Efficiency of the introduction of different Ca levels in broiler chicken diets as part of dietary fiber extrudates

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Abstract. One of the main tasks of industrial poultry farming is the introduction of new technologies that reduce the cost of obtaining products through the use of non-traditional feed components. The aim of the research is to study the effectiveness of the technology of extrusion processing of feed components at the stage of interaction with the biome of the poultry digestive system. It was found that the inclusion of extruded bran in the diet is accompanied by a significant increase in the number of Lactobacillus. Moreover, the most significant growth was observed in the intestines of a bird receiving an extrudate with an input of 20% calcium. This group was also characterized by a maximum growth of *Escherichia* 7.3 times in comparison with the control. The introduction of an extrudate with a content of 10% calcium is associated with a decrease in the number of *E. coli* in the cecum of chickens. Thus, pretreatment of non-starch polysaccharides together with calcium in the extruder stimulates abdominal digestion of chickens, which indicates the possibility of their use in industrial production.

1. Introduction
In the context of the rapid development of industrial poultry farming, the need for resource-saving technologies is increasing, which makes it possible to use non-traditional sources of feed components without compromising the productivity of poultry and the quality of the products obtained. In this regard, studies aimed at studying the effect of extrusion of dietary fiber with the inclusion of minerals on the poultry microbiome are of particular interest [1, 2]. As is known, a significant amount of non-starch polysaccharides in poultry feeding, due to the lack of digestive enzymes and a number of other reasons, negatively affects the feed intake and the growth rate of broiler chickens [3, 4]. However, it is known that non-starch polysaccharides in the diet are necessary for the development of the gastrointestinal tract, the formation and functioning of the bird microbiome [5–7]; activation and maintenance of local and systemic immunity of the body [8]. It was proved that the addition of indigestible carbohydrates stimulates the growth of the symbiotic microflora of Bifidobacteria and Lactobacillus [9, 10], which creates an optimal pH of the medium and suppresses pathogenic microflora [11, 12].

In turn, the addition of calcium to the diet avoids the inhibition of anaerobes in the use of diets with a high content of lipids [13]. It is known that high doses of calcium in the diet affect mineral metabolism [14, 15], can lead to decreased productivity [16, 17], and also increase the pH in the gastrointestinal tract [18].

It is possible to avoid a decrease in productivity when using sources of non-starch polysaccharides in feeding broiler chickens by using preliminary extrusion processing with the inclusion of mineral elements [19].

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There is insufficient information on the possibility of using extrusion technology to obtain feed components from non-starch polysaccharides with the inclusion of calcium. The aim of the study was to study the reaction of the biocenosis of blind gut of broiler chickens to the use of extruded feed with different levels of calcium.

2. Materials and Methods
2.1. Object of study
Chickens-broilers of Arbor-Aykres cross, intestinal microflora.

2.2. Experiment design
The studies were conducted on the basis of the vivarium of the Federal Research Center of BST RAS (the village of Chernorechye, Orenburg region). Animal services and experimental studies were performed in accordance with the instructions and recommendations of Russian Regulations, 1987 (Order No.755 on 08/12/1977 the USSR Ministry of Health) and “The Guide for Care and Use of Laboratory Animals” (National Academy Press Washington, DC 1996). The experiment was approved by the Ethics Committee of the Federal Research Center of BST RAS.

Based on the individual weighing data, 6 groups (n = 30) of broilers of daily age were formed by the method of analogue pairs. Starting from the age of three weeks, the birds were placed on the regime of the main accounting period.

The birds were fed with full feed. In the study, wheat bran was used as a source of non-starch polysaccharides. The extrusion process was carried out using a universal single screw press extruder PSh30/1 with a mixture humidity of 30 % (Russia).

The composition of the growth diet for birds aged 28 to 42 days included: wheat – 22.3 g/kg; corn 32.0 g/kg; wheat bran – 10.0 g/kg; soybean meal – 14.5 g/kg; sunflower meal – 7.0 g/kg; fish meal – 5.27 g/kg; sunflower oil – 5.0 g/kg; lysine monochlorohydrate – 0.01 g/kg; dl-methionine – 0.01 g/kg; l-threonine – 0.01 g/kg; table salt – 0.30 g/kg; monocalcium phosphate – 0.7 g/kg; feed chalk – 0.3 g/kg; limestone flour – 0.5 g/kg; baking soda (sodium bicarbonate) -0.10 g/kg; vitamin-mineral premix – 2.0 g/kg.

When composing the diet in all groups, 10 % of the grain portion was replaced with wheat bran. In the experimental groups, native bran was replaced by an extrudate with different CaCO$_3$ contents: the experimental group received an extrudate; Experimental group II received an extrudate with 10 % CaCO$_3$; Experimental group III received an extrudate with 15 % CaCO$_3$; Experimental group III received an extrudate with 20 % CaCO$_3$; V experimental group received an extrudate with 25 % CaCO$_3$.

To study the intestinal microflora, slaughter was performed at the 6th week of the experiment. After opening the bird, the contents of the cecum were placed in sterile eppendorf microtubes (NuovaAptaca S.R.L., Italy), and no later than 1 hour after the slaughter, they were delivered in refrigerator bags to the laboratory for inoculation.

2.3. Microbiological studies
Studies of the intestinal microbiota were carried out at the Testing Center of the Central Laboratory of BST RAS. For the study of anaerobes we used anaerostats AE-01 (Russia). The creation of the atmosphere necessary for the cultivation of microorganisms was carried out using chemical gas-generating packages. The study of microflora was carried out according to standard methods using the following media: Staphylococcus – Agar Baird-Parker; Proteus – Differential diagnostic agar for Proteus; Escherichia – Agar Endo; Salmonella – Bismuth Sulphite Agar; Lactobacillus – MRS Medium; Bifidobacterium – Bifidum medium; Clostridiumperfringens – Sulfite agar; Enterococcus (E. faecalis, E. faecium) – Enterococc Agar.
Statistical processing of the obtained data was carried out using the Statistica 10.0 software package (StatSoft Inc., USA), including the determination of the arithmetic mean value (M), standard error of the mean (m). The results were considered reliable at p < 0.05.

3. Results

The results of a study of the microflora of the contents of the blind processes of the intestines of broiler chickens receiving a diet with the inclusion of an extrudate against a background of different dosages of CaCO₃ showed pronounced changes among representatives of the anaerobic and aerobic components of the intestinal microbiome compared to the control (Table 1).

| Name of microorganism / units | Control | Experimental I | Experimental II | Group III | Experimental IV | Experimental V |
|-------------------------------|---------|----------------|----------------|-----------|----------------|----------------|
| Salmonella [CFU/g]            | -       | 62.3±9.02***(a) | 11.7±4.98**(b) | 94.7±8.97***(a) | 182.3±5.61***(a,b) | 202.7±7.06***(a,b) |
| Staphylococcus, 10⁶ [CFU/g]   | 15.67±1.89 | 13.4±0.64 | 22.4±0.66***(a,b) | 35.3±0.55***(a,b) | 38.5±0.61***(a,b) | 17.2±0.55***(b) |
| Lactobacillus, 10⁷ [CFU/g]    | 18.7±0.79 | 196.3±8.99***(a) | 51.2±7.09***(a,b) | 131.3±6.94***(a,b) | 723.0±7.00***(a,b) | 334.0±7.81***(a,b) |
| Clostridium perfringens, 10⁵ [CFU/g] | - | 116.7±4.98***(a) | 2.9±0.55**(a,b) | - | 4.1±0.72**(a,b) | |
| Enterococcus, 10⁷ [CFU/g]     | 14.2±0.52 | 16.5±0.72 | 51.4±0.50***(a,b) | 24.1±0.75***(a,b) | 65.2±0.72***(a,b) | 40.8±0.38***(a,b) |
| Escherichia, 10⁶ [CFU/g]      | 202.00±7.00 | 40.4±0.80***(a) | 1.03±0.07***(a,b) | 303.0±9.07***(a,b) | 1 490±60.83***(a,b) | 1 023±73.1***(a,b) |
| Bifidobacterium*              | -       | ++             | -              | +         | ++             | ++             |

* - np growth, **+** - weak growth, +++ - growth,
(a) - in relation to control group
(b) - in relation to experimental group I
*p<0.05, **p<0.01, ***p<0.001

As follows from the data obtained, the replacement of native bran in the diet of birds, extrudate contributed to the growth of Bifidobacterium in the first experimental group. In the II experimental group, stagnation of the growth of the Bifidobacterium culture was noted, with a 15 % CaCO₃ injection, weak growth was observed, and with an increase in concentrations up to 20–25 %, bacteria growth was observed to the level of the I experimental group. The growth pattern also depended on the inclusion of calcium in the extrudate, so in the experimental group I colonies of Bifidobacterium represented thickened comets, and in the experimental groups IV and V, the colonies were thinner in the form of "strands".

The introduction of bran extrudate into the diet led to a significant increase in the number of bacteria of the Lactobacillus spp family by 10.5 times (p < 0.001). The growth rate relative to the control group is maintained when the calcium component is included in the extrudate. In this case, the largest growth in numbers was observed in the IV experimental group. The gradation of the experimental groups according to the number of bacteria was as follows: II < III < V < IV. Growth rate of Lactobacillus spp. does not have a linear dependence on the amount of calcium introduced, so a 10–15 % injection of a mineral supplement in the II and III experimental groups led to a 3.8 and 1.49-times inhibition of bacterial growth relative to the I experimental group, respectively (p < 0.001). A further increase in concentration to 20 % significantly increased the growth of Lactobacillus spp. 3.7 times, 25 % of CaCO₃ in the extrudate reduces the bacteria of this family.

According to morphological properties, two species were distinguished: E. Faecalis (smooth, round, burgundy, shiny, at least 1 mm), E. Faecium (smooth, round, lilac-pink with a light rim, at least 1.5 mm in diameter). The control and experimental group I did not differ significantly in number. An increase in the number of Enterococcus colonies occurs in the series of experimental groups III < V < II < IV.
Pathogenic bacteria of the genus *Salmonella* were not detected in the contents of the blind processes of the intestines of the control group. While the introduction of extrudate into the diet was accompanied by an increase in *Salmonella* growth to 62.33 ± 9.02 CFU/g (p < 0.001). In relation to the experimental group I, only in the experimental group II there was a lower growth (6 times less). In the remaining groups, a dose-dependent effect was found, a further increase in the calcium component in the extrudate to 15–25 % stimulates *Salmonella* growth (Figure 1).

The growth of *Clostridium* bacteria in the control, the I and IV experimental group was not detected, the maximum growth was achieved in the II experimental group.

Replacement of bran with extrudate in the experimental group I led to a decrease in the number of *Staphylococcus*. An additional introduction of a calcium-containing additive into the extrudate was accompanied by a statistically significant increase in the number of bacteria, both with respect to the control group and relative to the experimental group I (Figure 1). An increase in the growth of *Staphylococcus* occurs in the series of experimental groups II <III <IV. In the experimental group IV, with the introduction of 20 % CaCO$_3$ into the extrudate, the amount of pathogen relative to the experimental group I increased 2.5 times (p < 0.001). A further increase in the dosage of CaCO$_3$ to 25 % has a depressing effect on the growth of *Staphylococcus*, the number of bacteria in the control and V experimental groups does not differ. The difference between the experimental group V and the experimental group I, receiving a diet without the introduction of calcium supplements was 27.8 % (p < 0.001).
The bacteria of the genus *Escherichia* in the II experimental group showed minimal growth, the introduction of extrudate in the I experimental group reduces the number of *Escherichia coli* relative to the control. The addition of a calcium-containing additive to the extrudate promotes the growth of obligate microflora. An increase in CaCO$_3$ dosages to 20 % in the IV experimental group contributed to the maximum growth of *Escherichia*, 7.3 times in comparison with the control.

Analysis of the absolute quantitative distribution within the groups shows that the introduction of extrudate into the diet of broiler chickens stimulates the quantitative growth of *Lactobacillus* and *Escherichia*. Moreover, the maximum growth was observed when 20 % calcium-containing additives were introduced into the extrudate. In the control, the ratio to *Escherichia* was 1:1 (Figure 2), in the experimental one, the ratio shifts toward 50:1, the growth of opportunistic microflora in this group was the lowest, the introduction of Ca into the diet increases the pH value depending on the concentration, the proportion of *Escherichia* increases to 4:1 in the experimental III, to 5:1 in the IV experimental group, an increase in the administered calcium by 25 % reduces the amount of *Lactobacillus*, the ratio is 3:1.

Bacteria of the Proteus and Shigella genuses were not found in the intestines in any of the groups.

4. **Discussion**

Analyzing the data obtained, it can be assumed that the replacement of native bran with extrudate is associated with an increase in the pathogenic *Salmonella* in the poultry intestine due to an increase in the amount of available carbohydrates in the chyme. One of the reasons restraining the growth of pathogenic macroflora in the intestines of the control group was the relatively significant content of dietary fiber and, as a consequence, a change in the acidity of the medium [20].

The presence of *Escherichia coli* in the blind processes of the control bird is probably associated with an inflammatory reaction due to the intake of a high fiber diet [21].

The results of our studies show that the cecum microbiome responds to the introduction of calcium into the extrudates by increasing the amount of endogenous microflora. Thus, an increase in the proportion of calcium in the extrudate led to a significant 3.7-fold increase in *Lactobacillus*, relative to the diet without calcium. This may be due to the response of microflora to the shift of the hydrogen index to the alkaline side, it is known that *Lactobacillus* along with *Bifidobacterium* fermenting difficultly hydrolyzable carbohydrates, contributing to acidification of the medium [22, 23].
Along with this, the growth of *Escherichia coli* in the experimental groups can be explained by the fact that, unlike anaerobes, they do not secrete extracellular polysaccharide hydrolases and therefore cannot use dietary fiber. There is an assumption [24] that since commensal and pathogenic strains of *Escherichia coli* colonize the colon by growth in the intestinal mucus [25, 26] *E. coli* depends on anaerobes present in the mucus, which can provide them with mono- and disaccharides and maltodextrins, which they need for growth. A high Ca content leads to an increase in the growth rate of *Escherichia coli* by 25 times relative to the control.

An analysis of the ratio of *Lactobacillus* and *E. coli* within groups showed that a high level of hardly hydrolyzable carbohydrates in the diet leads to almost the same growth rate of these bacteria. Replacing the dietary component with a more digestible one leads to a reduction in the population of *E. coli* by almost 50 times relative to *Lactobacillus*. The introduction of 15–25 % of calcium in the diet resumed the growth of *E. coli*. Small dosages of Ca in the extrudate up to 10 % cause a single bacterial growth.

With an increase in Ca, a colonization of the microbiocenosis by salmonella, staphylococcus, and other transient microorganisms occurred in the diet, which is confirmed by the works performed earlier [27].

*Clostridium* reacted with growth, only at a Ca dosage of 10 %. It is known that these bacteria depend on the level of Ca. Its low content in the diet increases the production of phytase and thus can improve the absorption of minerals, amino acids and increase the productivity of the bird as a whole [13, 28-30].

5. Conclusion

The Ca content in the diet is closely related to the microbiome of the intestine of the bird. The introduction of extruded feed into the diet with the inclusion of Ca is accompanied by an increase in obligate microflora, at doses dependent on the growth of conditionally pathogenic microflora. Thus, the increase in the calcium content in the extrudate should be carried out with caution, since changes in the microbiome can lead to the launch of metabolic disorders occurring at the initial stage in a subclinical form.

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