IJBPAS, June, 2016, 5(6): 1185-1194

ISSN: 2277–4998

International Journal of Biology, Pharmacy and Allied Sciences (IJBPAS)

'A Bridge Between Laboratory and Reader'

www.ijbpas.com

COW MASTITIS MILK AS A SOURCE OF BACTERIOCIN ACTIVE ENTEROCOCCI

LAUKOVÁ A[1*], STROMPFOVÁ V[1].

1: Institute of Animal Physiology Slovak Academy of Sciences

Šoltésovej 4-6, 040 01 Košice, Slovakia

[*]Corresponding address: E Mail: laukova@saske.sk; Tel.+421556330283,

Fax:+421557287842

ABSTRACT

Enterococcal counts from cows mastitis milk reached 1.59 ± 0.2 (log10) CFU/mL in average. Isolated strains were allotted to the species *Enterococcus faecium* (PCR). The average value of lactic acid in enterococci was 1.24 ± 0.11 mmol/L. *Ent* A gene was detected in all tested strains. Bacteriocin activity of enterococci lead to growth inhibition of *L. monocytogenes* P7562, CCM 4699 and *E. faecalis* EE5a by the qualitative method (inhibition zones 16-22 mm). *E. fecium* EF21 was selected for partial purification of its bacteriocin substance. The partially purified substance 21 (PPB) showed activity 25 600 AU/mL against the principal indicator *L. monocytogenes* P7562 (by the quantitative method). Moreover, the growth of *Staphylococcus aureus* SA5 from mastitis milk was inhibited, *L. monocytogenes* CCM 4699 and *E. faecalis* EE5a (6 400 AU/mL). PPB 21 is thermo-stable substance (activity 25 600 AU/mL) and it was stable at -20 ºC even after 2 months storage (25 600 AU/mL). First production of bacteriocin by EF21 strain was detected after 2 h of its broth cultivation (200 AU/mL); the highest activity was measured in its exponential growth phase of EF21 strain.

Key words: **Cow Mastitis milk, Enterococci, Bacteriocin, Activity**

INTRODUCTION

Mastitis represents the most economically important disease in dairy milk production worldwide. Among the most frequent agents causing mastitis are included *Streptococcus uberis, Str. pyogenes, Str. agalactiae, Str. dysgalactiae, Staphylococcus aureus* as well as some of coagulase-negative staphylococci **[1, 2, 3].** Wagner et al. **[4]** reported also excretion of *Listeria monocytogenes* in a subclinical

case of mastitis. In addition, the species *Enterococcus faecalis* or *E. faecium* were detected among microbiota in cows mastitis milk **[1].** Enterococci, especially the species *E. faecium* is known to produce antimicrobial active peptides-bacteriocins (mostly enterocins) which can inhibit the growth of more or less closely related bacteria **[5, 6].** There are bacteriocins which can serve as the alternatives to antibiotics in prevention or treatment of mastitis. Up to now bacteriocin/lantibiotic lacticin 3147 and nisin were experimentally applied to prevent mastitis **[7].** Moreover, ubericin A produced by *Streptococcus uberis* E was described by Heng et al**. [1]** or uberolysin produced by *Str. uberis* 42 **[8].** Our laboratory (Institute of Animal Physiology, Slovak Academy of Sciences, Košice, Slovakia) has been focused on bacteriocin (enterocin) studies since 1993 year. Many enterocins produced by *E. faecium* strains of different origin were characterized and applied in different environments such as food, food-producing or companian animals with beneficial **[9, 10, 11, 12].** Following our studies, the aim of this study was to check bacteriocin active enterococci isolated from cows mastitis milk (from herds in Slovakia) to select the candidate strain for further detail studies.

MATERIALS AND METHODS

Samples treatment, bacteria identification, lactic acid estimation

The isolates originated from 138 milk samples of 69 cows from different herds in Slovakia. Sampling was provided following the Guide for the Care of the Animals with acceptation of farmers and Slovak Veterinary and Food Administration. Samples were treated by the standard microbiological method (ISO 7899, International Organization of Standardization); appropriate dilutions were plated onto the selective media: M-Enterococcus agar (Becton and Dickinson, Cockeysville, USA) and Chromocult agar (Merck, Darmstadt, Germany). Plates were incubated in a 5 % gaseous (CO_2/air) atmosphaere at 37 ºC for 48 h. Presumptive colonies were picked up, checked for purity and genotyped by PCR (Techgene KRD thermocycler Techne, United Kingdom); the sequence of the primer pairs of *Enterococcus faecium* was as follows: 5‘-GCAAGGCTTCTTAGAGA-3‘ and 5‘-CATCGTGTAAGCTAACTTC-3‘ **[13].** Nine genotyped *E. faecium* strains were selected for the next analyses.

Lactic acid production was tested by the spectrophotometric method based on the conversion of lactic acid to acetaldehyde by heat from sulfuric acid. Acetaldehyde reacts with *p*-hydroxybiphenyl forming colour

complex. The value of lactic acid is expressed in mmoL/L.

Determination of *Enterocins* genes

Before testing bacteriocin activity of selected strains, the presence of genes for enterocin (Ent) production was tested. Taking into account our previous knowledge concerning the detection of *Ent* genes **[14],** *Ent* A, P, B and L50B were checked. The sequences of the primer pairs used for PCR-amplification of the structural *Ent* A, P, L50B, B genes are listed in Table 1. The reaction conditions for *Ent* A included 5 min denaturation at 95 °C, followed by 30 cycles of 30 s at 95 °C, 30 s at 58 °C, 30 s at 72 °C **[15];** then by 5 min at 72 °C and a cool down to 4 °C. For *Ent* P, L50B and B, 56 °C instead of 58 °C was used as the annealing temperature. Template (2 μL) was added to 8.75 μL of the reagent mixture which contained 0.5μL each of the primers, 1 μL of (10 mM) dNTPs, 1.5 μL of (5 mM) $MgCl_2$, 5 μL of 10 x reaction buffer, 0.25 μL of 1 U Taq polymerase (Invitrogen) and water to the total volume 50 μL. The sequences of the primer pairs used for PCR products were visualised by 2% agarose electrophoresis, containing 1 μg of ethidium bromide. Positive control strains for PCR reactions were *E. faecium* EK13= CCM7419 for *Ent* A (P) (9), *E. faecium* L50 strain for *Ent* L50B and B **[16, 17, 18].**

***In vitro* bacteriocin activity testing and partial purification of bacteriocin**

Bacteriocin activity was tested by the qualitative method **[19]** against the indicator bacteria (Table 3). Inhibition of indicator bacteria was expressed as an average of the inhibition zones size in mm. Based on inhibion activity, *E. faecium* EF21 was selected for partial purification procedure. Partial purification followed the method previously described by Mareková et al. **[9].** Briefly, 18 h culture of EF21 strain in MRS broth (300 mL, Merck) was centrifuged (MR1812, Jouan, France) for 30 min at 10, 000 *g* in order to remove the cells. After adjusting of the supernatant to pH 5.0 ammonium sulphate was added to the supernatant to obtain 40% (w/v) saturation. Mixture was stirred at 4 ºC for 24 h. After centrifugation (10, 000 *g* for 30 min), the pellet was resuspended in the minimal volume of sodium phosphate buffer (pH 6.5). The activity of PPB 21 was checked by the quantitative agar spot test **[20]** and expressed in Arbitrary units per mL (AU/mL); it responses to the highest dilution of PPB which inhibited the growth of indicator strains. There six principal indicator bacteria were used: *E. avium* EA5 (our isolate from piglet), *Listeria monocytogenes* P2024, P7562 (Veterinary Institute, Olomouc, Czech Republic), CCM4699 (Czech Culture Collection of

Microorganisms, Brno, Czech Republic), *E. faecium* EF 9a, *E. faecalis* EE5a (our isolates from rabbits, Table 3).

Thermo- stability and molecular mass of PPB

PPB samples were exposed to heating under the different temperatures (30 ºC for 1h, 2h, 60 ºC for 30 min, 1h, 80 ºC for 10, 30 min, 100 ºC for 5, 10 min). To test the stability of PPB during different storage conditions, PPB was stored at 20 ºC (laboratory temperature), 4 ºC and -20 ºC for 1, 2 weeks, 1, 2 months. The molecular mass of PPB was estimated by using Microcon YM-3 and YM-10 centrifugal filters (Amicon, Milipore Corporation, Bedford, Maryland, USA). Ultrafiltration with filters was carried out with 500 µL of PPB according to the instructures of manufacturers. After centrifugation, the filtrate and retentate were separated and tested by the agar spot test (the samples after heating and different storage as well) against the principal indicator strain *L. monocytogenes* P7562. Activity was expressed in AU/mL.

Bacteriocin production during EF21 strain growth

E. faecium EF21 was grown in MRS broth (Merck) in water bath with shaking (Julabo SW20, Germany). The growth of producer strain was checked by measuring the optical density ($OD_{600,}$ Specol 11, Jena, Germany) each 2-1 h, respectively and appropriate dilutions of samples were spread onto M-Enterococcus agar (Becton and Dickinson) to enumerate growing cells. At the same time the samples were taken, centrifuged and concentrate (Concentrator plus, Eppendorf AG, Germany) to estimate the bacteriocin activity as former mentioned. Moreover, pH of samples was measured during EF21 strain growth (pH Meter 3310, Jenway, England).

RESULTS

Enterococcal counts in cows mastitis milk samples grown on M-Enterococcus agar and on Chromocult agar were detected in the same level 1.59 ± 0.2 (log10) CFU/mL in the average. Nine strains were allotted to the species *E. faecium* by PCR method **(Table 2).** The average value of lactic acid in enterococci was 1.24 ± 0.11 mmol/L. The strains from the same cow reached both, the highest (1.44 ± 0.0 mmol/L) and the lowest (1.07± 0.0 mmo/L) values of LA **(Table 2).**

Ent A gene was detected in all tested strains. The other tested *Ent* genes were not present in our strains. Using the qualitative method for bacteriocin activity testing, the growth of *L. monocytogenes* P7562, CCM 4699 was inhibited by all isolates (inhibition zones 16-22 mm) as well as the growth of *E. faecalis* EE 5a strain (18-20 mm). However, by this method tested

strains did not show inhibition activity against *E. avium* EA5, *E. faecium* EF 9a as well as against *L. monocytogenes* P2024. *E. faecium* EF21 strain inhibited the growth of the indicators mentioned (inhibition zones 22 mm); it was selected for partial purification of its antimicrobial substance.

PPB of EF21 reached activity 25 600 AU/mL against the principal indicator strain *L. monocytogenes* P7562 (tested by the quantitative method, **Table 3**). Target of 36 indicator bacteria was used. The growth of SA5 strain (from mastitis milk) was inhibited as well as *L. monocytogenes* CCM 4699 and *E. faecalis* EE 5a with activity 6 400 AU/mL. The growth of other staphylococci as well as Gram-negative strains up to now used were not inhibited. However, the best stability of PPB 21 was detected under storage at -20 ºC and 4ºC; after 2 months still activity 25 600 AU/mL was determined; the least stable was PPB 21 at storage under laboratory temperature **(Table 4**). The great result is that PPB 21 was active with the initial activity, i.e. 25 600 AU/ml under all temperatures from 30 ºC up to 100 ºC from 5 min up to 2 h. During growth of EF21 strain, the highest activity was detected in the exponential growth phase (12 800 AU/mL). First production of bacteriocin substance was noted after 2 h of EF21 strain cultivation (200 AU/mL, **Table 5**). The molecular mass of PPB 21 is in the range 3-10 kDa.

DISCUSSION

The enterococcal counts from cows mastitis milk on both media were the same; up to 10^2 CFU/mL. It seems, that both selective media were enough sensitive to enumerate enterococci. However, Miranda et al. **[21]** reported more reduced development of the species *E. faecium* observed on Chromocult enterococci agar; there poultry samples were checked. Enterococci did not represent the principal causative agents of mastitis; however, they have been detected among microbiota of mastitis milk **[1].**Their occurrence could be explained e.g. by the external contamination of teets (hygiene aspect). However, the presence of enterococci in milk can be beneficial because of their bacteriocin activity. Their antimicrobial, preventive effect can be graduated by both, by bacteriocin and/or by lactic acid produced by them. *Ent* A has belonged to the first sequenced bacteriocins-enterocins **[15].** It is the most frequently detected Ent among the isolates of *E. faecium* species; *e.g.* among 368 strains of *E. faecium* of different origin, *Ent* A gene was found in 155 **[14].** Also De Vuyst et al. **[22]** reported the priority of *Ent* A gene occurrence in the collection of 426 Enterococcus strains. Those authors stated no relationship could

be established between the presence of *Ent* structural genes and the origin of the strain either. The majority of Ents characterized up to now are represented by small, thermo-stable peptides **[6].** Stability of bacteriocin substances is important factor at least from its application possibilities tools. For this reason, bacteriocin should be stable to improve its benefits. Therefore, stability of PPB 21 has indicated its for detail on molecular base established studies, but also for its further application. Based on our previous results with Ents applied in animal husbandry, they showed antimicrobial effect, but after their application increase of e.g. phagocytic activity was noted **[23].** After Ent M application (produced by *E. faecium* AL41 strain of environmental origin), phagocytic activity was increased even 3 weeks after its cessation **[23, 11].** Similarly, when Ent 4231 or Ent 7420 were applied **[24, 11],** the counts of clostridiae and coagulase-positive staphylococci were decreased. At present, there are limited groups which have worked with applications of Ents in animal husbandry and/or veterinary medicine. Up to know obtained results underline a real benefits of Ents. Recently, a new enterocin T belonging to Class IIa enterocins was described **[25]** produced by Enterococcus sp. from broccoli. Our results indicate that enterocins have possessed capacity to be used in mastitis prevention or agents limitation; in spite of *in situ* analysed bacteriocins produced by real mastitis pathogens. Of course, the other studies and experiments have to be processed.

In conclusion, bacteriocin active *E. faecium* strains (from 138 mastitis milk samples of 69 cows in Slovak farms) were isolated. Selected strain *E. faecium* EF21 produces small, thermo-stable substance with the highest production (12 800 AU/mL) in the exponential growth phase. Partially purified bacteriocin (PPB21) reached even activity 25 600 AU/mL against the indicator strain *L. monocytogenes* P 7562; against *S. aureus* SA5 activity was 6 400 AU/mL. The substance was active even after 2 months of storage at -20 ºC. Taking into account up to now obtained results, it could be probably belonged to Class II enterocins.

ACKNOWLEDGEMENTS

Results were achieved under financial support by the Agency for Science and Research Support-APVV, project ENZYBIO 0586-7. We are grateful to Dr. Juraj Elečko for milk sampling and transportation.

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Table 1: The sequences of the primer pairs used for PCR-amplification of the structural genes

Enterocins genes		
Ent A	F	5' – GGT ACC ACT CAT AGT GGA AA - 3'
R		5' – CCC TGG AAT TGC TCC ACC TAA - 3' (Aymerich et al. 1996)
Ent P	F	5' – GCT ACG CGT TCA TAT GGT AAT-3'
R		5' – TCC TGC AAT ATT CTC TTT AGC - 3' (Cintas et al. 1997)
EntL50B	F	5' – ATG GGA GCA ATC GCA AAA TTA - 3'
R		5' – TAG CCA TTT TTC AAT TTG ATC - 3' (Cintas et al. 1998)
Ent B	F	5' – CAA AAT GTA AAA GAA TTA AGA TCG - 3'
R		5' –AGA GTA TAC ATT TGC TAA CCC - 3' (Casaus et al. 1997)

Table 2: The strains of *Enterococcus faecium*, *Enterocin* genes and lactic acid values

Strain	Ent A	Ent P	Ent B	EntL50B	Lactic acid*
EF21	+	-	-	-	1.29 ± 0.08
EF31	+	-	-	-	1.39 ± 0.06
EF41	+	-	-	-	1.37 ± 0.03
EF61	+	-	-	-	1.29 ± 0.03
EF101	nt	nt	nt	nt	1.44 ± 0.00
EF102	+	-	-	-	1.07 ± 0.07
EF62	+	-	-	-	1.17 ± 0.25
EF1111	nt	nt	nt	nt	1.09 ± 0.02
EF34/PZ	nt	nt	nt	nt	1.06 ± 0.03

***Enterococcus faecium*; Lactic acid was expressed in mmol/L ± SD; nt: not tested; +:presence of *Ent* gene; -:absence of *Ent* gene**

Table 3: Indicator strains used in the quantitative bacteriocin method to test activity of PPB 21

Indicator	
P7562	25 600 AU/mL
P2024	-
CCM4699	6 400 AU/mL
E. faecalis 5a	6 400 AU/mL
S. aureus SA5	6 400 AU/mL

***Listeria monocytogenes; E. avium* EA5 was not inhibited, *S. aureus* – 4, S. *epidermidis* – 6 strains, *S. warneri*-10 strains from meat manufactures, *Enterococcus faecium* EF9a from faeces of rabbits; *Salmonella* sp. – 10 strians from faeces of rabbits were not inhibited by PPB** 21.

Table 4: Activity of PPB 21 under different temperatures during different storage

PPB21	LT	4 °C	-20°C
1w	6 400	12 800	25 600
2ws	1 600	12 800	25 600
1m	400	6 400	25 600
2ms	400	6 400	25 600

PPB-partially purified substance, LT-laboratory temperature; Activity is expressed in AU/mL; w, ws: week, weeks; m, ms:month, months;

Table 5: Bacteriocin production by *E. faecium* EF21 strain during its growth in MRS broth

	0h	2h	4h	6h	8h	10h	11h	24h
pH	6.57	6.55	6.37	5.35	4.94	4.77	4.73	4.48
OD_{600}	0.069	0.070	0.158	1.047	1.257	1.270	1.274	1.260
CFU	4.57	5.53	7.07	8.09	8.40	9.20	9.36	9.39
BA	0	200	800	1 600	3 200	6 400	12 800	12 800

BA:Bacteriocin activity expressed in AU/mL; CFU:bacterial counts expressed in colony forming unit per mL; OD_{600} : optical density at 600 nm;