
EF2E8	A, P	ng	2.111
EF4E6	A	ng	2.171
EF5E6	P	ng	2.130
EF11E9	P	ng	1.994
EF12E1	B	ng	2.177
EF23E1	A	ng	2.008
EF24E9	A	ng	2.145
EF25E8	P	ng	1.803
EF27E4	P, L50B	EA5, CCM4699*	2.135
EF28E4	A, P	ng	2.086
EF29E4	A	ng	1.947

Ents = enterocins, Bc activity = bacteriocin-enterocin-like activity, ng = negative (no growth inhibition)

Enterococcus faecium strains were hemolysis and gelatinase negative

**Enterococcus avium* EA5 – our laboratory, *Listeria monocytogenes* CCM4699 – Czech Culture Collection, Brno, Czech Republic; inhibition zones in the range 10–20 mm

Table 3. Enterocins genes and bacteriocin activity of hemolysis and gelatinase negative *Enterococcus faecalis* strains

Strain	Ents genes	Bc activity	Score value
EE1E1	A, P	ng	1.895
EE5E3	P	ng	2.340
EE8E1	ng	ng	2.272
EE10E4	ng	ng	2.106
EE11E4	P	ng	2.053
EE23E5	A, P	ng	1.992
EE25E1	P	ng	2.122
EE28E1	P	ng	2.315
EE29E3	ng	EA5*	2.193
EE33E4	P	ng	2.424
EE36E1	ng	LMG13568**	2.299

Ents = enterocins, Bc activity = bacteriocin-enterocin activity, ng = negative or no inhibition

**growth of EA5 was inhibited (zones size > 10 mm), **Listeria innocua* LMG 13568 (University of Ghent/Laboratory of Microbiology, Ghent, Belgium; inhibition zones 12 mm)

and/or secure probable species identification/probable species identification reaching the score 2.053–2.315.

Gelatinase positive phenotype was detected only in the strains EE5E4, EE10E7, EE22E1, EE22E5, EE31E2, EE36E4, and EE37E1 of the species *E. faecalis*. The other enterococci from ewes' lump cheeses showed negative hemolytic phenotype.

The most frequently detected enterocin gene was the gene for Ent P production (Tables 2, 3) followed by Ent A gene. The strains EF1E4, EF2E8, EF28E4, EE1E1, and EE23E5 showed presence of both, Ents A and P genes. Ent B gene was detected only in EF12E1 strain (Table 2); Ent L50B gene was also detected in one strain – EF25E8; however, in this strain Ent P was detected, too. Each of *E. faecium* strains possessed at least one out of four tested Ent genes, while in *E. faecalis* strains four strains were without Ents genes (Table 3). In spite of absence of Ents genes in EE29E3 strain, it showed inhibition activity (its growth was inhibited) against the indicator strain *E. avium* EA5 (inhibition zone > 10 mm); and EE36E1 strain inhibited growth of the indicator strain *L. innocua* LMG 13568 (inhibiton zone 12 mm). On the other hand, among *E. faecium* strains possessing Ents genes, inhibition (bacteriocin-like) activity was only noted in EF27E4 strain against EA5 strain and *L. monocytogenes* CCM4699 with inhibiton zone 10–22 mm.

DISCUSSION

As mentioned above, enterococci are ubiquitous microorganisms and belong to the obligatory gastrointestinal microbiota. In our study enterococci detected in ewes' lump cheeses possibly originated from faecal contamination during milking. Traditionally, the prevalence of enterococci e.g. in milk is considered a result of faecal contamination. For example Hill et al. (2012) reported enterococci in raw milk in the range 10^1–10^4 CFU/ml. The presence of enterococci in dairy foods has conflicting effects – they either represent a risk of foreign or intrusive flora indicating poor hygiene during milk handling and processing, or are beneficial to the production of unique traditional products, in protecting against diverse contaminants, and/or as probiotics (Bhardwaj et al., 2008). However, Birollo et al. (2001) showed that enterococci had a little value as hygiene indicators in the industrial processing of foods. Therefore, enterococci with beneficial properties naturally occurring in milk can act as starter cultures during manufacturing of milk products (Bhardwaj et al., 2008). Different species of enterococci can be detected in raw milk of dairy products (Vrabec et al., 2015) but both the species *E. faecium* and *E. faecalis* are still of the greatest importance. *E. faecalis* often predominates over *E. faecium* (Vrabec et al., 2015). Vrabec et al. (2015) also confirmed predominancy of *E. faecalis* e.g. in traditional Slovak Bryndza cheese, followed by *E. faecium*. Moreover, they also reported the species *E. gilvus*, *E. saccharolyticus*, *E. durans,* and *E. casseliflavus* detected by MALDI-TOF mass spectrometry. In our previous study, dominancy of the species *E. faecalis* and *E. faecium* in Bryndza cheese was confirmed, too, using the molecular method (Woodford et al., 1997).

Cytolysin, a protein toxin that causes a β-hemolytic reaction on erythrocytes, is the most important virulence factor attributed to the genus *Enterococcus*; absence of this gene and/or phenotype among the tested strains indicates their low pathogenicity potential (Ribeiro et al., 2011). Gelatinase is a proteolytic enzyme (extracellular metalloendopeptidase EC 3.4.24.30) that acts on a variety of substrates such as insulin-beta chain, collagenous material in tissues, vasoconstrictor endothelin-1, as well as sex-pheromones and their inhibitor peptides (Waters et al., 2003). Production of gelatinase for example increased pathogenicity in an animal model (Singh et al., 1998). Furthemore, gelatinase can cleave fibrin suggested to have important implications in virulence of *E. faecalis*, as the secreted protease can damage host tissue and thus allow bacterial migration and spread. Gelatinase coding gene is common among enterococci from different origins, but it is frequently silent due to deletions in the *fsr* operon resulting in the absence of gelatinase-positive phenotype (Semedo et al., 2003). As mentioned above, seven *E. faecalis* strains were gelatinase phenotype positive. Although *gelE* gene detection was not provided in this study, the seven gelatinase positive strains were excluded from the testing to possess genes for enterocins production.

Enterococci are able to produce antimicrobial peptides – enterocins (Franz et al., 2007); enterocins or the strains-producing enterocins are promising for dairy industry or veterinary medicine (Lauková, Czikková, 2001; Giraffa, 2003, Lauková et al., 2015a). The highest frequency of Ents genes A, P in our study correlates with the results of Strompfová et al. (2008) testing a target of 427 enterococci from different sources and Ent A and P genes were the most frequent. Also Ent A gene was exclusively found in *E. faecium* strains while Ents P, B, and L50B genes were detected in both species *E. faecium* and *E. faecalis*. As shown from our results and also previously reported, the detection of Ent structural gene does not mean the real inhibition activity/enterocin production. On the other hand, the lack of detectable antimicrobial activity does not mean the genes involved in bacteriocin production are defective; we can discuss about silent genes. Some factors influencing bacteriocin production such as media, temperature, environment, etc. were reported by Lauková (1992) or Qin et al. (2001). Although the limited target of enterococci from Slovak ewes' lump cheeses was tested for Ents genes occurrence and bacteriocin activity, the research has contributed to the general knowledge regarding the enterocin genes distribution.

However, it is necessary to keep in mind the obligatory assessment of enterococci and evaluation of each strain intended for commercial use. Such a strain must be authorized for health claims and strictly meet the requirements of EFSA Regulation (EC) No. 1924/2006 (Piskoríková, 2010) including a qualified presumption of the strain safety. But the most important is to assess the absence of transferable genes responsible for resistance and virulence factors. From the positive side, enterococci are closely involved in the production of traditional fermented foods and probiotic preparations (Franz et al., 1999). Morover, in our previous studies, beneficial effects of enterocins *in situ* in dairy products, meat products, feed and *in vivo* in animals were manifested by antimicrobial reduction of spoilage microflora, by stimulating unspecific immunity parameters (phagocytic activity), by increasing daily weight gain in food-animals, by anti-*Eimeria* effect, etc. (Lauková et al., 2012, 2015b; Pogány Simonová et al., 2015).

CONCLUSION

E. faecium and *E. faecalis* strains were isolated from Slovak ewes' lump cheeses. Negative hemolytic and negative gelatinase activity strains showed mostly

genes for enterocins P and A production; three strains showed inhibition activity against *E. avium*, *L. innocua,* and *L. monocytogenes*. *E. faecium* EF27E4 strain was selected for more detailed studies. To our knowledge, this study was the first to test the presence of enterocins genes in enterococci from Slovak cheeses.

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