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INTESTINAL MICROFLORA AND EXPRESSION OF IMMUNITY-RELATED GENES IN HENS AS INFLUENCED BY PREBIOTIC AND PROBIOTIC FEED ADDITIVES

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Abstract

It is known that probiotic and prebiotic feed additives improve the function of the intestines and lead to normal the processes of digestion of food for animals. Colonization of the gastrointestinal tract with beneficial microflora helps to reduce the negative impact of pathogenic or opportunistic pathogenic microflora, maintain optimal acidity of the gut, prevent dysbiosis, and stimulate local and general immune factors. However, the biological mechanisms for the implementation of such properties of these drugs are still not fully understood. We evaluated the effect of two Russian products, the multifunctional feed additive Profort® (Biotrof LLC, Russia), combining the qualities of an enzyme and probiotic, and the prebiotic feed additive Vetelact (SVC, Agrovetzashchita, Russia) on the quantitative and qualitative composition of the intestinal microbiota in egg layers to compare the effect of these feed additives on the intestinal microbiota with the expression of the β-defensin 9 (AvBD9), interleukin 8 (IL8), gallinacin-10 (Gal-10) and proenkephalin (PENK) genes that are associated with immune systems. Lohmann white LSL hens with an egg laying intensity of at least 95 % at the age of 25 weeks were used in the experiment (the conditions of vivarium, 2019). The hens were assigned to three groups (20 birds each). Feeding the birds was carried out with mixed feed, the feed specification were calculated according manual from Lohmann Tierzucht. Birds of the control group received only mixed feed. Birds of the experimental groups were also fed with biological additives for 28 days. The egg production was recorded daily, the egg laying intensity, egg weight and body weight were recorded weekly. After the termination of the experiment, the composition of the microbiota of the blind processes of the intestine was determined using NGS sequencing and the expression levels of the β-defensin 9 (*AvBD9*), interleukin 8 (*IL8*), gallinacin-10 (*Gal-10*), and proenkephalin (*PENK*) genes were assessed. It is known that β-defensin 9 and gallinacin-10 belong to the family of endogenous peptides, which are an important element of the innate immunity system and a link between innate (non-specific) and acquired (adaptive, specific) immunity, proenkephalin is one of six opioid peptides that regulate signaling between cells and affect many biological processes in vertebrates, including development, growth and reproduction, and interleukin 8 is one of the main pro-inflammatory chemokines formed by macrophages, epithelial and endothelial cells which also plays an important role in the innate immune system. It was established that the hens receiving probiotic had the highest egg productivity (3.33 % higher than the control, $p < 0.05$), while their final body weight was minimal. Feeding a prebiotic led to a 0.24-0.45 % ($p > 0.05$) decrease in egg production with the body weight 0.9 % ($p > 0.05$) higher compared to the control. Feeding the prebiotic contributed to an increase in the total number of microorganisms in the intestinal contents to 7.625±0.74 lg CFU/g (the microbial number in the control was 7.598±1.01 lg CFU/g), while the feeding with probiotic reduced the number of microorganisms to 7.565±0.56 lg CFU/g ($p > 0.05$). Both feed additives contributed to an increase in the number of bifidobacteria and cellulolytic bacteria in the intestine and reduced the total amount of pathogenic and undesirable microflora by 25-50 % vs. control. A decrease in the proportion

of pathogenic and undesirable microorganisms in the composition of microbiota naturally reduced the body's need for non-specific defense factors and pro-inflammatory cytokines. In the birds receiving feed additives, the expression of the β-defensin 9 gene was 3.3-5.0 times lower, and the interleukin 8 (*IL8*) gene expression level was reduced by 8-36 % compared to the control. Along with a decrease in the expression of β-defensin 9 and interleukin-8 genes, a 1.48-1.55-fold increase in the expression of the gallinacin-10 gene and 1.11-1.91-fold increase in proenkephalin were established, which is probably associated with strengthening the protective functions of the body. The selective effect of probiotic and prebiotic on the reproduction of various types of bacteria in the intestine, confirmed by the negative expression of genes associated with immunity, justifies the promise of using the studied products to increase the resistance of poultry and normalize functions of the immune system without compromising of poultry performance.

Keywords: commercial poultry, probiotic, prebiotic, intestinal microbiota, immune factors, β-defensin 9, interleukin 8, gallinacin-10, proenkephalin, genes expression

Modern immunologists appreciate application of bioactive feed additives favorable for normal intestinal microflora and stimulating body defenses as a better approach in counteracting infectious processes [1, 2].

The intestines of farm animals and poultry play an important role not only in the assimilation of food nutrients, but also in maintaining the body's immune defense [3, 4]. The barrier function of the intestinal microvilli of the cylindrical epithelium cannot fully protect the body from the invasion of pathogenic bacteria and viruses if the intestines are not colonized by a number of beneficial microorganisms. It has been proven that normal microflora stimulates the development of some cecum tissues in mammals [5]. The gut microbiota of birds performs numerous functions to maintain homeostasis and resistance. It takes part in the normal functioning of the cardiovascular, endocrine, hematopoietic, nervous and other systems. The intestinal microflora synthesizes amino acids, enzymes, antibiotics, vitamins, and other metabolites valuable for the macroorganism [2, 6]. Microbiota also plays a significant role in maintaining the body's defense system [7, 8].

The intestines are one of the main sites of invasion and habitat of pathogenic microorganisms. Therefore, the intestinal functionalities provide for a special mechanism which is extremely important for the fight against the pathogenic microflora [7] with two groups of factors involved. The first group includes physical barriers and special conditions of the internal environment [8], e.g. mucous layer and the protective properties of mucin, preventing penetration and attachment of microorganisms to the intestine villi, acidic pH in the small intestine, normal oxygen levels in the intestinal environment which prevent proliferation of anaerobes, etc. [9, 10]. The second group comprises immune factors, e.g. antimicrobial peptides (defensins), neuropeptides and interleukins, which regulate synthesis of mucin and intestinal immunoglobulins.

Mucins are glycosylated proteins with a molecular weight of up to 20 kDa, which play a key role in preventing pathogen penetration through the intestinal mucosa [11, 12]. There is a distinct relationship between intestinal microflora and the amount of mucin [13].

Antimicrobial peptides are the key components of innate immune system in animals. These peptides are capable of disrupting integrity of the membranes of microorganisms [14]. α-Defensins are characteristic only for mammals, while birds possess only β-defensins [15]. Four types of defensins called gallinacins have been identified in chickens. Gallinacins are specific towards *Campylobacter* sp., *Salmonella* sp., *Clostridia* sp., *Escherichia coli*, can suppress their growth, change the morphology and ultimately, cell lysis and death [16]. Gallinacins are expressed in the small intestine, liver, gall sac and spleen of chickens. Other recently studied chicken defensins possess tissue specificity, e.g. AvBD1, AvBD7 and AvBD9 are

expressed in goiter, AvBD8, AvBD10 and AvBD13 in the intestine, AvBD1 and AvBD7 in spleen [14, 17, 18].

Canadian researchers [19] showed that a probiotic containing *Lactobacillus acidophilus*, *L. casei*, *Streptococcus faecium*, *Saccharomyces cerevisiae* and organic acids positively influenced intestinal morphology in chickens (the duodenum of test birds was longer than in control). Expression of immunity-related genes gave conflicting results. Drinking probiotics for 7 or 14 days leads to an increase in the expression of AvBD3, IL6, IL10 genes, while in interleukin 12 (IL12) and γ-interferon (INF-γ) genes it was lower compared to control.

Proenkephalin, like other neuropeptides, not only affects regulation of the inflammatory process, but also coordinates cell-cell signaling and reduces the activity of cellular alkaline phosphatase [20-22]. The main function of proinflammatory interleukins (proinflammatory cytokines) is attracting additional leukocytes from the blood to the pathological focus to increase the resistance of the epithelial cells to the infection [14, 23].

Most recent studies on bioactive feed additives in poultry farming have evaluated the effects of these drugs on productivity, intestinal health, and gene expression in broiler chickens as the most convenient model. Laying hens, the production cycle of which exceeds 80 weeks, remained outside the scope of such studies, though the search for drugs that will preserve intestinal health and immunity in laying hens remains urgent.

This work is the first study in Russia showing effects of dietary pre- and probiotic supplementation not only on poultry gut microbiota via stimulation of beneficial microflora, but also on expression of immune-related genes.

This study aimed to assess the effect of prebiotic and probiotic preparations on productive performance, gut microbiota patterns, and the expression of immunity-associated genes in laying hens.

Materials and methods. For experiments (vivarium of Scriabin MVA, 2019), Lohmann white LSL cross female chickens aged 18 weeks were placed in individual cages to determine the rate of lay. Hens with an egg laying rate of at least 95 % at the age of 25 weeks were assigned into 3 groups of 20 bird each.

The control birds (C) were fed standard compound feed (basic ration, BR) in accordance with the recommendations for the cross, including wheat, sunflower and soybean meal, grass meal, sunflower oil, vitamins and mineral supplements. Feed additives were used for 28 days as per the manufacturers' instructions. Chickens of group I (test) received a lactulose (50%)-based prebiotic Vetelact (NEC Agrovetzashchita SP, Russia) on at 0.1 ml/kg body weight. Chickens of group II (test) received probiotic Profort® (Biotrof LLC, Russia) containing live cells of *Bacillus megaterium* B-4801 and *Enterococcus faecium* 1-35 (at least 7.0 lg CFU/g, 500 g/t feed).

Laid eggs were counted daily, the egg laying rate was calculated weekly. Egg weight and poultry body weight were determined weekly by individual weighing (electronic scales ME-R 326AFU, Mercury Equipment, China).

At the end of the experiment, 5 individuals from each group were euthanized. Cecal tissue fragments and cecal contents were sampled to assess expression of β-defensin 9, gallinacin-10, interleukin 8, proenkephalin genes and to profile microbiota composition.

Microbial DNA was extracted using QIAamp Power Fecal DNA Kit (Qiagen, USA) as per the manufacturer's protocol. DNA was quantified with a Qubit 3.0 fluorimeter (Thermo Fisher Scientific, Inc., USA).

Total microbial counts per unit volume were determined by quantitative PCR (qPCR, a Light Cycler® 96 System thermocycler, Roche, Switzerland) with

Maxima SYBR Green/ROX qPCR Master Mix (dye SYBR Green fluorescent detection, Termo Fisher Scientific, Inc., USA). The primers used were Eub338 5′-ACTCCTACGGGAGGCAGCAG-3′, Eub518 5′-ATTACCGCGGCTGCTGG-3′ (Eurogen, Russia). To quantify microbial community composition, extracted DNA was processed using 16S Metagenomik Kit and Ion 520 & Ion 530 ™ Kit-OT2 kit (Thermo Fisher Scientific, Inc., USA) as per the manufacturer's instructions and loaded on the chip for NGS sequencing (an Ion GeneStudio™ S5 System, Thermo Fisher Scientific, Inc., USA). In total, 2 million reads with 300-400 bp read length were obtained (211,000 reads on average per sample). DNA sequencing data analysis was performed with Ion Reporter network software (https://ionreporter.thermofisher.com/ir/).

In addition to a simple comparison of the microbiome profiles, the number of taxa weighted by relative abundance per the Shannon and Simpson diversity indexes was determined [24].

To assess gene expression, total RNA was extracted from cecal tissue fragments. RNA purity was estimated by classical agarose gel electrophoresis method (Mini-SubCell GT camera, Bio-Rad, USA). cDNA was synthesized from RNA template via reverse transcription (iScript kit for cDNA synthesis, Qiagen, USA). Analysis of gene expression, allowing detection of its activation upon a particular effect, was performed by real-time PCR (a LightCycler® 96 System thermocycler, Roche, Switzerland) with 2× Quanti Nova SYBR GREEN PCR kit (Qiagen, Austria). The reference genes were the "housekeeping" genes *TBP* (TATA-binding protein) and *ACTBL2L* (β-actin) (since the primer annealing temperature was different for the studied genes, two "housekeeping" genes corresponding to primers were taken). the cycle threshold (Ct) value was determined for each reference and analyzed genes. The data were processed by the Livak and Shmitgen method [25] with calculation of mean values of threshold cycles in the group and ΔCt (difference between threshold cycle values for the desired gene and the "housekeeping" gene), then of differences between ΔCt for test and control groups $\Delta\Delta Ct = \Delta Ct2 - \Delta Ct1$. Relative gene expression was calculated as the threshold cycle value normalized by control ($2^{-\Delta\Delta Ct}$) [25].

Mathematical and statistical analysis was carried out using standard methods of correlation and analysis of variance (Excel 2007 software). The means (*M*) and standard error of the mean (±SEM) were calculated. The results were checked for the significance of differences according to Student's *t*-test (https://gallery.shinyapps.io/dist_calc/). Differences were deemed statistically significant at $p < 0.05$. Each cDNA sample was examined in real-time PCR in triplicate. Assessment of biological diversity and processing of microbiota data was performed using Qiime 2.0 bioinformatics platform (https://qiime2.org/).

Results. According to the manufacturer's information, the Profort® probiotic feed additive is able to normalize microflora and increase the safety and productivity of poultry. The probiotic bacteria of Profort® synthesize lactic acid and vitamin B_{12} which stimulates regeneration of intestinal epithelium, participates in synthesis of nucleic acids and accelerates restoration of antioxidants in the body [26]. Studies on broilers showed that the growth rate in chickens fed with dietary Profort® was 6.9% higher than in the control group, while feed conversion was 3% better [27]. Prebiotic preparation Vetelact contains lactulose, the disaccharide of galactose and fructose, which is cleaved in colon into low molecular weight organic acids. These acids stimulate growth of beneficial bifidobacteria and lactobacilli, inhibit potentially pathogenic clostridia and Escherichia, stimulate intestinal motility, improve absorption of phosphorus and calcium salts, and promote excretion of ammonium ions [28]. The use of Vetelact increased broiler safety by 2.85% and body weight at the end of growing by 2.38-3.52% while

reducing the cost of feed per bird by 3.3-3.6% and per 1 kg weight gain by 5.8-7.1% [29].

In our tests, the body weight of chickens, egg production and egg weight did not differ significantly between all groups (Table 1).

1. Productivity of Lohmann White cross chickens fed with prebiotic Vetelact and probiotic Profort® (for each group $n = 20$, $M \pm$SEM, vivarium of Skryabin MVA, 2019)

Indicator	C (control)	Group I (prebiotic)	Group II (probiotic)
At the begining of the experiment (175 days of age)			
Live weight, g	1434.4±20.76	1451.1±26.27	1417.5±22.44
Egg weight, g	56.19±0.82	57.24±1.18	55.13±0.67
Egg production, pcs.	28.39±2.04	27.69±1.98	28.18±2.11
Egg-laying rate, %	95.23±0.82	95.26±0.83	95.36±0.82
At the end of the experiment (203 days of age)			
Live weight, g	1468.1±16.02	1482.13±29.31	1467.53±18.22
Egg weight, g	58.19±0.53	58.05±1.31	58.91±0.83
Egg production, pcs.	21.47±0.25	21.38±0.34	22.24±0.30
Egg-laying rate, %	93.35±1.09	92.93±1.48	96.68±1.32*

* Difference from control are statistically significant at $p < 0.05$.

Table 1 shows that feeding chickens of group II with Profort® led to 1.23% increase in egg weight ($p > 0.05$) and 3.3% increase in egg-laying rate ($p < 0.05$) compared to the control. Since birds of this group expended more energy or egg production, at the end of the experiment, they were 0.03% inferior to the control in average body weight ($p > 0.05$). Prebiotic Vetelact did not have a significant effect on productivity and even slightly decreased it (by 0.24-0.45%, $p > 0.05$) as compared to control. Moreover, the weight of chickens in the test slightly exceeded that in the control (by 0.9%, $p > 0.05$).

However, the prebiotic slightly increased the total number of microorganisms in the intestinal contents (7.625±0.74 log CFU/g) while the probiotic, on the contrary, reduced this indicator value to 7.565±0.56 log CFU g (in control 7.598±1.01 log CFU/g). However, the revealed differences between the groups were unreliable, being just a trend.

2. Microbial profiles (%) of cecal contents in Lohmann White cross chickens fed with prebiotic Vetelact and probiotic Profort® (for each group $n = 20$, $M \pm$SEM, vivarium of Skryabin MVA, 2019)

Taxon	C (control)	Group I (prebiotic)		Group II (probiotic)	
		total	Δ to control, %	total	Δ to control, %
Phylum *Actinobacteria* including:	0.08±0.03	0.12±0.10	+50.00	0.18±0.15	+125.00
order *Bifidobacteriales*	0.08±0.02	0.10±0.10	+20.00	0.07±0.05	–12.50
Phylum *Bacteroidetes*	32.00±2.20	27.10±1.80	–15.40	37.4±2.74	+16.80
Phylum *Firmicutes* including	52.40±2.40	55.00±2.70	+4.92	47.2±4.04*	–9.90
family *Lactobacillaceae*	32.20±3.80	36.90±4.50	+14.70	23.1±3.8*	–28.30
family *Clostridiaceae*	13.30±5.30	16.10±2.30	+20.60	21.4±2.1*	+60.40
family *Ruminococcaceae*	5.86±0.95	4.78±0.60	–18.40	7.08±0.92	+20.80
genus *Selenomonadales*	0.12±0.02	0.14±0.03	+16.70	0.21±0.15	+75.00
Phylum *Proteobacteria* including	15.20±2.31	17.70±1.06	+16.50	14.7±1.66	+39.10
family. *Enterobacteriacea*	0.75±0.17	0.35±0.09	–53.30	0.51±0.26	–32.00
Phylum *Synergistetes*	0.03±0.01	0.05±0.01	+66.60	0.03±0.01	0
Phylum *Tenericutes* including	0.06±0.03	0.04±0.02	–33.30	0.09±0.05	+50.00
family *Mycoplasmataceae*	0.04±0.03	0.00±0.00	–91.70	0.01±0.01	–75.00
Phylum *Spirochaetes*	0.01±0.00	0.00±0.00	–66.60	0.02±0.02	+100
Pathogens and undesirable microorganisms in total	0.88±0.10	0.44±0.07	–50.00	0.66±0.20	–25.00
Uncultured	0.23±0.20	0.02±0.01	–91.30	0.32±0.26	–3.10

* Difference from control are statistically significant at $p < 0.05$.

A comparison of microbial profiles of cecal bacterial community in the test and control groups (Table 2) revealed six main phyla, *Actinobacteria*,

Bacteroidetes, *Firmicutes*, *Proteobacteria*, *Synergistetes* and *Tenericutes*. The exception was bacteria of the phylum *Spirochaetes* absent in birds of the test group I. The bacteria of this phylum belong mainly to pathogenic and undesirable microflora, and in healthy individuals (control and test groups in the experiment) its content is allowed in minimal quantities. The genus *Lactobacillus* is important for intestinal microbiota, as it provides nutrients to the host and protects against opportunistic microflora, and bacteria of the *Bifidobacteriales* order can synthesize vitamins to supply the host body [6].

The abundance of *Actinobacteria* phylum increased by 50.00% in birds from group I fed with the prebiotic preparation (including 20.00% growth in counts of order *Bifidobacteriales*), the level of bacteria from family *Lactobacillaceae* increased by 14.70%, while the number of cellulolytic bacteria of family *Ruminococcaceae*, as well as pathogenic and undesirable microflora decreased by 18.40 and 50.00%, respectively. In birds from group II fed with a probiotic the number of bacteria of the phylum *Bacteroidetes* decreased by 12.50%, of *Lactobacillaceae* family by 28.30%, while the number of cellulolytic bacteria increased by 20.80%, and the abundance of pathogenic and undesirable microflora decreased by 25.00% (see Table 2). Publications on age-related changes in microbiota in laying hens have reported a decrease in the abundance of cellulosolytic bacteria with age and an increase in abundance of phylum *Bacteroidetes* and lactobacilli [30]. In experiments on chickens [6], feeding a phytobiotic with *Macleaya cordata* plant extract of led to an increase in the number of lactobacilli, a decrease in the abundance of pathogenic microflora, and a decrease in the expression of cytokine and immunoglobulin genes (IL-4, IFN-γ).

Thus, in our study, the prebiotic and probiotic had a multidirectional effect on the abundance of bifidobacteria and cellulolytic bacteria, but equally affected the decrease in the number of pathogenic and undesirable microorganisms.

Analysis of α-diversity of the chicken cecal microbiota using the Shannon index (3.27±0.10 in control, 3.16±0.10 in group I, and 3.40±0.04 in group II) and the Simpson index (0.84±0.02; 0.81±0.03 and 0.86±0.001, respectively) showed that the differences between the groups are not statistically significant ($p > 0.05$), which allows us to conclude only about a trend.

β-Defensins and gallinacins of birds play a vital role in innate antibacterial immunity [31, 32]. Defensins, being cationic peptides, are active against bacteria, fungi, enveloped and non-enveloped viruses. Immune cells use defensins to kill bacteria absorbed in phagocytosis [2].

Immune-related genes and primers that we used to study their expression in birds as influenced by dietary probiotic and prebiotic additives are shown in Table 3.

3. Primers used to assess the immune-related gene expression in Lohmann White cross chickens (vivarium of Skryabin MVA, 2019)

Gene, protein	Primer
«Housekeeping» genes:	
ACTBL2L (β-actin)	F: 5′-ATTGTCCACCGCAAATGCTTC-3′
	R: 5′-AAATAAAGCCATGCCAATCTCGTC -3′
TBP (TATA-binding protein)	F: 5′-GAACATCATGGATCAGAACAACA-3′
	R: 5′-ATAGGGATTCCGGGAGTCAT-3′
AvBD9 (defensin 9)	F: 5′-AACACCGTCAGGCATCTTCACA-3′
	R: 5′-CGTCTTCTTGGCTGTAAGCTGGA-3′
Gal-10 (gallinacin-10)	F: 5′-GCTCTTCGCTGTTCTCCTCT-3′
	R: 5′-CCCAGAGATGGTGAAGGTG-3′
PENK (proenkephalin)	F: 5′-GCTGGATGAGAACCATCTGC-3′
	R: 5′-AGCCTCCGTACCTCTTAGCC-3′
IL8 (interleukin 8)	F: 5′-GGAAGAGAGGTGTGCTTGGA-3′
	R: 5′-TAACATGAGGCACCGATGTG-3′

In birds fed with probiotic and prebiotic drugs, the expression of *AvBD9* gene reduced significantly (5.0 and 3.3 times, respectively) (Table 4). The expression of β-defensins in the intestine is induced by pro-inflammatory cytokines [26], as well as by microorganisms (for example, in humans, by *Escherichia coli*, *Helicobacter pylori* or *Pseudomonas aeruginosa*) [33]. In our tests, a decrease in the number of pathogenic microorganisms seemed to reduce the need for pro-inflammatory cytokines, which was reflected in the level of β-defensin 9 synthesis. This pattern was also characteristic of gene *IL8* expression but to a lesser extent (Table 5).

4. β-Defensin 9 gene *AvBD9* expression in cecal tissues of Lohmann White cross chickens fed with prebiotic Vetelact and probiotic Profort® (for each group $n = 20$, M±SEM, vivarium of Skryabin MVA, 2019)

Group	Ct *TBP*	Ct *AvBD9*	ΔCt	ΔΔCt	Values nirmalized by control ($2^{-\Delta\Delta Ct}$)
C (control)	25.91±0.77	29.34±0.84	3.44	0	1
I (prebiotic)	24.85±0.43	30.60±0.65	5.75	2.31	0.20*
II (probiotic)	22.92±0.47	28.14±0.92	5.22	1.78	0.29*

Note. ΔCt = Ct *AvBD9* – Ct *TBP*; ΔΔCt = ΔCt test – ΔCt contol; *TBP* (TATA-binding protein) — a «housekeeping» gene.
* Difference from control are statistically significant at $p < 0.05$.

5. Interleukin 8 gene *IL8* expression in cecal tissues of Lohmann White cross chickens fed with prebiotic Vetelact and probiotic Profort® (for each group $n = 20$, M±SEM, vivarium of Skryabin MVA, 2019)

Group	Ct *ACTBL2L*	Ct *IL8*	ΔCt	ΔΔCt	Values nirmalized by control ($2^{-\Delta\Delta Ct}$)
C (control)	16.99±0.58	23.19±0.41	7.08	0	1
I (prebiotic)	15.79±0.55	22.99±0.72	7.20	0.13	0.92
II (probiotic)	14.33±0.36	22.04±0.21	7.71	0.63	0.64*

Note. ΔCt = Ct *IL8* – Ct *ACTBL2L*; ΔΔCt = ΔCt test – ΔCt contol; *ACTBL2L* (β-actin) — a «housekeeping» gene.
* Difference from control are statistically significant at $p < 0.05$.

Haghighi et al. [34] showed that the expression of IL6, IL10, IL12 interleukin genes in broiler chickens increased upon *Salmonella typhimurium* infection, however, when feeding the probiotic, the expression did not differ from that in the uninfected control. The probiotic also affected the expression of the interferon gene (*INF*). Upon infection in the birds receiving probiotic, the expression was lower than in infected individuals not fed with probiotic [34]. In the report of Ateya et al. [35], feeding the experimental broiler chickens with probiotic, synbiotic and acidifier upon *Escherichia coli* infection led to a decrease in the expression of a number of proinflammatory factors (Il6, Il8, AvBD2, AvBD9), while the anti-inflammatory cytokine IL10 gene (*IL10*) showed a sharp increased expression when compared to uninfected control [35].

We found a positive correlation ($r = 0.442$, $p < 0.05$) between the number of bacteria from *Firmicutes* phylum and the *AvBD9* gene expression. A similar trend for *IL8* gene expression was reveled for the polynomial equation; the detected correlation turned out to be very low and negative ($r = –0.006$). In the work of Oakley and Kogut [36], the level of cytokine expression, as a rule, negatively correlated with the relative abundance of various members of the *Firmicutes* group and positively correlated with an abundance of proteobacteria. Correlations between the microbiome structure and the specific transcription of cytokine mRNA indicate the importance of gut microbiome for poultry health and productivity and can be a successful tool for identifying bacterial taxa with certain immunomodulating properties. In our studies, when feeding the prebiotic, the number of phylum *Firmicutes* microorganisms and proteobacteria increased by 5 and 16%, respectively ($p > 0.05$), while IL8 cytokine expression remained practically unchanged ($p > 0.05$). For the probiotic, we found a lower abundance of the same

microorganisms, by 10% ($p < 0.05$) and 3% ($p > 0.05$), respectively, with a decrease in IL8 gene expression by 36% ($p < 0.05$).

Under the influence of stimuli causing stress, and in response to factors enhancing stress response (corticotropin-releasing factor, cytokines, catecholamines, etc.), immunocytes begin to secrete opioids. These peptides activate peripheral opioid receptors and cause a feeling of analgesia, suppressing excessive excitation of sensory neurons and facilitating the secretion of neuropeptides. Opioid peptides, including proenkephalin, enkephalins, endorphins, are currently being studied more and more intensively [20-22]. In studies of scientists from South Korea, there was a significant variation in the expression of a number of genes, including the proenkephalin gene, in connection with processes of egg formation [37]. Table 6 shows the results of our analysis of the expression of the proenkephalin gene in chickens. It can be seen that, unlike cytokines, the expression of the proenkephalin gene (*PENK*) under the influence of the probiotic increased 1.11 times ($p > 0.05$), under the influence of the prebiotic 1.91 times ($p < 0.05$).

6. Proenkefalin gene *PENK* expression in cecal tissues of Lohmann White cross chickens fed with prebiotic Vetelact and probiotic Profort® (for each group $n = 20$, $M \pm$SEM, vivarium of Skryabin MVA, 2019)

Group	Ct *TBP*	Ct *PENK*	ΔCt	ΔΔCt	Values nirmalized by control ($2^{-\Delta\Delta Ct}$)
C (control)	25.91±0.77	22.63±0.83	–3.28	0	1
I (prebiotic)	24.85±0.43	20.64±0.45	–4.21	–0.93	1.91*
II (probiotic)	22.92±0.47	19.49±0.92	–3.43	–0.15	1.11

Note. ΔCt = Ct *PENK* – Ct *TBP*; ΔΔCt = ΔCt test – ΔCt contol. *TBP* (TATA-binding protein) — a «housekeeping» gene.
* Difference from control are statistically significant at $p < 0.05$.

The expression of the gallinacin-10 (*Gal-10*) gene also increased (Table 7), that is, despite the fact that this protein is quite close to β-defensin 9, the body response was the opposite. Note that other researchers also noted conflicting results for the expression of β-defensin 9 and β-defensin 3 in broilers [19, 35].

7. Gallinacin-10 gene *Gal-10* expression in cecal tissues of Lohmann White cross chickens fed with prebiotic Vetelact and probiotic Profort® (for each group $n = 20$, $M \pm$SEM, vivarium of Skryabin MVA, 2019)

Group	Ct *ACTBL2L*	Ct *Gal-10*	ΔCt	ΔΔCt	Values nirmalized by control ($2^{-\Delta\Delta Ct}$)
C (control)	16.99±0.58	23.19±0.41	5.36	0	1
I (prebiotic)	15.79±0.55	22.99±0.72	4.79	–0.56	1.48
II (probiotic)	14.33±0.36	22.04±0.21	6.75	–0.63	1.55

Note. Ct = Ct *Gal-10* – Ct *ACTBL2L*; ΔΔCt = ΔCt test – ΔCt contol; *ACTBL2L* (β-actin) — a «housekeeping» gene.

Our studies indicate (see Table 7) that the expression of gallinacin-10 gene when feeding prebiotic and probiotic increased 1.48 times ($p > 0.05$) and 1.55 times ($p < 0.05$), respectively. In total, 14 genes of defensins and gallinacins with various antimicrobial activity are revealed in chickens [38]. In broilers infected by *Salmonella enterica*, the expression of the gallinacin-10, gallinacin-11, gallinacin-13, and gallinacin-14 genes was suppressed, while the expression of defensins 1, 2, 7, 8, and 9 remained unchanged. The differential expression of defensins and gallinacins indicates the features of the participation of these genes in the immune response and a different response not only to pathogens, but also to food factors [39].

Normal microbiota which provides resistance to colonization and intestinal health, is a key condition for the proper development of the intestinal tract and the complete maturation of the immune system of the mucous membrane [40]. Our study is the first attempt to understand the interactions between commensal microbiota and the expression of regulatory cytokines in the cecum of

laying hens based on the identification of specific taxa the abundance of which significantly correlates with the expression of cytokine genes.

Thus, our experiments on healthy laying hens have confirmed the fact that dietary probiotic and prebiotic supplements positively affects the intestinal microflora with a minimal effect of these additives on productivity. Both tested feed additives contributed to an increase in the abundance of bifidobacteria and cellulolytic bacteria in the intestine and reduced the total number of pathogenic and undesirable microflora by 25-50%. The studied bioactive additives had a multidirectional effect on the functional activity of immune-associated genes (*AvBD9*, *IL8*, *PENK*, and *Gal*-10) with a general tendency to stabilize the state of the body and readiness to suppress the inflammatory process. The revealed trend of a significant increase in the number of vital bacteria and a similar decrease in pathogenic microflora in the intestine shows the promise of probiotic and prebiotic application to optimize immune functions, which will ultimately improve the health and productivity of poultry.

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