ORIGINAL ARTICLE / ÖZGÜN MAKALE

Turkish Journal of Immunology - Open Access
www.turkishimmunology.org

Turk J Immunol 2017; 5(1):5–12

The Increase in LEAP-2 mRNA Suggests a Synergistic Probiotics-Doxycycline Interaction in Chickens

LEAP-2 mRNA'sındaki Yükselme, Piliçlerde Probiotikler ile Doksisiklin Arasındaki Sinerjik İlişkiyi İşaret Etmektedir

Ivelina Pavlova[1], **Aneliya Milanova**[1]

Abstract

Background: Extensive interaction between gut microbiota and the host immune system has significant impact on chicken performance.

Objective: Therefore, the influence of doxycycline, administered with or without *Lactobacillus* spp., on PepT1 and LEAP-2 mRNAs expression in duodenum, jejunum and liver was investigated in Duc chickens.

Methods: One-day-old Duc broiler chickens were divided in four groups. Chickens without treatment served as controls. Five days after hatching the second group was treated with *Lactobacillus brevis, L.plantarum* and *L.bulgaricus* probiotics via feed for 15 days. The third group was treated with probiotics (as in group 2) and doxycycline (10 mg/kg bw, via drinking water for five days, started 15 days after hatching). The fourth group received antibiotic only as described above. Water and food (broiler starter without drugs) were supplied *ad libitum*. Samples from liver, duodenum and jejunum were collected at the end of the treatment with doxycycline (21 days after hatching).

Expression levels of LEAP-2 and PepT1 mRNAs were determined by qRT-PCR.

Results: Doxycycline administered alone or in combination with probiotics, provoked a statistically significant up-regulation of LEAP-2 mRNA in the liver and in the duodenum. Abundance of PepT1 mRNA was increased in the duodenum. Administration of doxycycline alone caused a moderate induction of LEAP-2 mRNA and down-regulation of PepT1 mRNA in the liver.

Conclusion: Up-regulation of the studied antimicrobial peptides provoked by combination of *Lactobacilli* and doxycycline might be beneficial in terms of host protection.

Keywords: Doxycycline, LEAP-2 mRNA, PepT1 mRNA, chicken, *Lactobacillus* probiotics

Öz

Genel Bilgi: Barsaktaki mikrobiata ile konağın bağışıklık sistemi arasında olan ilişki, piliçlerin verimliliğine önemli derecede etki eder.

Amaç: Laktobasil tipi bakterilerin verildiği ve verilmediği, doksisiklin verilen Duc piliçlerde duodenum, jejunum ve karaciğerde PepT1 ve LEAP-2 mRNA ifadeleri araştırıldı.

Yöntemler: 1 günlük Duc etlik civcivler 4 gruba ayrıldı. Tedavi verilmeyen civcivler kontrol grubu olarak alındı. Yumurtadan çıktıktan 5 gün sonra ikinci gruba Lactobacillus brevis verildi. L. plantarum ve L.bulgaricus probiotikleri 15 gün boyunca verildi. Üçüncü gruba ikinci gruba olduğu gibi, probiotik ile birlikte doksisiklin(yumurtadan çıktıktan sonra 15. günde içtikleri suya karıştırarak 10 mg/kg vücud ağırlığı olacak şekilde) uygulandı. Dördüncü grup, yukarıda tanımlandığı şekilde sadece antibiyotik aldı. Civvicler, suyu ve yiyeceği serbest olarak (ad libitum) aldı. Doksisiklin tedavisi bittikten sonra (yumurtadan çıktıktan 21 gün sonra) karaciğer, duodenum, jejunum ve karaciğerden örnekler alındı. LEAP-2 ve PepT1 mRNA düzeyleri qRT-PCR ile ölçüldü.

Bulgular: Tek başına ya da probiotikler ile birlikte verilen doksisiklin, karaciğer ve duodenumda anlamlı ölçüde LEP-2 mRNA'sı sentezine yıl açtı.PepT1 mRNA'sı artışı en çok duodenum'dan alınan örneklerde görüldü. Sadece doksisiklin verilmesi karaciğerde LEAP-2 mRNA üretiminde hafif bir artışa ve PepT1 mRNA düzeyinde düşüşe neden oldu.

Sonuç: Laktobasil ve doksisiklin kombinasyonu ile sentezi arttırılan antimikrobial peptidler, konağın infeksiyondan korunmasına yardımcı olabilir.

Anahtar Kelimeler: Doksisiklin, LEAP-2 mRNA, PepT1 mRNA, piliç, *Lactobacillus* probiotikler

[1]Department of Pharmacology, Physiology of Animals and Physiological Chemistry, Faculty of Veterinary Medicine, Trakia University, Stara Zagora, Bulgaria

Correspondence:
Ivelina Pavlova
Department of Pharmacology, Physiology of Animals and Physiological Chemistry, Faculty of Veterinary Medicine, Trakia University, 6000 Stara Zagora, Bulgaria

Tel: +35942699552
e-mail: ivelpp@abv.bg

Received: February 13, 2017
Accepted: April 13, 2017

doi: 10.25002/tji.2017.541

Introduction

Doxycycline is a second generation, semisynthetic broad spectrum antibiotic, used for many years in treatment of bacterial diseases in farm animals.[1] It has some advantages over

other tetracycline antibiotics such as higher bioavailability after oral administration together with lower affinity to Ca^{2+} ions, better penetration in tissues, and longer half-life of elimination, and lower MIC values against many Gram-positive and Gram-negative pathogens for poultry.[2–5] This antibacterial drug showed good stability in water solutions which is favorable for oral treatment of big groups of animals.[6] Doxycycline is widely used in poultry due to its efficacy in treatment of diseases caused by pathogenic bacteria such as *Staphylococcus pyogenes, Streptococcus spp., Escherichia coli, Enterobacter, Klebsiella, Haemophilus influenza, Pseudomonas aeruginosa, Chlamydia, Mycoplasma* and *Rickettsiae spp.*[7,8]

Apart from the problem with antibacterial resistance against tetracyclines[9,10], their administration is related to rapid changes in microbiota composition with increase in the number of microorganisms such as *Escherichia coli* and *Enterococcus* spp.[11] Administration of probiotics is often used for restoration of microbiota complexity after therapy with antibiotics.[12] Several studies characterized *Lactobacillus* spp. as suitable probiotic strains for poultry.[13,14] Probiotics used routinely in poultry lead to better body weight gain, lower feed conversion ratio (FCR) and decreased mortality.[15,16] Part of these effects can be attributed to the changes in the expression levels of transporter proteins such as Na^+/glucose cotransporter (SGLT-1) and P-glycoprotein (P-gp).[17] Additional benefit from probiotics supplementation to poultry feed is production of small molecules with antibacterial properties called bacteriocins and defensins.[18–20] Their influence on the function of the immune system through modulation of the microbiota in the gastrointestinal tract and through activation of Toll-like and NOD-like receptors has been described.[21]

LEAP-2 was described in poultry and it was recognized as an antimicrobial peptide with activity against *Salmonella* spp., *Streptococcus* spp., and *Staphylococcus* spp.[22,23] PepT1 is responsible for peptide absorption from gastro-intestinal tract which is related to feed utilization in poultry.[24] Its expression can be influenced by the microbiota in the gastro-intestinal tract and is down-regulated by *Cl. perfringens*.[25] Although increased knowledge on the effect of gastro-intestinal health and its impact on the performance of broilers there are no published data about the influence of probiotics and antibiotics on the expression of PepT1 and LEAP-2 mRNAs.

Therefore, the present study was designed as a pilot investigation of the effect of doxycycline administered with or without *Lactobacillus plantarum* 11, *Lactobacillus brevis* 51 and *Lactobacillus bulgaricus* 13 as well as the combination of these strains on the expression of PepT1 and LEAP-2 mRNAs in the duodenum, jejunum and liver of Duc broiler chickens.

Materials and Methods

Drugs

Doxycycline hyclate (Doxy-200 ws. Interchemie, Venray, Holland, Serial No: 255292/05–2015). Each 1 g of doxycycline contained 200 mg doxycycline hyclate.

Microbial cultures

Lactobacillus brevis 51, *L.plantarum* 11 and *L.bulgaricus* 13 (Microbial Collection-Laboratory of Genetics of Probiotic Bacteria, Institute of Microbiology, BAS) were used as probiotic strains. They were natural isolates from traditional dairy products and showed scientifically proven probiotic potential, a broad spectrum of antimicrobial activity and examined molecular mechanisms of the possible healthful effects.[26] They were characterized as candidate probiotics according to the in vitro criteria of WHO.[27,28] The *Lactobacillus* strains were cultured overnight at 37°C in De Man, Rogosa and Sharpe (MRS, Merck, Germany) broth under anaerobic condition (BBL® Gas Pak Anaerobic System Envelopes, Becton Dickinson). Exponential cultures of the investigated strains were used separately to inoculate 10% (w/v) sterile skimmed milk (Humana, Holdorf, Germany). After an overnight cultivation process at 37°C they were lyophilized, and stored at -20°C until administration to the chickens. Each mg of the lyophilized *L.brevis* 51 contained $1.6x10^6$ CFU/mg, *L.plantarum* 11 $1.06x10^6$ CFU/mg lyophilized product and *L.bulgaricus* 13–$0.25x10^3$ CFU/mg. The viability of the used strains lactobacilli was tested after the lyophilization and before the *in vivo* assays by a standard plate count procedure using MRS agar.

Animals and experimental design

The experimental procedure was approved by the Ethical committee at Trakia University, Stara Zagora (Reference No: 65/18.10.2013). The euthanasia of the chickens was done according to Regulation No: 20/01.11.2012.

Table 1. Experimental design of the study with Duc broiler chickens

Experimental groups	Probiotics in the feed	Doxycycline (10 mg/kg via water)	Sampling for qPCR analysis
Group 1	Not supplied	Not administered	On 21-st day after hatching, n=6
Group 2	Supplied for 15 days from 6-th to 20-th day after hatching	Not administered	On 21-st day after hatching, n=6
Group 3	Supplied for 15 days from 6-th to 20-th day after hatching	Treated for 5 days, from 16-th day to 20-th day after hatching	On 21-st day after hatching, n=6
Group 4	Not supplied	Treated for 5 days, from 16-th day to 20-th day after hatching	On 21-st day after hatching, n=6

One-day-old Duc chicks (n=120) were included in the experiment. They were equally divided in four groups, ten animals per pen, three pen for each group. They were kept according to the species requirements, at 24 h lighting regimen and room temperature between 26 and 28°C (temperature under the lights between 35 and 30°C according to the age of the chickens). Water and food (broiler starter without drugs) were supplied *ad libitum*. The chickens were clinically healthy and any signs of disease were not observed.

First group was used as control and it was not treated. Second group was treated with probiotics at a rate of 1 g of each probiotic strain per kg feed. Probiotics supplementation started 5 days after hatching and lasted 15 days. The probiotic strains were stored at -20°C until delivery to the chickens and they were added daily to the feed. Feed with probiotics was administered each morning between 7.30 and 8 h. Third group received a combination of probiotics as described above and doxycycline orally at a dose of 10 mg/kg body weight via drinking water. The treatment with doxycycline started 15 days after hatching and lasted five consecutive days. Chickens from the fourth group were treated with doxycycline only with the same dosage regimen as in the third group. Each day, the solutions in drinking water were freshly prepared between 7.30 and 8 h in the morning and between 16 and 17 h in the afternoon. The ingested dose of doxycycline did not differ from aimed dose of 10 mg/kg. The medicated water was administered *ad libitum*. Consumption of water was measured, and the body weight of the animals was registered. Feed consumption and body weight of the chickens were measured every day and average daily gain and FCR were calculated. These data were collected for all of the chickens included in the experiment.

Six chickens from each group (Table 1) were euthanized by cervical dislocation 126 hours after the beginning of the treatment with doxycycline. Tissue samples from liver, duodenum and jejunum were collected. They were snap-frozen in liquid nitrogen and stored at -70°C until RT-PCR analyses.

RNA isolation and RT-PCR analysis

Total RNA was isolated by using TriReagent for DNA and protein extraction (Genaxxon bioscience GmbH, Lot-No. 3T004856). Tissue samples were homogenized in 0.6 mL TRItidy G for 10–20 sec. The lysate was centrifuged at 12000 g for 10 min at 4°C and the upper aqueous phase was transferred to a new sterile tube which was incubated at 25°C for 5 min. Then 0.2 mL chloroform was added, the tubes were incubated for 30 min at 30°C and centrifuged at 12000 g for 15 min at 4°C. The upper aqueous phase was transferred in a sterile tube and 500 µL isopropanol was added. The organic phase with the DNA was centrifuged at 12000 g for 15 min at 4°C and the residual aqueous supernatant was removed. The pellets were washed with 1 mL 75% ethanol by vortexing and subsequently with absolute ethanol, then they were centrifuged at 7500 g for 5 min at 4°C. The ethanol was removed and after drying the RNA was dissolved in 20 µL DEPC-treated water. After that the tubes were incubated at 55–60°C for 10 min. RNA concentrations were measured with spectrophotometer A260/280 and stored at -70°C until reverse transcription. Single-stranded cDNA was synthesized from 3 µg total RNA using the First Strand cDNA Synthesis Kit (Fermentas Life Science) on a Quanta Biotech QB-96 (Quanta Biotech Ltd., Surrey, United Kingdom). The reaction mixture (total volume 20 µL) was incubated according to the manufacturer's instructions: for 60 min at 37°C, and then the enzyme

Table 2. Specific gene primers in poultry used in the study

Gene	NCBI accession number	Forward (F) and reverse (R) primers, 5′→3′	Ta, °C
LEAP-2	NM_001001606.1	F: CTCAGCCAGGTGTACTGTGCTT R: CGTCATCCGCTTCAGTCTCA	65
PepT1	AY029615.1	F: CCCCTGAGGAGGATCACTGTT	58.7
		R: CAAAAGAGCAGCAGCAACGA	
HPRT	NM_204848	F: GATATCCCACACTTCGAGGAG	65
H6PD	XM_425746.4	R: CGTTGCTGTCTCTACTTAAGCAG F: GAGAACCAGCACTTCTTAGAC R: GGGTTCAGCAACTCCACTG	64

LEAP-2, liver expressed antimicrobial peptide; PepT1, peptide transporter 1; HPRT, hypoxanthine phosphoribosyltransferase; H6PD, hexose-6-phosphate dehydrogenase; NCBI, the National Centre for Biotechnology Information; Ta, optimal annealing temperature.

was heat inactivated at 70°C for 5 min and the reaction mixture rapidly cooled to 4°C.

Specific primers for chicken PepT1 and LEAP-2 were used for RT-PCR (Table 2). Sybr Green method was applied for the real-time PCR analysis by using iQTM 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.[29] HPRT and H6PD (Table 1) were used as reference genes for the normalization of the expression levels of gene of interest.

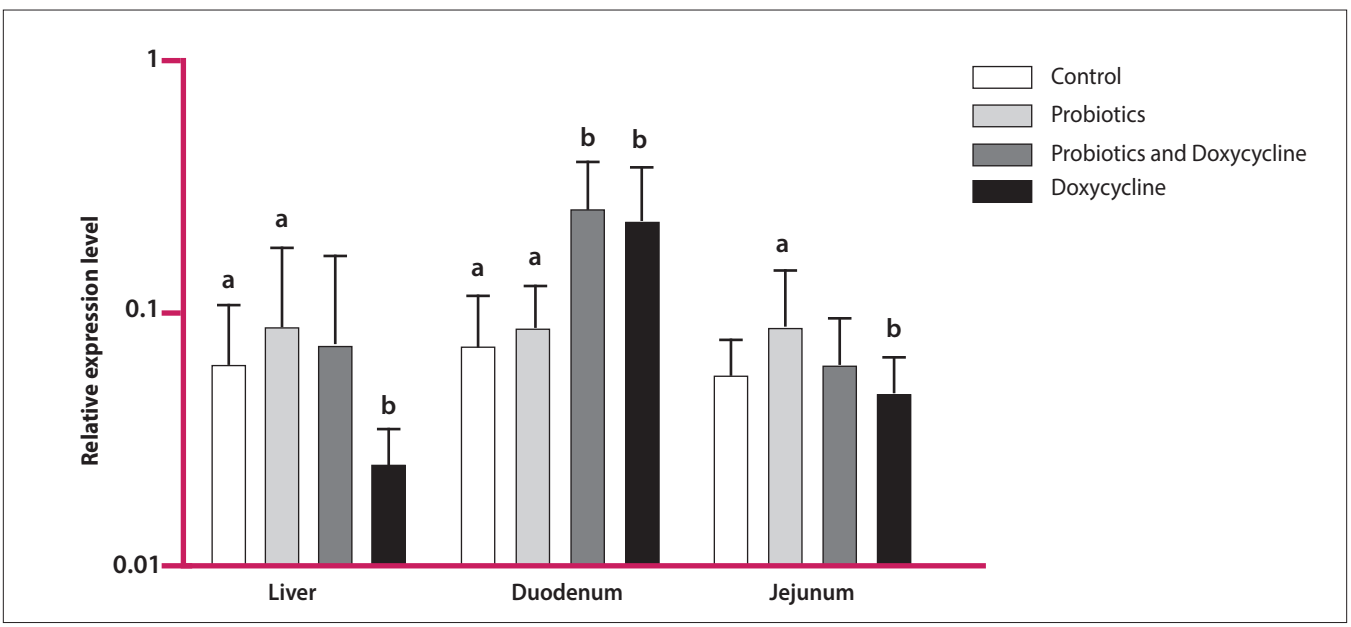

Figure 1. Relative expression levels of PepT1 mRNA in Duc broiler chickens, treated orally with doxycycline at a dose rate of 10 mg/kg via drinking water for 5 consecutive days with or without *Lactobacillus* probiotics. The experimenal groups were control (n=6), probiotics treated group (n=6), doxycycline and probiotics received chickens (n=6) and doxycycline treated group (n=6). Different latters indicate, statistically significant differences between groups for each tissue at P<0.05. The absence of letters and the same letters show lack of statistically significant differences between the groups.

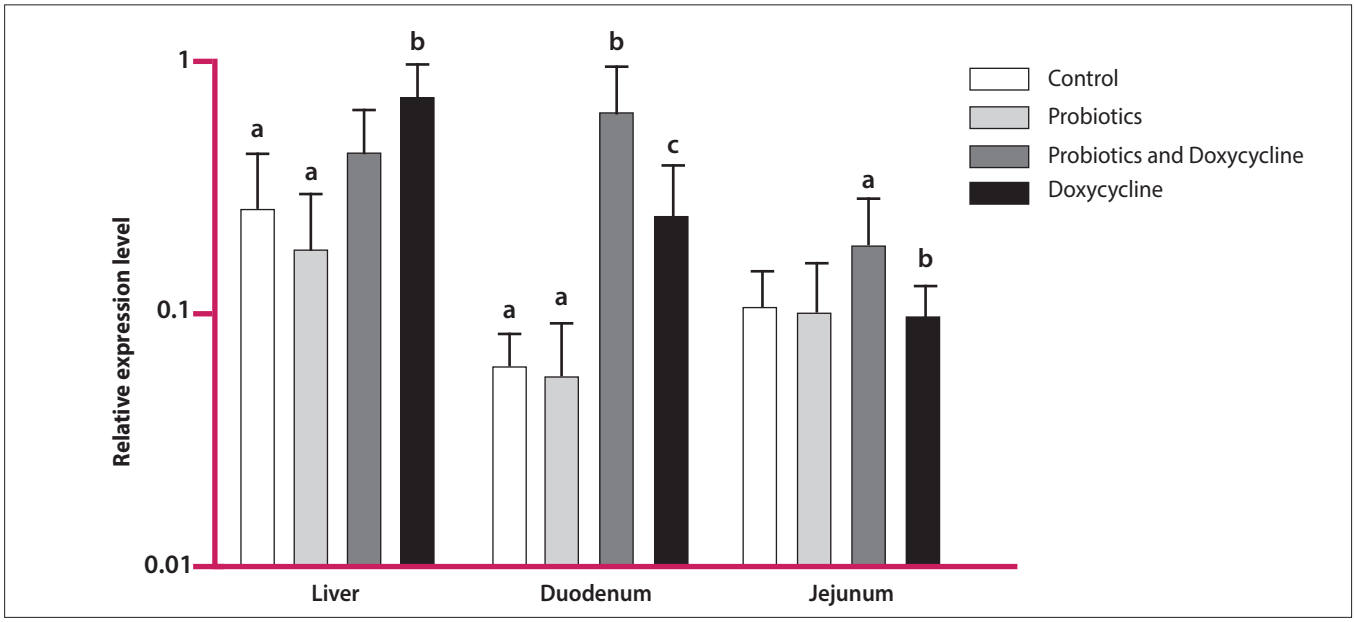

Figure 2. Relative levels of expression of LEAP-2 mRNA in Duc broiler chickens treated orally with doxycycline at a dose rate of 10 mg/kg via drinking water for 5 consecutive days with or without *Lactobacillus* probiotics. The experimental groups were control (n=6), probiotics treated group (n=6), doxycycline and probiotics received chickens (n=6) and doxycycline treated group (n=6). Different letters indicate satistifically significant differences between groups for each tissue at $P<0.05$. The absence of letters and the same letters show lack of statistically significant differences between the groups

Statistical analysis

The data were analyzed with Mann-Whitney nonparametric test (Prism 4.0 software) after a test for normal distribution. Effect of the different treatments on mRNA expression levels of the studied genes was evaluated. Data for each tissue were analyzed separately and comparison between different treatments was performed for each tissue. Differences were considered as statistically significant at $p<0.05$.

Results

PepT1 mRNA expression was found in the studied tissues in healthy Duc broilers (Figure 1). Administration of *Lactobacillus* probiotics resulted in a tendency to up-regulation of PepT1 mRNA in all investigated tissues. Combination of doxycycline and probiotics lead to its significant up-regulation in the duodenum if compared to the control and probiotics supplemented groups. The same changes were observed in the duodenum of doxycycline treated chickens. Opposite results were observed in the liver, where PepT1 mRNA was significantly down-regulated in doxycycline treated group in comparison to the controls and probiotics treated poultry. Doxycycline administration provoked significantly lower levels in the jejunum than the probiotics supplementation. Increased levels of PepT1 mRNA in the duodenum are accompanied by the lower FCR in the groups treated with doxycycline in comparison to the controls and group that received probiotics. The body weight at the end of the experiment was the lowest in control group (424.06±49.20 g) and the FCR was 1.84±0.28 kg/kg (coefficient of variation, CV=14.14%), followed by the probiotic treated group (435.75±41.53 g) with FCR 1.91±0.22 kg/kg and CV 11.62%. Chickens treated with doxycycline and probiotics had the highest body weight of 496.84±68.19 g and the lowest FCR (1.67±0.33 kg/kg, CV 13.66%) and these, that received the antibiotic only were 464.84±68.48 g with FCR 1.79±0.34 kg/kg and CV 16.87%.

Administration of doxycycline provoked significant increase in LEAP-2 mRNA expression in the liver. Levels of LEAP-2 mRNA expression in the duodenum were significantly up-regulated in both groups treated with the antibiotic (Figure 2). LEAP-2 mRNA expression in the jejunum was statistically up-regulated in probiotics-doxycycline treated group compared to the control and probiotics treated group. Its expression in doxycycline treated chickens was down-regulated to the levels of control and probiotics group. Similar results were observed in the duodenum.

Discussion

Published literature show a lot of data about pharmacokinetic properties and susceptibility of pathogenic bacteria to doxycycline in poultry. Influence of the treatment with this broad spectrum antibiotic on microbiota of gastro-intestinal tract with decrease in number of *Lactobacillus* spp. was also described.[30] These changes were related to the gain of body weight. Previous studies investigated the effect of heat stress of broiler chickens or administration of *Lactobacillus plantarum* in mice on the mRNA expression of nutrient transporters (including PepT1)[31,32] but the effect of *Lactobacillus* probiotics and doxycycline on the expression of PepT1 is not studied yet. Therefore, in this study effect of doxycycline treatment with or without *Lactobacilli* supplementation on the mRNA expression level of the peptide transporter PepT1 was investigated due to its importance in protein utilization and weight gain in broilers. PepT1 mRNA was found in the duodenum, jejunum and the liver of Duc broilers as has been previously described by Gilbert et al.[33] Administration of combination of *Lactobacillus plantarum* 11, *L.brevis* 51 and *L.bulgaricus* 13 in our study did not change the expression level of PepT1 mRNA in the studied tissues which was accompanied by a tendency to higher body weight and slightly higher values of FCR. *Lactobacillus plantarum* restored the decreased expression of PepT1 mRNA in the gastro-intestinal tract of mice with colitis to the levels of healthy animals.[31] The results in our study demonstrate that *Lactobacillus plantarum* 11, *L.brevis* 51 and *L.bulgaricus* 13 support the health in the intestines of Duc broilers through keeping the expression of PepT1 mRNA as in healthy controls. Statistically significant up-regulation of PepT1 mRNA in the duodenum and the tendency to increasing in the other two tissues in doxycycline-probiotics treated broilers can be discussed as positive influence on peptide absorption which resulted in the highest body weight and the lowest FCR in this group. Previous studies found the positive relationship of increased expression of PepT1 mRNA and feed utilization due to the role of PepT1 in absorption of di- and tripeptides.[33] Effect of doxycycline on PepT1 mRNA level can be attributed to its possibility to modulate the expression of the studied gene. The expression of PepT1 mRNA can be modulated by stress conditions in broilers or feed restriction as well as drug administration such as 5-fluorouracil and clonidine.[34–37] Furthermore, some frequently used pharmaceutical excipients like Tween 20 and Tween 80 exert inhibitory effects on the expression of PepT1.[38] Up-regulation of PepT1 mRNA, lower FCR ratio with higher body weight in the group of docycycline treated Duc broilers in comparison to controls can be explained by the effect of doxycycline administration. Doxycycline treatment has been associated with increased weight gain in humans due to changes in the microbiota in gastro-intestinal tract.[30] Our data about the influence of this antibiotic can be related not only to PepT1 mRNA expression but also to changes in the microbiota in the intestines of poultry.

Efficiency in the poultry industry depends on the health of the birds, respectively, on the function of the immune system. Antimicrobial peptides such as LEAP-2 are recognized as part of innate immunity and their expression can be changed by pathogens.[39] Infection with *Salmonella enteritidis* resulted in up-regulation of LEAP-2 mRNA but *Eimeria tenella* caused its significant down regulation.[40,41] Shao et al.[42] demonstrated that *Salmonella enteritidis* up-regulated LEAP-2 mRNA in the jejunum of broiler chickens and that supplementation of yeast ß-D-glucans restored expression of LEAP-2 mRNA to the levels of healthy birds. These levels were not affected in healthy chickens.[42] Unlike the effect of pathogens, supplementation of beneficial bacteria from *Lactobacillus* spp. to Duc broilers did not provoke changes in the expression of LEAP-2 mRNA. Similar data were observed in broilers, treated with *Lactobacillus acidophilus*, *Bifidobacterium bifidum* and *Enterococcus faecalis.*[43] Significant up-regulation of LEAP-2 mRNA in the duodenum of the chickens treated with doxycycline-probiotics combination and same tendency in the jejunum and liver can have positive impact on the innate immunity and disease resistance. Doxycycline administration resulted in significant up-regulation of LEAP-2 mRNA in the liver and in the duodenum in comparison to the controls and probiotics supplemented group. Its levels were further up-regulated in the intestines when the antibiotic was combined with probiotics. Although the lower expression in antibiotic administered animals if compared to the group treated with the combination doxycycline-probiotics, our data allow us to conclude that the benefit of doxycycline treatment can be enhanced by increase of antimicrobial peptide. It is difficult to compare the results from the current study due to absence of published data about such effects of doxycycline. *In vitro* investigation with Caco-2 cells and RAW 264.7 macrophages, and *in vivo* rat model of colitis supports the potential use of doxycycline in association with the probiotic *Saccharomyces boulardii* for the treatment of inflammatory bowel diseases due to

their anti-inflammatory effect.[44] In conclusion, finding combinations between probiotics and antibiotics that work synergistically in the stimulation of the host immune system would be a great relevance for the clinical practice in poultry husbandry. The results from this pilot study have to be further validated in functional studies and in experimental conditions with diseased animals in order to suggest the right doses of *Lactobacilli* and doxycycline.

Conclusion

Doxycycline administered alone and in combination with the three strains lactic acid bacteria led to significant up-regulation of PepT1 and LEAP-2 mRNAs in the duodenum in Duc broilers which contribute to better feed utilization and stimulation of innate immunity. These changes can have positive impact on health of broilers and they have to be further validated in models of economically significant bacterial diseases of chickens.

CO-AUTHOR CONTRIBUTIONS

The both co-authors contributed equally to the work being described.

ACKNOWLEDGEMENTS

The authors would like to thank to Prof. J. Fink-Gremmels, Utrecht University, the Netherlands for support in molecular analysis and to Assoc. Prof. Sv. Danova, Institute of Microbiology, BAS, Bulgaria, who kindly selected and prepared *Lactobacillus* spp. probiotics.

References

1. Chopra I, Roberts M. Tetracycline antibiotics: Mode of action, applications, molecular biology, and epidemiology of bacterial resistance. Microbiol Mol Biol Rev 2001;65:232–60. doi: 10.1128/MMBR.65.2.232-260.2001
2. Bryskier A, editor. Antimicrobial agents: Antibacterials and antifungals. Washington Dc, USA: ASM Press; 2005.
3. Agwuh KN, MacGowan A. Pharmacokinetics and pharmacodynamics of the tetracyclines including glycylcyclines. J Antimicrob Chemother 2006;58:256–65. doi: 10.1093/jac/dkl224
4. Russo E, Lucatello L, Giovanardi D, Cagnardi P, Ortali G, Di Leva V, Montesissa C. Approved medication of water with enrofloxacin to treat turkey colibacillosis: assessment of efficacy using a PK/PD approach. Vet Microbiol 2012;161:206–12. doi: 10.1016/j.vetmic.2012.07.028
5. Soliman AM, Aboubakr M, El-Hewaity M. Bioequivalence study of two oral doxycycline formulations (Doxysol® and Doxymed®) in healthy broiler chickens. Pharmacol Pharm 2015;6:1–8.
6. Marx JO, Vudathala D, Murphy L, Rankin S, Hankenson FC. Antibiotic administration in the drinking water of mice. J Am Assoc Lab Anim Sci 2014;53:301–6.
7. Zakeri B, Wright GD. Chemical biology of tetracycline antibiotics. Biochem Cell Biol 2008;86:124–36. doi: 10.1139/O08-002
8. Flammer K, Massey JG, Meek CJ, Papich MG. Assessment of plasma concentrations and potential adverse effects of doxycycline in cockatiels (Nymphicus hollandicus) fed a medicated pellet diet. J Avian Med Surg 2013;27:187–93. doi: 10.1647/1082-6742-27.3.187
9. Hu GZ, Pan YS, Wu H, Hu H, Xu R, Yuan L, Liu JH, Feng JK. Prevalence of tetracycline resistance genes and identification of tet (M) in clinical isolates of Escherichia coli from sick ducks in China. J Med Microbiol 2013;62:851–8. doi: 10.1099/jmm.0.051896-0
10. Warburton PJ, Ciric L, Lerner A, Seville LA, Roberts AP, Mullany P, Allan E. TetAB46, a predicted heterodimeric ABC transporter conferring tetracycline resistance in Streptococcus australis isolated from the oral cavity. J Antimicrob Chemother 2013;68:17–22. doi: 10.1093/jac/dks351
11. Videnska P, Faldynova M, Juricova H, Babak V, Sisak F, Havlickova H, Rychlik I. Chicken faecal microbiota and disturbances induced by single or repeated therapy with tetracycline and streptomycin. BMC Vet Res 2013;9:30. doi: 10.1186/1746-6148-9-30
12. Garrido-Mesa N, Utrilla P, Comalada M, Zorrilla P, Garrido-Mesa J, Zarzuelo A, Rodríguez-Cabezas ME, Galvez J. The association of minocycline and the probiotic Escherichia coli Nissle 1917 results in an additive beneficial effect in a DSS model of reactivated colitis in mice. Biochem Pharmacol 2011;82:1891–900. doi: 10.1016/j.bcp.2011.09.004
13. Kizerwetter-Swida M, Binek M. Selection of potentially probiotic Lactobacillus strains towards their inhibitory activity against poultry enteropathogenic bacteria. Pol J Microbiol 2005;54:287–94.
14. Shokryazdan P, Sieo CC, Kalavathy R, Liang JB, Alitheen NB, Faseleh Jahromi M, Ho YW. Probiotic potential of Lactobacillus strains with antimicrobial activity against some human pathogenic strains. Biomed Res Int 2014;2014:927268. doi: 10.1155/2014/927268
15. Lutful Kabir SM. The role of probiotics in the poultry industry. Int J Mol Sci 2009;10:3531–46. doi: 10.3390/ijms10083531
16. Peng Q, Zeng XF, Zhu JL, Wang S, Liu XT, Hou CL, Thacker PA, Qiao SY. Effects of dietary Lactobacillus plantarum B1 on growth performance, intestinal microbiota, and short chain fatty acid profiles in broiler chickens. Poult Sci 2016;95:893–900. doi: 10.3382/ps/pev435
17. Hooper LV, Wong MH, Thelin A, Hansson L, Falk PG, Gordon JI. Molecular analysis of commensal host-microbial relationships in the intestine. Science 2001;291:881–4. DOI: 10.1126/science.291.5505.881
18. Dicks LMT, Botes M. Probiotic lactic acid bacteria in the gastro-intestinal tract: health benefits, safety and mode of action. Benef Microbes 2010;1:11–29. doi: 10.3920/BM2009.0012
19. Nielsen DS, Cho GS, Hanak A, Huch M, Franz CM, Arneborg N. The effect of bacteriocin-producing Lactobacillus plantarum strains on the intracellular pH of sessile and planktonic Listeria monocytogenes single cells. Int J Food Microbiol 2010;141:S53–9. doi: 10.1016/j.ijfoodmicro.2010.03.040
20. Perumal V, Repally A, Dasari A, Venkatesan A. Partial purification and characterization of bacteriocin produced by Enterococcus faecalis DU10 and its probiotic attributes. Prep Biochem Biotechnol 2016;46:686–94. doi: 10.1080/10826068.2015.1135451
21. Abreu MT. Toll-like receptor signalling in the intestinal epithelium: how bacterial recognition shapes intestinal function. Nat Rev Immunol 2010;10:131–44. doi: 10.1038/nri2707

22. Townes CL, Michailidis G, Nile CJ, Hall J. Induction of cationic chicken liver-expressed antimicrobial peptide 2 in response to Salmonella enterica infection. Infect Immun 2004;72:6987–93. doi: 10.1128/IAI.72.12.6987-6993.2004
23. Townes CL, Michailidis G, Hall J. The interaction of the antimicrobial peptide cLEAP-2 and bacterial membrane. Biochem Biophys Res Commun 2009;387:500–3. doi: 10.1016/j.bbrc.2009.07.046
24. Gilbert ER, Li H, Emmerson DA, Webb KE Jr, Wong EA. Dietary protein composition influences abundance of peptide and amino acid transporter messenger ribonucleic acid in the small intestine of 2 lines of broiler chicks. Poult Sci 2010;89:1663–76. doi: 10.3382/ps.2010-00801
25. Guo S, Liu D, Zhao X, Li C, Guo Y. Xylanase supplementation of a wheat-based diet improved nutrient digestion and mRNA expression of intestinal nutrient transporters in broiler chickens infected with Clostridium perfringens. Poult Sci 2014;93:94–103. doi: 10.3382/ps.2013-03188
26. Danova S, Tropcheva R, Ivanovska N, Georgieva R, Dobreva-Yosifova G, Petrova M, Koleva P, Dermendvjieva J, Dimitonova S. Characterisation of Bulgarian lactobacilli as probiotics. In: Gulubov A, editor. Jubilee book: New trends in microbiology, 65th Anniversary of the Stephan Angeloff Institute of Microbiology. Sofia: 2012. p.177–92.
27. Anonymous Joint FAO/WHO Working Group Report on Drafting Guidelines for the Evaluation of Probiotics in Food. London, Ontario, Canada: April 30 and May 1, 2002. Guidelines for the Evaluation of Probiotics in Food. Available from: http://www.who.int/foodsafety/fs_management/en/probiotic_guidelines.pdf
28. Tropcheva R, Hristova J, Georgieva R, Salageanu A, Sgouras DN, Danova S. In vitro assessment of prebiotic utilization by dairy lactobacilli. Bulg J Agricult Sci 2013;19:105–7.
29. Vandesompele J, De Preter K, Pattyn F, Poppe B, Van Roy N, De Paepe A, Speleman F. Accurate normalization of real-time quantitative RT-PCR data by geometric averaging of multiple internal control genes. Genome Biol 2002;3(7):RESEARCH0034.
30. Angelakis E, Million M, Kankoe S, Lagier JC, Armougom F, Giorgi R, Raoult D. Abnormal weight gain and gut microbiota modifications are side effects of long-term doxycycline and hydroxychloroquine treatment. Antimicrob Agents Chemother 2014;58:3342–7. doi: 10.1128/AAC.02437-14
31. Chen HQ, Yang J, Zhang M, Zhou YK, Shen TY, Chu ZX, Zhang M, Hang XM, Jiang YQ, Qin HL. Lactobacillus plantarum ameliorates colonic epithelial barrier dysfunction by modulating the apical junctional complex and Pept1 in IL-10 knockout mice. Am J Physiol Gastrointest Liver Physiol 2010;299:G1287–97. doi: 10.1152/ajpgi.00196.2010
32. Sun X, Zhang H, Sheikhahmadi A, Wang Y, Jiao H, Lin H, Song Z. Effects of heat stress on the gene expression of nutrient transporters in the jejunum of broiler chickens (Gallus gallus domesticus). Int J Biometeorol 2014;59:127–35. doi: 10.1007/s00484-014-0829-1
33. Gilbert E, Li H, Emmerson D, Webb KE Jr, Wong EA. Developmental regulation of nutrient transporter and enzyme mRNA abundance in the small intestine of broilers. Poult Sci 2007;86:1739–53.
34. Rubio-Aliaga I, Daniel H. Peptide transporters and their roles in physiological processes and drug disposition. Xenobiotica 2008;38:1022–42. doi: 10.1080/00498250701875254
35. Hu XF, Guo YM, Huang BY, Bun S, Zhang LB, Li JH, Liu D, Long FY, Yang X, Jiao P. The effect of glucagon-like peptide 2 injection on performance, small intestinal morphology, and nutrient transporter expression of stressed broiler chickens. Poult Sci 2010;89:1967–74. doi: 10.3382/ps.2009-00547
36. Inoue M, Terada T, Okuda M, Inui KI. Regulation of human peptide transporter 1 (PEPT1) in gastric cancer cells by anticancer drugs. Cancer Lett 2005;230:72–80. doi: 10.1016/j.canlet.2004.12.023
37. Madsen S L, Wong EA. Expression of the chicken peptide transporter 1 and the peroxisome proliferator-activated receptor α following feed restriction and subsequent refeeding. Poult Sci 2011;90:2295–300. doi: 10.3382/ps.2010-01173
38. Otter M, Oswald S, Siegmund W, Keiser M. Effects of frequently used pharmaceutical excipients on the organic cation transporters 1-3 and peptide transporters 1/2 stably expressed in MDCKII cells. Eur J Pharm Biopharm 2017;112:187–95. doi: 10.1016/j.ejpb.2016.11.028
39. Su S, Miska KB, Fetterer RH, Jenkins MC, Wong EA. Expression of digestive enzymes and nutrient transporters in Eimeria acervulina-challenged layers and broilers. Poult Sci 2014;93:1217–26. doi: 10.3382/ps.2013-03807
40. Michailidis G. Expression of chicken LEAP-2 in the reproductive organs and embryos and in response to Salmonella enterica infection. Vet Res Commun 2010;34:459–71. doi: 10.1007/s11259-010-9420-3
41. Milanova A, Santos R, Lashev L, Koinarski V, Fink-Gremmels J. Influence of experimentally induced Eimeria tenella infection on gene expression of some host response factors in chickens. Bulg J Vet Med 2015;19:47–56. doi: 10.15547/bjvm.896
42. Shao Y, Wang Z, Tian X, Guo Y, Zhang H. Yeast β-d-glucans induced antimicrobial peptide expressions against Salmonella infection in broiler chickens. Int J Biol Macromol 2016;85:573–84. doi: 10.1016/j.ijbiomac.2016.01.031
43. Akbari MR, Haghighi HR, Chambers JR, Brisbin J, Read LR, Sharif S. Expression of antimicrobial peptides in cecal tonsils of chickens treated with probiotics and infected with Salmonella enterica serovar Typhimurium. Clin Vaccine Immunol 2008;15:1689–93. doi: 10.1128/CVI.00242-08
44. Garrido-Mesa J, Algieri F, Rodriguez-Nogales A, Utrilla M, Rodriguez-Cabezas M, Zarzuelo A, Ocete M, Garrido-Mesa N, Galvez J. A new therapeutic association to manage relapsing experimental colitis: doxycycline plus Saccharomyces boulardii. Pharmacol Res 2015;97:48–63. doi: 10.1016/j.phrs.2015.04.005