Relation to enterocins and herbal extracts of fecal hemolytic *Escherichia coli* from domestic ducks detected with MALDI-TOF mass spectrometry

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ABSTRACT

Surveillance studies have generally reported an increase in *Escherichia coli* strains resistant to major classes of antibiotics used for animals’ treatment. The aim of this study was to test the susceptibility of 25 strains (isolated from 30 domestic Mallard ducks—*Anas platyrhynchos*, both sex, aged 8 to 14 wk, taxonomically allotted to the species *E. coli* using MALDI TOF mass spectrometry system) to antimicrobials (antibiotics, enterocins, and herbal extracts). Testing was performed using the agar disc method and the agar diffusion method. A total of 19 *E. coli* strains were multiresistant to antibiotics; but 10 of those strains were susceptible to enterocins with an inhibition activity of 100 AU/mL. All strains were susceptible to herbal extracts. These results indicate further benefit application of enterocins and herbal extracts to prevent/reduce problems caused with *E. coli*. Moreover, additional studies are in process.

Key words: *Escherichia coli*, ducks, susceptibility, resistance, antimicrobials

INTRODUCTION

Poultry products can be source of foodborne pathogens and/or source of antimicrobial-resistant bacteria (Marshall and Levy, 2011). Their presence can result from selection pressure on bacteria due to the indiscriminate use of antimicrobials in aviculture as feed additives or as therapy. *Escherichia coli* is an incredibly diverse bacterial species with the ability to colonize and persist e.g., in environment and warm-blooded animal hosts, including poultry (Yan and Polk, 2004). Some strains can be, however, pathogenic and causing illnesses. They are categorized as either diarrheagenic *E. coli* or extraintestinal pathogenic *E. coli* (Kunert Filho et al., 2015). Bacteria can be intrinsically resistant to antibiotics, but they can also acquire resistance to antibiotics via mutations in chromosomal genes and by horizontal gene transfer. Bacterial strains carrying mobile genetic elements (plasmids, transposons, and integrons) can play important role in the dissemination of transmissible resistance genes. Surveillance studies have generally reported an increase in resistant *E. coli* strains occurrence, especially resistance to antibiotics used for the treatment of livestock and companion animals. Ducks can also be source of multiresistant *E. coli* (Blair et al., 2015). Increases in antimicrobial-resistant bacteria have generated significant concern on food safety. Animal-derived food can be contaminated e.g., by those multiresistant *E. coli* during slaughtering (Marshall and Levy, 2011; Asai et al., 2014). Therefore, food animals and their living environments are reservoirs of both resistant bacteria and resistant genes that could be transferred to human either by direct contact between animals and human or via the food production chain (Marshall and Levy, 2011). From this reason, farmers have interest in natural substances to prevent antibiotic resistance increase. Many Gram-positive bacteria, including some representatives of the phylum Firmicutes can be a source of bacteriocins (Franz et al., 2011). Bacteriocins are prokaryotic proteins or peptides with antimicrobial activity. Among them enterocins are bacteriocins produced mostly by enterococci, belonging to class II bacteriocins, which are thermostable with a broad antimicrobial spectrum (Franz et al., 2011; Simonová and Lauková, 2007; Cotter et al., 2013). Nowadays, there are many studies reporting inhibition activity of enterocins in animals (Lauková et al., 2004; Szabóová et al., 2008). Similarly, phytoadditives, especially oregano or sage, have been shown to present inhibition activity in vitro as well as in vivo (Szabóová et al., 2013). Both Oregano (*Origanum vulgare*) and sage (*Salvia officinalis*) belonged to the family Lamiacae. Both additives were found effective in reduction of clostridia or pseudomonads in broiler rabbits. Moreover, immuno-stimulative effect was also demonstrated...
The aim of our study was to test fecal hemolytic \textit{E. coli} from domestic ducks in relation to antimicrobials especially enterocins and herbal extracts (sage, oregano) to indicate their possible use to prevent bacterial contamination in domestic breeding and/or during slaughtering and meat processing as well.

**MATERIAL AND METHODS**

**Study Design**

A total of 22 mixtures of feces (n = 22) from 30 domestic Mallard ducks (\textit{Anas platyrhynchos}), both sex, aged 8 to 14 wk, and clinically healthy, were sampled in different private breeders. They were sampled continually; first sampling includes 11 animals (8 mixtures); second one sampling comprises 10 animals (7 mixtures) and third one sampling involved 9 ducks (7 mixtures). Ducks have been bred with straw litter. They have had grassed run and access to the waterfront. The diet consisted of ground barley, nettles, and abundant grazing which amounted to 50% of the feed. The animals had access to water ad libitum. The handling with animals followed rules of Ethic commision, and it was approved by Slovak Veterinary and Food Administration. Fresh feces were sampled in the duck coop by hand using gloves, immediately after being voided by the birds to prevent other contamination. Subsequently, fecal samples were put into sterile packs placed into a transport fridge and driven to our laboratory.

**Identification of Isolates Using MALDI-TOF Mass Spectrometry, Phenotypization and Hemolysis Testing**

Samples were treated according to the standard microbiological method (International Organisation for Standardization, ISO); 1 g of mixture feces was diluted in 9 mL of Ringer solution (pH 7; Merck, Darmstadt, Germany). Samples were stirred using a Stomacher–Masticator (Spain) and appropriate dilutions were plated onto Mac Conkey agar (Oxoid, United Kingdom) and incubated overnight. Fresh feces were sampled in the duck coop by hand using gloves, immediately after being voided by the birds to prevent other contamination. Subsequently, fecal samples were put into sterile packs placed into a transport fridge and driven to our laboratory.

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Identified strains were checked to form hemolysis (phenotype) on Trypticase soy agar (TSA, Becton and Dickinson) supplementd with 5% of defibrinated sheep blood. Inoculated media were incubated at 37°C for 24 to 48 h under aerobic conditions. Hemolytic phenotype was evaluated demonstrating type of clearing zones around the colonies.

**Antibiotic Profile**

Antibiotic susceptibility/resistance phenotype was performed according to the Clinical and Laboratory Standards Institute (CLSI, 2013) by the disc diffusion method on Mueller Hinton agar (Oxoid Ltd., Basingstoke, Hampshire, United Kingdom). Antibiotic discs (Becton and Dickinson; Lach-Ner, Czech Republic and Oxoid, United Kingdom) were as follows: penicillin (Pnc 10 IU), chloramphenicol (Chc 30 µg), tetracycline (Tct 30 µg), cefuroxime (Crx 30 µg), amikacin (Ak 30 µg), kanamycin (Kan 30 µg), aztreonam (Atm 30 µg), cephalothin (Kf 30 µg), phosphomycin (Phos 50 µg), cinoxacin (Cin 100 µg), ciprofloxacin (Cip 5 µg), and gentamicin (Cn 120 µg). Plates were cultivated at 37°C for 18 h. Evaluation of susceptibility/resistance was performed according to the manufacturer´s instruction. Size of inhibition zones was expressed in millimeter. For 2 antibiotics, ceftoxime (CTX 0.002 to 32 µg/mL) and ciprofloxacin (CIP 0.002 to 32 µg/mL), the antimicrobial gradient method using strips was used (BioMérieux, Marcy-l’Etoile, Etet, France) because discs were not disposable. Antibiotic strips were placed on the inoculated Mueller-Hinton agar (Oxoid) and incubated overnight. After incubation, strains were evaluated as susceptible/resistant according to EUCAST breakpoint table (The European Committee on Antimicrobial Susceptibility Testing, 2017). \textit{Escherichia coli} ATCC 25922 was used as control strain in both methods.

**Susceptibility to Enterocins and Herbal Extracts of Identified \textit{E. coli}**

Identified \textit{E. coli} were treated with partially purified bacteriocins (PPBs, enterocins; dose 10 µL of each) using the agar spot method (De Vuyst et al., 1996).
Briefly, TSA plates (1.5% agar, Becton and Dickinson) were overlaid with 0.7% TSA containing 200 µL of tested *E. coli* broth culture. Natural substances (10 µL) were dropped on the surface of 0.7% agar layer and incubated at 37°C. Enterocins used are listed in Table 1. They were characterized and prepared at our Laboratory of Animal Microbiology (Košice, Slovakia). The inhibition activity of PPBs was defined as the reciprocal of the highest dilution producing a distinct inhibition of an inhibition lawn, and it was expressed in arbitrary unit per milliliter of culture medium (AU/mL). The most susceptible indicator strain *Enterococcus faecium* EA5 (isolated from piglet) was used as control strain. Activity of PPBs against the most susceptible indicator (EA5) reached 3.200 up to 51.200 AU/mL (Table 1).

Moreover, susceptibility of identified *E. coli* to oregano and sage extracts (10 µL of both extracts, Calendula a.s., Nová Luboňová, Slovakia) was tested using the qualitative agar diffusion test and it was expressed in inhibition size zones (mm). Extract of oregano contained carvacrol 55 ± 3% (gas chromatography analysis; density:0.959 ± 0.002 g/cm³; refractive index:1.515 ± 0.001, Szabóová et al., 2012b). *Salvia officinalis* extract contained 24% of thujone, 18% of borneol, and 15% of cineole (Szabóová et al., 2012a).

**RESULTS**

The average count of presumptive *E. coli* was 4.6 ± 0.7 log10 CFU/g. A total of 25 strains were identified and belonged to the species *E. coli* by evaluation of protein spectra (MALDI-TOF MS system). Identification score values of 9 identified strains ranged from 2.300 to 3.000 (Ec Kˇc 2a, Ec Kˇc 22, Ec Kˇc 32, Ec Kˇc 5/A, Ec Kˇc 6a, Ec Kˇc 62, Ec Kˇc 66, Ec Kˇc 67, Ec Kˇc 68); other 16 strains were evaluated with score value in range from 2.000 to 2.299 (Figure 1). In addition, phenotypic properties confirmed allotment of 25 strains to the species *E. coli* showing the same reaction (e.g., for disacharides or enzymes) as the control strain *E. coli* ATCC 25922. Of 25, 24 *E. coli* tested formed β-hemolysis (Tables 2 and 3).

**Susceptibility Testing of *E. coli* to Antibiotics, Enterocins, and Herbal Extracts**

Identified *E. coli* showed 100% of resistance to penicillin and kanamycin. Tetracycline resistance (92%) and 84% of amikacin resistance were noted in tested strains. Strains were mostly resistant to cephalosporines; resistance to cefepime and cephalotin was of 76 to 80%; 16 strains were resistant to cefuroxime (64%). Resistance to quinolone antibiotic ciprofloxacin was low (16%). Strains *E. coli* Kč 1a and *E. coli* Kč 6a were resistant to 10 antibiotics; *E. coli* Kč 1b/2 and *E. coli* Kč 32 were resistant to 9 antibiotics. All strains were susceptible by intermediate reaction to cinoxacin (Tables 2 and 3). Approximately 32% of *E. coli* were resistant to cefotaxim and 56% of tested *E. coli* were resistant to ciprofloxacin.

Among 25 tested *E. coli*, 10 strains were susceptible at least to one enterocin. Four strains of *E. coli* were susceptible to enterocins Ent 131 and Ent 55. Three strains—Ec Kˇc 22, Ec Kˇc 42, Ec Kˇc 11 N; (Tables 2 and 3) were susceptible to Ent A(P) and Ent EM 41. On the other hand, 15 strains were resistant to enterocins. Multiresistant strain Ec Kč 3 was susceptible to enterocins; its growth was inhibited by Ent EM 41, Ent 131, and Ent 55. Strains Ec Kˇc 42 and Ec Kˇc 7 were susceptible to 2 Ents. Of 25, 7 strains were susceptible to one enterocin (Tables 2 and 3). Growth of tested strains was inhibited with inhibition activity of 100 AU/mL.

*Escherichia coli* isolated from ducks were susceptible to oregano and sage. Inhibition zones using oregano were bigger (up to 30 mm); after sage treatment, the inhibition zones measured up to 20 mm on average.

**DISCUSSION**

*Escherichia coli* of animal origin are increasingly associated with extraintestinal diseases in human. The aim of the research is increasingly common in avian *E. coli*; avian *E. coli* can cause diseases not only in poultry, but are often associated with extraintestinal *E. coli* in human causing mainly infection of urinary tract (Obeng et al., 2012). In some cases, human treatment is limited due to a high percentage of antimicrobial resistance (Laxminarayan et al., 2013). In
our study, the average count of \textit{E. coli} in ducks’ feces was $4.6 \pm 0.7 \log_{10} \text{CFU/g}$. Some authors presented occurrence of \textit{E. coli} in poultry in count 7.7 to 9.9 \log_{10} \text{CFU/g} (Murphy et al., 2005). Twenty-five strains were taxonomically allotted to the species \textit{E. coli} using the MALDI-TOF MS system. Although, 20 strains—in pairs (Ec Kˇc 3a—Ec Kˇc 4aN, Ec Kˇc 4a—Ec Kˇc 7a, Ec Kˇc 32—Ec Kˇc 68, Ec Kˇc 66—Ec Kˇc 70, Ec Kˇc 3b—Ec Kˇc 6a, Ec Kˇc 62—Ec Kˇc 11 N, Ec Kˇc 11 N—Ec Kˇc 69, Ec Kˇc 1aN—Ec Kˇc 2a, Ec Kˇc 2a—Ec Kˇc 7, Ec Kˇc 22—Ec Kˇc 1b/2, Figure 1) were identical on the basis of phylogenetic analysis, they differ in susceptibility to enterocins or to antibiotics. \textit{Escherichia coli} from ducks showed mostly antibiotic resistance; even multiresistant strains were determined. A high number of multiresistant \textit{E. coli} isolated from either meat or intestinal contents have also been reported by other authors (Adelowo et al., 2014; Koga et al., 2015). According to Hammerum and Heuer (2009), critically important antimicrobial agents for \textit{E. coli} are cephalosporines (mainly III and IV generation), quinolones, sulfonamides, and aminoglycosides. However, using cephalosporin antibiotics such as cefotaxime or quinolone antibiotics such as ciprofloxacin, a high number of \textit{E. coli} strains become susceptible to enterocins. Among aminoglycosides, 4% resistance to gentamicin, 100% resistance to kanamycin, and 84% resistance to amikacin was recorded. Tested \textit{E. coli} were also mostly resistant to tetracycline (92%), penicillin (100%), and to other cephalosporines (cefuroxim, cefalotin, and cefepime). High percentage of resistance to tetracycline and penicillin in \textit{E. coli} was also presented by Akond et al. (2009). In our study, 19 strains (76%) were multiresistant. This significant multiresistance in domestic breeding is striking, since no medication was applied to the breed. It could be probably explained with the fact that streams’ water (to which ducks are occasionally fed) can be a source of possible antibiotics’ transfer in water environment. Another possible condition is their free movement in the large area where they can come into contact with other domestic animals which can be a source of resistant bacterial strains. The significant increase of antibiotic-resistant bacteria and residua in meat products has evoked and has supported the research on new natural originated antimicrobials (enterocins, herbal extracts, etc.). Antimicrobial activity of enterocins has been confirmed in our previous in vitro and in vivo studies (Lauková et al., 1999, 2015; Ščerbová and Lauková 2016a,b). Moreover, tested trains were more susceptible to enterocins and herbal extracts than to antibiotics. A total of 19 \textit{E. coli} strains were recorded as multiresistant to antibiotics, but 10 of those strains were susceptible to enterocins and all strains were susceptible to herbal extracts. Although enterocins used are known to have a broad inhibition spectrum (Lauková et al., 1993, 2008,
### Table 2. Hemolytic activity, antibiotic profile, and susceptibility to enterocins of *Escherichia coli* from ducks.

| Strains   | CHC | TCT | CN | AK | ATM | CRX | KF | FEP | CIN | CIP | Sage | Oregano | Hemolysis | Ent | EM 41 | Ent 131 | Ent M | Ent 55 | Ent A(P) | Ent 9296 | Ent 412 | Ent 4231 |
|-----------|-----|-----|----|----|-----|-----|----|-----|-----|-----|------|----------|-----------|-------|-------|---------|-------|-------|----------|----------|-------|----------|
| Ec Kč 1a  | R   | R   | R  | R  | R   | R   | R  | S/20| I   | +   | +   | +       |           |       |       |         |       |       |          |          |       |          |
| Ec Kč 1b/2| R   | R   | S/17| R  | R   | R   | R  | S/25| S/25| +   | +   | +       |           |       |       |         |       |       |          |          |       |          |
| Ec Kč 2a  | S/20| R   | I   | R  | R   | R   | R  | I   | S/21| +   | +   | +       |           |       |       |         |       | 100   |          |          |       |          |
| Ec Kč 22  | S/20| R   | S/15| R  | R   | R   | R  | S/19| S/23| +   | +   | +       |           |       |       |         |       |       |          |          |       |          |
| Ec Kč 3a  | S/28| R   | S/17| R  | R   | R   | R  | S/21| I   | +   | +   | +       |           | 100   | 100   | 100     |       | 100   |          |          |       |          |
| Ec Kč 32  | R   | R   | S/16| R  | R   | R   | R  | S/25| S/21| +   | +   | +       |           |       |       |         |       |       |          |          |       |          |
| Ec Kč 3b  | S/25| R   | S/19| I  | S/22| I   | S/19| S/23| S/25| +   | +   | +       |           |       |       |         |       |       |          |          |       |          |
| Ec Kč 4a  | S/28| R   | S/25| R  | R   | R   | R  | S/28| S/22| +   | +   | +       |           |       |       |         |       |       |          |          |       |          |
| Ec Kč 4a  | S/25| R   | S/16| R  | R   | R   | R  | S/24| R   | +   | +   | +       |           |       |       |         |       |       |          |          |       |          |
| Ec Kč 4a  | S/25| R   | S/24| R  | R   | R   | R  | S/21| R   | +   | +   | +       |           |       |       |         |       |       |          |          |       |          |
| Ec Kč 7a  | S/20| R   | S/16| R  | I   | S/20| R  | S/25| R   | +   | +   | +       |           | 100   |       |         |       |       |          |          |       |          |
| Ec Kč 62  | S/20| R   | S/15| R  | I   | R   | R  | S/23| I   | +   | +   | +       |           |       |       |         |       |       |          |          |       |          |
| Ec Kč 63  | S/21| R   | S/16| I  | S/17| I   | I   | S/22| S/25| +   | +   | +       |           |       |       |         |       |       |          |          |       |          |
| Ec Kč 65  | S/21| I   | S/18| R  | S/23| R  | R   | S/23| S/22| +   | +   | +       |           |       |       |         |       |       |          |          |       |          |
| Ec Kč 66  | S/20| R   | S/16| R  | S/23| R  | R   | S/25| I   | +   | +   | +       |           |       |       |         |       |       |          |          |       | 100      |

All strains were susceptible to phosphomycine (Phos; inhibition zones from 17 to 30 mm); all strains were resistant to penicillin (Pnc) and kanamycin (Kan); ATB—antibiotics, Ec—*Escherichia coli*; Ent—enterocin; CHC—chloramphenicol, Tct—tetracycline, CN—gentamicin, AK—amikacin, ATM—aztreonam, CRX—cefuroxime, KF—cefalotin, FEP—cefepime, CIN—cinoxacin, CIP—ciprofloxacin; + hemolysis (phenotype) or herbal extract inhibit the growth of tested strains; –enterocins did not inhibit the growth of tested strains; R—resistance; e.g., S/21 means a size of inhibition zone in millimeter; I—intermediate reaction; activity of enterocins is expressed in arbitrary units per milliliter.

### Table 3. Hemolytic activity, antibiotic profile, and susceptibility to enterocins of *Escherichia coli* from ducks.

| Strains   | CHC | TCT | CN | AK | ATM | CRX | KF | FEP | CIN | CIP | Sage | Oregano | Hemolysis | Ent | EM 41 | Ent 131 | Ent M | Ent 55 | Ent A(P) | Ent 9296 | Ent 412 | Ent 4231 |
|-----------|-----|-----|----|----|-----|-----|----|-----|-----|-----|------|----------|-----------|-------|-------|---------|-------|-------|----------|----------|-------|----------|
| Ec Kč 67  | S/19| R   | S/17| R  | R   | S/20| S/18| R   | S/20| S/22| I   | +       | +        |       |       |         |       |       |          |          |       |          |
| Ec Kč 68  | S/21| R   | S/18| S/18| S/23| R   | R   | S/20| S/25| I   | +   | +       | +        |       |       |         |       |       |          |          |       |          |
| Ec Kč 69  | S/20| R   | S/16| R  | I   | R   | R   | R   | S/21| I   | +   | +       | +        |       |       |         |       |       |          |          |       |          |
| Ec Kč 70  | S/22| S/20| S/18| R  | S/25| I   | S/19| S/19| S/26| I   | +   | +       | +        |       |       |         |       |       |          |          |       |          |
| Ec Kč 1aN | S/22| R   | S/19| S/18| S/29| I   | I   | S/23| I   | I   | +   | +       | +        |       |       |         |       |       |          |          |       |          |
| Ec Kč 11N| S/20| R   | Int. | R  | R   | R   | R   | R   | S/24| S/21| +   | +       | +        | 100   |       |         |       |       |          |          |       |          |
| Ec Kč 66  | S/20| R   | S/16| R  | R   | S/23| R   | R   | S/25| I   | +   | +       | +        |       |       |         |       |       |          |          |       |          |

All strains were susceptible to phosphomycine (Phos; inhibition zones from 17 to 30 mm); all strains were resistant to penicillin (Pnc) and kanamycin (Kan); ATB—antibiotics, Ec—*Escherichia coli*; Ent—enterocin; CHC—chloramphenicol, Tct—tetracycline, CN—gentamicin, AK—amikacin, ATM—aztreonam, CRX—cefuroxime, KF—cefalotin, FEP—cefepime, CIN—cinoxacin, CIP—ciprofloxacin; + hemolysis (phenotype) or herbal extract inhibit the growth of tested strains; –enterocins did not inhibit the growth of tested strains; R—resistance; e.g., S/21 means a size of inhibition zone in millimeter; I—intermediate reaction; activity of enterocins is expressed in arbitrary units per milliliter.
E. coli extracts to prevent or reduce disorders caused with an approach using application of enterocins and herbal extracts. This indicates a new mostly resistant to antibiotics, they were susceptible to E. coli new approach to eliminate or to prevent control growth of with herbal extract cinnamaldehyde and EDTA to protein, usually a basic protein between 50 and 150 amino acid residues long that is loosely associated with the membrane (Cleveland et al., 2001). Effective combinative treatment of bacteriocin-lantibiotic ninis with herbal extract cinnamaldehyde and EDTA to control growth of E. coli strains from swine origin was reported by Des Field et al. (2017), which indicates a new approach to eliminate or to prevent E. coli agents.

CONCLUSION

Although identified species of hemolytic E. coli were mostly resistant to antibiotics, they were susceptible to enterocins and herbal extracts. This indicates a new approach using application of enterocins and herbal extracts to prevent or reduce disorders caused with E. coli.

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DISCLOSURE STATEMENT

There is no conflict of interest to declare.

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