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Original article

Characteristic and susceptibility to enterocins of enterococci in pheasants possessing virulence factor genes

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Abstract

With an increasing number of pheasants as gamebirds being reared each year, these species are becoming a more prominent part of the workload of many veterinary practices. Only limited information can be found concerning the microflora of common pheasants. A significant part of the obligate microflora consists of lactic acid bacteria, including enterococci. In this study, faeces were sampled from 60 pheasants aged 16-17 weeks. Enterococcal counts reached 5.48±1.9 (log10) CFU/g. Strains (17) were taxonomically classified to the genus Enterococcus using the Maldi-Tof identification system; they were allotted to the species *E. hirae* (58.8%), *E. faecium* (23.5%) and *E. faecalis* (17.7%) by highly probable species identification or by secure genus identification/probable species identication. Species allocation was also confirmed using conventional biochemical tests. Most strains formed β-hemolysis. Gelatinase active phenotype was found in three *E. faecalis* strains. Enterococci were β-glucuronidase negative, mostly trypsin negative with slight or moderate production of α-chymotrypsin. EH52b and EF42 strains possessed the highest potential for pathogenicity. Average value of lactic acid was 1.78±0.33 mmo/L. Most strains were tetracycline resistant (82.4%). Polyresistant *E. faecalis* strains with positive gelatinase phenotype and possessing virulence factor genes confirmed using PCR (*gel*E, efaAfs, *ccf cob, cpd)* were sensitive to enterocins (activity 1600-25 600 AU/mL).

Key words: faecal enterococci, common pheasant, characterization, susceptibility, enterocin

Introduction

With increasing numbers of pheasants as gamebirds being reared each year, these species are becoming a more prominent part of the workload of many veterinary practices (Welchman 2008). The common pheasant taxonomically belongs in the Kingdom Animalia, Phylum Chordata, Class Aves, Order Galliformes, Family Phasianidae, Genus Phasianus, species *Phasianus colchicus.* Pheasants are bred for meat and, as mentioned above, also as hunting-sport gamebirds. Moreover, pheasant meat represents a culinary delicacy which is supplied to many restaurants. In general, farmers breed a basic pair (male and female), which produces eggs. They collect the eggs and put them in a nest incubator. Chicks aged 6-8 weeks

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are placed into aviaries, where there is a significant loss of birds. The biggest risks for hens are rain and cold weather. Farmers strive to keep their animals in a good healthy state. Normal intestinal microflora is very important in the development of the immune system of hosts. Only limited information can be found concerning the microflora of common pheasants. A significant part of the obligate microflora consists of lactic acid bacteria (LAB). Enterococci comprise the third-largest genus of LAB after the genera *Lactobacillus* and *Streptococcus* (Franz et al. 2011). They are widespread bacteria commonly found in the intestines of humans and other animals (Devriese et al. 1995). From the taxonomical point of view, enterococci belong in the Kingdom Bacteria, Division *Firmicutes*, Class Cocci, Order Lactobacillales, Family Enterococcaceae, Genus Enterococcus (de Vos et al. 2009). Currently, up to 54 enterococcal species are validly described. On the basis of 16S rRNA gene similarity they fall into seven species groups involving different species: *E. avium* group, *E. cecorum* group, *E. dispar* group, *E. faecalis* group, *E. faecium* group, *E. gallinarum* group, *E. saccharolyticus* group. Strains of some species e.g. *E. faecalis* or *E. hirae* can be potential pathogens and they can cause diseases. On the other hand, some strains can possess beneficial properties; e.g. some *E. faecium* strains have been identified as bacteriocin-producing and probiotic strains (Foulquié Moreno et al. 2006, Strompfová and Lauková 2007, Pogány Simonová et al. 2013). This study therefore focuses on selected properties of enterococci from faeces of common pheasants to produce new insights, and to extend the limited information so far available; moreover, *E. faecalis* strains possessing virulence factor genes were studied for their sensitivity to enterocins with the aim of showing further possible utilization of enterocins, e.g. for elimination of pathogenic agents in aviaries.

Material and Methods

Sampling, bacterial isolation and identification

Faeces from pheasants in the semi-wild environment of western Slovakia (located in aviaries with free movement, aged 16-17 months) were sampled. They were fed commercial feed mixture BZ2 plus for broilers (Hrajnovka, Bratislava, Slovakia) and had access to water *ad libitum*. Fresh faeces were collected in the aviary (from 60 birds) by hand using gloves immediately after being voided by birds; they were put into 6 sterile packs (each pack contained faeces from approximately 10 birds), placed into a transport fridge and driven to our laboratory. Here the samples were treated according to the standard microbiological method of ISO (International Organization for Standardization) using appropriate dilutions; 1g of faecal mixture into 9 mL of Ringer solution (Merck, Germany); samples were stirred using a Stomacher (Masticator, Spain) and diluted. Sampling, bird handling and care were carried out with the approval of the Slovak Veterinary and Food Administration and the breeder. To enumerate enterococci, appropriate dilutions were plated on M-Enterococcus agar (Difco, Maryland, USA, ISO 15214) and incubated at 37°C for 24-48 h. Bacteria were enumerated as an average count of colonies grown in the highest dilution per sample and expressed in colony – forming units per gram of sample (CFU/g ±SD). Fifty randomly picked – colonies were inoculated on M-Enterococcus agar (Difco) to check their purity and submitted for species identification. Species identification was effected by means of the Maldi BioTyper TM identification system (Bruker Daltonics USA) based on analysis of bacterial proteins using Maldi-Tof mass spectrometry. Lysates of bacterial cells were prepared according to the producer's instructions (Bruker Daltonics, 2011) prior to identification. In addition, phenotypic characterization was performed using the commercial BBL Gram-positive Crystal kit (Becton and Dickinson, Cockeysville, USA) to confirm the species following the properties according to de Vos et al. (2009).

Hemolysis forming and gelatinase activity

Hemolysis was detected by streaking the cultures on MRS agar (Difco) supplemented with 5% of defibrinated sheep blood. Plates were incubated at 37°C for 24-48 h under semi-anaerobic conditions. The presence or absence of clearing zones around the colonies was interpreted as β-hemolysis and negative gama-hemolysis respectively (Semedo 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 h at 37°C), plates were flooded with a 15% solution (HgCl2 in 20% HCl). Loss of turbidity halos around colonies was then checked at 4°C (Kanemitsu et al. 2001).

Lactic acid production, enzyme production

Lactic acid was analysed using the validated spectrophotometric method and expressed in mmol/L. This method is based on the conversion of lactic acid to acetaldehyde by heat from sulfuric acid. Acetal-

dehyde reacts with 4-hydroxybiphenyl, forming a color complex.

Enzymatic activity of enterococci was tested using the commercial API-ZYM system (BioMerieux, France) following the manufacturers recommendations. Enzymes evaluated were: alkalic phosphatase, esterase (C4), esterase lipase (C8), lipase (C14), leucin arylamidase, valin arylamidase, cystin arylamidase, trypsin, α-chymotrypsin, acidic phosphatase, Naftol-AS-BI-phosphohydrolase, α-galactosidase, β-galactosidase, β-glucuronidase, α-glucosidase, β-glucosidase, N-acetyl-β-glucosamonidase, α-manosidase, α-fucosidase. Inocula (65 µL) 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 were assigned for each reaction according to the color chart supplied with the kit.

Antibiotic susceptibility testing

Antibiotic susceptibility was evaluated using the disk diffusion method (Clinical and Laboratory Standards Institute method-CLSI, 2012) involving eight antibiotics: ampicillin, gentamicin (Amp, Gm, 10 µg), penicillin (Pnc, 10IU), erythromycin (Ery,15 µg), chloramphenicol, kanamycin, vancomycin, tetracycline (Chc, Kan, Van, Tct, 30 µg, Becton and Dickinson, Cockeysville, USA; Oxoid, Great Britain; Lach-Ner, Czech Republic). *E. faecium* CCM 4231 (Lauková et al. 1993) was the control strain. The strains were cultivated in Brian Heart Infusion (Oxoid) overnight at 37°C. 100 µL was plated onto MRS agar enriched with defibrinated sheep blood (Becton and Dickinson) and disks were applied. The evaluation was done according to the manufacturers instructions; the inhibitory zones were expressed in mm. Antimicrobial-free agar plates were included as a control for obligatory growth. The use of the antimicrobial agents was decided according to the manufacturers guidance.

PCR detection of virulence factor genes in *Enterococcus faecalis* strains

Screening for eight virulence factor genes *gel*E (gelatinase), *agg* (aggregation), *cylA* (cytolysin A), *efaAfs* (adhesin *E. faecalis*), *esp* (surface protein), *ccf*, *cob, cpd* (sex pheromone) was performed using PCR amplification with primers and conditions reported previously by Ribeiro et al. (2011).

Susceptibility of *Enterococcus faecalis* strains to enterocins

Sensitivity of *E. faecalis* strains to enterocins (Ents) was tested using a quantitative agar spot test (De Vuyst et al. 1996) using Brian Heart agar (Becton and Dickinson, Cockeysville, USA). The following Ents were used: Ent EM41, Ent55, Ent4231, Ent EK13=Ent A (P), EntM and Ent2019. Producing strains as well as Ents were isolated and characterized at our laboratory. Semi-purified substances of Ents represent small, thermo-stable peptides. They were prepared as previously described by Lauková et al. (2012b) for EntEM41 (produced by *E. faecium* EM41 from ostrich-Emu); Strompfová and Lauková (2007) for Ent55 (produced by chicken isolate *E. faecium* EF55); Lauková et al. (1993) for Ent4231 (produced by ruminal *E. faecium* CCM4231)*;* Mareková et al. (2003, 2007) for Ents EK13=(A, P) and EntM produced by environmental isolates *E. faecium* EK13=CCM7419 and AL41; Simonová and Lauková (2007) for Ent2019 produced by rabbit isolate *E.faecium* 2019-CCM7420. Inhibitory activity was defined as the reciprocal of the highest dilution producing an inhibitory zone against the indicator strain and expressed in Arbitrary Units per ml (AU/mL) against the principal indicator strain *E. avium* EA5 (from our laboratory). Activity of Ents used reached up to 25 600 AU/mL.

Results

The average count of enterococci in the faeces of pheasants reached 5.48 ± 1.9 (log10) CFU/g. Among 50 strains submitted for identification, 17 strains were taxonomically classified to the genus Enterococcus and to the species *E. hirae* (58.8%), *E. faecium* (23.5%) and *E. faecalis* (17.7 %). The rest of the colonies were excluded from testing on the basis of the results of their purity control or evaluating them as identical on the basis of their Maldi-Tof score value. Ten strains were allotted to the species *E. hirae*, four isolates were detected as *E. faecium* and three strains as *E. faecalis.* Most strains were allotted to the species level by highly probable species identification (13 of 17; 2.300-3.000 or 2.000-2.300) involving eight *E. hirae*, three *E. faecium* and two *E. faecalis* strains. Three strains (2 *E. hirae*, 1 *E. faecalis*) were evaluated through secure genus identification/probable species identication. Only *E. faecium* EF42 was evaluated with a score of 1.949 indicating probable genus identification. All species showed positive reaction concerning the acidification of lactose, sucrose, trehalose, fructose. *E. hirae* and *E. faecalis* showed dubious

Table 1. Number of strains with negative enzymatic activity or with positive activity (in nmoL).

Enzyme	0 nmoL	5 nmoL	10 nmoL	20 nmoL	30 nmoL	40 nmoL
A. phosp.	13	4	0	0	0	0
Esterase	1	0	0	11	4	0
Est. Lip.	0	0	6	7	4	0
Lipase	15	2	0	0	0	0
Leu. aryl.	0	7	2	2	2	4
Val. aryl.	7	4	6	0	0	0
Cys.aryl.	6	4	4	3	0	0
Trypsin	11	3	2	1	0	0
Chymotr.	0	9	3	2	3	0
Ac. phosp.	4	10	3	0	0	0
Naftol	0	15	2	0	0	0
α-gal.	16	1	0	0	0	0
β-gal.	4	9	2	0	1	1
β-glucur.	0	0	0	0	0	0
α-gluc.	10	6	1	0	0	0
β-gluc.	7	5	3	0	1	1
N-acetyl	12	2	1	2	0	0
α-man.	15	2	0	0	0	0
α-fuc.	16	1	0	0	0	0

Alkalic phosphatase, esterase (C4), esterase lipase (C8), lipase (C14), leucin arylamidase, valin arylamidase, cystin arylamidase, trypsin, α-chymotrypsin, acidic phosphatase, Naftol-AS-BI-phosphohydrolase, α-galactosidase, β-galactosidase, β-glucuronidase, α-glucosidase, β-glucosidase, N-acetyl-β-glucosaminidase, α-manosidase, α-fucosidase

reaction for arabinose, but arabinose in *E. faecium* was positive. Glycerol was posive in all strains and hydrolysis of arginine and easculin as well. Among the 17 strains, the most frequently detected species was *E. hirae.*

Six strains did not form hemolysis (*E. hirae* EH33, EH43, EHb41, *E. faecalis* EE62, EE63, EE64). Eleven strains formed β-hemolysis. Gelatinase active phenotype was found in three *E. faecalis* strains (EH62, EH63, EH64).

Table 1 summarizes the numbers of tested strains with negative as well as positive enzymatic activity. Among the 10 *E. hirae* strains, nine were alkalic phosphatase negative; EH52b showed slight production (5 nmoL); *E. faecium* EF42 and *E. faecalis* EE63, EE64 as well. The rest of these two species were alcalic phosphatase negative. Similarly, lipase was negative in all strains (excluding EH52b and EF42-5 nmoL). Esterase Lipase reaction was moderate in the tested strains (10-30 nmoL). Among the 17 strains, only EF42 and EF61 had slight positive α-manosidase (5 nmoL). A slight reaction was also noted in acidic phosphatase, Naftol-AS-BI-phosphohydrolase, α-glucosidase and valin arylamidase;the strains possessed these enzymatic activities but only slight values were measured (5-10 nmoL). Strains were α-glucosidase negative (only EH52b was found with activity 5 nmoL). Values for β-glucosidase were variable (Table 1). Enterococci were mostly N-acetyl-β-glucosaminidase negative. The value of esterase was 20 nmol for 11 strains, in 4 strains 30 nmoL and 1 strain (EF62) 5 nmol. Luecin arylamidase was produced by all strains (5-40 nmoL). Eleven strains showed positive reaction for cystin arylamidase (5-20 nmoL). We focused particularly on β-glucuronidase; however, surprisingly, our strains were negative; α-galactosidase and α-fucosidase were also negative. Most strains did not produce trypsin. Production of α-chymotrypsin was slight or moderate. Strains EH52b and EF42 possessed the highest pathogenicity potential because of the highest values of produced enzymes compared with the other tested strains. The average value of lactic acid was 1.78 ± 0.33 mmol/L. Lactic acid production was balanced among the tested strains.

The isolates were sensitive to vancomycin, penicillin, ampicillin and chloramphenicol. They were also sensitive to Ery; only *E. faecium* EF42 was Ery^R. On the other hand, they were kanamycin resistant. Resistance to gentamicin showed up in eight strains of the

Table 2. Virulence factor gene testing in *Enterococcus faecalis* strains.

Virulence factors genes	*E. faecalis* EE62	*E. faecalis* EE63	*E. faecalis* EE64
gelE	+	+	+
efaAfs	+	+	+
ccf	+	+	+
cob	+	–	+
cpd	+	–	+

Genes *esp, agg, cylA* were not found in tested strains. + positive; – negative.

Table 3. Sensitivity to enterocins of *Enterococcus faecalis* strains.

	Ent EM41	Ent55	Ent4231	EntEK13=EntA(P)	EntM	Ent2019
E. faecalis EE62	12800	25600	1600	25600	12800	25600
EE63	25600	25600	25600	25600	3200	25600
EE64	25600	25600	25600	25600	25600	25600

Activity of enterocins against *E. faecalis* strains is expressed in Arbitrary Units per mL (AU/mL). Producing strains are *E. faecium* EM41 from ostrich-Emu-Ent EM41; Ent55 produced by chicken isolate *E. faecium* EF55; Ent4231 produced by ruminal *E. faecium* CCM4231*;* Ents EK13=(A, P), EntM produced by environmental isolates *E. faecium* EK13=CCM7419 and AL41; Ent2019 produced by rabbit isolate *E.faecium* 2019-CCM7420. Activity of Ents used reached up to 25 600 AU/mL.

17 (47.05%); 5 of *E. hirae*, one of *E. faecium* and two of *E. faecalis*. Most strains were Tct^R (14 strains of 17; 82.4%). Among the Tct^R strains, eight belonged to *E. hirae* and three to *E. faecium* and *E. faecalis. E. faecalis* isolates were found to be polyresistant. Minimal inhibitory concentration was in accordance with antibiotic concentrations in the disks used. Kan^R is typical among especially *E. faecium* strains (chromosomally encoded); however, in our study *E. hirae* and *E. faecalis* were Kan^R. The majority of strains were Tct^R. In general, the enterococci isolated were mostly antibiotic sensitive.

Polyresistant *E. faecalis* strains, which also have a positive gelatinase phenotype, were found in tests to harbour genes for eight virulence factors (Table 2). PCR confirmed the presence of genes *gel*E, adhesin *E. faecalis* (efaAfs) in all *E. faecalis* strains*.* The sex pheromone gene *ccf* was harboured in all three *E. faecalis* strains. Genes *cob* and *cpd* (sex pheromone) were detected only in two strains, EE62 and EE64. Genes *esp* (extracellular surface protein), *agg* (aggregation substance) and *cylA* (cytolysinA) genes were not found in the tested strains. Enterococci can possess the hemolytic phenotype even when no *cyl* gene is detected; in our strains*,* the *cylA* gene did not occur, although 11 strains formed β-hemolysis. In spite of the presence of β-hemolysis, *cylA* genes were not detected in our isolates. Proteases (e.g. gelatinase) are also confirmed in enterococcal pathology. In our *E. faecalis* strains which possessed positive gelatinase phenotype, the *gelE* gene was also detected. The strains were free of *agg* and *esp* genes and they were *efaAfs* gene positive. The strains harboured *ccf*, (2 of 3) *cop* and *cpd* sex pheromone genes.

Although the *E. faecalis* strains found in this study lacked *agg*, *esp* or *cylA* genes, they possessed *gelE, efaAfs, ccp, cop, cpd* virulence factor genes, so they were tested for their sensitivity to enterocins. *E. faecalis* strains were sensitive to all Ents with inhibitory activity reaching 1 600-25 600 AU/mL (Table 3). However, they were more sensitive to Ents 55, 2019 and EK13=A(P); 25 600 AU/mL) than to EntEM41, Ent4231 and/or EntM.

Discussion

The counts of faecal enterococci (5.48 ± 1.9 log10 CFU/g) detected in common pheasants were comparable with the counts of enterococci determined in other poultry (Lauková and Michlovičová, 2009). The strains were allotted to the species *E. hirae, E. faecium* and *E. faecalis.* However, among the 17 strains, the most frequently detected species was *E. hirae.* This is similar to the distribution of enterococci in faeces of e.g. ostriches (Kandričáková et al. 2012). There, *E. hirae* was also prevalent, followed by the species *E. faecium* and *E. mundtii*. However, Goncalvez et al. (2010) detected vancomycin resistant strains *E. durans* and *E. gallinarum* on a farm in southern Portugal. The identified enterococci represent an obligatory species also detected in other poultry (Lauková and Mich-

lovičová, 2009), but they have been detected in other animals as well, such as rabbits or dogs (Simonová and Lauková 2004, Marciňáková et al. 2006). In general, the most detected species in animal faeces is *E. faecium* (Lauková et al. 1993, Lauková et al. 1996, Devriese et al. 1995, Strompfová and Lauková 2007, Lauková et al. 2008). The phenotypic identification was in accordance with those claimed for type strains for the species reported in de Vos et al. (2009). The detected species belong to two groups on the basis of classification involving 16SrRNA gene similarity (Franz et al. 2011): in the *E. faecium* group with the species *E. faecium*, *E. canis*, *E. durans*, *E. hirae* and *E. mundtii* and in the *E. faecalis* group with the species *E. faecalis*, *E. caccae*, *E. haemoperoxidus*, *E. moraviensis*, *E. silesiacus*, *E. termitis*, *E. ureasiticus*, *E. quebencensis*.

The enzyme β-glucuronidase is produced by cells of the liver, kidney or spleen but also by certain intestinal bacteria (Walaszek 1990). Mroczynska et al. (2013) reported their possible production by *E. faecalis*; our tested strains *E. hirae*, *E. faecium* and *E. faecalis* did not produce it; a slight production of β-glucosidase was found in 10 strains (5-10 nmoL); only one strain, *E. hirae* EH52b, produced a high level of this enzyme (40 nmoL). In general, the enterococci reported here showed low pathogenicity except the strains EH52b and EF42.

As mentioned above, enterococci belong among lactic acid producing Firmicutes. Lactic acid production ability presented here is comparable with that in ruminants or other poultry-derived strains (Lauková 1996, Lauková and Michlovičová 2009).

In our study, the majority of strains were Tct^R. We did not observed plasmid content in our isolates, but this could be explained probably by conjugal transfer via plasmid because no therapeutical treatment was applied here. Resistance to antibiotics among *E. faecalis* is frequent; e.g. resistance to Gm has already been reported by Rice et al. (1991). An important finding in our study was no Van resistant isolates; several reports exist concerning Van resistant enterococci from animals (Woo Kyung Jung et al. 2007). However, in general, the enterococci isolated were mostly antibiotic sensitive.

Enterococci can possess a hemolytic phenotype even when no *cyl* gene is detected (Franz et al. 2003). In our case, the *cylA* gene did not occur although 11 strains formed β-hemolysis. Hemolytic/cytolysin activity has been found to be a natural bifunctional component of *E. faecalis* (Gilmore et al. 1990). Cytolysin is considered a virulence factor of *E. faecalis* strains in one animal model (Singh et al. 1998). Despite the presence of β-hemolysis, *cylA* genes were not detected in our isolates. Proteases (e.g. gelatinase) are also confirmed in enterococcal pathology. In our *E. faecalis* strains which possessed a positive gelatinase phenotype, *gelE* gene was also detected. Virulence factor is an effector molecule which enhances the ability of a microorganism to cause disease beyond that intrinsic to the species backround (Mundy et al. 2000). Typical examples for enterococci are mainly aggregation substance, gelatinase, or extracellular surface protein. Agg is a pheromone-inducible surface protein of *E. faecalis* which promotes aggregate formation during bacterial conjugation (Clewel 1993). Esp is an extracellular surface protein which plays a role in adhesion and evasion of the immune response of the host. Its occurrence is related especially to infection-derived *E. faecalis* isolates (Waar et al. 2002). Our strains were free of *agg* and *esp* genes. *EfaAfs* genes were reported as harboured in *E. faecalis* strains of different origin, e.g. clinical and food origin; our strains were *efaAfs* gene positive; this adhesin can probably act as an adaptation to specific environments (Cobo Molinos et al. 2008).

Enterocins are ribosomally synthetized peptides with antimicrobial activity against more or less related bacteria (Franz et al. 2011). *E. faecalis* strains were sensitive to the tested Ents. Sensitivity to Ents of *E. faecalis* strains possessing virulence factor genes indicates the possibility of using Ents for elimination of pathogenic agents, particularly since *in vivo* antimicrobial activity of Ents has been previously reported (Audisio et al. 1999, Lauková et al. 2004, Lauková et al. 2012b, Levkut et al. 2009). Moreover, this provides an opportunity to inhibit the growth of antibiotic-resistant bacteria which so often occur nowdays. Further, more detailed, tests are in progress.

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