Studying the microflora of broilers to assess the effectiveness of using new feed additives

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Abstract. The creation of new feed additives, compositions of original substances (vegetable or chemical origin) requires a modern assessment of their safety for the animal body and effective use in the production of agricultural products. In the experiment, the control group received a basic diet (BD); I experimental – BD with lived microorganisms based on bacterial strain Bacillus cereus in a dose of 4 ml/kg b.w./day; II experimental – BD + Bacillus cereus + vegetable substance (9 µg/kg b.w./day); III experimental – BD + vegetable substance (9 µg/kg per day). The Bacillus cereus strain was found to reduce the number of antimicrobial agents resistant forms of bacteria, whereas the use of vegetable substance did not significantly change the overall picture. At the same time, it should be noted that there is a need for further studies to evaluate active substances of medicinal plant extracts and their synergistic effect, to create promising feed substances.

1. Introduction

The antimicrobial sensitivity had become a global problem, and hence the World Health Organization had given priority to addressing this issue. In addition to the search for new antimicrobial agents and combinations thereof, the use of specific bacteriophages and other alternatives, there is the possibility of using beneficial live microorganisms (probiotic). Probiotics available on the market are generally recognized as safe (GRAS) for human and animal consumption [1, 2]. However, in recent years, the transmission of genes from probiotic bacteria to pathogens in the intestine indicates that the safety of probiotics is also becoming an urgent problem [3-5]. Therefore, it is necessary to develop adequate means and technologies to control the general condition of animals and receive safe products from them. The studies showed multiple drug resistance of lactic acid bacteria to a number of classes of antibiotics, including macrolides, betalactams and aminoglycosides [6–8].

However, it is noted that probiotics do not contribute to the spread of antibiotic resistance and can even reduce it [9, 10]. It was shown that the use of B. subtilis BYS2 is possible to stimulate growth, improve innate immunity and increase disease resistance in broilers [11]. In this regard, the probiotic agents based on Bacillus sp. strains capable of surviving under harsh conditions of fodder production seem quite interesting [12].

Synergistic substances may include biologically active substances of plants [13] forming part of extracts [14, 15], including from the group of coumarins [16, 17].

In this context, the present study was aimed at assessing the effectiveness of using new feed additives for poultry.
2. Materials and methods
Substances: live microorganisms - Bacillus cereus IP 5832 (BC) – Laboratories AVENTIS (France); vegetable substance - 7-hydroxycoumarin (Co), C9H6O3, (Aldrich).

The study covered 120 heads of 7-day-old broiler chickens (4 groups, n=30). The control group received a basic diet (BD); I experimental – BD with probiotic preparation based on bacterial strain BC IP 5832 in a dose of 4 ml/kg b.w./day; II experimental – BD + BC IP 5832 + Co (9 μg/kg b.w./day); III experimental – BD + Co (9 μg/kg per day). Feeding and watering of birds was carried out by a group method.

During this experiment, the antibiotic resistance of bacterial cultures was evaluated on broilers and the content of the intestine was taken at 7, 14, 21 days. The resulting cultures were evaluated by the diffusion method for antibiotic sensitivity (cefotaxime-Ctk, ciprofloxacin-Cip, levomycetin-Lev, doxycycline-Dox, ofloxacin-Of, ampicillin-Amp, ceftazidim-Caz, ceftriaxone-Cro).

Numerical data were processed by the one-way analysis of variance (ANOVA) and the results were presented as mean values for groups and standard errors of the mean.

3. Research results and discussion
The assessment of the overall spectrum of antibiotic sensitivity of bacteria derived from all four groups of birds showed that the largest growth inhibition zone was found for Ctk (23±2 mm), Amp (24±3 mm) and Cro (25±2 mm). Nevertheless, a sufficient degree of variability is observed for this group of agents (standard deviation makes 10.66, 12.44 and 11.61 mm) (Fig. 1).

![Figure 1. Mean diameter of growth inhibition zones of strains from four groups of birds under study](image)

Five resistant strains were characterized for cefatoxime, of which three strains also showed resistance to Cro, and the dependence of these indices was characterized by a reliable correlation coefficient r=0.84 (P<0.001). Four strains not correlating with the previous ones were resistant to Amp, which is caused by a different type of structure of the given agent belonging to the penicillin group rather than the cephalosporins. On the other hand, the representative of cephalosporins of generation III – Caz – was found to be much less effective against these groups of bacteria and the average growth inhibition was 15±3 mm. Among the identified cultures, 12 strains were resistant to the agent, but a positive relationship was found between the diameters of Caz inhibition zones when compared in pair with Ctk and Cro (in both cases r=0.66 at P<0.001).
A number of antibiotics demonstrated a lower level of growth inhibition of bacterial cultures, namely the group of fluoroquinolone agents – Cip and Of, for which the average diameter of the inhibition zone was 19±2 mm and 18±2 mm. These values had a standard deviation of 11.35 mm and 8.60 mm, respectively, and the sensitivity of strains to Cip and Of correlated with each other (r=0.69 at P<0.001).

The least sensitivity of bacterial cultures was demonstrated for Lev (17±2 mm), to which nine strains were resistant, and Dox (12±2 mm) – 14 resistant strains. The latter was characterized by the least variability (standard deviation made 7.19 mm), as well as the absence of any correlation with other agents. In contrast, Lev has a weak positive bond with Caz (r=0.58 at P<0.01).

Some studies demonstrated the presence of a wide range of antibiotic resistance genes in the members of normal microflora, including betalactams, tetracyclines, aminoglycosides. It is also noted that this does not depend strongly on the type of nutrition (traditional or organic), which is caused by the initial presence of this pool of genes in the genome of microbiota [18]. In some cases, the frequency of resistance to certain antibiotic groups in certain species is as high as 100 %, for example, Salmonella genus and Enterobacteriaceae family as a whole with respect to vancomycin and methicillin [19]. It is also noted that the genes of resistance to vancomycin and betalactam antibiotics are more typical for broilers rather than for laying chickens, and are also often deterministic in plasmids, including E. coli [20]. In general, there is a tendency to increase the number of polyresistant strains [21]. Besides, there is evidence of genetic proximity of genes determining antibiotic resistance found in bacteria of the intestinal tract of humans, chickens and pigs [22].

The dynamic assessment of the sensitivity of bacterial cultures of the four studied groups of birds showed that there was a general tendency to increase the sensitivity of strains to antibiotics on the 14th day of a bird’s life, which was subsequently replaced by a decrease to a reliably indistinguishable level in all groups. The greatest variation was observed in the control group, where on the seventh day the average diameter of the inhibition zone was 9.5±2 mm, and on the 14th day – 38±6 mm, but on the 21st day it decreased to 20±2 mm (Fig. 2).

![Figure 2. Dynamics of bacteria sensitivity to antibiotics in the study groups during 21 days](image)

When comparing the data on the diameter of bacterial strain inhibition zones obtained on the 14th day in the studied groups of birds, it was found that the Student criterion has a reliable level of significance for comparison pairs: control – II group (t=2.70 at df=14, P<0.05) and control – III group (t=2.42 at df=14, P<0.05).
Such a surge in number and sensitivity on the second week of a bird’s life is connected with the stage of intestinal microflora formation [23]. During this period, the activity of amylases, proteinases and lipases is increased, which is partially associated with an increase in the number of individual groups of bacteria, including Lactobacillaceae and Enterobacteriaceae, while for Bacillaceae this dependence has the opposite value [24].

The analysis of resistance to individual antimicrobial agents among isolated cultures of strains from different groups of birds revealed that for a number of antibiotics, the effect was slightly variable (Table 1). Thus, in case of cefatoxime, Cro and Dox, the diameters of the inhibition zones in all groups are close to each other and have no significant differences. On the other hand, fluoroquinolone antibiotics showed a dependence in increasing the sensitivity of bacterial strains obtained from birds additionally receiving probiotic cultures compared to the control group (for Cip it was 27±3 mm against 20±6 mm and for Of – 27±2 mm against 16±2 mm, t=3.55 at P<0.01). On the other hand, the presence of Co in the diet (alone or together with probiotics) leads to increased stability, even higher than in the control group. At the same time, the difference in diameters of culture inhibition zones from group I compared to bacterial isolates of group II and III was characterized by a significant Student criterion, which for Cip made 2.27 and 2.83 at P<0.05, and for Of – 3.30 at P<0.01 and 3.10 at P<0.05.

Finally, the assessment of the dynamic pattern of antibiotic resistance of bacterial strains in each of the groups of birds showed the influence of probiotic factor and the presence of small molecules. Thus, in the control group, the strains obtained on the 7th day of the experiment showed resistance to antimicrobial agents in 75 % cases, but on the 14th day this value dropped sharply to 25 % with remaining resistance to Dox and Of. On the 21st day significantly more strains were isolated, among which 28 % of antibiotic resistance cases were reported. In the II group of birds, the diet of which combined probiotic strain and Co, the cultures were isolated on the 7th day, for which 50 % resistance of cases were recorded, and on the 21st day this indicator fell to 31 %, with resistance being mainly to levomycitin, Dox and Caz. For the III experimental group of birds, there was a dramatic increase of bacterial content, but the number of resistant forms at different stages of the study was comparable to the II group. Nevertheless, at the general resistance to Dox, the strains received from the III experimental group of birds showed the resistance to fluoroquinolones – Cip and Of.

The use of probiotics as a nutritional supplement in the diet of farm animals can reduce the growth rate of antibiotic resistance due to the natural antagonism of used microorganisms [25]. With respect to chickens, the use of probiotics also leads to an increase in the resistance of infections caused by Salmonella spp., as well as a decrease in the frequency of idiopathic diarrhoea [26]. On the other hand, being bacteria the probiotic agents can also participate in the change of antibiotic sensitivity in normal and pathogenic intestinal microflora [27]. Besides, probiotic strains capable of producing bacteriocins and other secretory factors (BC) can exert pressure on bacteria having antibiotic resistance in order to reduce competitive load [28]. However, the combination with plant factors acting as antibiotic

Table 1. Diameters of growth inhibition zones of identified cultures of strains of different groups (mm)

| Group | Ctk | Cip | Lev | Dox | Of  | Amp | Caz | Cro |
|-------|-----|-----|-----|-----|-----|-----|-----|-----|
| Control | 21±5 | 20±6 | 18±4.5 | 12±2 | 16±2 | 28±6 | 13±6 | 26±5 |
| I group | 22±6 | 27±3 | 18±6 | 13±4 | 27±2 | 28±3 | 18±3 | 24±7 |
| II group | 23±2.5 | 17±3 | 10±5 | 11±4 | 18±2 | 19±4 | 11±6 | 24±2 |
| III group | 27±4 | 13±4 | 22±2 | 11±3 | 10±5 | 23±3 | 21±4 | 24±6 |
analogues [29] can change the pattern towards selection of stable forms, in particular due to selection of efflux-containing strains of bacteria.

4. Conclusion
The results of the study demonstrate the presence of an “innate” spectrum of resistance among the members of the intestinal flora of broilers. However, changing the diet by applying new substances allows the profile shifting towards fewer resistant forms, which has a beneficial effect on reducing the probability of transferring the resistance gene pool through the food chain. The including plant-based substances, as well as the change in the nature of the nutrient substrate, may slightly change the situation due to the possibility of molecules affecting the molecular targets of microflora cells.

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Conflict of Interest: The authors declare that they have no conflict of interest.

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