OCCURRENCE OF *ENTEROCIN* GENES IN ENTEROCOCCI FROM SLOVAK MILK PRODUCT ŽINČICA*

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Žinčica is a popular Slovak dairy product made from ewes' milk. It is a by-product resulting during ewes' lump cheese processing. Microbiota in Žinčica have rarely been studied, especially enterococci; however, they can produce beneficial substances – bacteriocins. In this study, the presence of *Enterocins* (*Ents*) genes were analysed in enterococci from Žinčica and partially also the inhibition activity. Samples of Žinčica were collected from different agrofarms producing ewes' lump cheese (34) in Central Slovakia. In the enterococci tested, *Ent* P gene was the most frequently detected (in 6 out of 7 enterococci), followed by *Ent* A and *Ent* L50B genes. *Ent* B gene was detected only in *E. faecium* 30E1. On the other hand, *E. faecalis* 31E2 did not contain *Ent* genes, although it showed inhibition activity against the indicator strains *Enterococcus avium* EA5, *Staphylococcus aureus* SA5, *Listeria monocytogenes* CCM4699 (inhibition zone sizing up to 20 mm). *E. faecium* 30E1 contained genes of four *Ents*; however, it showed no inhibition activity. Growth of the four indicators was inhibited due to the antimicrobial activity of *E. faecium* 32E1 with *Ent* P gene detection. This is the first study reporting on the occurrence of *Ent* genes in enterococci from Žinčica.

Traditional milk product, enterococci, bacteriocin, inhibition

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INTRODUCTION

Ewes' milk is a substance used in the manufacture of traditional dairy products in Slovakia such as ewes' lump cheese produced in chalets (OA EC 2010/C20/9), Slovak Bryndza-Liptauer cheese (OA EC 2007/C232/10) or Žinčica (Kolosta et al., 2014). Žinčica is a by-product of ewes' lump cheese production (Laukova et al., 2019). Whey from ewes' milk is heated to 90–95°C with continuous shaking. At this temperature, proteins in the whey start coagulating and locate on the surface of the whey. After the coagulation, the proteins are skimmed from the surface and cooled to acidifying temperature (50°C). After further shaking, the vessel is kept covered up till the next day to acidify the product at least to pH 5.2. High quality Žinčica has to be lightly acid with a delicious lactic acid taste similar to that of ewes' milk. Dairy products from ewes' milk have a long tradition in Slovakia and they are very popular among consumers; they have benefits on human health (Herian, 2014).

Enterococci are a controversial group of bacteria. On the other hand, they represent one of the largest lactic acid-producing genera from the phylum Firmicutes. Fifty-eight species have been validated (Euzeby, 1997; last updated 2018, www.bacterio.net/enterococcus.html). Many enterococci, especially those isolated from food (Giraffa, 2003; Franz et al., 2011) or food-producing animals (Laukova et al., 1993), possess probiotic/beneficial properties and/or produce

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

	Primers	Lenght of target genes (bp)	References
Ent A	F5'R5'-CCC TGG AAT TGC TCC ACC TAA-3' R5'-CCC TGG AAT TGC TCC ACC TAA-3'	452	Aymerich et al. (1996)
Ent P	F5'-GCT ACG CGT TCA TAT GGT AAT-3' R5'-TCC TGC AAT ATT CTC TTT AGC-3'	216	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'	159	Casaus et al. (1997)
Ent L50B	F5'-ATG GGA GCA ATC GCA AAA TTA-3' R5'-TAG CCA TTTT TTC AAT TTG ATC-3'	280	Cintas et al. (1998)

antimicrobial proteinaceous substances – bacteriocins, mostly enterocins (Laukova et al., 1993; Franz et al., 2007). In our previous studies (Laukova et al., 2001; Laukova, Czikkova, 2001), a beneficial effect of enterocins was shown e.g. in Slovak Bryndza (Liptauer cheese) experimentally infected with *Listeria innocua* and treated with enterocin CCM4231. A difference by one order of magnitude was noted indicating the inhibition effect against *Listeria* due to enterocin CCM4231 (Laukova, Czikkova, 2001). Moreover, an antagonistic effect of enterocin CCM4231 against *L. monocytogenes* Ohio (experimentally infected in Saint-Paulin cheese) was reported (Laukova et al., 2001). Previously the enterococci determined from Žinčica (Laukova et al., 2019) were hemolysis negative and mostly absent of the virulence factors genes typical for enterococci (Laukova et al., 2019). Moreover, these strains were mostly susceptible to antibiotics tested for their phenotype (Laukova et al., 2019). Therefore, we decided to check isolated enterococci for bacteriocins/enterocins genes. Strains of the species *Enterococcus faecium*, *E. faecalis*, *E. durans*, *E. mundtii* are known to have bacteriocin activity or bacteriocin genes which in enterococci are represented mostly by enterocins (Marekova et al., 2003; Franz et al., 2007). Enterocins are proteinaceous substances with antimicrobial activity against more or less related bacteria (Franz et al., 2007). In our study, four enterocin genes were analysed, and the inhibition/antagonistic activity against four main indicator strains was tested: *Enterococcus avium* EA5 (our strain isolated from faeces of piglet), *Staphylococcus aureus* SA5 from mastitis milk (our isolate), *Listeria monocytogenes* CCM4699, clinical isolate from Czech Culture Collection of Microorganisms (Brno, Czech Republic) and *L. innocua* LMG13568 from Belgium Coordinated Collection of Microorganisms in Ghent (Belgium). The aim of this study was to contribute with results to the basic research. Genes for *Enterocins* production have not been evaluated in enterococci from Žinčica, but bioactive enterococci possessing mostly *Enterocins* genes *Ent* A and *Ent* P were detected in ewes' lump cheese (Laukova et al., 2012; Laukova, Strompfova, 2016).

MATERIAL AND METHODS

Isolation and idendification of enterococci

Samples of Žinčica were supplied by different agrofarms producing ewes' lump cheese (34) in Central Slovakia. They were treated by the standard microbiological method (ISO 7899-2:2000). A volume of 100 µl was spread on Slanetz-Bartley agar plates (Merck, Darmstadt, Germany) and incubated at 37°C for 24–48 h (Laukova et al., 2019). Grown colonies were controlled for purity and identified using a matrix-assisted laser desorption ionisation time-of-flight mass spectrometry (MALDI-TOF MS) based on protein 'fingerprints' (MALDI-TOF MS; Bruker Daltonics, Billerica, USA) (Alatoom et al., 2011), performed using a Microflex MALDI-TOF mass spectrophotometer (Microflex, Leipzig, Germany) as was described in our previous study by Laukova et al. (2016). Moreover, polymerase chain reaction (PCR) confirmation was provided using the primers and protocol according to Woodford et al. (1997).

Detection of structural genes for Enterocin production

The *Enterocins* (*Ents*) genes (*Ent* A, P, B, L50B) used were selected based on our previous results (Strompfova et al., 2008) showing the most frequently detected *Ents* genes in different enterococci. The primer 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) (Table 1) and PCR conditions were as follows: 5 min denaturation at 95°C, 30 cycles for 30 s at 95°C, 30 s at 58°C, 30 s at 72°C; 5 min at 72°C, and 94°C. Annealing temperature for Ent P, L50B

Table 2. *Enterocins* genes occurrence and inhibition activity in enterococci from Žinčica

Strain	*Ent* genes	Indicator strains inhibited	Size of inhibition zone (mm)
E. faecium 30E1	A, P, L50B, B	no	–
E. faecium 32E1	P	EA5, SA5, 4699, 13568	20, 10, 20, 20
E. faecium 34E1	A, P	no	–
E. faecium 34E5	A, P, L50B	no	–
E. faecalis 30E4	P	EA5	20
E. faecalis 31E2	no	EA5, SA5, 4699	20, 10, 20
E. faecalis 35E1	A, P, L50B	no	–

genes for Enterocins A, P, L50B, B: *Enterococcus avium* EA5, *Staphylococcus aureus* SA5, *Listeria monocytogenes* CCM4699, *L. innocua* LMG13568; no = growth of indicator strains was not inhibited

and B was 56°C instead of 58°C. The PCR product was visualized by 2% agarose electrophoresis (1 µg ethidium bromide).

E. faecium EK13/CCM7419 (Marekova et al., 2003) was used as positive control for Ent A, P; *E. faecium* L50 (Cintas et al., 1997, 1998) for Ent L50B and B. Template (2 µl) was added to 8.75 µl of the reagent mixture which contained 0.5 µl of each primer, 1 µl (10 mmol l^{-1}) of nucleotide triphosphates containing deoxyribose called dNTPs (Invitrogen, USA) 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).

Antagonistic activity testing

The qualitative method according to Skalka et al. (1983) was used to test the antagonistic activity of the strains with *Ents* genes. The principal indicator strains such as *Enterococcus avium* EA5 (isolate from faeces of piglet, our laboratory), *Staphylococcus aureus* SA5 (our isolate from mastitis milk), *Listeria monocytogenes* CCM 4699 (clinical isolate from Czech Culture Collection of Microorganisms -CCM, Brno, Czech Republic), *L. innocua* LMG13568 (kindly supplied by professor Luc De Vuyst, University of Brussels, Belgium; Belgium Coordinated Collection of Microorganisms, Ghent, Belgium) were used. Antagonistic activity was expressed as clearing inhibition zones around the producing strain (in mm). *E. avium* EA5 is our most susceptible strain used in bacteriocin activity testing. The rest of indicator strains were used as representatives of frequent milk contaminants. Briefly, the Brain heart agar plates (Difco Laboratories Inc., USA) inoculated with tested enterococci were incubated overnight at 37°C. Then, the plates were overlaid with 2.5 ml of soft agar (0.7%) seeded with 200 µl of overnight cultures of indicator organisms (absorbance at 600 nm – OD600; 0.4–0.6). The plates were incubated overnight and widths of the clear inhibition zones were measured (in mm). *Enterococcus faecium* CCM4231 (our isolate deponed in CCM) was used as the bacteriocin-producing positive control.

RESULTS AND DISCUSSION

Seven out of 27 strains isolated from Žinčica were allotted to two species, *Enterococcus faecium* and *E. faecalis* (Table 2) using the taxonomic analysis (MALDI-TOF spectrometry, phenotypization) as previously reported by Laukova et al. (2019). Žinčica is a popular product associated with ewes' lump cheese production. It has a beneficial effect on human organism (Herian, 2014). In literature there is only limited information regarding the microbiota in special Slovak lactic acid milk products, especially in Žinčica. Enterococcal species detected in Žinčica (*E. faecium* and *E. faecalis*) belong to those species in the genus *Enterococcus* which were described at first (Franz et al., 2011).

E. faecalis 31E2 strain was *Ents* genes free (Table 2). Gene for *Ent* P was detected in most strains (except EE31E2). In the strains *E. faecium* 32E1 and *E. faecalis* 30E4 only *Ent* P gene was detected (Table 2). *Ent* A gene was found in four strains: *E. faecium* 30E1, 34E1, 34E5 and *E. faecalis* 35E1. Gene for *Ent* L50B was detected in two *E. faecium* strains (30E1, 34E5) and in *E. faecalis* 35E1. *Ent* B gene (for Ent B production) occurred only in *E. faecium* strain 30E1 (Table 2). The most frequent was *Ent* P gene (in six enterococcal strains) followed by *Ent* A gene (in four strains) and then by *Ent* L50B gene. However, in spite of *Ent* genes detection in *E. faecium* strains 30E1, 34E1 and 34E5, they did not show any inhibition

activity against four main indicator strains (Table 2). Although *E. faecalis* 35E1 possessed *Ent* A, P, L50B genes, it did not show an inhibition activity against the indicator strains. On the other hand, *E. faecalis* 31E2 did not have *Ent* genes; however, the growth of three indicator strains (*E. avium* EA5, *S. aureus* SA5 and *L. monocytogenes* CCM4699) was inhibited by its substance (size of inhibition zones up to 20 mm). The growth of all indicators was inhibited due to antimicrobial activity produced by *E. faecium* 32E1 strain (it has *Ent* P gene). Similarly, the inhibition of EA5 indicator was demostrated due to *E. faecalis* 30E4 possessing *Ent* P gene (Table 2). *E. faecium* 32E1 strain containing *Ent* P gene seems to be the most active strain; inhibition of four indicators was noted with inhibition zones sizing 10–20 mm (Table 2). Inhibition zones sizing 10 mm were measured against *S. aureus* SA5 strain due to all bacteriocin active enterococcal strains. The growth of *E. avium* EA5 and listeriae was inhibited with inhibition zones 20 mm. The main indicator *E. avium* EA5 and listeriae as well were more susceptible to inhibition activity of the tested enterococci than *S. aurues*. The species *E. avium* belongs to the same genus (*Enterococcus*) as the tested strains, although in *E. avium* group; listeriae are known to be predominantly inhibited using enterococcal bacteriocins – enterocins (F r a n z et al., 2007).

Enterococci identified from Žinčica contained mostly *Ent* A and *Ent* P genes which were also found in 368 strains of *E. faecium* and 59 strains of *E. faecalis* of different origin reported by S t r o m p f o v a et al. (2008). Among 81 food-derived *E. faecium* and *E. faecalis* strains (from fermented sausages), the most frequently detected was *Ent* A gene (in 24 strains) followed by *Ent* P gene (in 23 strains), *Ent* L50B gene (in 5 strains) and *Ent* B gene (in 4 strains) (S t r o m p f o v a et al., 2008). The most frequently detected *Ents* genes (*Ent* P, A genes) noted also in our study are in accordance with those detected in enterococci from ewes' lump cheeses (L a u k o v a et al., 2016). In addition, only *Ent* A gene was present in nine *E. faecium* strains isolated from mastitis milk (L a u k o v a , S t r o m p f o v a , 2016). M a r k o v a , M a r k v a r t o v a (2017) reported bacteriocin-producing enterococci of different species such as *E. faecium*, *E. faecalis*, *E. durans*, *E. gilvus* isolated from cheeses made from unpasteurised milk and two sourdoughs. However, gene for bacteriocin (unspecified) production was present only in *E. faecium* strains isolated from cheeses (M a r k o v a , M a r k v a r t o v a , 2017). They did not test inhibition activity. Oppositely, L a u k o v a et al. (2012) presented *E. durans* strains from sheep lump cheese with predominant occurrence of *Ent* P gene; however, in that study the inhibition substances only from two strains (*E. durans* ED7E9 and ED26E7) inhibited the same indicator strains as are those used in the current study (*E. avium* EA5, *L. monocytogenes* 4699 and *L. innocua* LMG13568) with inhibition activity ranging from 100 to 800 arbitrary units per ml (AU ml^{-1}). Some strains with detected *Ent* genes did not inhibit the growth of indicator strains. It does not mean that they were defective; we can suppose silent genes (Qin et al., 2001) or, of course, more indicator strains should be included in testing. Detection of *Ent* structural genes in strains may not always correspond with their inhibition activity. On the other hand, the strains, in which *Ent* genes were not detected, can show inhibition activity against indicator strains. It could be explained by the fact that the strains may contain an *Enterocin* gene type which we did not test. Similarly as in the enterococci from Žinčica, L a u k o v a et al. (2016) reported inhibition (antimicrobial) activity probably due to antimicrobial substances produced by other enterococci which were, however, lacking *Ent* gene. *Ent* genes detected in enterococci isolated from Žinčica usually belong to Class II enterocins, which are thermo-stable small peptides, mostly with a broad inhibition spectrum (F r a n z et al., 2007).

Although a limited target of enterococci from Žinčica was tested for *Ents* genes occurrence and bacteriocin activity, the study contributes to the general knowledge regarding the *Enterocin* genes distribution. Moreover, it presents novel information regarding the properties of enterococci isolated from Žinčica. In further studies we will continue in testing the inhibition activity and attempt to purify the most active substance resulting from the tested strains.

CONCLUSION

Ent P gene was detected in 6 out of 7 enterococci tested. It was the most frequently detected *Ent* gene in enterococci isolated from Žinčica followed by *Ent* A gene and L50B gene. *Ent* B gene was found only in *E. faecium* 30E1. On the other hand, *E. faecalis* 31E2 did not possess *Ent* genes, although it showed inhibition activity against *E. avium* EA5, *S. aurues* SA5 and *L. monocytogenes* CCM4699 (inhibition zone sizing up to 20 mm). *E. faecium* 30E1 contained genes for four *Ents*; however, it did not show antibacterial activity against indicator bacteria. Oppositely, growth of four indicator strains was inhibited due to antimicrobial activity of *E. faecium* 32E1 which contained *Ent* P gene. This is the first study reporting on the occurrence of *Ent* genes in enterococci from Žinčica. A study of antimicrobial activity including a larger group of indicator bacteria is in progress.

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