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INTESTINAL MICROBIOTA AND BROILER PERFORMANCE UPON ADMINISTRATION OF PHYTASE TO INCREASE PHOSPHORUS DIGESTIBILITY AND NUTRIENT UTILIZATION FROM FEED

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Abstract

Phosphorus is an essential element in the nutrition of humans, animals, and plants. Due to the short growth period (34-42 days of age), fast growth of live bodyweight and skeleton the problem of mineral nutrition and balance (including calcium and phosphorus) is especially urgent for broiler chicks (*Gallus gallus* L.). The predominant form of phosphorus in vegetable feed ingredients is phytate which is an antinutritive factor for poultry and cannot be digested. As a result the supplementation of diets for poultry with phytases, enzymes degrading the indigestible phytate complexes, has gradually become a common practice worldwide. The recent data of Russian (O.V. Trufanov, 2011; E.V. Anchikov, 2012) and foreign authors (S.W. Kim et al., 2018; C.L. Walk et al., 2019; O.O. Babatunde et al., 2019) evidenced that the supplementation of diets with reduced phosphorus content with different doses of phytase improves daily weight gains, phosphorus content in blood serum, tibial strength, and tibial contents of minerals. The efficiency of supplementation of diets for broilers (cross Cobb 500) with reduced by 0.1 % phosphorus content with two innovative new-generation phytase preparations (Feedbest-P and Berzyme-P, produced by Sibbiopharm Co., Russia) was studied; the data of pioneer research of the effects of different phytase preparations on the cecal microbial community are also presented. The trials were performed in the Center for Genetics & Selection Zagorskoye EPH (Moscow Province) in 2018. In the first trial the diets for experimental treatments were supplemented with phytase preparation 1 with activity 10,000 FTU/g (20, 40, and 60 ppm); in the second trial phytase preparation 2 was used with activity 50,000 FTU/g (6, 12, and 30 ppm). The supplementation of diets with preparation 1 increased live bodyweight in broilers at 37 days of age by 2.7; 3.0 and 3.7 % (respective to aforementioned doses) in compare to non-supplemented control treatment; feed conversion ratio (FCR) in these treatments was better by 2.9; 4.0 and 4.6 %, respectively. The respective improvements with different doses of preparation 2 were 1.3; 3.1 and 2.0 % and 1.9; 5.6 and 3.7 %. Positive effects on the digestibility of dietary nutrients, deposition of calcium and phosphorus, mineral contents in the skeleton were found with both phytase preparations. The investigation of cecal microbiota using T-RFLP (terminal restriction fragment length polymorphism) method revealed the significant increase in the pool of cellulolytic bacterial species in the phytase-fed broilers; the increases in the species of *Eubacteriaceae*, *Clostridiaceae*, *Lachnospiraceae*, *Ruminococcaceae,* and

Bacteroidetes were also found. The most of the identified bacterial species in cecal population were ascribed to the phylums *Firmicutes*, *Bacteroidetes*, *Actinobacteria*, *Proteobacteria* and *Fusobacteria*.

Keywords: *Gallus gallus*, phytase, compound feeds, broiler chicks, productive performance, cecal microbiota

Enzyme preparations have a high potential for practical use in feed production. Recently, up to 90 % of poultry feeds are enriched with phytases, the enzymes that break down indigestible phytate containing complexes. The use of phytases allows better absorption of plant food P by poultry, reduces the level of inorganic phosphates in diets and the excretion of phosphorus in droppings [1-3]. Young birds, in particular broiler chickens, are especially in dire need of phosphorus. Selection for growth rate has caused the development of chicken skeleton to lag behind the formation of muscle tissue which often results in leg abnormalities of non-infectious etiology [4].

The market of commercial bacterial and fungal phytases for animal husbandry is quite diverse and saturated. The activity of phytase preparation determines its dose in feeds, thermostability, costs and effectiveness of practical use [5]. Phytase-enriched premixes or direct enrichment of animal feed in the farm can be applied which requires due equipment to mix feeds, premixes and supplements.

Dietary phytase can affect the physical and chemical properties of the chyme in the gastrointestinal tract, especially pH, which leads to shifts in the profiles and activity of the gut microbiota. Phytases show highest activity at acidic pH of 3 to 6, i.e., phytases can release phosphorus already in crop and gizzard [6]. From 0 to 12,000 FTU/kg live weight of broilers, upon a decrease in the amount of calcium and phosphorus, contributed to a significant increase in the average daily weight gains in chick, blood phosphorus, and tibia strength and ash content [7]. However, phytase can adversely impact the absorption of lysine, cysteine, aspartic acid, glycine, methionine, tryptophan, and serine [8]. Regardless of poultry age, diet and products the birds are farmed for, phytase increases the availability and amount of phytic phosphorus in the jejunum contents. Phytase acts more effective in 14-22-day old chickens when bone tissue formation and growth intensify [9]. The size of mineral component particles also affects the effectiveness of dietary phytase [10]. Upon a combined use of phytase with xylanase or β-glucanase, broiler chickens showed a decrease in feed intake and *Escherichia coli* colonization of the intestines [11] without detectable changes in the digestive system.

The microbiocenosis of poultry intestines is a fairly rich and complex community of symbiotic microorganisms, comprising bacteria, archaea, micromycetes, protozoa, and viruses [12-15]. Bacteria are the main members of chicken intestinal microbiome [16]. The 16S rRNA gene sequencing technique revealed 13 bacterial phyla in the intestinal microbial ecosystem of chickens, with *Firmicutes*, *Bacteroidetes*, and *Proteobacteria* dominating (> 90%). Of more than 900 equivalents of operational taxonomic units (OTUs) found in the intestines of chickens 117 OTUs belong to known bacterial genera. The effect of dietary phytases on the microbial ecosystem of the gastrointestinal tract is extremely poor studied.

In this work, the alterations of cecum microflora in broiler chickens when phytase of different activity were added to the feed were first studied by the T-RFLP (terminal restriction fragment length polymorphism) method which has a number of advantages compared to methods of classical bacteriology. It was shown that the Russian biopreparations Feedbest-R and Berzyme-R provide competitive advantage of the chicken gut normoflora, while the counts of conditionally pathogenic and pathogenic members of the microbial community decrease. Feedbest-R and Berzyme-R increase P utilization from feeds with low digestible phosphorus, improve protein and fat digestibility, as well as utilization of nitrogen, amino acids, calcium, phosphorus and trace elements (iron, manganese, copper, zinc).

Our subjective was to investigate effects of two Russian phytase containing preparations on broilers' productive performance, bone mineralization, nutrient digestibility and use, and cecum microbiome composition.

Material and methods. Experiment 1 and experiment 2 were performed in vivarium (Zagorsk EPH Selection and Genetic Center, Sergiev Posad, Moscow Region, 2018) on birds from 1 day to 37 days of age, and from 1 day to 36 days of age, respectively. For each experiment, Cobb 500 broilers (*Gallus gallus* L.) were assigned into four groups of analogues in live weight (control group I, test groups II-IV, 35 birds per group, 280 birds in total). Chickens were kept in AviMax cell batteries (Big Dutchman, Germany) as per stated technological parameters [17]. For the first 5 days, chickens of all groups received the same starter compound feed. The feeds corresponded to the age norms for the cross (from day 6 to day 21 and from day 22 until the end of the experiment). In the control groups, the nutrients content the feeds were equal, in the experimental groups, the amount of digestible phosphorus was reduced by 0.1%. The enzyme was mixed with feed in a stepwise fashion. In experiment 1, the Feedbest-R (LLC PO Sibbiofarm, Berdsk, Russia) doses were 20, 40 and 60 g/t feed for groups II, group III and group IV, respectively. In experiment 2, Berzyme-R (PO Sibbiofarm LLC, Berdsk, Russia) doses were 6, 12 and 30 g/t feed for groups II-IV, respectively. The activity of Feedbest-R is standard (10,000 FTU/g), while of Berzyme-R it is 5-fold (50,000 FTU/g).

The investigated parameters were mortality, live weight at 1, 7, 14, 21 and 36-37 days of age (from day 21, females and males were weighed separately), feed conversion and average daily weight gain. At the age of 28-36 days, physiological balance experiments were carried out, in which the protein, fat, fiber digestibility and the balance of nitrogen, calcium, and phosphorus were determined. For this, 3 birds (analogues in live weight) from each group were kept in a cage to determine the amount of consumed feed and the amount and chemical composition of poultry manure. At the end of the experiments, the chemical composition of the tibia, pectoral and foot muscles were determined. After slaughter, anatomical cutting of carcasses was carried out and the slaughter yield of the gutted carcass was determined [18].

Cecal content was sampled for microbial analysis after slaughter of chickens at 36 days of age (three replicates from each group) strictly aseptically [18] and immediately frozen.

Microbial composition of the cecal content was assayed by T-RFLP (term-minimal restriction fragment length polymorphism) method. Total DNA was extracted with DNA Purification Kit (Fermentas, Inc., Lithuania) as per the manufacturer's recommendations. PCR (Verity DNA Amplifier, Life Technologies, Inc., USA) was performed with eubacterial primers 63F 5′-CAGGC-CTAACACATGCAAGTC-3′ labeled at the 5′-end (D4 WellRED fluorophore, Sigma-Aldrich, Inc., USA) and 1492R 5′-TACGGHTACCTTGTTACGACTT-3′ to amplify the 16S pRNA gene fragment (positions from 63 to 1492, numbering indicated for *Esherichia coli* 16S pRNA gene) in the following mode: 3 min at 95 °C (1 cycle); 30 s at 95 °C; 40 s at 55 °C; 60 s at 72 °C (35 cycles); 5 min at 72 °C. Labeled amplicons of 16S pRNA gene DNA were purified by a standard method [19]. Concentration of purified fragments was determined (a Qubit 2.0 fluorimeter, Invitrogen, Germany) according to the manufacturer's recommendation. HaeIII, HhaI, and MspI (Fermentas, Lithuania) were used for PCR amplicon restriction endonuclease analysis (the amplicon amount of 30-50 ng). Cleaved DNA fragments were sequenced (CEQ 8000, Beck-man Coulter, USA). Fragment Sorter program (available from http://www.oardc.ohiostate.edu/trflpfragsort/index.php) were used for phylogenetic affiliation.

Mathematical and statistical processing was carried out by standard methods of analysis of variance [20] using Microsoft Excel 2010 software. We used parametric (Student's *t*-test) and nonparametric (Wilcoxon-Mann-Whitney method) statistical methods. Mean values (*M*) and standard errors of the mean (±SEM) were calculated. Biological diversity was evaluated using the Shannon and Simpson indices in the Past program (http://folk.uio.no/ohammer/past/). The causal relationship between cecal microflora composition and phosphorus utilization was assessed using Pearson correlation coefficients, which makes it possible to establish direct relationships between the variables by their absolute values [20]. Correlation indicators were analyzed for microorganisms comprising over 1% of the total microbial counts in the community.

Results. Feedbest-R and Berzyme-R supplements increased the absorption of phytic phosphorus, the digestibility and use of other feed nutrients. In experiment 1, the Feedbest-R increased the use of phosphorus by 4.2% in group II, by 6.6% in group III and by 7.1% in group IV compared to control (Fig. 1, A). In test groups II, III and IV, the feed dry matter digestibility was 1.2; 2.8 and 3.0 % higher, respectively, and fat digestibility was 3.0; 3.7 and 3.9% higher, as compared to control.

Phytates bind positively charged metal ions, the macro- and microelements (calcium, zinc, iron, manganese, magnesium ions), as well as proteins, amino acids, and starch, reducing their bioavailability [9]. In the experiment 1, the phytase contributed to a better use of calcium in groups II, III and IV, by 1.8; 3.0 and 3.5%, respectively. The phytase has improved the digestibility of animal protein upon a sufficiently low its amount in the diet. Protein digestibility in groups II, III and IV was higher than in the control by 1.2; 1.4 and 1.5%. The test broilers also utilized feed nitrogen better (2.2-2.8%) than the control birds. The digestibility of essential amino acids lysine and methionine was 3.0-3.5 and 3.1-4.0% higher, respectively.

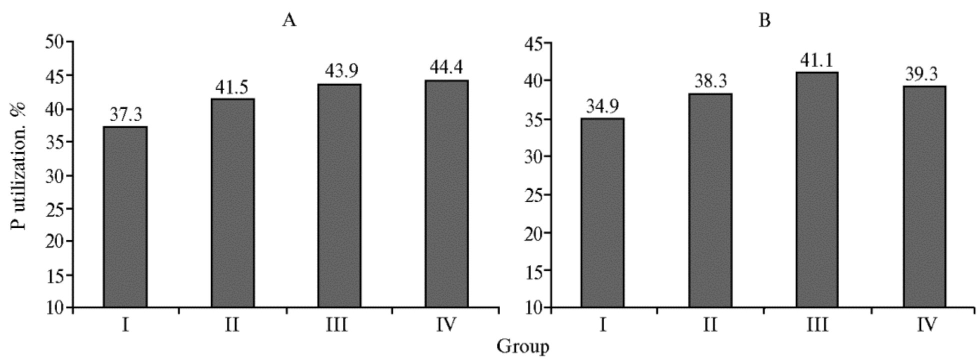

Fig. 1. Phosphorus utilized by Cobb 500 broilers (*Gallus gallus* L.) upon different dosage of dietary phytase preparations Feedbest-R (A) **and Berzyme-R** (B). For feed composition in groups, see the Material and methods section (n = 35 per group; vivarium of the Zagorsk EPH Breeding and Genetics Center, Sergiev Posad, Moscow Region, 2018).

As a result of improved phosphorus and calcium utilization under the influence of phytase, the accumulation of these trace elements in tibias showed a tendency to rise. In addition, birds of all experimental groups showed a higher concentration of iron (by 2.54-2.91 mg%), manganese (by 0.05-0.13 mg%), copper (by 0.06-0.013 mg%), and zinc (0.45-1.16 mg%) in the bones.

Due to higher digestibility and utilization of feed nutrients, the live weight of 37-day-old broilers was 2.7; 3.0 and 3.7% higher in groups II, III, and IV compared to control (group I), being 1.6; 2.1 and 2.5% higher in females and 3.6; 3.9 and 4.8% higher in males. Feed conversion in these groups was 2.9; 4.0 and 4.6% better than in control.

The Berzyme-R-enriched feeds (experiment 2) also positively affected the physiological state of the birds (see Fig. 1, B). In test groups II, III and IV, as compared to control, the use of phosphorus was 3.4; 6.2 and 4.4% higher, the use of calcium was 1.5; 3.2 and 2.3% higher, respectively, the digestibility of feed dry matter increased by 0.8-2.6%, of fat by 1.1-3.2%. The feed protein digestibility and the nitrogen utilization in the test groups were 0.9-2.9 and 1.3-2.8% higher, respectively.

Significant differences in the crude ash content in the tibias were not observed among broilers from different groups. There was a slight tendency towards an increase in calcium and phosphorus accumulation in the skeleton of poultry from the test groups. As in experiment 1, the iron, manganese, copper, and zinc levels were higher in tibia of broilers fed phytase than in the control. The body weight of 36-day-old broilers increased in test groups II, III and IV by 1.3; 3.1 and 2.0%, respectively, compared to the control. Moreover, the body weight in the groups turned out to be 1.14; 3.0 and 2.9% higher in the females and by 1.5; 3.3 and 1.3% higher in males.

The key commercial criterion of broilers is the slaughter yield of meat of gutted carcasses. According to the results of both experiments, this indicator was higher in the experimental groups. Chemical analysis of broiler pectoral and foot muscles showed the absence of significant differences between the experimental and control groups in terms of moisture, crude protein, fat, ash.

T-RFLP analysis revealed 78±3.9 to 108±5.4 bacterial phylotypes in the cecal microflora of chickens in experiment 2 (Table). A more pronounced taxonomic diversity and complexity of the microbial communities was noted in the control group not fed the phytase additive, as well as in the group III fed Berzyme-R at a dose of 12 g/t feed. This indicates the heterogeneity of the compositions of the microbiocenosis, the accumulation of entropy and some disorganization in these groups as compared to test groups II and IV.

Biodiversity of microorganisms in the cecum of Cobb 500 broilers (*Gallus gallus* L.) depending on the dosage of a dietary phytase preparation Berzyme-R (*n* = 35 per group; *M*±SEM, Vivarium of Breeding and genetic center Zagorsk EPH, Sergiev Posad, Moscow province, 2018)

Indicator	Group I	Group II	Group III	Group IV
Number of phylitypes	101.0±4.60	107.0±6.20	78.0±3.90	108.0±5.40
Community dominance index	0.04±0.002	0.33±0.018	0.04±0.002	0.31±0.014
Shannon index	3.57±0.180	1.46±0.062	3.67±0.150	1.64±0.050
Simpson index	0.96±0.038	0.67±0.020	0.96±0.041	0.69±0.047
Margalef index	21.2±1.50	11.4±0.49	16.7±0.72	13.6±0.59

N o t e. For description of the groups see section Material and methods.

A significant proportion of detected microorganisms could not be attributed to any existing taxon (Fig. 2). The unidentified bacteria ranged from 4.7±0.3 to 12.3±0.52%, depending on the group. The number of such microorganisms was the largest in group IV. Other researchers have also earlier identified a high proportion of uncultured bacteria in the composition of the gut microbiota of chickens [21].

The composition of the identified gut microorganisms as a whole was similar in all groups of broilers (see Fig. 2). In most samples, the bacteria were assigned to the phyla *Firmicutes*, *Bacteroidetes*, *Actinobacteria*, *Proteobacteria*, and *Fusobacteria*. Bacteria from *Firmicutes* phylum were dominant in all cases. These data partially agree with the results obtained previously [22-24]. The 16S rRNA gene sequencing showed that the members of phyla *Firmicutes* and *Proteobacteria* dominate in gut microbial communities of broilers, reaching over 90% of the analyzed sequences [25].

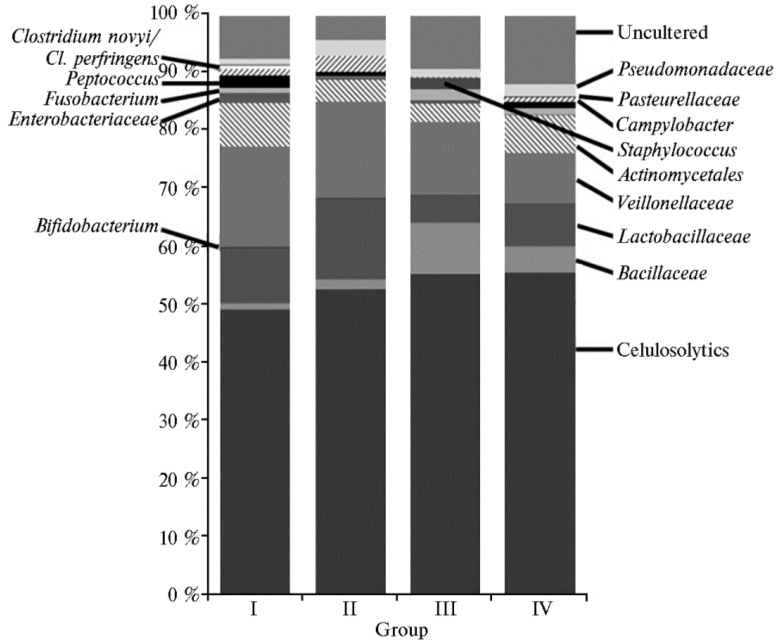

Fig. 2. Profiles of Cobb 500 broiler (*Gallus gallus* L.) cecal microbiocenosis depending on the dosage of dietary phytase preparations (the profiles are based on the T-RFLP analysis data). For description of the groups see section Material and methods.

Interestingly, the addition of phytase promoted an increase in a pool of cellulolytic bacteria of the families *Eubacteriaceae*, *Clostridiaceae*, *Lachnospiraceae*, *Ruminococcaceae* and phylum *Bacteroidetes*. These microorganisms play an important role in bird's digestion, since they produce a number of digestive enzymes, including cellulases, which allows the macroorganism to effectively use the energy of feeds rich in fiber. We revealed a 1.2-2.3-fold decrease in the abundance of order *Actinomycetales* by 1.2-2.3 times and a 2.8-14.4-fold decrease in *Enterobacteriaceae* family in broilers receiving a concentrated phytase preparation as compared to the control group. The *Enterobacteriaceae* family comprises pathogenic species of the genera *Escherichia*, *Klebsiella*, *Enterobacter*, *Serratia*, *Citrobacter* etc., therefore, a decrease in the number of this bacterial group may indicate a correction of dysbiotic disorders in the intestines of broilers.

The *Lactobacillaceae* family was the most abundant (13.8±0.62%) in chickens of group II. The main metabolite of lactobacilli of family *Lactobacillaceae* is lactate which reduces the pH of the chime and thus suppresses pathogenic forms. Also, the smallest portion of the genus *Fusobacterium* members, among which pathogens of inflammatory diseases are often found, was characteristic of the chickens in group II. In addition, the number of bacteria *Clostridium novyi*/*Cl. perfringens*, representatives of the genus *Staphylococcus* and the family *Pasteurellaceae* were below the level of reliable determination by T-RFLP method. Nevertheless, these microorganisms were detected in the intestines of chickens, i.e. staphylococci in group III (1.88%) and group IV (0.19%), clostridia in the control

(0.73%), pasteurella in the control (0.09 %) and group IV (0.23%). Importantly, such pathogens as *Staphylococcus aureus* and *Pasteurella multocida* are often found among bacteria of the genus *Staphylococcus* and the family *Pasteurellaceae. Clostridium novyi* and *Cl. perfringens* are often associated with gastroenteritis and lameness in chickens [26, 27].

The Pearson correlation coefficients confirmed the relation between the abundance of functionally significant representatives of the cecal microbial community and the dosage of dietary phytase. An increase in the assimilation of phosphorus had a reliable direct correlation with an increase in the number of cellulolytic bacteria (r = 0.98 at $p \leq 0.05$), in particular, with more abundant members of the phylum *Bacteroidetes* (r = 0.99 at $p \leq 0.001$) and the family *Lachnospiraceae* (r = 0.84 at $p \leq 0.05$). Also, the higher P availability was associated with lower counts of bacteria from *Veillonellaceae* (r = –0.84 at $p \leq 0.05$) and *Enterobacteriaceae* (r = –0.92 at $p \leq 0.01$) families.

The modulating effect of phytase on the cecal microflora can be associated with an increase in the availability of phosphorus, a change in pH, as well as other physicochemical parameters in the lumen of the gastrointestinal tract. Despite the fact that information about changes in gut microbial communities of chickens under the influence of phytase added to feed is extremely limited, there are papers indicating that the use of this enzyme affects the microbiome structure. A. Ptak et al. [25] showed an increase in the number of *Lactobacillus* sp. and *Enterococcus* sp. when chickens received feed supplemented with phytase.

The researchers indicate the unequal efficacy of different phytase preparations [28-31]. Summarizing the results of our experiment, we can state that both Russian phytase preparations (Feedbest-R and Berzyme-R), when added to broiler feed, allows for lower concentration of inorganic phosphorus in the diet, while increasing bird productivity. The phytases we tested can increase the average daily in live weight gain of broilers and improve feed conversion similar to that reported by other researchers [5, 32]. As per P.H. Selle et al. [29], the use of dietary phytase significantly increased the calcium (32.2%) and phosphorus (28.0%) utilization in the ileum of broilers that received wheat-type feed. In our experiments, exogenous phytases contributed to a more efficient utilization of feed nutrients, as well as calcium and phosphorus. This is explained by the cleavage of phytates [32], which not only serve as a reservoir of phosphorus, but also bind a significant part of microelements, proteins, carbohydrates, amino acids, turning them into complex insoluble conglomerates. Phytases improve protein digestibility and the use of feed amino acids thus reducing endogenous loss of amino acids [33]. Better skeleton mineralization in chickens under the influence of phytases noted in our experiments was also reported earlier [34-36].

Thus, phytase activity of Feedbest-R and Berzyme-R preparations is high, which allows a 0.1% decrease in the digestible phosphorus in feed for broilers. Moreover, Feedbest-R and Berzyme-R improve the digestibility of protein, fat, and the use of nitrogen and amino acids of the feed. The digestibility of calcium and phosphorus increases by 3.4-7.1% depending on the dosage of the additives, and trace elements (iron, manganese, copper, zinc), as well as protein and amino acids are better used. Application of phytase-containing supplements increases bone mineralization. A concentrated phytase-based preparation fed to broilers changed the qualitative and quantitative composition of the cecal microbiome. The normal flora mainly possessed a competitive advantage, whereas the number of conditionally pathogenic and pathogenic bacteria decreased

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