Influence of *Bacillus subtilis* and Acetic Acid on Cobb500 Intestinal Microflora

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Abstract
The beneficial modes of probiotic action include regulation of intestinal microbial homeostasis, stabilization of the gastrointestinal barrier function expression of bacteriocins and interference with the ability of pathogens to colonize and infect the mucosa. Organic acids as feed additives have been used to reduce or eliminate pathogenic bacteria and fungal contamination, control microbial growth and reduction of microbial metabolites. The aim of this study was to determine the effect of *Bacillus subtilis*, acetic acid and their combination on the intestinal microflora of broiler chickens (Cobb 500). The experiment was carried out on 4 groups each contains 100 chicks as follows: control (without addition), treatment 1 (acetic acid), treatment 2 (*Bacillus subtilis*) and treatment 3 (acetic acid+*Bacillus subtilis*). Six samples from each group were selected as a sample (mixed sex). The highest average number of log CFU.g^{-1} *Lactobacillus* sp. was in the treatment 3–7.11 log CFU.g^{-1} and the lowest was in the control group–6.85. The highest average number of log CFU.g^{-1} *Enterococcus* sp. was in the treatment 2–7.17 log CFU.g^{-1} and the lowest was in the control group–5.65. In both observing additions of *Bacillus subtilis* and acetic acid increase the number of log CFU.g^{-1} *Lactobacillus* sp. and *Enterococcus* sp. compared with control group. The lower average number of log CFU.g^{-1} coliform bacteria was in the treatment 2–5.9 log CFU.g^{-1} and the higher was in control group–6.98. The additional supplement decreased the number of log CFU.g^{-1} coliform bacteria in the treatment groups compared with the control.

Keywords: acetic acid, *Bacillus subtilis*, Cobb500, intestinal microflora

1. Introduction

The gastrointestinal tract (GIT) of warm-blooded animals is densely populated by bacteria. Composition and density of the microbiota can vary a lot among individuals because it is markedly affected by the bacterial composition of the inoculum received at birth or hatch, the structure of the host intestinal epithelium and the diet [1, 2]. In chicken, the main sites of bacterial activity are the crop and the caeca and, to a lesser extent, the small intestine [3].

Bacterial density reaches at maturity 10^3-10^5 bacterial cells per gram of digesta in the proximal small intestine (duodenum) because it is characterized by rapid flow of the highly fluid digesta, while the distal small intestine (jejunum and ileum) harbors $>10^9$ bacteria cells per gram of digesta [4, 5].
The caeca contain a more diverse community of bacteria, including species of the genera *Bacteroides*, *Bifidobacterium*, *Clostridium*, *Enterococcus*, *Escherichia*, *Fusobacterium*, *Lactobacillus*, *Streptococcus* and *Campylobacter*, and reaching $>10^{11}$ cell/g of digesta [1, 4].
Various types of feed additives have been evaluated under commercial conditions and in experimental trials with the objective to achieve

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improvements on growth performance and the best economic return [6].

Many definitions have been proposed for the term probiotic. The most recent one is "live microorganisms which, when administered in adequate amounts, confer a health benefit on the host" [7].

Many studies have demonstrated that DFMs showed beneficial effects on lowering nutritional competition in the gut for nutrients and mucosal binding sites, producing substances that kill or inhibit the growth of pathogenic bacteria [8], maintaining normal gut microflora [9].

Organic acids are considered to be any organic carboxylic acid with the general structure of R-COOH, and hence include fatty acids and amino acids. Organic acids are shortchained acids (C1–C7) and are either simple monocarboxylic acids such as formic, acetic, propionic and butyric acids, or are carboxylic acids bearing an hydroxyl group (usually on the α carbon) such as lactic, malic, tartaric, and citric acids [10].

Activity of organic acids to reduce pH in the lower gut is highly desirable, because the lower gut is colonized by many anaerobic opportunistic pathogens. Reduction of the intestinal pH might depress the growth of pathogenic bacteria, reduce subclinical infections [11] and support the proliferation and growth of beneficial bacteria [12].

2. Materials and methods

The broilers originated from one line (Cobb 500). Four hundred chicks were randomly distributed to 4 dietary treatments (Tr1, Tr2, Tr3 and Tr4). Housing conditions were under the breed standard and EU welfare. The fattening duration was 42 days. Water and diets were available ad libitum. The basic diet prepared by factory in Slovakia (Table 1) was used as a control (without addition), treatment 1 (acetic acid), treatment 2 (*Bacillus subtilis*) and treatment 3 (acetic acid+*Bacillus subtilis*). Probiotics namely B. *subtilis* was used in dosage 500 g per t of feed. Vinegar with 5% malic acid was added 10 ml per l to drinking water. The birds were weighed and six broilers (mixed sex) of the mean body weight were selected from each dietary treatment and subjected to a 12 h fasting. Consequently, they were stunned, manually slaughtered, eviscerated and intestinal microflora (caecum) was analyzed.

Table 1. Composition of basic diets (%)

Component	Starter	Grower	Finisher
Wheat	33.83	35.33	36.82
Maize	34.00	36.00	37.00
Soybean meal	22.70	22.00	20.00
Fish meal 71%	5.00	2.00	-
Lime stone	1.00	1.00	1.10
Monocalcium phosphate P 22.7%	0.90	0.80	1.00
Salts	0.10	0.15	0.20
Sodium biocarbonate	0.15	0.20	0.25
Lysine HCL	0.10	0.10	0.29
Methionine	0.15	0.22	0.29
Bergafat	1.50	1.60	2.50
Clinacox 0.5%[1]	0.02	-	-
SACOX 12%[2]	-	0.05	-
EUROMIX BR 0.5%[3]	0.50	0.50	0.50

[1]*Standardized feed oils based on vegetable oils, total fat content in (%) min. 98.5*

[2]*Clinacox 0.5% Active Ingredient: Each kg contains 5grams of diclazuril. As an aid in the prevention of coccidiosis caused by Eimeria acervulina, E. brunetti, E. maxima, E. mitis, E. necatrix and E. tenella inbroiler chickens.*

[3]*SACOX is 12% Micro Granulated salinomycin sodiumbesides strong control of coccidiosis The approveddose range is 50 to 70 mg/kg complete feed in the EU.*

[4]*EUROMIX BR 0.5% the active substances perkilogram of premix: vitamin A 2 500 000 IU; vitamin E 20 000 mg; vitamin D3 800 000 IU; niacin 12 000 mg; d-pantothenic acid 3 000 mg; riboflavin 1 800 mg; pyridoxine 1 200 mg; thiamine 600 mg; menadione 800 mg; ascorbic acid 20 000 mg; folic acid 400 mg; biotin 40 mg; kobalamin 8.0 mg; choline 100 000 mg; betaine 50 000 mg; Mn 20 000 mg; Zn 16 000 mg; Fe 14 000 mg; Cu 2 400 mg; Co 80 mg; I 200 mg; Se 50 mg.*

Monitored microbiological parameters: The number of CFU (colony forming units) of *Enterococcus* sp. on Slanetz-Bartley agar after 48 to 72 hours incubation at 37°C. The number of CFU of *Lactobacillus* sp. on MRS agar after the incubation took from 48 to 72 hours at the temperature 37°C. The number of CFU of coliform bacteria on McConkey agar after 48 to 72 hours at 37°C. In evaluating the results we used the plate dilution method. Basic dilution was: 1 g Chym+99 ml saline (0.85% NaCl) by decimal dilution system.

Basic dilution (10^{-1}), we prepared by mixing 5 g sample and 45 ml saline or 10 g sample and 90 ml of normal saline. The basic dilution was prepared further by decimal dilution system. Samples have been incubated on the surface or embedded. The inoculated Petri dishes were cultivated in an incubator, bottom up. Temperature and time, was adjusted according to the group of cultivated microorganisms. After the cultivation, we counted colonies grown on culture medium in Petri dishes. To calculate CFU.g^{-1} (Colony Forming Units), we used the following formula (which takes into account Petri dishes of two consecutive dilutions): N=ΣC/[(n1+0.1n2).D] ΣC-the sum of characteristic colonies on selected dishes n1-number of dishes of 1 dilution used to calculate n2-number of dishes of 2 dilution used to calculated-dilution factor is identical to the 1st dilution used. [17] For the inoculation we used dilutions 10^{-4}, 10^{-5} and 10^{-6}. The results were evaluated according to basic statistical characteristics.

3. Results and discussion

In our experiment, we monitored the population of microorganisms in the digestive tract of broiler chickens. Number of Lactobacillus sp. (Figure 1) varied in the experimental group in the range from 6.85 log CFU.g^{-1} to 7.11 log CFU.g^{-1}. The highest average number of log CFU.g-1 Lactobacillus sp. was in the Treatment 3 group with acetic acid+*Bacillus subtilis*-6.39 log CFU.g^{-1}.

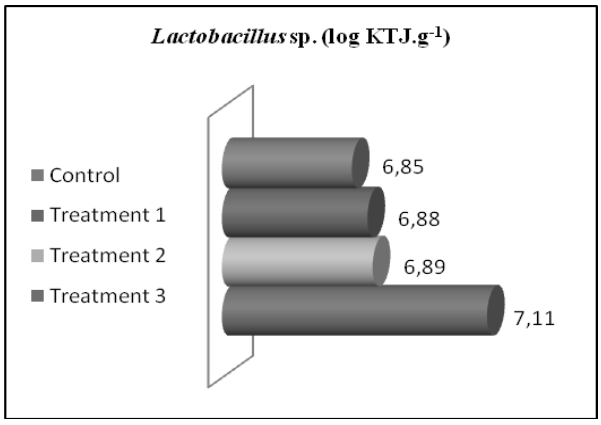

Figure 1. Average numbers of *Lactobacillus* sp. in intestinal tract

The study conducted by [13] who obtained no significant differences between the control and probiotic treatments regarding total anaerobs, total aerobs, coliforms and *Bacteroides* spp.in cecum of broilers, but found significantly higher concentrations of *Bifidobacterium* spp., *Lactobacillus* spp., and gram-positive cocci.

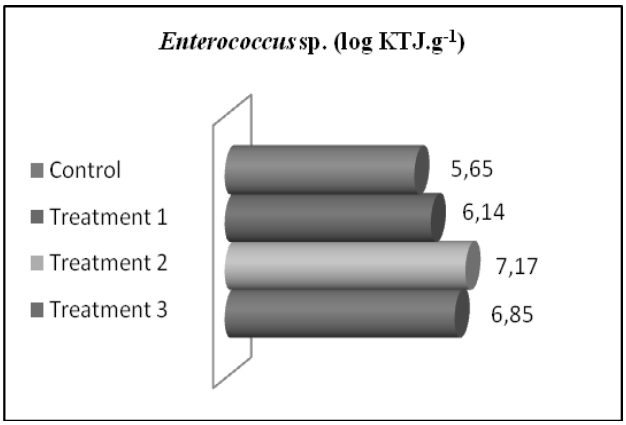

Figure 2. Average numbers of *Enterococcus* sp. in intestinal tract

The number of *Enterococcus* sp. (Figure 2), was in the range from 5.65 to 7.17 log CFU.g^{-1}. The highest average number of log CFU.g^{-1} of *Enterococcus* sp. was observed in the Treatment 2 group (7.17 log KTJ.g^{-1}), where the chickens were fed with probiotics added feed.

The number of Coliform bacteria (Figure 3) ranged from 5.9 to 6.98 log CFU.g^{-1}. The lowest average number of log CFU.g^{-1} Coliform bacteria was recorded in the group Treatment 2 (5.9 log CFU.g^{-1}).

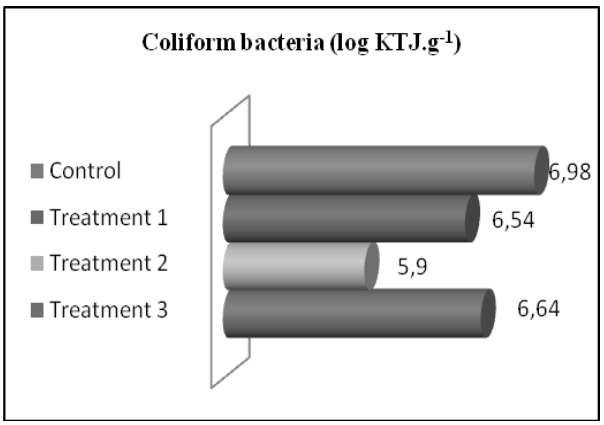

Figure 3. Average numbers of Coliform bacteria in intestinal tract

Numerous reports indicated that the addition of probiotics in feed, either solely or in combination with other feed additives like prebiotics, could regulate the intestinal microflora in order to

increase the concentration of the beneficial bacteria such as Lactobacillus ssp. and Streptococcus ssp. and inhibit the reproduction of harmful bacteria in the gut [14]. Other studies using different probiotics have shown no significant influence on the gut microbial composition [15].

4. Conclusions

The highest average number of log CFU.g^{-1} *Lactobacillus* sp. was in the acetic acid+*Bacillus subtilis* treatment–7.11 log CFU.g^{-1}. The highest average number of log CFU.g^{-1} *Enterococcus* sp. was in the *Bacillus subtilis* treatment–7.17 log CFU.g^{-1}. The lower average number of log CFU.g^{-1} coliform bacteria was in the *Bacillus subtilis* treatment–5.9 log CFU.g^{-1}.

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