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Original article

Escherichia coli strains from ostriches and their sensitivity to antimicrobial substances

J. Ščerbová, A. Lauková

Institute of Animal Physiology, Slovak Academy of Sciences,
Šoltésovej 4-6, 040 01 Košice, Slovak Republic

Abstract

Ostriches are bred especially for their high-quality meat. There is a lack of knowledge concerning the ostrich's microflora. *Escherichia coli* is a commensal microorganism of the poultry intestine, ostriches included. However, some strains may become pathogenic. This study was therefore undertaken to detect coliform bacteria in ostrich faeces and to test their antibiotic profile and sensitivity to enterocins. Faeces (n=54, 18 mixture samples from 3 different age groups of 140 ostriches) were sampled to isolate coliform bacteria. The counts of coliform bacteria varied from 5.69 ± 2.4 log10 CFU/g to 5.73 ± 2.4 CFU/g. Pure colonies were identified using MALDI-TOF MS mass spectrometry and confirmed by phenotypization. Seventy-one strains were allotted to the species *E. coli*. Sixty-four of those 71 strains caused hemolysis. They were mostly polyresistant to antibiotics. Thirty-two polyresistant strains of *E. coli* were sensitive to enterocins. These strains were most sensitive to Ent 9296 (26 strains). Moreover, Ent EM41 produced by *E. faecium* EM41 (isolated from ostrich faeces) inhibited the growth of 20 strains, reaching activity of 100 AU/ml. Our results indicate the possibility of enterocins being used for prevention/reduction of coliforms. Of course, *in vivo* studies are also being processed.

Key words: *Escherichia coli,* ostriches, sensitivity, antibiotic, enterocin

Introduction

Escherichia coli is a commensal microorganism in the intestine of human and warm-blooded animals, including poultry (De Vos et al. 2009). Poultry species are widely known as food-producing animals. Animal-derived food can be contaminated during slaughtering e.g. by *E. coli*. Pathogenic serotypes of *E. coli* cause a variety of lesions in immunocompromised hosts as well as in poultry (Kunert et al. 2015). *E. coli* of animal meat origin has been associated with extraintestinal infections in humans, e.g. urinary tract infections (Hammerum and Heuer 2009). Poultry farming is an effective livestock sector which is increasingly focused on the less traditional varieties of food-producing animals e.g. ostriches (*Struthio*). There are several farms which breed ostriches mainly for their high-quality meat. Adult ostriches are resistant to health disorders. However, young birds can be threatened by bacteria such as hemolytic *Escherichia*

Correspondence to: A. Lauková, e-mail: laukova@saske.sk, tel.: +421 55 7292964

coli, *Campylobacter* sp., *Salmonella* sp. or parasites especially during their transfer from nests to the farm area (Cooper 2005). Although *E. coli* belong to the obligatory microflora of the intestinal tract of poultry (ostriches included), certain pathogenic strains cause colibacillosis. Colibacillosis is often the first reason for morbidity and mortality in poultry (Gross 1994, Cooper 2005, Newell et al. 2010). Antimicrobial therapy is an important tool in reducing both the incidence and mortality associated with avian colibacillosis (Blanco et al. 1997). However, surveillance studies have generally reported an increase in the number of *E. coli* strains resistant to major classes of antibiotics used for the treatment of livestock and companion animals. Given the widespread use of non-human highly important antimicrobial agents (cephalosporines, fluorochinolones, aminoglycosides, sulfonamides), this generates a reservoir of resistant bacteria which may result in widely increased antimicrobial resistance (Lanz et al. 2003, Kadlec and Schwarz 2008). For this reason, treatment or prevention of bacterial infections becomes less successfull. *E. coli* with antimicrobial resistance genes (isolated from animals) represent a reservoir for human infection. In the past, antimicrobial agents were extensively used in animal therapy, for prophylaxis and metaphylaxis, and in some geographical regions for growth promotion. Many of them belong in the same family of antimicrobial compounds used for treatment (Witte 1998). Drug-resistant commensal *E. coli* may constitute a significant reservoir of antibiotic resistance determinants which can spread to bacteria pathogenic for animals and/or humans (Hammerum and Heuer 2009). In intensively reared food-producing animals antibiotics may be administered to whole flocks rather than individual animals. For this reason, farmers increasingly turn to the use of natural substances to prevent or to avoid an increase in antibiotic resistance.

The aim of every breeder is to maintain a healthy farm and thereby to obtain healthy and safe food. This study is focused on indicating alternative ways to reduce and/or prevent *E. coli* contamination. Bioactive substances, e.g. bacteriocins with antimicrobial activity produced by some bacteria can be used. Enterocins (bacteriocins) are proteinaceous substances with a wide antimicrobial spectrum against mostly Gram-positive but also against Gram-negative bacteria, and are produced by different strains of *Enterococcus faecium* (Lauková et al. 2004, Franz et al. 2007). Enterocins can successfully reduce intestinal bacteriosis (Lauková et al. 2004, Strompfová et al. 2006, Szabóová et al. 2011). In this study, the antibiotic profile of *E. coli* strains (isolated from ostrich feaces) was tested to discover their *in vitro* sensitivity to enterocins. Sensitivity of *E. coli* from ostriches to enterocins has never been tested yet. The enterocins used were produced by enterococci isolated and studied at our laboratory.

Materials and Methods

Isolation of coliforms

One hundred and forty ostriches from three aged groups were sampled simultaneously (n=18, faecal mixture samples from each group) on a farm in Slovakia during a half a year period: 56 birds aged three weeks (group 1), 42 ostriches aged 6-9 weeks (group 2), and 42 ostriches aged 12-16 months (group 3). The ostriches were fed with commercial feed mixture 1567 Pštros MINI-Energys (De Heus a.s., Czech Republic) and they had water intake *ad libitum*. Fifty-four faecal mixture samples were collected with the farmer's agreement (approved by Slovak State Veterinary and Food Administration). Fresh faeces were collected in the ostrich pen by hand using gloves, immediately after being voided by the birds to prevent other contamination. They were put into sterile packs (n=18 from each group) placed into a transport fridge and taken to our laboratory. Samples were treated according to the standard microbiological method of the International Organisation for Standardization (ISO) using appropriate dilutions; 1 g of faeces into 9 ml of Ringer solution (Merck, Darmstadt, Germany); samples were stirred using a Stomacher-Masticator (Spain) and diluted. Mac Conkey agar (Oxoid, United Kingdom) was used to isolate coliform bacteria. Plates were incubated at 37°C for 24 h. Bacterial number was calculated as an average count of colonies grown in the highest dilution per sample and expressed as colony-forming units per gram of sample (CFU/g ±SD). Randomly picked colonies (119) from 54 faecal samples were checked for purity and submitted to further analysis.

Identification of isolates and hemolysis test

Presumptive colonies were identified using the MALDI-TOF mass spectrometry (MS) based on proteiň fingerprintš (Alatoom et al. 2011, MALDI-TOF MS Bruker Daltonics), performed using a Microflex MALDI-TOF mass spectrophotometer. A single colony from Mac Conkey agar was mixed with matrix (α-cyano-4-hydroxycinnamic acid and trifluoroacetic acid), the suspension was spotted onto a MALDI plate and ionized by nitrogen laser (wave-length 337 nm, frequency 20Hz). The results were evaluated using the MALDI-TOF MS Biotyper 3.0 (Bruker

Daltonics USA) identification database. Taxonomic allottment was evaluated on the basis of highly probable species identification (value score 2 300-3 000) and secure probable species identification/probable species identification (2 000-2 299). Positive controls were those involved in the identification system. Identical colonies evaluated using the MALDI-TOF MS score value were excluded.

Phenotypization of strains was performed using the BBL Crystal Enteric/Nonfermenter ID System (Becton and Dickinson, USA). The strains were prepared according to the producers instructions. Evaluation was carried out with BBL Crystal Mind software (Becton and Dickinson, USA). *E. coli* ATCC 2592 served as control strain.

Hemolysis phenotype in the identified strains was checked using Trypticase soy agar (Becton and Dickinson, USA) supplementd with 5% of defibrinated sheep blood. Plates were incubated at 37°C for 24-48 h under aerobic conditions. Presence of hemolysis was demonstrated by clearing zones around the colonies and evaluated as reported by Semedo-Lemsaddek et al. (2003, 2013).

Antibiotic resistance profile

The identified *E. coli* were tested for their antibiotic phenotype profile. Antibiotics were selected according to recommendations including in the commercial tests for antibiotic (sensitivity) resistance analysis in coliforms. Antimicrobial resistance testing was performed using the disk diffusion method against antibiotics as included in the Clinical Laboratory Standard Institute guidelines (CLSI 2011). The standard disk diffusion method was performed using Mueller-Hinton agar (Bio-Rad, France) with disks provided by Lach-Ner (Czech Republic) and Oxoid (United Kingdom). A total of eight antibiotic disks such as penicillin (10 IU), erythromycin (15 µg), chloramphenicol (30 µg), tetracycline (30 µg), (Lach-Ner, CZ), gentamicin (120 µg), aztreonam (30 µg), cefalotin (30 µg), and nalidixic acid (30 µg, Oxoid) were used. Concentrations of antibiotic disks followed the recommendations of the commercial test suppliers for *E. coli*/coliform testing. The zone diameter for individual antimicrobial agents was then assessed as susceptible, intermediate and resistant according to the interpretation table of antibiotic disks producers. Antimicrobial-free agar plates were included as a control for obligatory growth of the strain tested. *Escherichia coli* ATCC 2592 served as a positive control strain.

Sensitivity to enterocins

The identified *E. coli* were treated with semi-purified substances of enterocins using the quantitative agar diffusion method (De Vuyst et al. 1996). They were isolated and characterized at our laboratory (Institute of Animal Physiology, Laboratory of Animal Microbiology, Slovak Academy of Sciences, Košice, Slovakia). Enterocins produced by the following strains were used: *E. faecium* EM41 from ostrich – Ent EM41 (Lauková et al. 2012a), environmental strain *E. faecium* AL41=CCM8558 and EK13 =CCM7419 produced by Ent M and Ent A, P (Mareková et al. 2003, 2007), ruminal *E. faecium* CCM4231-Ent 4231 (Lauková et al. 1993), chicken isolate *E. faecium* EF55 – Ent 55 (Strompfová and Lauková 2007), *E. faecium* EF412 from horse faeces-Ent 412 (Lauková et al. 2008), *E. faecium* from silage – Ent 9296 (Marciňáková et al. 2004). The 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 (tested against the principal – the most sensitive indicator strain) *Enterococcus avium* EA5 (isolate from piglets) used as positive control. Activity of semi – purified enterocins against the most sensitive indicator (EA5) was as follows: Ent 55 – 51 200 AU/ml; Ent EM41, Ent412, Ent 9296, Ent A(P) – 25 600 AU/ml; Ent M – 6 400AU/ml; Ent4231 – 3 200 AU/ml. Testing was performed using Trypticase soy agar (1.5%; 0.7%) and broth (Becton and Dickinson, USA). Incubation was carried out at 37°C for 18-24 h.

Competitive studies

The former media were also used for competitive studies using batch cultivation. *E. coli* strain Ec 221/c (isolated from ostrich faeces), resistant to antibiotics and sensitive to enterocins under *in vitro* testing was selected for this study. Among the enterocins, Ent M, Ent EM41, Ent 412 were used based on our previous results from *in vivo* studies in husbandries, where reduction of coliforms was noted (Lauková et al. 2012b). Control sample (CS) and experimental samples (ES) were inoculated with 0.1% overnight broth culture of Ec 221/c strain. Cultivation was performed in water batches with shaking at 37°C for 24 hours. Semi-purified enterocins were added to the ES at the start of cultivation (ES1) and in the logarithmic growth phase (ES2) of Ec 221/c strain. Samples (1.5 ml) including the zero sample were taken every one/two hours. Optical density (OD_{600}) was measured spectrophotometrically with a Specol 11 meter (Carl Zeiss, Jena, Germany). Bacterial counts

Table 1a. Hemolysis test, antibiotic profile and sensitivity to enterocins of *Escherichia coli* strains.

Strain	Ent EK13	Ent M	Ent 4231	Ent EM41	Ent 9296	Ent 55	Ent 412	Hem	CHC 30	Tct 30	Ery 30	Pnc 10IU	Gen 120	KF 30	Na 30
Ec111/c	–	100	100	–	100	–	100	+	S^{23}	R	R	R	S^{14}	R	R
Ec112/c	100	100	100	100	100	–	100	+	S^{14}	R	R	S^{23}	S^{16}	R	R
Ec131/c	–	–	–	–	–	–	–	+	S^{17}	R	R	R	R	R	R
Ec132/c	–	100	100	100	100	–	–	+	S^{20}	R	R	R	R	R	R
Ec141/c	–	–	–	–	–	–	–	+	S^{21}	R	R	R	R	S^{16}	R
Ec151/c	–	–	–	–	–	–	–	+	S^{21}	R	R	R	R	R	R
Ec161/c	–	–	–	–	–	–	100	+	S^{16}	R	R	R	R	S^{15}	R
Ec172/c	100	100	100	100	100	100	100	+	S^{24}	R	R	R	S^{16}	R	R
Ec1111/c	–	–	–	–	–	–	–	+	S^{23}	R	R	R	R	R	R
Ec1112/c	–	–	–	–	–	–	–	+	S^{25}	R	R	S^{23}	R	R	R
Ec1121/c	–	–	–	–	–	–	–	+	S^{19}	R	R	R	R	R	R
Ec1122/c	–	–	–	–	100	–	–	+	S^{18}	R	R	R	S^{15}	R	R
Ec1181/c	100	100	100	100	100	100	100	+	S^{25}	R	R	R	S^{19}	S^{15}	R
Ec1182/c	100	100	–	–	100	100	100	+	S^{21}	R	R	R	R	R	R
Ec221/c	100	100	100	100	100	100	100	+	S^{22}	R	R	R	R	R	R
Ec222/c	–	–	–	–	–	–	100	+	S^{17}	R	R	R	R	S^{15}	R
Ec231/c	–	–	–	–	–	–	–	+	S^{14}	R	R	R	S^{14}	R	R
Ec261/c	–	–	100	100	100	100	–	+	S^{20}	R	R	R	R	R	R
Ec262/c	–	–	–	–	–	–	–	+	R	R	R	R	R	R	R

All strains were sensitive to aztreonam (Atm -30 µg, range of inhibitory zones from 20 to 25); Chc – chloramphenicol (30 µg), Tct – Tetracycline (30 µg), Ery – Erythromycin, (15 µg), Pnc – Penicillin (10 IU), Gen – Gentamicin (120 µg), CEF – Cefalotin (30 µg), NA – Nalidixic acid (30 µg), S^{No}-sensitive and size of inhibitory zone in mm, R-resistant, inhibitory activity of enterocins is expressed in Arbitrary Units per ml, + hemolysis (phenotype), – enterocins did not inhibit the growth of tested strains, Initial activity of enterocins used: Ent 55-51 200 AU/ml, Ent 412, EK13=A, (P) EM41 Ent 9296-25 600 AU/ml, Ent M-6 400 AU/ml, Ent 4231-3 200 AU/ml; e. g. S^{23} means a size of inhibiting zone

Table 1b. Hemolysis test, antibiotic profile and sensitivity to enterocins of *Escherichia coli* strains.

Strain	Ent EK13	Ent M	Ent 4231	Ent EM41	Ent 9296	Ent 55	Ent 412	Hem	CHC 30	Tct 30	Ery 30	Pnc 10IU	Gen 120	KF 30	Na 30
Ec271/c	100	100	100	100	100	–	–	+	S^{18}	R	R	R	R	R	R
Ec272/c	100	100	100	100	100	100	100	+	S^{18}	R	R	R	S^{13}	R	R
Ec281/c	–	–	–	–	100	–	100	+	S^{18}	R	R	R	S^{14}	R	R
Ec282/c	–	–	–	–	–	–	–	+	S^{18}	R	R	R	S^{16}	R	S^{19}
Ec213/c	–	–	–	–	–	–	–	+	S^{18}	R	R	R	S^{15}	R	R
Ec2152/c	–	–	–	–	–	–	–	+	S^{20}	R	R	R	S^{16}	R	R
Ec341/c	100	–	–	100	100	–	100	+	R	R	R	R	S^{19}	R	S^{21}
Ec352/c	–	–	–	–	–	100	100	+	S^{25}	S^{24}	S^{28}	R	S^{13}	R	R
Ec371/c	–	100	–	100	100	100	100	+	R	R	R	R	R	R	R
Ec372	–	–	–	–	–	–	–	+	R	R	R	R	S^{21}	R	S^{19}
Ec381/c	–	–	–	–	–	–	–	+	S^{19}	R	R	R	R	R	R
Ec3131/c	–	–	–	–	–	–	–	+	S^{18}	R	R	R	R	S^{18}	S^{17}
Ec3132/c	–	–	–	–	–	–	–	+	R	R	R	R	R	R	S^{22}
Ec3181/c	–	–	–	–	–	–	–	+	R	R	R	R	R	R	S^{20}
Ec112/Ps	–	–	–	–	–	–	–	+	S^{22}	S^{18}	R	R	R	S^{24}	S^{19}
Ec182/Ps	–	–	–	–	–	–	–	+	S^{21}	R	R	R	R	S^{23}	S^{18}
Ec221/Ps	–	–	–	–	–	–	–	+	S^{20}	S^{19}	R	R	R	S^{18}	S^{21}
Ec213/Ctr	–	–	–	–	–	–	–	+	S^{20}	R	R	R	S^{13}	S^{19}	R
Ec112/Cl	–	–	–	–	–	–	–	+	S^{22}	S^{17}	R	R	R	S^{22}	S^{20}

All strains were sensitive to aztreonam (Atm – 30 µg, range of inhibitory zones from 20 to 25); Chc – chloramphenicol (30 µg), Tct – Tetracycline (30 µg), Ery – Erythromycin, (15 µg), Pnc – Penicillin (10 IU), Gen – Gentamicin (120 µg), CEF – Cefalotin (30 µg), NA – Nalidixic acid (30 µg), S-sensitive, R-resistant, inhibitory activity of enterocins is expressed in Arbitrary Units per ml, + hemolysis (phenotype), – enterocins did not inhibit the growth of tested strains, Initial activity of enterocins used: Ent 55-51 200 AU/ml, Ent 412, EK13=A,P, EM41 Ent 9296-25 600 AU/ml, Ent M-6 400 AU/ml, Ent 4231-3 200 AU/ml; e. g. S^{18} means a size of inhibiting zone

Table 1c. Hemolysis test, antibiotic profile and sensitivity to enterocins of *Escherichia coli* strains.

Strain	Ent EK13	Ent M	Ent 4231	Ent EM41	Ent 9296	Ent 55	Ent 412	Hem	CHC 30	Tct 30	Ery 30	Pnc 10IU	Gen 120	KF 30	Na 30
Ec132/Cl	–	–	–	–	–	–	–	–	S^{17}	S^{17}	R	R	R	S^{21}	S^{18}
Ec162/Cl	–	–	–	–	–	–	–	+	S^{19}	S^{18}	R	R	S^{14}	S^{23}	S^{19}
Ec222/Cl	–	–	–	–	–	–	–	–	S^{23}	S^{18}	R	R	S^{14}	S^{22}	S^{19}
Ec311/Cl	–	–	–	–	–	–	–	+	R	R	R	R	R	S^{20}	S^{23}
Ec3151/Cl	–	–	–	–	–	–	–	+	S^{21}	S^{17}	R	R	S^{14}	S^{24}	S^{20}
Ec3153/Cl	–	–	–	–	–	–	–	–	S^{21}	S^{18}	R	R	S^{17}	S^{22}	S^{20}
Ec121/Sch	–	–	–	–	–	–	–	+	S^{19}	S^{18}	R	R	R	S^{25}	S^{19}
Ec371/Cl	–	–	–	–	–	–	–	+	S^{20}	S^{18}	R	R	S^{14}	S^{21}	S^{20}
Ec332/Cl	–	–	–	–	–	–	–	+	S^{20}	S^{15}	R	R	S^{15}	S^{23}	S^{22}
Ec317/Cl	–	–	–	–	–	–	–	–	S^{20}	S^{18}	R	R	S^{15}	S^{25}	S^{20}
Ec142/Cl	–	–	–	–	–	–	–	+	S^{20}	S^{16}	R	R	R	S^{20}	S^{18}
Ec271/Cl	–	–	–	–	–	–	–	+	S^{23}	R	R	R	R	S^{20}	S^{20}
Ec322	100	100	100	100	100	–	100	–	S^{20}	S^{17}	R	R	S^{13}	R	R
Ec323	100	–	100	–	100	–	100	+	S^{20}	S^{15}	R	R	R	R	R
Ec361	100	100	100	100	100	–	100	+	S^{14}	R	R	R	R	R	R
Ec362	100	100	100	100	100	–	100	+	S^{19}	S^{15}	R	R	S^{13}	R	R

All strains were sensitive to aztreonam (Atm – 30μg, range of inhibitory zones from 20 to 25); Chc – chloramphenicol (30 μg), Tct – Tetracycline (30 μg), Ery – Erythromycin, (15 μg), Pnc – Penicillin (10IU), Gen – Gentamicin (120 μg), CEF – Cefalotin (30 μg), NA – Nalidixic acid (30 μg), S-sensitive, R-resistant, inhibitory activity of enterocins is expressed in Arbitrary Units per ml, + hemolysis (phenotype), – enterocins did not inhibit the growth of tested strains, Initial activity of enterocins used: Ent 55-51 200 AU/ml, Ent 412, EK13=A,P, EM41 Ent 9296-25 600 AU/ml, Ent M-6 400 AU/ml, Ent 4231-3 200 AU/ml; e. g. S^{16} means a size of inhibiting zone

Table 1d. Hemolysis test, antibiotic profile and sensitivity to enterocins of *Escherichia coli* strains.

Strain	Ent EK13	Ent M	Ent 4231	Ent EM41	Ent 9296	Ent 55	Ent 412	Hem	CHC 30	Tct 30	Ery 30	Pnc 10IU	Gen 120	KF 30	Na 30
Ec371	–	–	–	–	–	–	–	+	S^{20}	S^{16}	R	R	S^{13}	R	R
Ec391	100	100	100	100	100	–	100	–	S^{15}	R	R	R	R	R	R
Ec392	–	–	–	–	–	–	–	–	S^{20}	S^{17}	R	R	S^{13}	R	R
Ec111	–	–	–	–	–	–	–	+	S^{22}	S^{20}	R	R	S^{16}	R	R
Ec112	–	–	–	–	–	–	–	+	S^{29}	S^{25}	R	R	S^{25}	R	S
Ec141								+	S^{20}	S^{15}	R	R	S^{16}	R	S
Ec152	–	–	–	–	–	–	–	+	S^{21}	S^{17}	R	R	S^{14}	R	S
Ec171	–	–	–	–	–	–	–	+	S^{20}	S^{18}	R	R	S^{14}	R	S
Ec115	100	100	100	–	100	–	100	+	S^{19}	S^{15}	R	R	S^{13}	R	S
Ec22	100	100	100	100	100	–	100	+	S^{20}	S^{16}	R	R	R	R	S
Ec241	100	100	100	–	–	–	–	+	S^{24}	S^{20}	R	R	R	R	S
Ec242	100	100	100	100	100	–	100	+	S^{22}	S^{21}	R	R	S^{15}	R	S
Ec272	–	–	–	–	–	–	–	+	S^{20}	S^{15}	R	R	S^{14}	R	S
Ec215	100	100	100	100	100	–	100	+	S^{15}	R	R	R	R	R	S
Ec2182	100	100	100	100	100	–	100	+	S^{19}	S	R	R	R	R	S^{18}
Ec311	100	100	100	100	100	–	100	+	S^{19}	S^{16}	R	R	R	R	S^{21}
Ec312	100	100	100	100	100	100	100	+	R	R	R	R	S^{15}	R	S^{17}

All strains were sensitive to aztreonam(Atm – 30 μg, range of inhibitory zones from 20 to 25); Chc – chloramphenicol (30 μg), Tct – Tetracycline – (30 μg), Ery – Erythromycin, (15 μg), Pnc – Penicillin (10 IU), Gen – Gentamicin (120 μg), CEF – Cefalotin (30 μg), NA – Nalidixic acid (30 μg), S-sensitive, R-resistant, inhibitory activity of enterocins is expressed in Arbitrary Units per ml, + hemolysis (phenotype), – enterocins did not inhibit the growth of tested strains, Initial activity of enterocins used: Ent 55-51 200 AU/ml, Ent 412, EK13=A,P, EM41 Ent 9296-25 600 AU/ml, Ent M-6 400 AU/ml, Ent 4231-3 200 AU/ml; e. g. S^{16} means a size of inhibiting zone

were monitored using the standard microbiological dilution method and appropriate dilutions were spread onto Mac Conkey agar (Oxoid, United Kingdom); viable cells were expressed in CFU/ml. Final sampling was carried out at the end of the cultivation (after 24 hours). The competitive studies were repeated once.

Results

Coliform bacteria isolated from 140 ostriches were well-balanced in each age group, reaching 5.75 ± 2.4 log10 CFU/g in group 1, 5.69 ± 2.4 CFU/g in group 2, and 5.73 ± 2.4 CFU/g in group 3. The MALDI-TOF MS system allotted 71 strains of 119 isolated colonies

from 140 ostriches and 54 faecal mixture samples to the species *Escherichia coli* with score values 2000-2250 (37 strains), 2251-2350 (19 strains), 2351-2500 (15 strains). Forty-eight colonies from 119 were found to be indentical based on MALDI-TOF MS score evaluation, and they were excluded from further testing. In addition, evaluation of phenotypic properties also confirmed allottment of 71 strains to the species *E. coli* showing the same reaction (e.g. for disacharides or enzymes) as the control strain *E. coli* ATCC 25922. The majority of tested strains (64 from 71) caused hemolysis.

The phenotype of antibiotic resistance profile of the identified *E. coli* was tested for eight antibiotics (Table 1a,b,c,d). Most strains were resistant to antibiotics. However, all strains were sensitive to aztroneam (inhibitory zone size from 21 to 28 mm) and only seven strains were resistant to chloramphenicol. Seven strains were biresistant, 15 strains were resistant to three antibiotics. The rest of the strains were polyresistant which means resistant to four antibiotics (16 strains), five antibiotics (17 strains), six antibiotics (14 strains) or seven antibiotics (two strains-Ec262/c, Ec371/c) from the eight antibiotics used. Erythromycin resistance was found in 69 strains (97.2%), while 39 strains (54.9%) were resistant to tetracycline, 98.6% strains of *E. coli* (70 strains) showed phenotypic resistance to penicillin, 50.7% (36 strains) to gentamicin and nalidixic acid, and 69% were resistant to cefalotin (49 strains). Because of the large number of resistant strains (phenotype detected) they were tested for their sensitivity to enterocins.

Although the growth of 39 of the 71 *E. coli* strains tested (including also those resistant to antibiotics) was not inhibited by the enterocins used, the growth of 32 strains (45%) was inhibited (Table 1a,b,c,d). The majority of strains (26) were inhibited by Ent 9296 (37%, produced by *E. faecium* EF 9296 from silage) followed by Ent 412 (produced by *E. faecium* EF 412 from horse faeces). Ent 412 inhibited the growth of 25 *E. coli* strains (35%); among those strains seven were isolated from ostriches of age group 1, eight of group 2 and 10 of group 3. Ent M (produced by the environmental strain *E. faecium* AL41=CCM 8558) and Ent 4231 (produced by ruminal strain CCM 4231) inhibited the growth of 22 *E coli* strains (30.9%); in the case of Ent M, the growth of eight strains from ostriches of group 1 was inhibited, followed by eight strains of group 2 and five strains of group 3. Using Ent 4231, the growth of seven strains from birds in each of age groups 1 and 3 and eight strains from group 2 were inhibited. Ent A (P) produced by environmental *E. faecium* CCM 7419 inhibited the growth of 21 *E. coli* strains (29.6%); six strains from age group 1, and eight from each groups 2 and 3. Twenty *E. coli* strains (28.2%) were sensitive to Ent EM 41 (ostrich isolate); among these were five *E. coli* strains from ostriches in age group 1, eight from group 2, and seven from group 3. Ent 55 (produced by EF 55 strain from chicken crop) inhibited the growth of nine *E. coli* strains (12.7%); three from each group. In general, the growth of tested strains was inhibited, reaching inhibitory activity 100 AU/ml. Polyresistant *E. coli* strains (resistant to 4-6 antibiotics; Ec312, Ec 172/c, Ec 1181/c, Ec221/c, Ec272/c) were inhibited by all enterocins. Among 32 strains sensitive to enterocins, they were sensitive at least to one Ent and/or their growth was inhibited by three, four or five and (as indicated) by seven enterocins. Strains inhibited by enterocins were resistant to at least four antibiotics (Table 1a,b,c,d). Antibiotic resistant strain *E. coli* Ec 221/c was sensitive to all tested enterocins (100 AU/ml). This strain was submitted for competititve studies.

In competitive studies Ent M and Ent EM41 did not inhibit the growth of the tested strain. Bacteriostatic effect caused by Ent 412 was observed in ES1 and ES2. In both experimental groups, decrease in Ec 221/c strain was recorded with a difference of 1.13 log cycle (CS – 9.23 log10 CFU/ml, ES1 a ES2 – 8.1 log 10 CFU/ml, Fig. 1). However, in ES1 compared to CS at 5 hours, a difference of 0.67 log cycle was found, in ES2 compared to CS at 10 h there was a difference of 0.15 log cycle, at 24 h ES1 and ES2 compared to CS produced a difference of 0.61, 0.67 log cycle respectively; this all means a tendency to inhibit the Ec221/c strain growth.

Discussion

Limited knowledge concerning the microflora of ostriches has been shown up to know. We focused on representatives of the genus *Escherichia* as a frequent causative agent in poultry (ostriches included) in order to study their antibiotic resistance profile and sensitivity to enterocins. Seventy-one strains were taxonomically allotted to the species *Escherichia coli* using by MALDI-TOF MS system. According to De Vos et al. (2009), the species *Escherichia coli* is allotted to the Class Gammaproteobacteria, to the Order Enterobacteriales, to the Family Enterobacteriaceae and to the Genus *Escherichia*. According to Hammerum and Heuer (2009) the following antimicrobial agents are regarded as critically important for *E. coli*: cephalosporins (third and fourth generation), quinolones, sulfonamides, and aminoglycosides. Aminoglycosides are frequently used for treatment of animals, including poultry (Hammerum and Heuer 2009). In our case, *E. coli* were biresistant or poly-

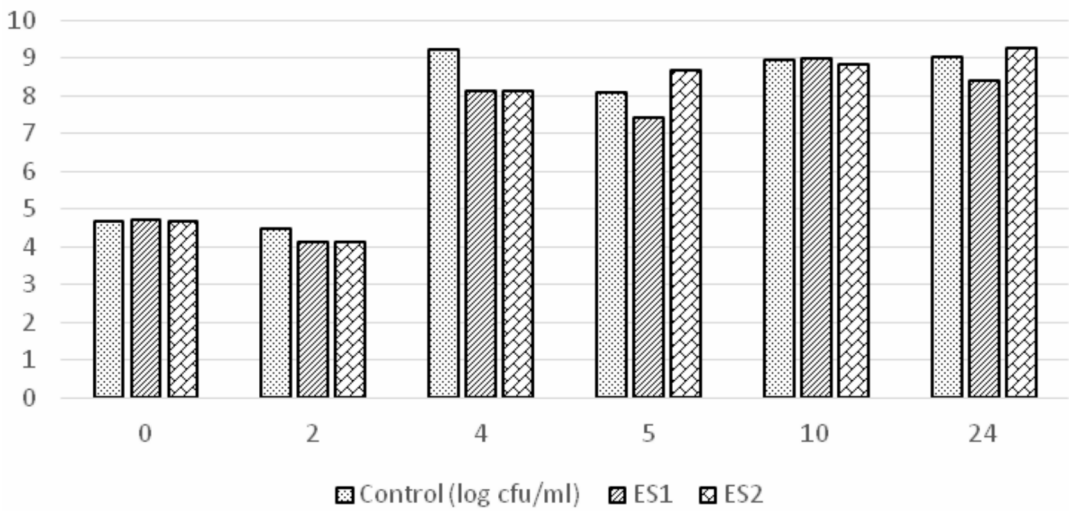

Fig. 1. Competitive study of *Escherichia coli* Ec221/c (isolated from faeces of ostrich) with enterocin Ent 412.
ES1 – enterocin addition at the start of the experiment; ES2 – Ent in log phase of strain growth ES1 and ES2 to C, at 4 hour (difference 1,13 log cycle); ES1 to C (difference at 5 hours 0.67 log cycle), ES2: C at 10 h(difference 0.15 log cycle), at 24 h ES1 and ES2: C (difference 0.61, 0.67 log cycle), which means a tendency to decrease Ec221/c strain

resistant. The antibiotic profile of *E. coli* showed high antibiotics resistance. Antibiotic resistance is an emerging problem worldwide. In the case of aminoglycosides (gentamicin), 50.7% resistance has been reported. Resistance of isolated strains to cephalosporines (first generation – cefalotin) is higher (67.6%). Chinolones represented by nalidixic acid reached resistance of 50.7%. The identified *E. coli* strains are highly resistant to tetracycline (54.9%), 97.2% of strains are resitant to erythromycin, and 98.6% are resistant to penicillin. A high percentage of *E. coli* strains resistant to erythromycin and penicillin in poultry was also reported by Akond et al. (2009). Similar findings on multiple drug resistance of *E. coli* have been reported by Guerra et al. (2003) or Zhao et al. (2005). Food supply, including poultry products, may transmit drug-resistant *E. coli* to humans. Due to long-term and frequent use of antibiotics in poultry breeding, resistance in *E. coli* may persist in the avian intestinal tract for a long time even in the absence of antibiotics (van de Bogaard et al. 2001). In our study the farmer declared no antibiotic use. Moreover, the strains were treated with another type of antimicrobial agent, with bacteriocins-enterocins. Many strains (especially species *E. faecium*) are able to produce enterocins (Lauková et al. 1993, Franz et al. 2007). As mentioned above, our laboratory has focused on the study of enterocins for several years. Knowing the inhibitory range of enterocins is very important for their possible application. This is, why enterocins produced by our *E. faecium* strains possessing also probiotic properties were used in this study. The growth of *E. coli* was inhibited by Ents including hemolysis-positive strains and polyresistant strains. The enterocins used were able to inhibit 45% of the tested strains (32 strains). *In vitro* Ents ability to control the growth of Gram-negative *E. coli* was confirmed. The Ents used belong among the bacteriocins with a broad inhibitory spectrum (Franz et al. 2007). Their use to inhibit *E. coli* is a promising indication for their further application mainly when problems with antibiotic resistance and/or residues in meat of food-producing animals exist. Sensitivity of *E. coli* strains (especially those positive for hemolysis and polyresistance) to Ents indicates the possibility of using Ents for elimination of pathogenic agents. Moreover, the antimicrobial activity of Ents used here has been previously reported *in vitro* and *in vivo* studies, e.g. in rabbits and in chickens as well (Strompfová et al. 2006, Pogány Simonová et al. 2009, Szabóová et al. 2011, Lauková et al. 2012a,b). This represents a chance to inhibit the growth of recently often-detected antibiotic-resistant bacteria. In our study, many of the strains causing hemolysis were sensitive to Ents, while they showed resistance to antibiotics as well. On the other hand, the growth inhibition of 32 strains by Ents is reported, and at the same time they were polyresistant. Antimicrobial activity of bacteriocins, including enterocins, is often influenced by various other factors, for example the medium used, the presence of fat droplets in the case of meat material and sensitivity of the indicator strain (Nes et al. 2002). Frequent variation in the results of inhibitory activity when testing enterocins can be construed as the fact that the individual bacteriocins recognize specific receptor sites on the target cells (Kjos et al. 2009). Com-

petitive studies show a tendency of Ents to decrease *E. coli* growth. The bacteriostatic effect of Ents has already been presented in several studies (e.g. Pogány Simonová et al. 2009). It is necessary to underline that in our case a large target of Gram – negative *E. coli* was tested. The importance of the results obtained is that sensitivity to enterocins of Gram-negative *E. coli* from ostriches has never been tested before. The results presented here promote the use of Ents primarily for prevention. It has been repeatedly confirmed that enterocins unrelated to the source of the enterocin-producing strain are effective. Poultry farming represents a livestock sector where probiotic preparations are frequently used and where probiotic bacteria (mainly of the species *E. faecium)* have already been successfully applied (Lauková et al. 2003, Levkut et al. 2009). In our previous work inhibitory effect against coliform bacteria was shown not only *in vitro* (Lauková et al. 1993), but also *in vivo*, e.g. in Japanese quails (Lauková et al. 2004) or in rabbits (Pogány Simonová et al. 2009, Szabóová et al. 2011). The possibility of eliminating and preventing poultry disorders using probiotic microorganisms and/or their bioactive substances, i.e. enterocins is indicated not only by their antimicrobial activity; they are also able to beneficially affect host immunity through the stimulation of non-specific immunity parameter-phagocytic activity, as well as reducing mortality and increasing daily weight gain, all without inducing oxidative stress (Lauková et al. 2012a,b).

In conclusion, our results suggest that Ents (or their producers which also possess probiotic properties) can inhibit *E. coli* contamination in poultry farms, including ostriches. Despite the preliminary character of these results, they contribute to the knowledge of ostrich microflora and enterocins and provide good prospects for the further use of Ents. Of course, additional i*n vivo* studies are also currently in progress.

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