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Original article

Domestic fowl of ducks, a source of faecal bioactive *Enterococcus hirae* strains

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Abstract

Faecal *Enterococcus hirae* from domestic ducks were studied for their bioactivity to select bioactive strain for more detailed study with its probable use in poultry and also to bring novelty in basic research. After defecation, faeces (n=23, faecal mixture of 40 ducks) were sampled from domestic ducks in eastern Slovakia; birds were aged from eight to 14 weeks. *E. hirae* strains were identified using Matrix-assisted laser desorption/ionization time-of flight mass spectrometry with a highly probable species identification score (2.300-3.000) or a secure genus identification/ /probable species identification score (2.000-2.299), confirmed by polymerase chain reaction and phenotypization in accordance with the properties for the type strain *E. hirae* ATCC 9790. Strains were hemolysis negative (γ-hemolysis), and did not have active enzyme stimulating disorders. *Enterocin* genes were detected in three strains out of seven. Three out of four *Enterocin* genes were detected in Kč1/b (*Ent* A, P, L50A); the most frequently detected was the *Ent* P gene. The strains inhibited indicator strains *E. faecalis*, listeriae, but also *Escherichia coli* and *Buttiauxiella* strains. Lactic-acid producing *E. hirae* were mostly susceptible to antibiotics. Based on parameter evaluation, *E. hirae* Kč1/b, Kč6 can be additionally studied to select the type of bioactive substance.

Key words: ducks, *Enterococcus hirae*, mass spectrometry, PCR, bioactivity, properties

Introduction

Ducks *(Anas platyrhynchos f. domestica)* belong to Order Anseriformes, and to Family *Anatidae*. In Slovakia, this order represents widespread fowl. The duck belongs to the group of food-producing animals because its meat is tasty and is a culinary delicatesy (Haščík et al. 2009). Therefore, the health and quality of ducks and their meat is of interest to breeders, owners and consumers. Enterococcosis has been reported in a variety of avian species worldwide. Enterococcal species isolated from birds with clinical disease have been reported e.g. the representatives of the species *Enterococcus avium*, *E. durans*, *E. faecalis*, *E. faecium* and *E. hirae* (Morishita 2017). Strains of *E. hirae* were isolated from the duodenal epithelium of chicks with diarrhoea. Based on analysis of 16S rRNA (16S ribozomal ribonucleic acid, Franz et al. 2011) gene similarity, the species *E. hirae* was allotted to the *E. faecium* group of enterococci. On one hand, *E. hirae* can cause bacteriaemia in mammals or birds (Dicpinigaitis et al. 2015); strains can possess

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hemolysin which may play a role in the pathogenesis of blood-borne enterococcal infections. On the other hand, enterococci are also obligatory microbiota in the intestinal tract of poultry and other birds (Devriese et al. 1991). Some enterococcal species, e.g. *E. faecium*, and *E. durans,* were reported to have beneficial, probiotic properties or to be able to produce antimicrobial substances-bacteriocins/enterocins, which can inhibit undesirable microbiota; this means that microbiota in the host are optimized (Lauková et al. 1993, Franz et al. 2007, 2011). Up to now, enterocins were classified to several classes: Class I represented by lantibiotic enterocins, Class II including thermo--stable, small peptides with three subgroups: enterocins of the pediocin Family, enterocins synthesized without a leader peptide and other linear non pediocin-like enterocins, Class III involves cyclic antibacterial peptides, and Class IV involves large proteins (Franz et al. 2007). The majority of enterocins characterized up to now belonged to Class II enterocins including hiracin (Hassan et al. 2015). Production of bacteriocins is one of the beneficial properties of the identified strain, often showing a beneficial effect in animals (Ciganková et al. 2004, Umu et al. 2016, Lauková et al. 2017a).

Therefore, the aim of this study was to test the properties of faecal *E. hirae* strains isolated from domestic ducks. We were more focused on finding bioactive strains and bringing novelty to basic research with possibilities of selection a bacteriocin-producing strain for more detailed study with its probable use in poultry breeding. In relation to this aim, after taxonomic allotment, the following parameters were analysed: hemolysis, lactic acid production, enzymatic activity, structural genes for enterocin production, bioactivity (inhibiton activity).

Materials and Methods

Sampling and strain isolation

Ducks were located in ducks aviaries with straw bedding with the possibility of moving freely to a meadow and having a water area available. They were fed ground barley, nettle and a sufficient amount of green pasture; this represented 50% of the diet. They had water *ad libitum*. Immediately after defecation**,** the faeces (n=23, faecal mixture of 40 ducks) were sampled from domestic ducks in eastern Slovakia with the agreement of the duck owners. The birds were aged from eight to 14 weeks. Faecal samples in sterile bags were transferred to our laboratory for microbial analyses. They were treated according to the standard microbiological method – the International Organization for Standardization (ISO); 1g of faeces was stirred in Ringer solution (pH 7.5, Merck, Darmstadt, Germany) using the Stomacher-Masticator (Spain) and diluted in Ringer solution (Merck, ratio 1:9). The appropriate dilutions were spread on M-Enterococcus agar (Difco Detroit, USA; ISO-7899). The plates were incubated at 37°C for 24-48 h. The purity of presumed colonies was checked on Brain heart agar (Difco) enriched with 5% of defibrinated sheep blood. Pure colonies were submitted for identification.

Strains identification

Strain identification was performed using Matrix assisted laser desorption/ionization time of-flight mass spectrometry based on protein "fingerprints" (MALDI-TOF Mass Spectrometry, Bruker Daltonics, Maryland, USA), with a Microflex MALDI-TOF mass spectrometer. Briefly, a single colony from M-Enterococcus agar (Difco) 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 (wavelength 337 nm, frequency 20 Hz). Lysates of bacterial cells were prepared according to the producer's instructions (Bruker Daltonics, USA). Results were evaluated using a MALDI Biotyper 3.0 (Bruker Daltonics, USA) identification database. Taxonomic allotment was evaluated on the basis of highly probable species identification (value score 2. 300 - 3. 000) and secure genus identification/probable species identification (2. 000 - 2. 299). Positive controls were those involved in the identification system. Identical colonies evaluated by the MALDI-TOF score value were excluded. Strains were also genotyped using the polymerase chain reaction method - PCR (Techgene, United Kingdom) followed by agarose electrophoresis in 0.8 % agarose gel (Sigma-Aldrich, Darmstadt, Germany) buffered with 1xTAE (Tris Acetate EDTA buffer, Merck) containing 1 µg/ml of ethidium bromide (Sigma). The molecular mass standard (Promega, Wisconsin, USA) was used according to the manufacturer's instructions. Deoxyribonuclease acid – DNA (template) from each strain was isolated by the rapid alkaline lysis method (Baelae et al. 2001). The sequences of the primer pairs used for PCR-amplification of *Enterococcus hirae* were 5´-TCTTGATGCCGATG-3´ and 5´-ATCCTTCGCGGAAT-3´ (Invitrogen, Massachusetts, USA, El-Kharroubi et al. 1991) following a protocol according to Woodford et al. (1997). *E. hirae* DCH5 (Sanchéz et al. 2008) was the positive control. Additionally, the strains were phenotyped using a commercial BBL Crystal Gram-positive ID System kit (Becton and Disckinson, Cockeysville, USA);

the control strain was *E. hirae* DCH5 (Sanchéz et al. 2008). For the following analysis, the identified strains were maintained on M-Enterococcus agar (Difco) and stored using the Microbank™ system (Pro-Lab Diagnostic, Richmond Hill, Ontario, Canada).

Hemolysis and lactic acid production

Hemolysis was detected by streaking the cultures on De Man-Rogosa-Sharpe (MRS) agar (Difco) supplemented with 5% defibrinated sheep blood. The plates were incubated at 37°C for 24-48 h under semi-anaerobic conditions. Presence/absence of clearing zones around the colonies was interpreted as β-hemolysis and negative γ-hemolysis respectively (Semedo-Lemsaddek et al. 2013).

Lactic acid (LA) was analysed using the validated spectrophotometric method and expressed in millimols per liter (mmol/l). This method is based on the conversion of lactic acid to acetaldehyde by heating sulfuric acid. Acetaldehyde reacts with 4-hydroxybiphenyl, forming a color complex.

Evaluation of enzyme production

Enzymatic activity was tested using the commercial API-ZYM system (Biomérioux, Marcy L'Etoile, France) following the manufacturer's recommendation. Evaluated enzymes involved in Table 2 were: 1: alkaline phosphatase, 2: esterase (C4), 3: esterase lipase (C8), 4: lipase (C14), 5: leucine arylamidase, 6: valine arylamidase, 7: cystine arylamidase, 8: trypsin, 9: α-chymotrypsin, 10: acid phosphatase, 11: naftol-AS-BI-phosphohydrolase; 12: α-galactosidase, 13: β-galactosidase, 14: β-glucuronidase, 15: α-glucosidase, 16: β-glucosidase, 17: N-acetyl-β-glucosamonidase, 18: α-manosidase, 19: α-fucosidase. Inocula (65 µl-microliter) of McFarland standard one suspensions were pipetted into each well of the kit. Enzymatic activities were evaluated after 4 h of incubation at 37°C and after the addition of Zym A and Zym B reagents. Color intensity values from 0 to 5 and their relevant value in nanomoles (nmol) were assigned for each reaction according to the color chart supplied with the kit.

Antibiotic phenotype analysis

Antibiotic phenotype in identified *E. hirae* strains (100 µL of an 18-h culture of each strain) was tested by the qualitative agar disc diffusion method on Columbia agar (Becton and Dickinson) enriched with 10% of defibrinated sheep blood (Clinical and Laboratory Standards Institute method-CLSI 2016). Thirteen antibiotic discs (Oxoid, Basingstoke, United Kingdom, Lach-Ner, Czech Republic) were applied: clindamycin (2 µg), novobiocin (5 µg), ampicillin (10 µg), penicillin (10IU), erythromycin (15 µg), azithromycin (15 µg), streptomycin (25 µg), chloramphenicol (30 µg), rifampicin (30 µg), tetracycline (30 µg), vancomycin (30 µg), kanamycin (30 µg), and gentamicin (120 µg). After incubation at 37ºC for 18 h, the strains were evaluated as resistant or sensitive according to the manufacturer's instruction; the inhibition zone was expressed in mm. Antimicrobial free agar plates were included as a control for obligatory strain growth. The use of the antimicrobial agents was decided according to the manufacturer's guidance. *Enterococcus hirae* ATCC 9790 was a positive control.

Structural genes for enterocin and hiracin production

Enterocin (*Ents)* genes tested were: *Ent* A (P), L50A, L50B and *hiracin* JM79. They have been detected most frequently in different enterococci previously (Strompfová et al. 2008); in *E. hirae* the *hiracin* gene was analysed (Sanchéz et al. 2008). Primer sequences for PCR amplification of *Ents* genes were according to Aymerich et al. (1996) for Ent A, according to Cintas et al. (1997, 1998) for Ent P, L50A and L50B as follows: 5 minutes (min) denaturation at 95°C, 30 cycles of 30 secondes (sec) at 95°C, 30 sec at 58°C, 30 sec at 72°C; 5 min at 72°C and 94°C. Annealing temperature for Ent P, L50B and L50A was 56°C instead 58°C. Primers for the *hiracin* gene (Sanchéz et al. (2008) and the protocol were as follows: 2 min denaturation at 97°C, 35 cycles of 45 sec at 94°C, 30 sec at 59°C, 3 minutes at 72°C. Annealing for hiracin was 72°C. PCR product was visualized by 2% agarose electrophoresis (1 µg ethidium bromide). Positive controls and primers used were: *E. faecium* EK13/CCM7419 (Mareková et al. 2003) for Ent A (P); *E. faecium* L50 (Cintas et al. 1998) for Ent L50B, L50A and for *E. hirae* DCH5 (kindly provided by Dr. Carmen Herranz from the University of Veterinary Medicine, Madrid, Spain). Template (2 µ) was added to 8.75 µl of the reagent mixture which contained 0.5 µl of each primer, 1 µl of (10 mmol/l) dNTPs (deoxynucleotide sets, Invitrogen) and water to a total volume of 50 µl. The primer for hiracin was that for hiracin JM79 (Sánchez et al. 2008). DNA (template) was extracted by the rapid alkaline lysis method (Baelae et al. 2001).

Bioactivity testing

Bioactivity (antimicrobial/inhibition) activity of the strains possessing *Ents* genes was tested using the qualitative agar diffusion method according to Skalka et al. (1983) against the principal indicator (the most susceptible strain *Enterococcus avium* EA5,

isolated from piglet, our laboratory). However, together 85 indicator strains were tested out of five different genera. Beside the EA5 strain, the other indicators were *Listeria monocytogenes* CCM 4699 (Czech Culture Collection, Brno, Czech Republic), 15 *L. monocytogenes* strains from different meat products (Katarína Sýčevová, Veterinary Institute, Olomouc, Czech Republik), *L. innocua* LMG13568 (University of Brussels, Belgium), 32 *Enterococcus faecalis* strains (faeces, appendix of rabbits, our strains), *Staphylococcus aureus* SA5 (our strain from mastitis milk), 10 *S. aureus* (trout intestines), two *E. hirae* (Kč5 from duck and EHS from serval (wild cat, our isolates). *Escherichia coli* (20) were isolated from the faeces of ostriches and five strains, *Buttiauxiella agrestis* (BA2/109/1, BA4/1/2/2), *B. ferragutiae* BF1/1/2 from red deer; *B. gaviniae* BG 8/143/1 from roe deer and BG 5/107/1 from red deer (our isolates). Briefly, Brain heart agar plates (Difco) inoculated with tested enterococci were incubated overnight at 37°C. Plates were overlaid with 2.5 ml of soft agar (0.7%) seeded with 200 µl of overnight cultures of indicator organisms (OD_{600}=0.4-0.6). After overnight incubation widths of the clear inhibition zones were measured (in mm). The evaluation score was assessed using the size of the inhibition zones, from five to 10 mm (+), 10-20 (++) and more than 20 (+++). *Enterococcus faecium* CCM 4231 (our ruminal strain) was used as a bacteriocin-producing positive control.

Results

Seven *E. hirae* strains were identified from the faeces of 40 domestic ducks *(Anas platyrhynchos f. domestica*) using MALDI-TOF mass spectrometry. The evaluated score value for *E. hirae* Kč1/b was 2. 289, for Kč2/b it was 2. 333; Kč4 reached a score of 2.193. *E. hirae* Kč5 had a score of 2.29, Kč5a was evaluated with a score of 2. 401, *E. hirae* Kč6 had 2. 072 and Kč7 had 2. 183. Strain Kč2/b was evaluated on the basis of highly probable species identification (2. 300-3. 000) and the other six strains were evaluated on the basis of secure genus identification/probable species identification (2. 000-2. 299). Moreover, allotment of strains to the *E. hirae* species was confirmed also by PCR. In addition, phenotypic properties were mostly in accordance with those for the type strain *E. hirae* ATCC 9790 in Bergey's Manual (De Vos et al. 2009); reaction to the esculin test was positive; arginin, glucose, lactose, fructose and trehalose fermentation were positive, sucrose showed a variable reaction and mannitol reaction was negative. *E. hirae* strains were evaluated with γ-hemolysis meaning hemolysis did not occur.

E. hirae Kč2/b, Kč5 and Kč5a were not tested for *Ents* genes. Strain Kč7 was free of tested *Ents* genes. The *E. hirae* Kč1/b strain had three out of four *Ent* genes (*Ent* A, P and L50A); Kč4 and Kč6 strains possessed the *Ent* P gene and Kč6 also the *Ent* A gene. The *Ent* L50B gene was not detected in *E. hirae* strains and the *Ent* L50A gene was present only in the Kč1/b strain. Among *Ents* genes, the *Ent* P gene (in three out of four tested strains) was the most frequently detected.

E. hirae from ducks did not show inhibition activity against the principal strain *E. avium* EA5. However, the growth of indicator *E. hirae* EHS (from serval) was inhibited by all strains tested besides *E. hirae* Kč6 (Table 1) In *E. hirae* Kč5a and Kč4 inhibition zones measured 14 mm (++), in the other strains inhibition zones reached up to 10 mm (+). *E. hirae* Kč1/b showed inhibition activity against all 32 *E. faecalis* strains with inhibition zones wider than 20 mm in 10 strains (+++) and 22 strains were inhibited with inhibition zones ranging from 10 to 20 mm (++) (Table 1). *E. hirae* Kč6, Kč7 were active with inhibition activity against 31 *E. faecalis*; *E. hirae* Kč6 had inhibion zones mostly in size from 10 to 20 mm (++, 25 strains), three strains were inhibited with zones wider than 20 mm (+++) and three strains were with zones up to 10 mm (+). Inhibition activity of Kč7 reached more than 20 mm against three strains (+++); 27 strains of *E. faecalis* were inhibited with inhibition zones sized 10-20 mm (++) and one strain up to 10 mm (+, Table 1). Kč5a was active against two strains of *E. faecalis* (inhibition zones + and ++, 18mm); Kč2/b, Kč4 and Kč5 did not inhibit the growth of *E. faecalis* strains. The growth of *S. aureus* strains was not inhibited. Listeriae were not inhibited by the strains Kč2/b, Kč4, Kč5, and Kč5a. *E. hirae* Kč1/b, Kč6 and Kč7 possessed bacteriocin-like activity inhibiting the growth of 12 out of 15 listeriae (Table 1); the growth of *L. innocua* LMG 13568 was not inhibited, *L. monocytogenes* CCM 4699 (clinical strain) was inhibited by Kč6 and Kč7 (inhibition zones sized 12, 15 mm, ++). Kč1/b was active against 12 strains of *L. monocytogenes* (out of 15 used) with inhibition zones more than 20 mm (+++) in 10 strains and in the 10-20 mm (++) range regarding two strains. Kč6 showed activity against 13 listeriae (inhibition zones from 10 to 20 mm against 11 listeriae (++) and two strains were inhibited with inhibition zones more than 20 mm (+++); Kč7 also showed inhibition activity against 13 strains; inhibion zones in 8 strains measured from 10 to 20 mm (++); in five strains inhibition zones reached more than 20 mm (+++). Regarding the *E. coli* used as the indicator strains (20), the growth of at least eight strains was inhibited due to the bioactivity of *E. hirae* (Table 1). *E. hirae* Kč7 was active against 17 *E. coli* out of 20, nine strains with a zone

Table 1. Bacteriocin activity of *Enterococcus hirae* strains isolated from domestic ducks.

	Strains						
	Kč1/b	Kč2/b	Kč4	Kč5	Kč5a	Kč6	Kč7
E. hirae							
EHS	1/1*	1/1	1/1	1/1	1/1	1/0	1/1
E. faecalis							
32 strains	32/32	32/0	32/0	32/0	32/2	32/31	32/31
L. m.+L.i.							
15	17/12	17/0	17/0	17/0	17/0	17/13	17/13
E. coli							
20	20/14	20/8	20/15	20/14	20/12	20/16	20/17
B. agrestis							
2	2/1	2/1	2/2	2/2	2/2	2/2	2/2
B. gaviniae							
2	2/1	2/1	2/1	2/2	2/1	2/1	2/1
B. ferragutiae							
1	1/0	1/0	1/0	1/0	1/1	1/1	1/1

* number of tested strains and number of inhibited strains. Range of activity: + means 5-10 mm size of inhibition zone; ++ means 10-20 mm size of inhibition zone; +++ more than 20 mm size of inhibition zone; The growth of *E. hirae* Kč5 (used as an indicator) was not inhibited by the tested strains as well as 10 *Staphylococcus aureus* strains. Moreover, also *E. avium* EA5 was not inhibited by the strains tested. L.m. –*Listeria monocytogenes*, L.i.-*Listeria innocua*; B.-*Buttiauxiella*, *E. coli*-*Escherichia coli*; *E. faecalis*--*Enterococcus faecalis*

of 10-20 mm (++) and eight strains with a zone up to 10 mm (+). *E. hirae* Kč6 was active against 16 strains of *E. coli*; six *E. coli* were inhibited reaching zones sized 10 to 20 mm (++); 10 strains had zones up to 10 mm (+). Kč4 was active against 15 *E. coli* with inhibition zones up to 10 mm (+) in all. However, this strain did not inhibit listeriae, *E. faecalis* strains or *S. aureus*. *E. hirae* Kč2/b and Kč5 were active against 14 strains of *E. coli* (zones more than 20 mm against one strain); zone sized 10-20 (++) was reached against six strains and in seven strains the zone was up to 10 mm (+). In Kč5a, 12 out of 20 *E. coli* strains were inhibited with zones up to 10 mm (+). *B. gaviniae* and *B. agrestis* were mostly inhited (+) (Table 1) as well as *B. ferragutiae*; in Kč7 inhibition zone was 11 mm (++). In general, the most bioactive were *E. hirae* Kč7, Kč6 and Kč1/b; they inhibited 45 Gram-positive indicators out of 60, and 21 out of 25 Gram-negative (Kč7); Kč6 inhibited 44 out of 60 Gram-positive indicators, and 20 out of 25 Gram-negatives; Kč1/b was active against 45 out of 60 Gram-positive and 16 out of 20 Gram-negative indicators. The least active *E. hirae* were Kč2/b, Kč4, Kč5 (they inhibited only one out of 60 indicators) and Kč5a inhibited three out of 60 indicators. In general, the *E. hirae* tested showed better bioactivity against Gram-negative indicators (Table 1); altogether the growth of at least 10 out of 25 indicators was inhibited (Table 1).

Testing the enzymes generally associated with intestinal disorders (α-chymotrypsin, β-glucuronidase, β-glucosidase, N-acetyl-β-glucosaminidase) was negative in *E. hirae* strains or results were within the limit (Table 2). The beneficial enzyme β-galactosidase was, however, also negative.

Lactic acid (LA) values were well balanced and they ranged from 1.31 ± 0.14 to 1.75 ± 0.32 mmol/l. The average value of LA was 1.51 ± 0.32 mmol/l. *E. hirae* Kč2/b reached the highest value of LA (1.75 ± 0.32 mmol/l) followed by Kč1/b strain (1.71 ± 0.30). The lowest value of LA was measured in the Kč4 strain (1.31 ± 0.14). *E. hirae* Kč5, Kč5/a, Kč6 and Kč7 produced the following amounts of LA:1.53 ± 0.23, 1.40 ± 0.10, 1.48 ± 0.21, 1.43 ± 0.19 mmol/l.

E. hirae were mostly susceptible to antibiotics. All strains were susceptible to vancomycin, kanamycin, novobiocin, penicillin, azithromycin,chloramphenicol, erythromycin. However, *E. hirae* Kč2/b, Kč4, Kč5 and Kč5a were resistant to gentamicin, clindamycin and streptomycin. The Kč4 strain was also resistant to tetracycline and Kč1/b to streptomycin. *E. hirae* Kč4 was polyresistant, meaning it was resistant to four antibiotics (streptomycin, clindamycin, tetracycline and gentamicin). *E. hirae* Kč5a was bi-resistant (clindamycin, gentamicin) and *E. hirae* Kč6 and Kč7 were susceptible to 13 tested ATBs.

Table 2. Enzymatic profile of *Enterococcus hirae* strains isolated from domestic ducks.

	Strains						
	Kč1/b	Kč2/b	Kč4	Kč5	Kč5a	Kč6	Kč7
1	5	5	5	5	5	5	5
2	30	30	10	30	20	10	20
3	30	20	20	30	30	20	20
4	0	0	0	0	0	0	0
5	10	10	0	30	10	5	0
6	0	0	0	0	0	0	0
7	10	10	0	10	0	0	0
8	0	0	0	0	0	0	0
9	10	10	0	10	10	5	0
10	10	20	20	30	10	10	0
11	10	20	20	10	10	10	0

Enzymes 12-19 (α-galactosidase, β-galactosidase β-glucuronidase, α-glucosidase, β-glucosidase, N-acetyl-β-glucosamonidase, α-manosidase, α-fucosidase) were negative in strains of *E. hirae* except β–glucosidase in Kč1/b, Kč7 which showed activity of 20 nmol/l, and β-galactosidase in Kč5/a with 5 nmol.

Discussion

The species *Enterococcus avium*, *E. durans*, *E. faecalis*, *E. faecium* and *E. hirae* have been reported as the most detected enterococcal species in birds with clinical disease (Morishita 2017). However, Murphy et al. (2005) detected various microbiota in wild New Zealand *Mallard ducks* using 16S rRNA analysis. They found *E. caecorum* as the most frequently detected species from the genus Enterococcus; this species belongs to the *E. cecorum* group of enterococci (based on 16S rRNA analysis) reported by Franz et al. (2011). In our previous studies regarding poultry-derived enterococci, *E. hirae* was the dominant species in faeces of ostriches and pheasants (Lauková et al. 2016, 2017b).

As formerly mentioned, the species *E. hirae* belong to the genus Enterococcus and to the group *E. faecium* (Franz et al. 2011). Moreover, this genus (allotted to the phylum Firmicutes) belongs among lactic acid producing bacteria. This could probably explain, why LA values measured in the tested *E. hirae* strains were similar or lower than those for ruminant-derived *E. faecium* strains reported by Lauková (1999). In addition, Kandričáková (unpublished data) reported similar LA values measured in faecal strains of *E. hirae* from ostriches and pheasants.

Bacterial glucuronidase plays a role e.g. in colon cancer and this enzymatic activity was negative in our *E. hirae* strains. On the other hand, β-galactosidase helps to ferment lactose and it was also negative. Enzyme activity testing shows that our bioactive strains do not have positive reaction for enzymes stimulating disorders.

Surprisingly, *E. hirae* possessing an inhibition activity inhibited the growth of Gram-negative strains. Although a lower number of Gram-negative indicators was used, their growth was mostly inhibited. Enterocins (bacteriocins mostly produced by enterococci, especially by the species *E. faecium)* are known to reduce coliforms in *in vivo* experiments. Pogány Simonová et al. (2009) reported a decrease of *E. coli* in broiler rabbits after bacteriocin-producing, probiotic strain application. The important result is *Buttiauxiella* strain inhibition; up to now no information was reported for *Buttiauxiella* in association with this analysis.

The most bioactive were Kč1/b, Kč5a, Kč6 and Kč7 strains; however, the Kč4 strain also inhibited 15 out of 20 coliforms and other Gram-negative strains. It could be interesting to repeat testing with raw bacteriocin using a quantitative method and additional indicators to provide for more detail in the studied strain. However, the *hiracin* gene was not detected. On the other hand, the bioactive strain Kč7 did not possess *Ents* genes; it can probably produce a different bioactive substance. Genes for the production of Ent A, P and L50A were detected in the Kč1/b, which was also bioactive against indicators, and this strain is also susceptible to ATBs similarly to *E. hirae* Kč6. In our case the tested antibiotic phenotype resulted in mostly susceptible bacteria. Splichalová et al. (2015) isolated *E. hirae* from coraciiform birds which were susceptible to vancomycin and erythromycin; no genes were detected there. Moreover, Kandričáková et al. (2015) reported faecal *E. hirae* from pheasants, not hemolytic, and they were susceptible to vancomycin, erythromycin, ampicillin, chloramphenicol and penicillin, they did not produce trypsin and produced similar values

of LA as the strains in our study (1.78 ± 0.33 mmol/l).

Bioactive bacteria have been found in different environments (Nes et al. 2014). Bacteriocin-producing (bioactive) enterococci have been isolated from the faeces and gastrointestinal tract of animals. Bioactive substances (enterocins) originated from Gram-positive bacteria require many more genes for their active production than do those of Gram-negative bacteria (Nagao et al. 2006). Enterococcal bacteriocins are known to have an antimicrobial spectrum against more or less related bacteria (Nes et al. 2014) involving also coliforms, pseudomonads and salmonallae (Lauková et al. 2004, 2017). Moreover, raw or pure enterocins were found to stimulate unspecific immunity by increasing phagocytic activity in broiler rabbits or laying hens (Pogány Simonová et al. 2013, Lauková et al. 2017a) or even due to Ent A, a reductive effect was demonstrated in gnotobiotic Japanese quails experimentally infected with the *Salmonella Enterica* serovar Duesseldorf (Lauková et al. 2004). Karrafová et al. (2015) reported a beneficial effect on IgA production and secretion in the intestines of chickens challenged with *S. Enteritidis* after application of the Enterocin M- producing strain *E. faecium* AL41. To know that the other enterococcal species are bioactive widens the horizons of basic science and also provides a chance to find a probably new bioactive substance from a new source. For example, Gupta et al. (2016) purified the Enterocin LD3 from *E. hirae* LD3, originating from fermented food. In our study listeriae were mostly inhibited.

In conclusion, having in mind the bioactive strains Kč1/b, Kč6, Kč7, which do not form enzymes stimulating disorders and which are mostly susceptible to antibiotics and also possessing *Ents* genes (besides Kč7), this information enriches the horizons of basic science and indicates these strains in more detailed studies of bioactive substances.

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