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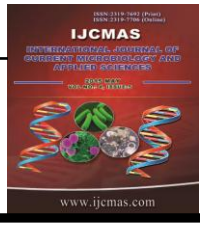

Original Research Article

Staphylococci Detected in Faecal Samples of Common Pheasants and their relation to enterocins

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A B S T R A C T

Limited information are disposable concerning the microflora of common pheasants. Methicillin resistant staphylococci (MRS) can be potential causative agents in pheasants; meat of pheasants is used as food which can threaten health of consumers. This study has been focused on distribution of staphylococci in faeces of pheasants and sensitivity of the isolates to antimicrobials of proteinaceous character-enterocins. Mixture of faecal samples (60 birds aged 16-17 weeks) were tretated by the standard microbiological method, plated on Mannitol Salt agar and Baird-Parker agar to isolate staphylococci. Pure isolates were identified by Maldi-Tof system and phenotyped. Their sensitivity was tested not only to antibiotics but also to enterocins. Staphylococci in faeces of pheasants reached 3.79±0.9 (log10) CFU/ml. Twelwe strains were taxonomically allotted to five species: *Staphylococcus cohnii* subsp. *cohnii*, S. *lentus*, *S. hominis*, *S. succinus* subsp. *succinus*, and *S. hyicus*. Biochemical tests confirmed the species allottation (positive catalase, fructose, trehalose, lactose, mannitol, sucrose; tests of arginine dihydrolase, urease, alkalic phosphatase were variable). These coagulase-negative strains (except SHy53) produced lactic acid (1.210 ± 0.10 mmol/l in average). The most of strains were sensitive to antibiotics except polyresistant strains SCo61 and SHy53. However, tested strains, methicillin resistant *Staphylococcus cohnii* SCo61 incuding were sensitive to enterocins (100-25 600 AU/ml).

Introduction

Common pheasant taxonomically belongs to the Class Aves, Order Galliformes, Family Phasianidae, Genus Phasianus, species *Phasianus colchicus.* It is well-known gamebird; one of the most hunted birds over the world. Besides that, pheasants are also breeded for meat, which has high nutritive value; the content of high quality protein and low fatness. It has been considered to belong to healthy animal products (Kuzniacka *et al.*, 2007; Lukasziewicz *et al.*, 2011). Meat of pheasant belongs to the culinary delicacy which is supplied to the many restaurants. Therefore, the most important role for farmers is to maintain good health status of pheasants. Obligatory

intestinal microflora is very important in the development of the immune system; animals can resist the invading of pathogenic or spoilage bacteria and enhance the ability to resist infection (Xu Shulin and Shen Xiuli, 1998). Up to now, only limited information can be found concerning the microflora of common pheasants. Xu Shulin and Shen Xiuli (1998) reported bifidobacteria, lactobacilli, bacteroidacae as dominant microflora in pheasants aged 10-30 days. In our previus studies, lactobacilli and enterococci were detected in faeces of pheasants (Kandričáková, 2014; Kandričáková and Lauková, 2014). These bacteria belong to lactic acid–producing Firmicutes. However, staphylococci mostly those coagulase-negative (CoNS) also form a part of obligatory microflora in pheasants (Lauková *et al.*, 2012a). From spoilage bacteria, *Campylobacter jejuni* was detected in pheasants (Nebola *et al.*, 2007). In this study, we have been focused on the genus *Staphylococcus* from two general aspects; to spread information related to the basic microbiology as well as to test antibiotic phenotype in the isolates with the impact on methicillin resistance. Methicillin resistant staphylococci (MRS) can be potential causative agents in pheasants husbandry which in relation to the fact that their meat is used as food can be a risk for health of consumers. Therefore, sensitivity of staphylococci to antimicrobials of proteinaceous character-bacteriocins/enterocins (Franz *et al.*, 2007) was also tested. Bacteriocins (enterocins) can be beneficially applied in husbandry from at least two points; their beneficial effect has already been reported in poultry-hens (Lauková *et al.*, 2015a), Japanese quails (Lauková *et al.*, 2004), ostriches (Lauková *et al*., 2015b), rabbits (Pogány Simonová *et al.*, 2009; Lauková *et al*., 2012b, Szabóová *et al.*, 2011); their usage acceptance is not related to evaluation by EFSA and they do not leave residues in meat.

Materials and Methods

Isolation of staphylococci

Faeces from 60 birds aged 16-17 weeks (mixture samples) were sampled in the semi-wild environment of West Slovakia. Pheasants fed commercial feed mixture BZ 2 plus (Hrajnovka, Bratislava, Slovakia) and had access to water *ad libitum.* They were located in the aviaries with free movement. Handling and all care was provided in relation with acceptance of Slovak Veterinary and Food Administration and breeder. Samples were treated by the standard microbiological method-International Organization for Standardization-ISO; appropriate dilutions (1g of faeces) were diluted in Ringer solution (Merck, Germany), mixed using the Stomacher (Masticator, Spain) and plated on Mannitol Salt agar (Difco-ISO14385) or Baird-Parker agar with supplement (Difco, Maryland, USA, ISO-6888) to isolate staphylococci. Plates were incubated at 37º C for 24-48 h. Bacteria were counted as an average count of colonies in the highest dilution per sample and expressed in colony forming unit per gram of sample (CFU/g ± SD). Nineteen randomly picked up colonies were controlled for their purity and submitted for the species identification.

Species identification by Maldi-Tof system and phenotypic tests

Staphylococci were identified by Maldi BioTyper TM identification system (Bruker Daltonics, 2008) based on analysis of bacterial proteins by Maldi–Tof mass spectrometry. Lysates of bacterial cells were prepared according to the instruction of producer (Bruker Daltonics, 2008) prior to

identification. In addition, biochemical (phenotypic) characterization was performed by the use of the commercial BBL Gram-positive Crystal kit (Becton and Dickinson, Cockeysville, USA) to confirm the species allotation following the properties and reference-type strains according to Bergeys Manual (2009). Formation of acid from fructose, lactose, sucrose, trehalose, mannitol was evaluated, arginine deamination, urease, alkalic phosphatase, catalase and coagulase reactions were performed.

Antibiotic phenotype, lactic acid production

Antibiotic susceptibility was evaluated by the agar diffusion method (Clinical and Laboratory Standards Institute method-CLSI, M07-A9, 2012) involving 13 antibiotics from three suppliers (Becton and Dickinson, Cockeysville, USA; Lach-Ner, Czech Republic and Fluka, AG Switzerland). Following antibiotic disks were used according to the manufacturers guides and clinical practise: novobiocin, neomycin (Nb, Neo-5 µg), lincomycine (Lin-2µg), methicillin, ampicillin, tobramycin, gentamicin (Met, Amp, Tob, Gnt-10 µg), penicillin (Pnc-10IU), erythromycin (Ery-15µg), chloramphenicol, vancomycin, tetracycline (Chc, Van, Tct-30 µg) and phosphomycine (Fos-200 µg, Fluka).

Strains were cultivated in Brian Heart Infusion-broth (pH7.0, Oxoid) overnight at 37º C. Volume 100 µl was plated onto Brian heart agar (Becton and Dickinson) and disks were applied. Results were evaluated according to the manufacturers instructions; the inhibitory zones were expressed in mm. Antimicrobial free agar plates were included as a control to check obligatory growth of the strains.

Lactic acid was analysed by 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. Acetaldehyde reacts with 4-hydroxybiphenyl, forming a color complex.

Relation of *Staphylococci* to antimicrobials-enterocins

Sensitivity of staphylococci to five semi-purified enterocins (Ents) was tested by the quantitative agar spot test (De Vuyst *et al*., 2004) using Brian Heart agar (Becton and Dickinson, Cockeysville, USA). Following Ents were used: EntEM41, Ent55, EntM, Ent EK13=*Ent* A (P), 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 Lauková *et al*. (2012b) for EntEM41 (produced by *E. faecium* EM41 from ostrich; Strompfová and Lauková (2007) for Ent55 (produced by chickens isolate *E. faecium* EF55*;* 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 unit per ml (AU/ml); the principal indicator strain used was *E. avium* EA5 (from our laboratory). Activity of Ents was up to 25 600 AU/ml for EM41 and EK13=EntA(P); 12 800 for Ent2019, for EntM 6 400 AU/ml and 51 200 AU/ml for Ent55.

Results and Discussion

Staphylococal species identification, lactic acid production

Staphylocci detected in faecal samples of pheasants reached 3.79±0.9 (log10) CFU/ml. Among 19 randomly picked up colonies, checked for their purity, 12 strains were taxonomically identified, two strains were excluded on the basis of value score identity and five strains were not pure; they were excluded from identification. Based on Maldi-Tof mass spectrometry, 12 isolates were allotted to five different staphylococcal species as follows: one strain *Staphylococcus cohnii* subsp. *cohnii* SCo61, two strains S. *lentus* (SL31, SL32) and *S. hominis* (SHo13, SHo54), six strains *S. succinus* subsp. *succinus* (SU52, SU61, SU62, SU63, SU64, SU65) and one strain *S. hyicus* SHy53. Most strains showed score value in the range 2.000-2.299 or 2.300-3000 (Table 1); five strains showed score value in the range 1.700-1.999.

Following the characteristics typical for the identified species such as catalase positive reaction, acid formation from fructose, trehalose, lactose, mannitol, sucrose, they were positive as indicated in Bergeys Manual (2); reactions of arginine dihydrolase, urease and alkalic phosphatase were variable. Strains (excluding *S. hyicus* SHy53) were coagulase-negative and catalase-positive.

Lactic acid values in mmol/l are summarized in Table 1. Average value of lactic acid (LA) produced by identified staphylococci was 1.210 ± 0.10 mmol/l. The most production of LA was detected by *S. cohnii* strain SCo61 (1.895 ± 0.015 mmol/l). The least LA value was measured in *S. succinus* SU65 (0.670± 0.100 mmol/l).

Staphylococcal species in relation to antibiotics

S. hominis SHo13 was sensitive to all (13) antibiotics tested (Table 2). One strain was polyresistant-*S. cohnii* SCo61; it showed resistance towards novobiocin, lincomycine, erythromycin, tetracycline, phopshomycine and methicillin. Among six *S. succinus* strains, SU65 was resistant to three antibiotics-to Lin, Tct and Nb. The other five strains *S. succinus* were resistant only to Nb. *S. succinus* SU52 was sensitive with only dubious reaction to vancomycin. Coagulase-positive strain *S. hyicus* SHy53 was biresistant; resistant to two antibiotics-Lin and Ery. Both strains *S. lentus* SL31, SL32 were resitant to Lin; *S. hominis* SHo54 was resitant to Ery. Strains were mostly sensitive to antibiotics (Atb); they were sensitive to tobramycine, ampicillin, gentamicin, penicillin and chloramphenicol. Only one Met^R strain was detected-*S. cohnii* Sco61. Most strains were sensitive to methicillin, tetracycline, vancomycine and phosphomycine. Six novobiocin-sensitive strains were recorded (SL31, SL32, SHo54, SU52, SHy53, SHo13). On the other hand, six strains were resistant to Nb (SCo61, SU61, SU62, SU63, SU64, SU65, Table 2); three strains (SCo61, SHo54, SU65) were resistant to Ery and two strains were Tct resistant (SCo61, SU65).

In vitro sensitivity of staphylococcal species to enterocins

Staphylococci were sensitive at least to 1 of 5 Ents used (inhibitory activity was in the range 100-25 600 AU/ml). Met^R strain *S. cohnii* subsp. *cohnii* SCo61 was sensitive to five Ents (Ent EM41, Ent55, EntM, EntA (P), Ent2019) used with activity 400-12 800 AU/ml. *S. hominis* SHo54 was sensitive to four Ents (Ent55, EntM, EntA (P), Ent2019) except Ent EM41, 100-400 AU/ml, Table 3).

S. lentus SL31 was sensitive to three Ents (except EntEM41, Ent55 produced by ostrichs and poultry *E. faecium* strains, Table 3). The growth of coagulase-positive *S. hyicus* SHy53 was inhibited only by Ent2019 (100AU/ml, Table 3); it means it was sensitive to Ent2019 and resistant to other Ents. Sensitivity to Ents among six strains *S. succinus* subsp. *succinus* was variable (Table 3); they were sensitive at least to two Ents (SU63, SU64, SU65 with activity 100 AU/ml, table 3) and mostly to four Ents (inhibitory activity range 100-1 600 AU/ml). *S. succinus* subsp. *succinus* SU52 and SU61 were sensitive to three different Ents; SU52 was inhibited by Ents reaching the highest activity up to 25 600 AU/ml). But simultaneously, *S. succinus* subsp. *succinus* strains were less sensitive compared to the other species (except SU52 strain).

It can be stated, that the total counts of staphylococci in faecal samples of pheasants are similar to those counts referred for the other poultry; e.g. Marciňáková *et al*. (2005) reported 4.78 ±0.17 (log10) CFU/g staphylococci in faeces of turkeys with the most part of coagulase-negative staphylococci (CoNS). In faeces of Japanese quails were staphylococci counted in the amount 2.50±0.13 (log10) CFU/g (Lauková *et al.*, 1991). In this study, species identification was in accordance with higly probable species identification (two strains), secure genus and probable species identification (five strains) and probable genus identification (five strains, Bruker Daltonics, 2008). Based on 16S rRNA sequences (Takashi *et al.*, 2008) strains allotted to five different staphylococcal species can be involved to four clusters/groups: *S. haemolyticus* group including the species *S. hominis*, *S. hyicus* group with the representant *S. hyicus*, *S. saprophyticus* group with the species *S. cohnii* and *S. succinus*, *S. sciuri* group including here detected species *S. lentus*. Former mentioned staphylococcal clustering involves 11 clusters. Among 12 strains, 11 are coagulase-negative staphylococci; only *S. hyicus* represents coagulase-positive staphylococci. Biochemical tests also confirmed the species allottation by catalase positive reaction, acid formation from fructose, trehalose, lactose, mannitol, sucrose. Tests of arginine dihydrolase, urease and alkalic phosphatase were variable (Bergeys Manual, 2009). *S. hyicus* Shy53 was coagulase-positive. Key biochemical properties were in accordance with the representants of the detected species described in Bergeys Manual (2009). In ostriches Kandričáková (2014) reported faceal staphylococi allotted to eight species such as *S. equorum*, *S. warneri*, *S. epidermidis*, *S. haemolyticus*, *S. xylosus*, which were not detected in pheasants, but the species S. *succinus*, *S. cohnii*, *S. hominis* were detected in both, pheasants and ostriches. Moreover, in ostriches also *Kocuria carniphila* was detected (Kandričáková, 2014). Important result is absence of *S. aureus* in examined faecal samples of common pheasants; *S. aureus* belongs to coagulase-positive staphylococci and it is supposed to be disorder stimulating agent. The other identified species (excluding *S. hyicus*) belong to coagulase-negative staphylococci.

Comparing LA values produced by detected staphyloccci from pheasants, they are very similar to those measured in staphylococci from ostriches (Kandričáková, 2014). There, LA values produced by the different staphylococcal species were in the range 0.99 ±0.17 (*S. hominis* SHo3112) to 2.23±0.17 (*S. xylosus* SX242) mmol/l. Staphylococci are involved in LAB, but usually their LA production is significantly lower compared e.g. to enterococci and

lactobacilli. Maybe LA activity by the genera is dependent on the strain origin as shown in our study. However, here are not important differences in LA production among staphylococal species.

Methicillin-resistant CoNS (MRCoNS) constitute a potential health risk in food-derived animals and from this point of view they can have impact on consumers. Therefore, to eliminate the problem or to diminish it, it has been looking for especially „natural" ways. Enterocins are antimicrobial substances of proteinaceous character (Franz *et al*., 2007) which have been indicated to inhibit some bacteria. Inhibition of Met^R *S. cohnii* Sco61 isolated from faeces of pheasant reported here is promising result. Based on it we would like to test effect of Ents *in vivo* against this strain in model experiment (direct infection with it in animal model). Together done, the other staphylococci were more Atb sensitive (except SHy53, Sco61). However, e.g. Mártonová *et al.* (2008) reported staphylococci resistant to Amp and Pnc isolated from brown hare. On the other hand, staphylococci susceptible to Pnc, Amp, Gn or Van were reported by Zdolec *et al*. (2012) similarly as in our study (however, they isolated staphylococci from meat products of wild boar). In spite of the majority of sensitive strains to enterocins in this study, *S. hyicus* SHy53 was inhibited only by Ent2019 (100AU/ml); it showed high resistance to Ent. Studying mode of action of strains resistant to Ents is in paramount importance of scientists and nisin is only one bacteriocin (lantibiotic) in which it was explained (Kramer *et al*., 2004).

Table.1 Identification score and range of strains and lactic acid production

Strains	Score value	Range	LA (mmol/l)
S. cohnii			
Sco61	1.892	+	1.895 (0.015)
S.lentus			
SL31	1.975	+	0.985 (0.460)
SL32	1.946	+	1.265 (0.210)
S. hominis			
SHo13	1.809	+	1.185 (0.290)
Sho 54	2.349	+++	1.340 (0.290)
S. succinus			
SU52	2.276	+++	1.400 (0.105)
SU61	2.036	++	1.285 (0.120)
SU62	2.165	++	1.325 (0.230)
SU63	2.081	++	1.195 (0.270)
SU64	2.071	++	0.850 (0.240)
SU65	2.153	++	0.670 (0.100)
S. hyicus			
SHy53	1.923	+	1.200 (0.140)

Average LA value -1.210 (0.10) mmol/l; Range:range of identification: (+++) 2.300- 3000; (++) 2.000-2.299; (+) 1.700-1.999; 0.000-1.699-not reliable identification; LA-lactic acid is expressed in mmol/l ± SD.

Table.2 Antibiotic profile of identified staphylococci

	Nb^{5}	Met^{10}	Lin^{2}	Ery^{15}	Tct^{30}	Van^{30}	P^{200}
SCo61	R	R	R	R	R	S	R
SL31	S	S	R	S	S	S	S
SL32	S	S	R	S	S	S	S
SHo54	S	S	S	R	S	S	S
SU52	S	S	S	S	S	d	S
SU61	R	S	S	S	S	S	S
SU62	R	S	S	S	S	S	S
SU63	R	S	S	S	S	S	S
SU64	R	S	S	S	S	S	S
SU65	R	S	R	S	R	S	S
SHy53	S	S	R	S	S	S	S

All strains were sensitive to neomycin (Neo-5 µg), to tobramycin, ampicillin, gentamicin (Tb, Am, Gn, 10 µg) as well as to penicillin- Pn10 IU and to chloramphenicol (Chc-30 µg). *S. hominis* SHo13 was sensitive to all tested antibiotics. S-sensitice, R-resistant, d-dubious; Nb-novobiocin-5µg, Met-methicillin-10µg, Lin-licomycine 2 µg, Tct, Van-tetracycline and vancomycin-30 µg, P-phosphomycine-200 µg

Table.3 Sensitivity of staphylococci to enterocins (activity of enterocins is expressed in AU/ml)

Strains	Enterocins				
	EM41	Ent55	EntM	EntA(P)	Ent2019
S. cohnii					
Sco61	400	800	6 400	12 800	12 800
S.lentus					
SL31	-	-	100	800	400
SL32	400	800	800	3 200	400
S. hominis					
SHo13	800	800	100	100	400
Sho 54	-	400	400	100	200
S. succinus					
SU52	800	25 600	-	25 600	-
SU61	-	400	-	800	100
SU62	100	400	400	1 600	-
SU63	-	100	-	100	-
SU64	-	-	-	100	100
SU65	-	100	-	100	-
S. hyicus					
Shy53	-	-	-	-	100

EntEM41 produced by *Enterococcus faecium* EM41 from ostrich, Ent55 produced by *E. faecium* EF55 from chciken, EntM, Ent A(P) produced by *E. faecium* AL41 and EK13-CCM7419 of environrnental origin, Ent2019 produced by *E. faecium* EF2019-CCM7420 from rabbits faeces; – no inhibition

However, Atb polyresistant strain and moreover Met^R *S. cohnii* SCo61 was sensitive to enterocins used- its growth was inhibited by Ents. It is promising result for further use of Ents to treat Met^R strains or to cure/prevent their occurrence by Ents. As former mentioned, we have beneficial experiences with antimicrobial effect of Ents in *in vitro* conditions (Lauková *et al*., 2003) as well as *in vivo* experiments in farms/husbandries; not only count of staphylococci were reduced but also e.g. coliforms and in poultry even tendency to reduce *Salmonella* spp. and *Campylobacter* spp. was noted (Lauková *et al.,* 2004; Pogány Simonová *et al*., 2009; Ščerbová and Lauková, 2014).

Effect of enterocins were directed not only against spoilage bacteria but also to stimulate the immunity in the host organism by an increase of phagocytic activity as reported in the case of Ent2019 or EntM application in rabbits (Pogány Simonoá *et al.,* 2009; Lauková *et al.,* 2012b). Moreover, Husáková *et al.* (2014) reported positive effect of EntM-producing strain *E. faecium* AL41 in broiler chickens pretreated with AL41 strain and infected with *Salmonella* Enteritidis. They recorded an increase in concentration of secretory IgA (sIgA) in the intestine flush of *E. faecium* group of chickens at day 4 after *Salmonella* infection.

It can be concluded, among 19 randomly picked up colonies of faecal samples of common pheasants, 12 strains were taxonomically identified by Maldi-Tof mass spectrometry and phenotypization and allotted to five different staphylococcal species; it indicates species variability of staphylococci in the digestive tract of pheasants. The most value of LA was produced by *S. cohnii* strain SCo61. Strains, Met^R SCo61 including were sensitive to enterocins. Our results contribute to the basic research as well as to application possibility of enterocins to control microbiota in animals.

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