Influence of Additional Level of Probiotics on Intestinal Microbiota in Broiler Chickens

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Abstract
The digestive tract of broiler chickens contains a highly complex microbiota, generally consisting of body beneficial bacteria and, in some cases, of pathogen bacteria. To reduce the quantity of pathogen bacteria, the structure of this microbiota can be changed by using some feed additives, such as probiotics. The purpose of the experiment was to establish the influence of the additional level of probiotics on the intestinal microbiota in broiler chickens. Probiotics administration (*Lactobacillus paracasei* J.R., *Lactobacillus rhamnosus* 15b, *Lactobacillus lactis* y and *Lactobacillus lactis* FO was made in different combination and at different age of broilers, respectively hatching until 42 days and only in the last week growth. In addition to probiotics, two of the experimental groups used two synthetic aminoacids, namely L threonine and DL methionine, used in excess of the broiler chicken requirements. At the end of the experiment (42 days), caecal content samples were taken. After isolating the caecum, the caecal content was sampled in sterile cryotubes and frozen to -80°C pending DNA extraction. The resulting DNA was subsequently used to detect the differences between the groups with and without probiotics by examining the microbiota composition. At the beginning, the phylogenetic differences were analyzed to establish dominant genders. The investigations performed showed that the use of probiotic microorganisms in various combinations entails changes in the intestinal microbiota. The pyrosequencing method was used to quantify the abundance of microorganisms with probiotic potential in the microbiota composition. Also, the number of microorganisms with probiotic potential changes after the use of probiotic microorganisms.

Keywords: broiler chickens, intestinal microbiota, probiotics, pyrosequencing, dominant genders

1. Introduction

Understanding the intestinal microbiota in chicken is important for the maximizing of production, the welfare of animals and food safety. An important measure taken in raising broiler chickens in intensive system consists in reducing the conversion of food. It is known that food, the genetic material, he age, the state of health influence the microbiota of the host animal. The intestinal microbiota is a complex ecosystem with wide metabolic activity [1-3]. Microbiota plays an important part from the moment of hatching until their slaughtering. The intestinal bacteria are important in keeping an immune system active, in modulating the intestinal functions and in the protection against the pathogen agents [4, 5]. In the natural growth systems of the animals, the digestive tract is populated by a microflora which in time has consolidated a beneficial influence on the host. The artificialized and intensive growth with unilateral and simplified feeding, with the use of different replacements and medicines

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adding to the stress factors results in the quantitative diminishing of the intestinal microflora and even the disappearance of bacterial types [6]. Lately, the improvement of the animals' nutritional and sanitary balance by administering beneficial bacterial cultures currently makes the object of in depth studies, the use of the probiotics becoming a habit [7]. It also plays an important part in the digestion of the nutrients [8, 9]. The types *Lactobacillus* and *Enterococcus* are types present in important quantities (of the order 10^4 – 10^8/g) in the digestive microflora structure of swine, poultry and calves. Their effect consists in preventing the colonization of the intestine by the pathogen germs favored by the stress factors such as the drastic change of the diet, antibiotherapy etc. This barrier effect through which the existing flora opposes the implantation of a new germ may be explained by means of the competition to the available of nutrients as well as through the production by the probiotic microflora of some metabolites having toxic effect on the pathogen germs, as for example on the volatile fatty acids; the occupation by the probiotic flora of the fixation sites on the surface of the digestive mucous membrane [10]. The caecum of the chicken contains a complex microbiome made up mostly of bacteria. The studies performed until now reveal the fact that the lactobacilli (>10^4 CFU/g) as well as the clostridia (10^2-10^4 CFU/g) are not, as it may appear, so abundant compared to the anaerobic species (10^{10}-10^{11} CFU/g) in the chicken caecum [6, 10, 11]. In chicken, the digestive microbiome influences the intestinal morphology having a direct impact on the efficiency of the nutrients absorption.

2. Materials and methods

The experiment performed to establish the effect of some probiotic bacteria on the intestinal microbiota in the broiler chickens was conducted throughout six weeks, in 2014. The used biological material was broiler chickens of the hybrid Ross 308. They were raised until the age of 6 weeks using a combined feed recommended in the guide for the raising of the above mentioned hybrid. The chickens were divided into six experimental groups and a control group according to the organizing scheme in the table below. The difference between the groups consisted in a number of administered probiotic bacteria (*Lactobacillus paracasei* J.R., *Lactobacillus rhamnosus* 15b, *Lactobacillus lactis* y and *Lactobacillus lactis* FO) and the administration periods (table 1). In addition to probiotics, two of the experimental groups used two synthetic aminoacids, namely L threonine and DL methionine, used in excess of the broiler chicken requirements. At the end of the experimental period three chickens were sacrificed from each group. Intestinal content was sampled from the caecum. The organizing scheme of experiment is presented in table 1.

The caecal content samples were taken according to the S.O.P. 1, concerning the diminishing of CFU/ml, drafted within the project „The role of probiotic bacteria in preventing Campylobacter jejuni colonization of poultry" code: PN-II-RU-TE-2012-3-0092., financed by UEFISCDI Stage I /2013. After the isolation of the caecum, the caecal content was sampled in sterile cryotubes and frozen down to -80°C pending DNA extraction.

DNA extraction from the caecal content samples

For the DNA extraction we used the kit QIAamp DNA Stool Mini Kit (QIAGEN, cat. no. 51504), according to the producer's instructions.

The DNA extraction method:

- 200 mg of cecal content were weighed in a sterile tube and 2 ml of Buffer ASL were added, after 1 min. of vortexing 1.6 ml of lysate was sampled and transferred into a new tube, the samples were incubated for 5 min at 70°C;
- After the incubation, the samples were centrifuged at 14,000 rpm and 1.2 ml of supernatant were sampled in a new tube, over which a tablet of InhibitEX Tablet was added;
- After the disolving of the tablet, vortexing and incubation for 1 min at room temperature, the samples were centrifuged for 3 min. at 14,000 rpm and the supernatant was transferred in a new tube, being centrifuged for 3 min at 14,000 rpm for the sedimentation of the leavings on the tablet;
- 15µL of K proteinase, 200 µL of supernatant and 200 µl of Buffer AL were pipetted in a separate tube, the samples were vortexed and incubated at 70°C for 10 min;
- After incubation, 200 µL of ethanol 96-100% were added, they were vortexed and the sample was passed on the QIAamp columns, it was

centrifuged for 1 min. at 14 000 rpm, the column being passed in a new collection tube;
- 500 µl Buffer AW1 were added on the column, it was centrifuged for 1 min. at 14 000 rpm, the filtrate was eliminated;
- 500 µl Buffer AW2 were added on the column, it was centrifuged for 3 min. at14 000 rpm, the filtrate was eliminated, the centrifugation was repeated for 2 min., and the column was passed in a new collection tube;
- 200 µl Buffer AE were added on the column, directly over the filter and it was incubated for 1 min at room temperature for the ADN eluting;
- They were centrifuged at 14,000 rpm for 1 min and the extracted DNA concentration was established.

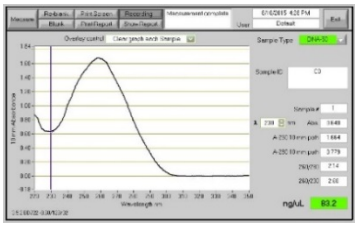
The establishment of the DNA quantity was made with a NanoDrop spectrophotometer, using 2 µL of the sample.

Table 1. The experiment organization scheme

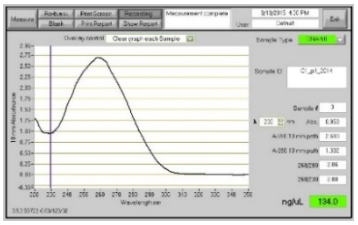
Group	Experimental year	Administered probiotics
0	2014	**L0** for which the chickens were fed with a combined feed where no probiotics and synthesis aa were introduced except for the values of the necessary amounts presented in the guide for the raising of the hybrid Ross 308;
1	2014	**L1** for which the chickens were fed with a combined feed where a probiotics premix was introduced (*Lactobacillus paracasei* J.R., *Lactobacillus rhamnosus* 15b) starting with day 0;
2	2014	**L2** for which the chickens were fed with a combined feed where a probiotics premix was introduced (*Lactobacillus paracasei* J.R., *Lactobacillus rhamnosus* 15b, *Lactobacillus lactis* y and *Lactobacillus lactis* FO) starting with day 0;
3	2014	**L3** for which the chickens were fed with a combined feed where a probiotics premix was introduced (*Lactobacillus paracasei* J.R., *Lactobacillus rhamnosus* 15b) only during the last week of growth from 35 to 42 days;
4	2014	**L4** for which the chickens were fed with a combined feed where a probiotics premix was introduced (*Lactobacillus paracasei* J.R., *Lactobacillus rhamnosus* 15b, Lactobacillus lactis y and Lactobacillus lactis FO) starting with the last week of growth (35 - 42 days);
5	2014	**L5** for which the chickens were fed with a combined feed where a probiotics premix was introduced (*Lactobacillus paracasei* J.R., *Lactobacillus rhamnosus* 15b, *Lactobacillus lactis* y and *Lactobacillus lactis* FO) to which two L threonine and DL methionine synthesis aa's added with values exceeding the necessary amounts provided by the raising guide by 25% starting with day 0;
6	2014	**L6** for which the chickens were fed with a combined feed where a probiotics premix was introduced (*Lactobacillus paracasei* J.R., *Lactobacillus rhamnosus* 15b, *Lactobacillus lactis* y and *Lactobacillus lactis* FO) to which two L threonine and DL methionine synthesis aa's added with values exceeding the necessary amounts provided by the raising guide by 25% only during the last week of growth and namely from 35 to 42 days.

3. Results and discussion

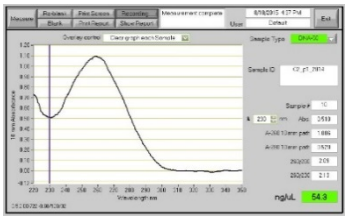
Imagine software was used to analyze the results of the mycroarry analysis and present in figure 1 and the DNA quantity extracted from the experimental samples was present in the table 2.



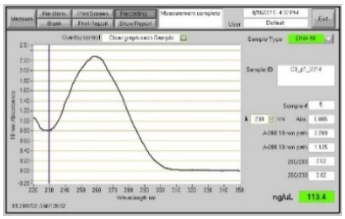
Group 0, experiments 2014 Control



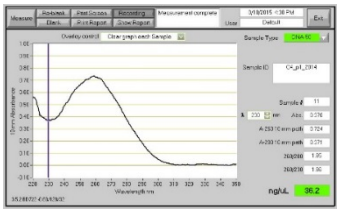
Group 1, experiments 2014, *Lactobacillus paracasei* J.R., *Lactobacillus rhamnosus* 15b, starting with day 0



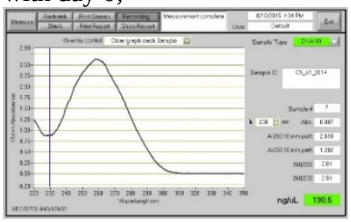
Group 2, experiments 2014 *Lactobacillus paracasei* J.R., *Lactobacillus rhamnosus* 15b, *Lactobacillus lactis* y and *Lactobacillus lactis* FO starting with day 0;



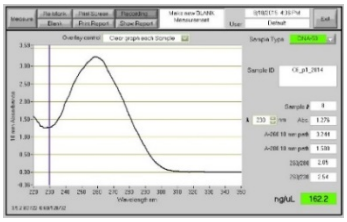
Group 3, experiments 2014 *Lactobacillus paracasei* J.R., *Lactobacillus rhamnosus* 15b only during the last week of growth



Group 4, experiments 2014 *Lactobacillus paracasei* J.R., *Lactobacillus rhamnosus* 15b, *Lactobacillus lactis* y and *Lactobacillus lactis* FO starting with the last week of growth



Group 5, experiments 2014 *Lactobacillus paracasei* J.R., *Lactobacillus rhamnosus* 15b, *Lactobacillus lactis* y and *Lactobacillus lactis* FO to which two L threonine and DL methionine synthesis aa added



Group 6, experiments 2014 *Lactobacillus paracasei* J.R., *Lactobacillus rhamnosus* 15b, *Lactobacillus lactis* y and *Lactobacillus lactis* FO to which two L threonine and DL methionine synthesis aa added

Figure 1. Imagine software results obtained from the DNA extraction from the caecal content samples

Table 2. The DNA quantity extracted from the experimental samples

Experimental group	DNA quantity
Group 0, 2014	83 ng/µl (eluted in 200 µL)
Group 1, 2014	134 ng/µl (eluted in 200 µL)
Group 2, 2014	54 ng/µl (eluted in 200 µL)
Group 3, 2014	113 ng/µl (eluted in 200 µL)
Group 4, 2014	38 ng/µl (eluted in 200 µL)
Group 5, 2014	130 ng/µl (eluted in 200 µL)
Group 6, 2014	162 ng/µl (eluted in 200 µL)

The resulting DNA was used subsequently in order to detect the differences between the groups with probiotics and the ones without by analyzing the composition of the microbiota. In the beginning, the phylogenetic differences were investigated in order to establish the dominant genders (Figure 2 chart A and Chart B).

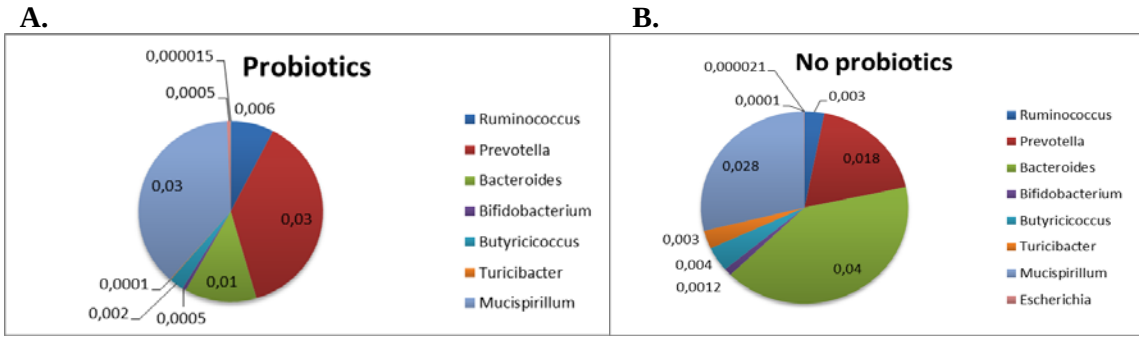


Figure 2. The composition of the microbiota by comparison between the groups with probiotics to the ones without probiotics

We can see from the above chart that the weight of the probiotic potential bacteria in the structure of the microbiota is the following: *Ruminococus* 0.006%, *Prevotella* 0.03%, *Bacteroides* 0.01%, *Bifidobacterium* 0.0005%, *Butyricicoccus* 0.002%, *Turicibacter* 0.001% and *Mucispirillum* 0.03%. The microbiota of the broiler chickens that did not receive probiotics in their food has a totally different structure. This aspect can be noticed in the chart below. After that, we used the pyrosequencing method in order to quantify the abundance of the microorganisms with probiotic potential from the composition of the microbiota (Figure 3, charts: A, B, C, D, E, F, G, H, I).

Figure 3 points to the relative abundance of the types *Ruminococcus (A), Prevotella (B), Bacteroides (C), Bifidobacterium (D), Butyricicoccus (E), Turicibacter (F), Mucispirillum (G), Echerichia (H)* and *Streptococcus (I)* as resulted from the pyrosequencing tests. Figure 3 points out the fact that all the non-pathogen bacteria were in the greatest quantity in the experimental option 4 where four bacterial types with probiotic potential were administered during the last week of growth of the broiler chicken, respectively week six. After Li, Y, et al (2016) [12] the probiotic *B. subtilis* CGMCC 1.1086 can effectively improve the growth performance and FCR of broilers via the beneficial modulation of caecal microbiota. Alun Carter, *et al.* 2017 [11] demonstrated that the oral administration of a combined probiotic comprising *L. salivarius* 59 and E. Faecium PXN33 resulted in reduced colonization of poultry by S. Enteritidis S1400. Torok *et al.* (2011) [3] identified sequences related to *Lactobacillus salivarius, L. aviarius, L. crispatus, Faecalibacterium prausnitzii, E. coli, Gallibacterium anatis, Clostridium lactatifermentans, Ruminococcus torques, Bacteroides vulgatus,* and *Alistipes finegoldii* from ileal and cecal samples.

Fuller M. F., (2004) [6] states that the main factors influencing the number and type of bacterial species from the chickens' cecum are age, feed and use of microbial feed additives.

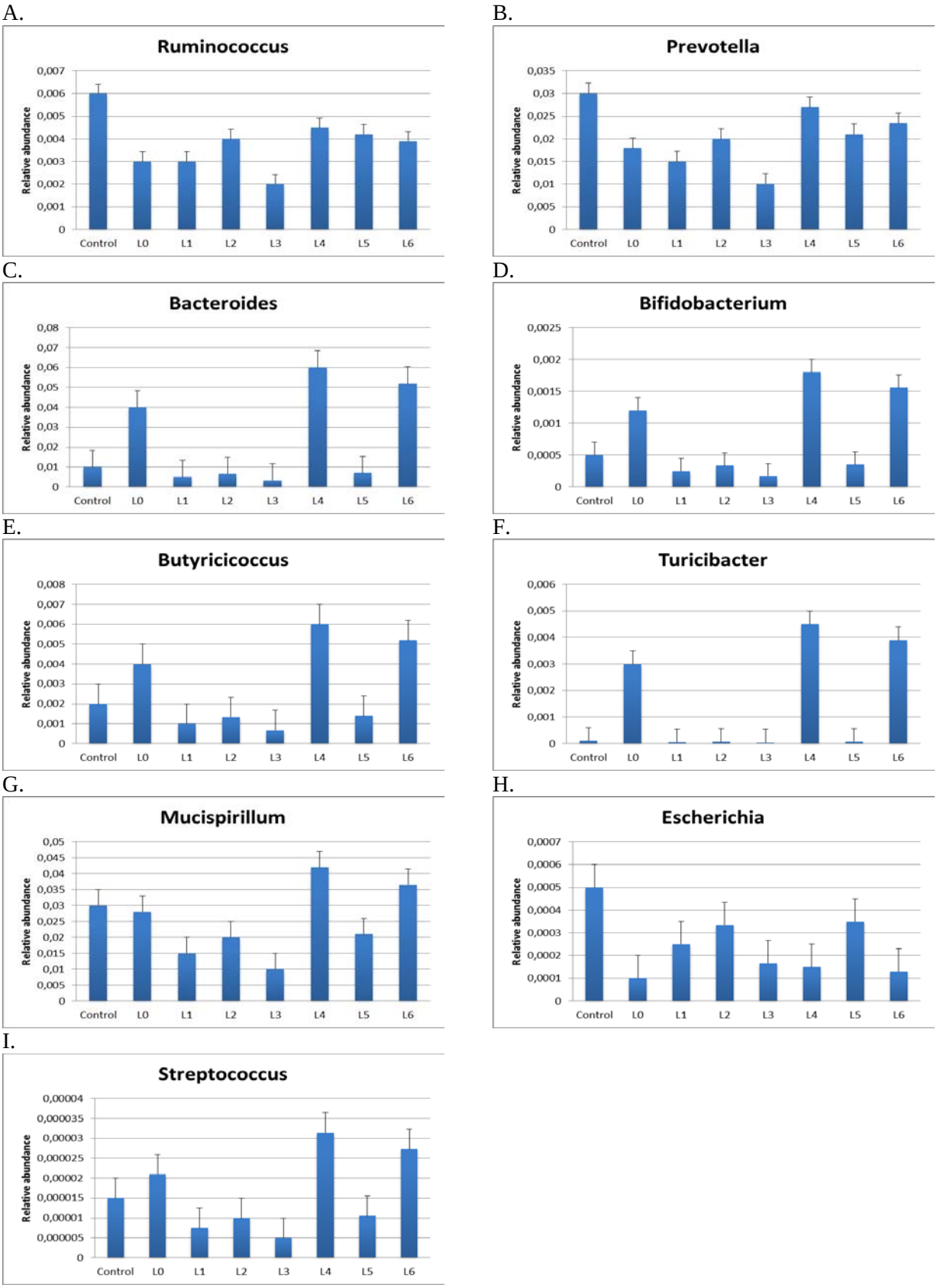


Figure 3. The composition of the microbiota by comparison between the groupes which received probiotics to the ones without probiotics.

4. Conclusions

The administration of probiotics in the broiler chickens diets influences the structure of the microbiota in all the characterized genders. The fact that they act by means of competition in diminishing the ability of the bacteria with pathogen potential to colonize the digestive tract is conclusive.

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