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Original article

DIFFERENT EFFECT OF DOXYCYCLINE AND ENROFLOXACIN ON CATHELICIDIN-3 mRNA EXPRESSION IN CHICKENS WITH OR WITHOUT PROBIOTICS SUPPLEMENTATION

I. PAVLOVA & A. MILANOVA

Department of Pharmacology, Veterinary Physiology and Physiological Chemistry, Faculty of Veterinary Medicine, Trakia University, 6000 Stara Zagora, Bulgaria

Summary

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The function of immune system of poultry has a significant impact on poultry husbandry sustainability. Therefore the aim of this study was to investigate the effect of lactic acid bacteria administered with enrofloxacin or doxycycline on expression levels of antimicrobial peptide cathelicidin-3 (CATH3) at mRNA level in the duodenum, jejunum and liver of broilers. A day-old Ross (n=24) and Duc (n=24) chickens were included in experiments with enrofloxacin and doxycycline, respectively. They were divided into four groups (n=6) for each experiment: control, supplemented with probiotics (15 days via feed, 5 days after hatching), treated with either enrofloxacin or doxycycline (10 $mg.kg^{-1}$ for 5 days, via drinking water) and treated with antibiotic and probiotics. Expression levels of CATH3 mRNA in liver, duodenum and jejunum were determined by RT-PCR and were statistically evaluated by Mann-Whitney test.Administration of probiotics led to insignificant down-regulation of CATH3 mRNA in the investigated tissues. The combination of doxycycline with probiotics led to statistically significant down-regulation of CATH3 mRNA in the duodenum ($P<0.01$). Statistically significant up-regulation of mRNA of the studied gene was found in the jejunum of enrofloxacin treated Ross chickens. The data suggest the existence of an interaction between antibiotics and innate immunity. Further evaluation in infected poultry would shed more light on the pharmacodynamics of antibacterials.

Key words: CATH3 mRNA, doxycycline, Duc and Ross broilers, enrofloxacin, *Lactobacillus* spp.

INTRODUCTION

The challenges to modern poultry husbandry are related to the production of functional and safe food for consumers. Probiotics are routinely applied as feed supplements for poultry because their use is associated with better feed utilisation (low levels of conversion of feed), with stimulation of immunity in birds and with

reduced incidence of diseases (Lee *et al*., 2011; Allen *et al*., 2013). Despite the positive effects of probiotics on the development of immunity of poultry, in some cases antimicrobial therapy is necessary. Probiotics can be co-administered with antibiotics to reduce the adverse effects from the administration of antibacterial drugs (Ouwehand *et al*., 2014). Therefore, knowledge of the mechanism of interaction between probiotics and antibiotic is required in order to apply the correct combination. The selection of strains as probiotics is based on strict criteria such as sensitivity to antibiotics, possibility of transfer of resistance in pathogenic strains, stability in the gastrointestinal tract of animals and its colonisation (EU/SCAN, 2005; Sen *et al*., 2012).

Furthermore, understanding of the effects of interactions between probiotics and antibiotics on the animals is very important for their proper clinical administration. The published literature describes desirable effects of probiotics such as stimulation of immune system of birds and stabilisation of integrity of gastrointestinal barrier (Murugesan, 2013). Data in humans suggest that probiotics can stabilise the barrier functions of the intestine by increasing the production of antimicrobial peptides from epithelial cells such as cathelicidins and beta-defensins (Theodorakopoulou *et al*., 2013). Antimicrobial peptides are considered key factors in innate immunity by providing a first line of defense of the body against potential pathogens in mammals and birds (Michailidis, 2010). Cathelicidins are antimicrobial peptides and expression of four cathelicidins was described in poultry so far (Lynn *et al*., 2004; van Dijk *et al*., 2005; 2007). These are the cathelicidin-1 or fowlicidin-1 (Lynn *et al*., 2004), chicken myeloid antimicrobial peptide 27 (CMAP27) or fowlicidin-2 (van Dijk *et al*., 2005), third chicken cathelicidin fowlicidin-3 (Xiao *et al*., 2006) and cathelicidin-B1 (Goitsuka *et al*., 2007). Cathelicidins were found predominantly in epithelial cells in the gastrointestinal tract and kidney, and in myeloid tissue (Sang & Blecha, 2009). High to medium levels of expression of the cathelicidin-1 were described from the crop to the small and large intestines of chickens. The liver levels of cathelicidin-1 were low and those of the cathelicidin-2 were higher (Lynn *et al*., 2004; van Dijk *et al*., 2005). Their activity against Gram-positive and Gram-negative microorganisms and also against pathogenic fungi and nematodes has been demonstrated (Scocchi *et al*., 2005). In addition to these properties, cathelicidins possess immunomodulatory activity, such as chemotaxis, binding and inactivation of toxins (Agerberth *et al*., 2000; Scott *et al*., 2002). Their expression can be down- or up-regulated by infection of poultry with pathogenic bacteria such as *Salmonella* spp. (Akbari *et al*., 2008; van Dijk *et al*., 2009). The application of probiotics in *Salmonella*-infected chickens was also associated with up-regulation of the expression of these antimicrobial peptides (Akbari *et al*., 2008). Some studies found that their activity was reduced in presence of serum and was not changed by physiological solutions of sodium chloride (van Dijk *et al*., 2011). Despite the increasing number of investigations on cathelicidins there are no data concerning the effect of antibiotics on their expression.

Effects of interactions between antibiotics and probiotics on innate immunity are not well understood. Taking into account the insufficient knowledge about the expression of cathelicidins, the current study aimed to describe the effect of enrofloxacin and doxycycline on the expres-

sion levels of CATH3 mRNA in the duodenum, jejunum and liver of broiler chickens.

MATERIALS AND METHODS

Drugs

The broilers were treated with enrofloxacin hydrochloride (Baytril 5%, KP076SN, 02.2014 Bayer Animal Health GMBH Leverkuzen, Germany) and doxycycline hyclate (Doxy-200 ws., 255292/05-2015, Interchemie, Venray, Holland). Enrofloxacin was administered as a freshly prepared water solution according to the manufacturer instructions at a dose of 1 $mL.L^{-1}$ of drinking water. Doxycycline was administered at a dose of 1 g of drug formulation (200 mg active compound) in 4 L of drinking water.

Lactic acid bacteria

Freeze-dried *Lactobacillus brevis* 51 (LK-IMicB), *L. plantarum* 11 (LK-IMicB) and *L. bulgaricus* 13 (LK-IMicB) were provided by the Institute of Microbiology at the Bulgarian Academy of Sciences (LK-IMicB – Microbial Collection of Genetics of Lactic Acid Bacteria and Probiotics, laboratory, Assoc. Prof. Svetla Danova, DSc). The strains are natural isolates of the traditional dairy products and were characterised as candidate probiotics (Danova *et al*., 2012, Tropcheva *et al*., 2013), according to *in vitro* WHO criteria (FAO/WHO, 2002). They were previously characterised as strains with high tolerance of transit and were selected as probiotics able to maintain their viability in the gastro-intestinal tract of chickens. The strains were cultured in skimmed milk (Humana, Holdorf, Germany), lyophilised and stored at –20 °C until the beginning of the experiments. Lyophilised bacteria of *L. brevis* 51 were at a concentration of 1.6×10^{6} $CFU.mg^{-1}$ lyophilised product, *L. plantarum* – 1.06×10^{6} $CFU.mg^{-1}$ and *L. bulgaricus* – 0.25×10^{3} $CFU.mg^{-1}$. These strains showed a broad spectrum of antibacterial activity (Danova *et al*., 2012).

Animals and experimental design

The experiments included one-day old Ross 308 broilers (n=24) and Duc broiler chickens (n=24). The animals were obtained from Bovans Bulgaria Ltd., hatchery, Chirpan. The experiments were approved (Protocol No 65/18.10.2013) by the Ethical commission for animal experiments at Trakia University, Stara Zagora. The chickens had free access to feed and water.

Experiment I. Treatment with probiotics and enrofloxacin. Ross 308 broilers (n=24) were equally divided in four groups. The first group was untreated and served as a control (n=6). The second group (n=6) was supplemented with probiotics. The third group (n=6) was treated with probiotics and enrofloxacin. The fourth group (n=6) was treated with enrofloxacin only. The enrofloxacin was administered at a dose of 10 $mg.kg^{-1}$. The treatment started 15 days after hatching and lasted five days. The medicated water was prepared *ex tempore* and was administered each morning between 7.30 and 8.00 h and between 16 and 17 h in the afternoon. The probiotics were administered with the feed for 15 consecutive days at a dose of 1 $g.kg^{-1}$ feed from each strain (*L. brevis, L. plantarum* and *L. bulgaricus*), five days after hatching. They were stored at –20 °C as lyophilised products until their addition to the feed. The feed with probiotics supplementation was daily prepared and administered to the broilers.

Experiment II. Treatment with probiotics and doxycycline. Duc broiler chickens (n=24) were divided in four groups. The first group consisted of 6 untreated chickens and was used as a control. The second group was supplemented with probiotics (n=6). The third group (n=6) was treated with a combination of probiotics and doxycycline. Doxycycline only was administered to the fourth group (n=6). The antibiotic was administered via drinking water at a dose of 10 $mg.kg^{-1}$. The medicated water was prepared *ex tempore* between 7.30 and 8.00 h and between 16 and 17 h. The probiotics were administered according to the procedure described above, as in experiment with enrofloxacin.

The chickens were euthanised by cervical dislocation 126 h after the start of the treatment with either of antibiotics enrofloxacin or doxycycline (Regulation No 20/01.11.2012). Tissue samples from liver, duodenum and jejunum were taken for RT-PCR analysis and they were snap frozen in liquid nitrogen and kept at –70 °C until analysis.

Isolation of total RNA

Total RNA was isolated by using TRItidy G (Cat. No. A4051, Genaxxon bioscience GmbH, Germany). In sterile tubes with 0.6 mL TRItidy G were added 35 mg tissue samples from liver or 50 mg tissue samples from duodenum or jejunum, respectively. The samples were homogenised with Tissue tearor (Model 985370-395. Biospec Products Inc.) for 10–20 s. They were centrifuged at 12000×g for 10 min at 4 °C. TRItidy G reagent was placed in a sterile tube and the samples were incubated at 25–30 °C for 5 min. Chloroform (0.2 mL) was added to the lysate and the samples were shaken for 15 s and incubated at 30 °C for 10 min. The lysate was centrifuged at 12000×g for 15 min at 4 °C and the water phase was moved to a new sterile tube. Then 500 µL isopropanol was added and the samples were kept for 15 min at 4 °C. After that they were centrifuged at 12000×g for 15 min at 4 °C and the supernatants were carefully removed. One mL 75% ethanol was added to the pellets of mRNA, the samples were vortexed and then centrifuged at 7500×g for 5 min. The supernatant was removed and 1 mL 100% ethanol was added to the samples. The tubes were centrifuged again at 7500×g for 5 min at 4 °C. The ethanol was removed carefully. After evaporation of the ethanol for a few minutes at room temperature, 20 µL DEPC-water was added to the pellets and the samples were incubated at 55–60 °C for 10 min. The samples were placed on ice and the quality and quantity of total RNA was determined by ultraviolet absorbance at 260 and 280 nm. The samples were stored for a short period at – 70 °C prior to cDNA synthesis.

cDNA synthesis by reverse transcription

First-strand cDNA was synthesised using the RevertAid^{TM} First Strand cDNA Synthesis Kit (Fermentas Life Science, Cat. No. k1621, Thermo Fisher Scientific Inc., USA) according to the manufacturer's instructions. To a master mixture containing oligo (dT)18 primer and M-MuLV reverse transcriptase, 3 µg total RNA dissolved in sterile nuclease-free water was added. The reaction mixture (total volume 20 µL) was incubated for 60 min at 37 °C, and then the enzyme was heat inactivated at 70 °C for 5 min and the reaction mixture rapidly cooled to 4 °C. Reverse transcription was performed on a Quanta Biotech QB-96 Gradient Thermal Cycler (Quanta Biotech Ltd., Surrey, United Kingdom). The cDNA was diluted 1:3 in sterile RNase-free water. The samples

were stored at –70 °C until qRT-PCR analysis.

qRT-PCR analysis

Specific primers for chicken CATH3, hexose-6-phosphate dehydrogenase (H6PD) and hypoxanthine phosphoribosyltransferase (HPRT) were used (Table 1). HPRT and H6PD were used as reference genes for the normalisation of the expression levels of gene of interest. Sybr Green method was applied for the real-time PCR analysis by using iQ^{TM} Sybr Green Supermix (Cat. No. 170-8885, Bio- Rad, Hercules, CA). iCycler iQ system (Bio-Rad, Hercules, CA) was used for RT-PCR and MyiQ System Software, v. 1.0.410 (Bio-Rad Laboratories Inc.) was applied for analysis of results. Each reaction went through a PCR cycle with a denaturation step at 95 °C for 20 s, an annealing step specific for each set of primers for 30 s and an elongation step at 72 °C for 30 s. After 35 cycles a melting curve was obtained by increasing the temperature with 0.5 °C every 10 s from 6 °C to 95 °C demonstrating the formation of only one product. Efficiencies for each reaction were estimated by LinRegPCR 7.0 software. Relative gene expression level was assessed using the algorithm described by Vandesompele *et al*. (2002).

Statistical analysis

Mann-Whitney test (Prism 4.0, GraphPad Software, Inc., La Jolla, CA, USA) was used to evaluate the data for statistically significant differences in mRNA levels of CATH3 between the untreated controls, probiotic supplemented chickens and poultry supplemented with antibiotics administered with or without probiotics. Level of significance was set at $P<0.05$.

RESULTS

CATH3 mRNA was found in the tissue samples of duodenum, jejunum and liver in both DUC (Fig. 1) and Ross 308 (Fig. 2) broiler hybrids. Inter-individual variations in the levels of expression were found. Administration of lactic acid bacteria led to insignificant down-regulation of CATH3 mRNA in the investigated tissues of DUC and in Ross 308 chickens.

The combination of doxycycline and probiotics led to statistically significant down-regulation of CATH3 mRNA in the duodenum ($P<0.01$). Administration of doxycycline did not provoke changes in the levels of CATH3 mRNA. Statistically significant up-regulation of mRNA of the studied gene was found in the jejunum of enrofloxacin Ross 308 chickens in com-

Table 1. Specific primers for chickens used for qRT-PCR analysis

Gene	NCBI number	Forward/reverse primers 5′→ 3′	Ta, °C
CATH3	NM_001311177.1	F: GCTGTGGACTCCTACAACCAAC R: TGGCTTTGTAGAGGTTGATGC	55
H6PD	XM_425746.4	F: GAGAACCAGCACTTCTTAGAC R: GGGTTCAGCAACTCCACTG	64
HPRT	NM_204848	F: CGTTGCTGTCTCTACTTAAGCAG R: GATATCCCACACTTCGAGGAG	65

CATH3 – gene encoding cathelicidin-3 (Achanta *et al*., 2012); H6PD – gene, encoding hexose-6-phosphate dehydrogenase; HPRT – gene, encoding hypoxanthine phosphoribosyltransferase 1; NCBI – the National Centre for Biotechnology Information; Ta – optimal annealing temperature.

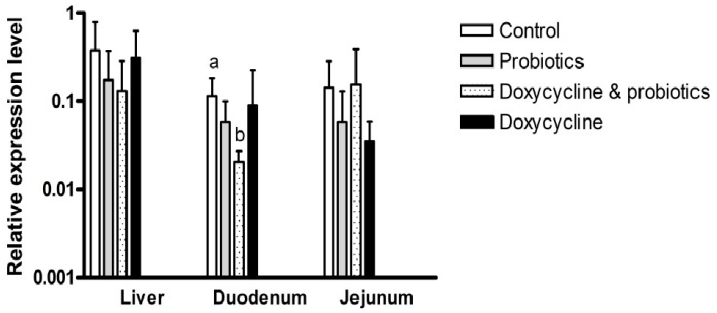

Fig. 1. Expression of CATH3 mRNA in the liver, duodenum and jejunum normalised against reference genes H6PD and HPRT in healthy Duc broilers, treated with doxycycline via drinking water for 5 days at a dose of 10 mg.kg^{-1}, supplemented (n=6) or not (n=6) with lactic acid bacteria; *a* and *b* represent statistically significant difference at P<0.05.

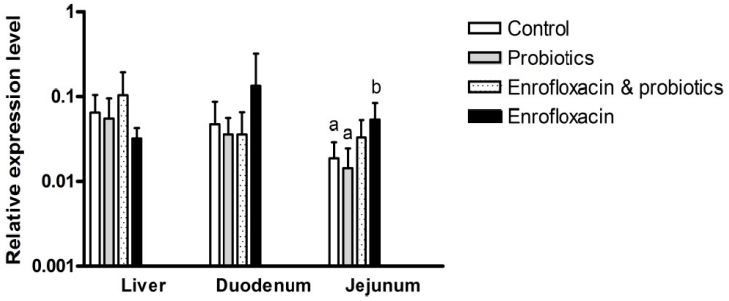

Fig. 2. Expression of CATH3 mRNA in the liver, duodenum and jejunum normalised against reference genes H6PD and HPRT in healthy Ross 308 broilers, treated with enrofloxacin via drinking water for 5 days at a dose of 10 mg.kg^{-1}, supplemented (n=6) or not with lactic acid bacteria; *a* and *b* represent statistically significant differences at P<0.05.

parison to the control group (P<0.05) and to the group treated with probiotics (P<0.01).

DISCUSSION

Cathelicidins have a role in protecting animals from infections till the development of adaptive immunity. Therefore their expression and function are important for the health of chickens during the first days after hatching. Cathelicidins are peptides with bactericidal properties, including against bacteria that show resistance to some antibiotics (Bommineni *et al*., 2007; van Dijk *et al*., 2011). Their expression in chickens was affected by factors such as bacterial diseases and by administration of probiotics (Akbari *et al*., 2008). Broad spectrum antibiotics have a significant impact on the microbiota in the gastro-intestinal tract but their effect on the innate immunity of poultry in terms of antimicrobial peptides was not evaluated.

CATH3 mRNA was described in many tissues of broilers (Achanta *et al*., 2012). In the cited study the highest expression levels were found in the lungs, caecal tonsils, proventriculus and the

heart, followed by these in the intestinal epithelium from duodenum to colon and in the liver. Our data showed that CATH3 mRNA was expressed in the duodenum, jejunum and liver in both Ross 308 and Duc hybrids with relatively high inter-individual variations. Administration of probiotics *L. brevis, L. plantarum* and *L. bulgaricus* led to insignificant down-regulation of CATH3 mRNA similarly to the effect of *Lactobacillus acidophilus, Bifidobacterium bifidum* and *Enterococcus faecalis* (Akbari *et al*., 2008). Similarly, co-administration of organic acids and probiotic strains *Lactobacillus casei, L. acidophilus, Streptococcus faecium* and *Saccaromyces cerevisiae* did not provoke changes in CATH3 mRNA levels (Rodriguez-Lecompte *et al*., 2012). The probiotics, as a part of commensal bacteria, contribute to maintaining intestinal health and suppress the inflammation. Thus they do not provoke changes in the expression levels of CATH3 antimicrobial peptide. In contrast, the pathogenic bacteria such as *Salmonella enterica* serovar Typhimurium up-regulated CATH3 mRNA (Akbari *et al*., 2008; Meade *et al*., 2009). These changes were neutralised by probiotics administration, an effect attributed to their properties for inhibition of *Salmonella enterica* and of its adhesion to the intestinal cells of the host (Akbari *et al*., 2008; Meade *et al*., 2009). Microorganisms such as *Campylobacter jejuni* provoke down-regulation of CATH3 mRNA and β-defensins which leads to successful colonisation of the gastro-intestinal tract of chickens by this pathogen (Meade *et al*., 2009). Co-administration of probiotic strains *L. brevis*, *L. plantarum* and *L. bulgaricus* with broad spectrum antibiotics such as enrofloxacin and doxycycline does not have a negative impact on innate immunity as determined by CATH3 mRNA levels. The combination probiotics-doxycycline led to significant down-regulation of CATH3 mRNA only in the duodenum which can be attributed to absence of inflammation in the gastro-intestinal tract provoked by pathogenic bacteria. Unchanged expression levels in the probiotics-enrofloxacin treated group can be similarly discussed. The results from another study demonstrated that doxycycline, administered with or without lactic acid bacteria up-regulated the expression of mRNA levels of another antimicrobial peptide LEAP-2 in the jejunum and in the duodenum and jejunum, respectively and stimulation of the innate immunity in broilers can be expected (Pavlova *et al*., 2015). These data allow us concluding that doxycycline did not have similar effects on the expression levels of different antimicrobial peptides in broilers. The treatments with enrofloxacin resulted in significant induction of CATH3 mRNA in the jejunum and to a lesser extent in the duodenum which can be considered as positive influence on factors of innate immunity. These data suggest that further investigations on cathelicidins and defensins in infected animals are needed to better understand the interaction of antibiotics with innate immunity.

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Correspondence:

Prof. Anelia Milanova
Department of Pharmacology, Veterinary Physiology and Physiological Chemistry,
Faculty of Veterinary Medicine,
Trakia University,
6000 Stara Zagora, Bulgaria
e-mail: akmilanova@gmail.com