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Effect of chitosan complexes on the bacterial community of cecum and productivity of broiler chickens

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Abstract. The search and development of natural biological additives that have a comprehensive effect as immunostimulants and improve digestion in poultry is relevant. This study was carried out at the Selection and Genetics Center "Zagorskoe EPH". The control and experimental groups were formed of the 1-day-old Ross-308 cross broilers (35 heads in each). Six groups were formed. The broilers of the group No.1 (control) received basic feed (BF) with the addition of feed antibiotic Maxus. The group No.2 (control) received BF without feed antibiotic. The broilers of the group No.3 (experimental) received BF and the KH-1 chitosan complex. The group No.4 (experimental) received BF and the KHM chitosan complex with the addition of copper nanoparticles. The group No.5 (experimental) received BF and drinking preparation based on the KH-Aqua chitosan complex. The group No.6 (experimental) received BF and drinking preparation based on the KH-Aqua chitosan complex enriched with copper nanoparticles. The bacterial community of the gut cecum was analyzed using the molecular genetics method of next-generation sequencing (NGS). The addition of chitosan complexes (both supplemented with copper nanoparticles and in the drinking form) made it possible to obtain high livability of broilers with increased live body weight and decreased feed consumption per 1 kg of live body weight gain. The live body weight of 35-day-old broilers in the experimental groups was 2.96-5.70% higher than that of the control with a 5.86-8.23% decrease in feed consumption per 1 kg of live body weight gain. The results of NGS showed that the effect of chitosan complexes on the regulation of the composition of the microbiome of broilers' cecum was predominantly positive. There was an up to 4.4-fold increase in the content of representatives of the normoflora, bacteria of the family Lactobacillaceae. The number of bacteria of genus Helicobacter, among which pathogens are often found, in the experimental groups was 2.6-33.3 times lower than in the group received antibiotics. So, the chitosan complexes were proved to be valuable supplements for poultry.

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1 Introduction

In the poultry industry and feed production, much attention is paid to the reduction or complete elimination of the use of feed antibiotics. The major danger of feed antibiotics is that when ingested, even in microdose, they cause antibiotic resistance in pathogenic microorganisms. So, a new generation of biological supplements, including chitosan complexes, is now replacing antibiotics. Feed additives can become an alternative to antibiotics, but the key challenge is to achieve and maintain stable results under industrial conditions.

Increasing the productivity of poultry through the use of various drugs, including feed antibiotics and preservatives, in diets results in the accumulation of degradation products of these drugs in the body, and disrupts the intestine normoflora. The EU countries have banned the use of antibiotics in feeding animals and poultry. In Russia, an action plan was adopted to combat the development of antibiotic resistance. So, the search and development of natural biological additives that have a comprehensive effect as immunostimulants and improve digestion in poultry is relevant [1]. Now the feed market offers several biopreparations which solve this problem to various degrees. These are mainly probiotics, prebiotics, synbiotics, organic acids, 1-monoglycyrides and other biological agents to replace feed antibiotics. Several companies have a positive experience in replacing feed antibiotics with natural additives that can improve the immune status of broilers and their productivity.

Chitosan is a derivative of the polysaccharide chitin. It is formed from N-acetyl-D-glucosamine links found in insects, marine diatom algae, fungi and crustaceans by deacetylation, demineralization, deproteinization and decolorization [2-4]. The studies on the use of chitosan as a feed additive in animal diets have shown different results [5-9]. At the same time, many studies consider chitosan as an additive with multifunctional activity, for example, as an antimicrobial agent against foodborne pathogens [10-13]. So, it can be used as an alternative to feed antibiotics to improve growth performance and intestinal function, as well as to reduce ammonia excretion during broiler breeding. Moreover, chitosan can form complexes with microelements and converse them into more digestible forms.

Copper also has a bacteriostatic action with antioxidant protection through superoxide dismutase and lysyloxidase and is a component of several immune system enzymes. One of the most important advantages of copper nanoparticles compared to antibiotics in the treatment of various bacterial diseases in animals is that they do not lead to bacterial resistance. It can be assumed that the combination of properties of copper nanoparticles and chitosan will result in the creation of highly effective antimicrobial drugs with adaptogenic, immunomodulatory and antioxidant properties of prolonged action for oral use as an alternative to antibiotics [14].

This work aims to study the composition of the bacterial community of cecum using the molecular genetics method of next-generation sequencing (NGS), zootechnical, physiological, and biochemical parameters of broilers when chitosan complexes KH-1 and KHM with and without copper nanoparticles were included in feed or in water without addition of feed antibiotic.

2 Materials and methods

Studies were carried out at the Selection and Genetics Center “Zagorskoe EPH” in 2021. The control and experimental groups were formed of the Ross-308 cross broilers of 1 to 35-day-old (35 heads in each). The broilers were kept in cage batteries of the P-15 type.

The amount of floor space per bird; light, temperature, humidity, feeding and watering regimes in all age periods were the same for all groups and met the recommendations of the Federal Scientific Center “All-Russian Research and Technological Institute of Poultry” (VNITIP).

Poultry was fed with bulk mixed fodder with nutritional value according to VNITIP norms. Broilers received Starter mixed feed on days 1-14, Grower feed on days 15-21; and Finisher feed on days 22-35.

Chitosan complexes were produced by Agrochitin Co. with a degree of deacetylation of about 90% and pre-tested in mixed fodder for broiler chickens at the household farm of Lyskovsky district of Nizhny Novgorod region.

The levels of chitosan complexes addition into feed and water and copper nanoparticles have been established in previous studies.

Six groups were formed from day-old conditioned chicks by random sampling.

The scheme of the experiment on broiler chickens is shown in Table 1.

Table 1. Scheme of experiments on broiler chickens, n=35

Group	Number of heads	Feeding Features
No.1 control	35	The basic feed balanced by all nutrients in accordance with the 2019 VNITIP norms (BF), with the addition of 100 g/t of feed antibiotic Maxus (BF-1)
No.2 control	35	BF without the addition of feed antibiotic
No.3 experimental	35	BF and the KH-1 chitosan complex at a rate of 100 g/t
No.4 experimental	35	BF and the KHM chitosan complex with the addition of copper nanoparticles at a rate of 100 g/t
No.5 experimental	35	BF and drinking preparation based on the KH-Aqua chitosan complex at a rate of 1 mL/L of water during the whole period of rearing
No.6 experimental	35	BF and drinking preparation based on the KH-Aqua chitosan complex enriched with copper nanoparticles at a rate of 1 mL/L of water in the periods from days 1 to 5; 11 to 13 and 24 to 28. In the periods from days 6 to 10, 14 to 23, and 29 to 35, only the KH-Aqua chitosan complex was fed.

In the course of the experiment, the following zootechnical parameters were assessed:

- Livability of livestock by counting mortality and identifying its causes, %;
- Live body weight of chickens at the age of 1, 14, 21 and 35 days by individual weighing of all flocks, by groups;
- Average daily live body weight gain, g;
- Feed consumption, for the entire growing period, kg per head;
- Feed consumption per 1 kg of live body weight gain at the end of the experiment, kg;
- Digestibility and utilization of basic nutrients of mixed fodder by the results of the physiological experiment at the age of 30-35 days;
- Chemical composition of broiler meat (protein, fat, ash), %;
- Cholesterol content in blood, in each experimental group, at the end of experiment, %;
- Yield of pectoral muscles, %;
- Slaughter yield, %;
- Content of ash (%), calcium (%), phosphorus (%), copper (mg %), manganese (mg %), zinc (mg %) in tibia.

The composition of the bacterial community of the intestinal cecum was analyzed using the Next Generation Sequencing method. Samples were taken at the end of the experiment under aseptic conditions.

Total DNA from the samples was isolated using the Genomic DNA Purification Kit (Fermentas, Inc., Lithuania) according to the provided instructions. Amplification for subsequent NGS sequencing was performed using the Verity DNA amplifier (Life Technologies, Inc., USA) using the eubacterial primers 343F (5´-CTCCTACGGRRSGCAGCAG-3´) and 806R (5´-GGACTACNVGGTWTCTAAT-3´), flanking the V1V3 region of the 16S rRNA gene. Methagenomic sequencing was performed on a MiSeq genomic sequencer (Illumina, Inc., United States) with the MiSeq Reagent Kit v3 (Illumina, Inc., USA). The maximum length of the sequences obtained was 2x250 bp. Chimeric sequences were excluded from the analysis using the USEARCH 7.0 program (http://drive5.com/usearch/). Processing of the obtained reads using the bioinformatic platform CLC Bio GW 7.0 (Qiagen, Netherlands) included overlapping, quality filtering (QV > 15), and primer trimming. Taxonomic affiliation of microorganisms to the genus was determined using the RDP Classifier program (http://rdp.cme.msu.edu/).

Mathematical and statistical processing of the results was performed using Microsoft Office Excel 2003 and R-Studio (Version 1.1.453) (https://rstudio.com) software packages. Mean values (M) and standard errors of the mean (±SEM) were determined. The results of statistical analysis were considered significant at $p \leq 0.05$.

3 Results and Discussion

The content of microelements in the main components of feed, by the age periods of broilers rearing (1-14, 15-21 and 22-35 days) was: 62.4 mg, 64.7 mg,72.8 mg of iron; 31.1 mg,30.7 mg and 31.9 mg of zinc; 42.4 mg, 41.3 mg and 37.7 mg of manganese; 6.5 mg, 6.2 mg and 6.9 mg of copper; 0.13 mg, 0.12 mg and 0.11 mg of iodine; 0.15 mg, 0.13 mg and 0.14 mg of selenium in 1 kg of mixed feed without premix addition.

The main zootechnical indicators of broilers are presented in Table 2.

Table 2. Zootechnical indicators of broiler chickens

Characteristics	**Group**					
	1c	**2c**	**3**	**4**	**5**	**6**
Livability, %	100.0	100.0	100.0	100.0	100.0	100.0
Live body weight, (g) at the age of (days)						
1	44.40 ±0.22	44.23 ±0.20	44.14 ±0.27	44.19 ±0.21	44.38 ±0.27	44.30 ±0.19
14	466.60 ± 5.56	447.11 ± 6.07	485.66** ± 5.02	475.66 ± 5.91	492.09*** ± 5.66	498.26*** ± 5.33
21	890.20 ±8.26	852.11 ±9.93	943.77*** ±7.72	912.40*** ±9.52	966.26*** ±8.64	968.14*** ± 8.60
35 (average)	2033.78	1968.36	2116.28**	2093.94	2147.33	2149.67
males	2159.44 ± 22.06	2082.61 ± 19.34	2265.56** ± 14.05	2240.44 ± 16.57	2323.72*** ± 14.13	2326.94*** ± 17.21
females	1908.11 ± 18.23	1854.11 ± 14.86	1967.00** ± 12.93	1947.44 ± 10.44	1970.94*** ± 12.35	1972.39*** ± 10.32
Feed consumption	3.326	3.316	3.322	3.329	3.330	3.326

per head for the whole period, kg						
Feed consumption per 1 kg of live body weight gain, kg	1.672	1.725	1.603	1.624	1.583	1.580
Average daily live body weight gain, g	56.84	54.98	59.20	58.56	60.08	60.15
Yield of pectoral muscles to the gutted carcass, %	24.11	22.66	24.19	24.00	24.22	24.29
Slaughter yield, %	72.11	71.37	72.44	72.14	72.77	72.95

** $P \leq 0.01$; *** $P \leq 0.001$

The data in Table 2 shows that the livability of poultry in all groups was 100%.

The live body weight of broilers in the experimental groups was 476 - 498 at 14 days of age, 944 - 968 g at 21 days of age, and 2116 - 2150 g at 35 days of age, which exceeded by 1.94 - 6.78%, 2.49 - 8.76%, and 2.96-5.70% the control group No.1, respectively, and by 6.39-11.44%, 7.08-13.62%, and 6.38-9.21% the control group No.2

At the age of 35 days, the live body weight of females in the experimental groups was 2.06-3.37% higher and that of males by 3.75-7.76% higher compared to control group No.1, and by 5.03-6.38% and 7.58-11.73% higher compared to control group No.2. When chitosan complexes were used, the average daily gain of live body weight in experimental broilers was 58.56- 60.15 g and exceeded that of the control group No.1 by 3.03-5.82%, and that of the control group No.2 by 6.51-9.40%.

A higher live body weight of young chicks ensured high feed conversion by the end of rearing. During the entire period of rearing, the feed consumption per 1 kg of live body weight gain in the experimental groups were 1.580-1.624 kg, which was 2.87-5.32% lower than those of the control group No.1, and 8.17-8.23% lower than those of the control group No.2.

It should be noted that zootechnical indicators were higher in broilers of experimental group No.6, and lower in broilers of control group No.2, which did not receive feed antibiotic and chitosan complexes. In all experimental groups of broilers, the main zootechnical indicators were higher than in the chickens that received feed antibiotic.

Protein digestibility in the experimental groups of chickens that received chitosan complexes was 89.0-91.1%, which was 0.6-3.1% higher than that of control group No.1, and 3.01-5.44% higher than that of control group No.2.

The utilization of feed nitrogen in the experimental groups was within the physiological parameters for young animals of this age (51.9-52.8%), exceeding the control group No.1 by 2.77-4.55%, and the control group No.2 by 4.43-6.24%.

Lysine and methionine availabilities from the experimental feed were 82.4-82.9% and 81.2-81.9%, respectively, exceeding these values in broilers of control groups by 0.4-3.2% for lysine and by 0.7-2.5% for methionine.

The digestibility of fat from mixed fodder by broilers of experimental groups was 76.4-76.7% and it was 0.60-1.99% higher than in the control group No.1, and 3.24-3.65% higher than in the control group No.2.

Calcium and phosphorus utilization in experimental broilers was higher than in control groups by 0.4-2.6% and 0.5-2.8%.

The analysis of chemical composition of the breast muscles showed that when chitosan complexes were added to mixed fodders and water, there was a tendency to increase the dry matter and protein level in the breast muscles of experimental chickens as compared with that of control groups by 0.36-0.78% and 2.08-2.29%. The fat level was lower in experimental groups by 0.44-0.48% as compared with control group No.1 and by 0.52-0.56% as compared with control group No.2. The ash level in the pectoral muscles of experimental broilers was 0.41-0.53% higher compared to control group No.1 and 0.60-0.72% higher compared to control group No.2.

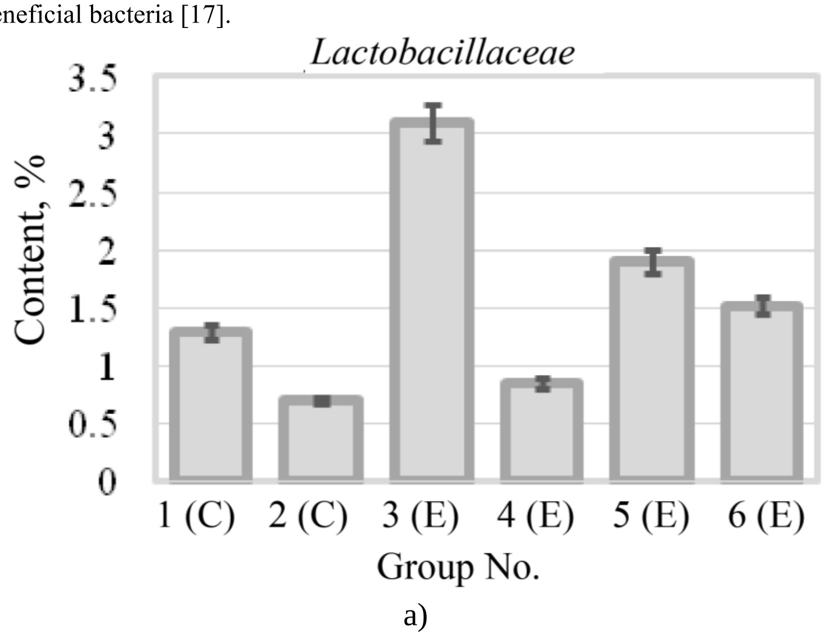
The study of mineralization of the bones of broiler chickens showed that the ash content in the tibia of the 35-day-old broiler chickens in all experimental groups was 45.77-46.92%, the calcium content was 17.42-17.48%; the phosphorus content was 8.49-8.93%; the copper content was 0.244-0.330 mg %, the manganese content was 0.37-0.45 mg %; the zinc content was 13.20-14.40 mg %. There were no significant differences in these indicators between the experimental groups and the control group No.1.

Chickens receiving mixed feed without addition of feed antibiotic (control group No.2) had statistically significant lower levels of ash, calcium and phosphorus in tibia than chickens of control group No.1 and experimental groups Nos.3-6. The highest deposition of copper in the tibia was observed in broilers of experimental group No.4, who additionally received the KHM chitosan complex with the addition of copper in the form of nanoparticles at a rate of 100 g/t.

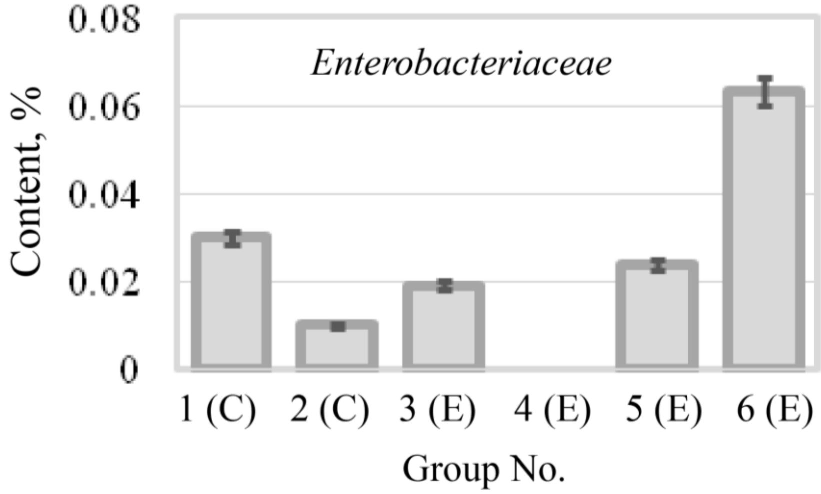
When chitosan complexes were used in feed and water, there was a tendency for cholesterol levels in experimental chickens in groups Nos.3,5 and 6 to decrease by 18.12%; 31.54% and 24.16% compared with control group No.1 at $P \leq 0.01$ and $P \leq 0.001$.

The results of the next-generation sequencing showed that the composition of the chyme microbiome of the cecum of broilers' intestines differed between the control and experimental groups at $p \leq 0.05$ (Fig. 1).

It was shown that in experimental groups Nos. 3, 4 and 6, the content of bacteria of Lactobacillaceae family was up to 4.4 times higher than in control groups Nos. 1 and 2 ($p \leq 0.05$). This probably had an effect on the improvement of zootechnical parameters of broilers, since these microorganisms can produce significant amounts of lactic and acetic acids, which reduce the pH values in the gastrointestinal tract [15], compete with pathogens for nutrients and epithelial sites for adhesion [16]. Earlier similar results were obtained by Huang R. L. et al. [17]. The introduction of chitosan into the diet was associated with improved nutrient utilization by the host and promoted the growth of the number of beneficial bacteria [17].



a)



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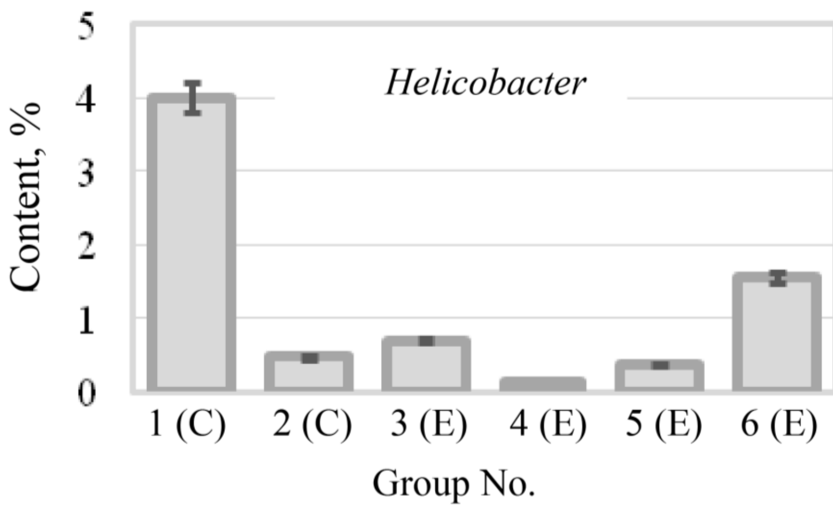

c)

Figure 1: Contents of different groups of microorganisms in the chyme of the cecum of broilers' intestines determined by NGS method: a) Lactobacillaceae, b) Enterobacteriaceae, c) Helicobacter

The number of bacteria of the genus Helicobacter in experimental groups Nos. 3-6 was 2.6 to 33.3 times lower than in the control group No.1 with the introduction of feed antibiotic ($p \leq 0.05$) (Fig. 1). The presence of these microorganisms in the gastrointestinal tract of birds is undesirable. Detection of H. pullorum in the intestine has been associated with gastroenteritis in farm birds, including chickens, turkeys, and guinea fowl [18]. The increased abundance of these microorganisms in the intestines of birds who received antibiotic with feed can be explained by the fact that Helicobacter species are known to be resistant to antibiotics. The resistance arises due to mutations resulting in changes in base in the 23S ribosomal RNA subunits and due to mutations in the rdxA gene, which encodes a novel nitroreductase [19]. At the same time, the positive results in groups with addition of chitosan complexes correlate with the works of Zheng L. Y. and Zhu J. F. [20]. The researchers suggested that high-molecular-weight chitosan can form a polymeric membrane around the bacterial cell, preventing the cell from receiving nutrients. Low-molecular-weight chitosan can penetrate into cells and disrupt the physiological activity of the bacterial cell.

The content of bacteria of the family Enterobacteriaceae was quite low in the control and experimental groups and did not exceed 0.063±0.004%. In experimental group No.4 these microorganisms were absent. Nevertheless, in group No.6 with the addition of the KHM-Aqua chitosan complex enriched with copper nanoparticles to the chickens' diet, the number of these bacteria exceeded that in control group by 6.3 times ($p \leq 0.05$). Previously, similar results were obtained by Zhang Y. et al. [21] in pig livestock. The abundance of E. coli in the contents of ileum and cecum tended to increase at high levels of copper in the diet, for 3 weeks. Copper-resistant E. coli isolates were selected against a background of high copper concentrations. This resulted in an improved level of adaptation to high copper concentrations, which contributed to the colonization advantage of E. coli. Interestingly, Hölzel C. S. et al. [22] showed that high levels of copper in pig liquid manure were associated with increased resistance of E. coli strains to β-lactam antibiotics. This is quite logical, since it was demonstrated by the example of Enterococcus faecium that the resistance genes to copper (tcrB), to macrolides (ERM), and to glycopeptides (vanA) are located on the same plasmid [23].

4 Conclusion

Thus, we found that chitosan complexes are valuable additives for poultry. High livability of broilers with increased live body weight and decreased feed consumption per 1 kg of live body weight gain was achieved by the addition of the KH-1 and KHM chitosan complexes with supplementation of nanoparticles in the amount of 100 g/t, and drinking chitosan complex KHM-Aqua at a rate of 1 mL/L of water during the entire period of chickens growing, while excluding the feed antibiotic, and applying chitosan complex KHM-Aqua enriched with copper nanoparticles at a rate of 1mL/L of water in the periods of 1 to 5, 11 to 13 and 24 to 28 days; and drinking of chitosan complex KHM-Aqua from the days 6 to 10, 14 to 23 and 29 to 35. So, the live body weight of 35-day broilers in the experimental groups was higher than that in the control groups by 2.96-5.70% with a decrease in feed costs per 1 kg of live body weight gain by 5.86-8.23%.

The use of chitosan complexes provides an average daily increase in live body weight of 58.56 - 60.15 g, which is higher than in the control group No.1 by 3.03-5.82% due to improved digestibility and utilization of nutrients in the feed and lower fat deposition in meat.

When chitosan complexes were added to feed and water to broilers, there was a tendency to increase the protein level in pectoral muscles by 2.08-2.29% and to decrease the fat in pectoral muscles by 0.44-0.48% compared with control group No.1 and by 0.52-0.56% compared with control group No.2. Cholesterol levels in the blood of chickens in groups Nos.3,5 and 6 were statistically significantly reduced by 18.12%, 31.54% and 24.16% compared with the control group. The calcium and phosphorus depositions in the tibia increased by 17.42-17.48% and by 8.49-8.93%, respectively.

Higher zootechnical parameters of broilers were obtained when using mixed fodders without feed antibiotic with the addition of drinking preparation based on the KH-Aqua chitosan complex enriched with copper nanoparticles at a rate of 1 ml/l water in the periods of 1 to 5, 11 to 13 and 24 to 28 days. On days 6 to 10; 14 to 23 and 29 to 35 the broilers received the KH-Aqua chitosan complex.

The results of next-generation sequencing showed that the effect of chitosan complexes on the regulation of the composition of the microbiome of the cecum of broilers' intestines was predominantly positive. There was an up to 4.4 increase in the content of representatives of the normoflora, bacteria of the family Lactobacillaceae. The number of bacteria of the genus Helicobacter, among which pathogens are often found, in the experimental groups was from 2.6 to 33.3 times lower than in the group with the introduction of feed antibiotic. Probably, such shifts in the composition of the bacterial community influenced the improvement of zootechnical parameters of broilers. In the group received chitosan complex enriched with copper nanoparticles, the number of Enterobacteriaceae bacteria was 6.3 times higher than in the control group without supplements. It is likely that in the groups with copper supplementation, there was an improvement in the level of adaptation to high copper concentrations, which contributed to some colonization advantage of Enterobacteriaceae. Nevertheless, the overall content of these microorganisms in all groups remained insignificant. Therefore, judging by the values of zootechnical indicators negative effects on the body were not noted.

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