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EXPRESSION OF GENES OF IMMUNE RESPONSE AND ADAPTATION AND CECAL MICROBIOME COMPOSITION IN MALES AND FEMALES OF CHICKENS (*Gallus gallus* L.) IN CM5 AND CM9 PREPARENTAL LINES OF SMENA 9 CROSS

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Abstract

The Cornish and Plymouth Rock breeds form the basis of modern specialized meat crosses of chickens. The selection of the paternal line of the Cornish CM5 breed of the new Russian cross of meat chickens Smena 9 is carried out mainly on the basis of meat productivity, while the maternal line of the Plymouth Rock CM9 breed is primarily for reproductive efficiency and viability at a lower live growth rate than that of the CM5 line masses. In the present study, we revealed for the first time that hens and roosters of the parent stock of lines CM5 and CM9 of the novel cross Smena 9 differ in the expression of some immunity and adaptation genes, as well as in the composition of the microbiome and its putative metabolic pathways. Differences are related to genotype and sex. The aim of the work was to compare the level of expression of immunity genes and genes associated with adaptive potential, respectively, in the tissues of the bursa in the liver, as well as the composition and functions of the microbiome of the caecum of the intestine in chickens and roosters of the CM5 and CM9 lines. The experiments were carried out in the vivarium of the Zagorsk EPH (Moscow Province, 2022) on the parent stock of chickens and roosters of the CM5 and CM9 lines of 39 weeks of age, kept under identical conditions and receiving the same diet. From each line and gender, tissue samples were taken from five individuals with a close live weight. Analysis of gene expression in tissue samples was performed using quantitative reverse transcription PCR (RT-qPCR). Total RNA was isolated using the Aurum™ Total RNA mini kit (Bio-Rad, USA). PCR amplification was performed using SsoAdvanced™ Universal SYBR® Green Supermix (Bio-Rad, USA) and a detecting amplifier DTlight (DNA-Technology, Russia). In the liver tissues, we analyzed the expression of genes associated with the adaptive potential: genes *CAT1* of the transporter of cationic amino acids 1, *HSF1* and *HSF2* — transcription factors of heat shock proteins 1 and 2, *SOD* — superoxide dismutase, *Gpx1* — glutathione peroxidase, *HO-1* — heme oxygenase-1. In the tissues of the bursa, the expression of genes associated with immunity was analyzed: genes *IL8* — interleukin-8, *IRF7* — regulatory factor interferon 7,

PTGS2 — prostaglandin endoperoxide synthase, *AvBD1, AvBD2, AvBD9* and *AvBD10* — β-defensins 1, 2, 9 and 10, *Casp6* — caspase 6. A primer for the β-actin gene (ACTB) was used as a reference control. The relative level of expression was assessed by the $2^{-\Delta\Delta CT}$ method. Total DNA for analysis of the composition of the microbiome was isolated using the Genomic DNA Purification Kit (Thermo Fisher Scientific, Inc., USA). The caecal bacterial community was assessed by NGS sequencing on the MiSeq platform (Illumina, Inc., USA) with primers for the V3-V4 region of the 16S rRNA gene. The reconstruction and prediction of the functional content of the metagenome, gene families, and enzymes was carried out using the PICRUSt2 software package (v. 2.3.0). Mathematical and statistical processing of the results was carried out by the method of multivariate analysis of variance (ANOVA) in Microsoft Excel XP/2003 and R-Studio (v. 1.1.453). The results obtained showed an increase in the expression of the *HSF1* and *HSF2* genes in CM5 cocks compared to other groups ($p \leq 0.05$), in particular, the difference with CM9 cocks was 68 and 218 %, respectively ($p \leq 0.05$). The expression of the *HSF1* and *HSF2* genes within the CM5 line in roosters was 1.6 and 3.0 times higher, respectively, than in hens ($p \leq 0.05$). Significant activation of the expression of antimicrobial peptides and pro-inflammatory genes occurred in CM9 cocks compared to CM5 cocks and hens ($p \leq 0.05$). The expression of *AvBD2, AvBD9, AvBD10, IL8* and *PTGS2* genes in CM9 cocks increased 7.6-, 5.3-, 2.1-, 6.3- and 1.5-fold ($p \leq 0.05$), respectively, compared to CM5 cocks. NGS sequencing showed that the microbiome of the caecum of the CM9 hens and roosters contained bacteria of the superphylum *Elusimicrobiota* (0.32±0.11 and 0.49±0.19 %, respectively). These microorganisms did not occur in CM5 roosters while in the SM5 hens, their proportion was 0.04±0.01 %. Significant ($p \leq 0.05$) differences were found between the groups in 25 genera, in some genera, it depends on the genotype, in others — on the sex of the bird. For example, in cocks of the CM5 line, the abundance of microorganisms of the genera *Barnesiella*, Clostridia_UCG-014 and *Frisingicoccus* was 17.2, 2.0 and 4.9 times higher ($p \leq 0.05$), respectively, than in males of the CM9 line. Members of the genus *Desulfovibrio* were present in the intestines of CM5 and CM9 cocks (0.25±0.08 and 0.73±5.6 %). However, we did not find these microorganisms in the intestines of hens of both lines. Based on the results of bioinformatics reconstruction and functional annotation of NGS sequencing data, we identified 357 putative metabolic pathways in the gut microbial community, 65 of which differed ($p \leq 0.05$) between test groups. Genotype- and sex-specific modifications in gene expression, as well as in the structure and function of the gut microbiome, may provide adaptation of a macroorganism under changing conditions.

Keywords: broiler cross, Smena 9, caecum, microbiome, NGS sequencing, predicted metabolic pathways, gene expression, immunity, adaptations, bursa, liver

Test results [1] showed that poultry of the new domestic cross Smena 9 can be successfully used in broiler production. The Cornish and Plymouth Rock breeds form the basis of modern specialized meat crosses of chickens [2]. To date, little is known about the characteristics of the lines SM5 and SM9 of the Smena 9 cross; there are only data relating mainly to zootechnical parameters. The main selection characteristics of the paternal line Cornish CM5 are live weight, muscularity of the chest and legs [3]. However, because of a significant increase in live weight and changes in the exterior of Cornish birds, a decrease in the fertilization of eggs and hatching of chickens is observed. The low fertility of hatching eggs from chickens of selective grandparent and parent flocks is the main reason for the decrease in the yield of broilers from each parent pair. On the contrary, the main selection characteristics of the maternal line of Plymouth Rock SM9 in comparison with the paternal line of Cornish SM5 are superiority in egg production, egg weight, chick yield, timing of puberty, viability at a lower rate of increase in live weight of young animals and worse feed conversion [4]. Let us recall that the Plymouth Rock breed was originally created for dual use (meat and egg production) [5].

However, it is known that different chicken phenotypes are determined by complex traits that are controlled by many genes. Gene expression studies in poultry have shown that some zootechnical parameters, including productivity, may be associated with various cellular mechanisms, including mitochondrial oxidative stress, inflammatory response, protein degradation, stress responses, growth hormone signaling, cell cycle and apoptosis, fatty acid transport [6]. The bursa is a central and unique organ of humoral immunity in birds, in which the expression

of many genes occurs, primarily those associated with immunity [7], as well as a target organ for a number of pathogenic microorganisms. Liver tissue plays a central role in the adaptive response to stresses [8] to which birds are often exposed in intensive poultry farming environments. It has been proven that the liver expresses many genes that help the body deal with changes in environmental conditions, for example, temperature, and oxygen content in the air [9].

The search for candidate genes in chicken breeding often focuses on selection for growth rate [10] and conformation [11], while resistance to disease and stress has received much less attention. Thanks to the functions of the immune and digestive systems (in particular, thanks to the expression of genes associated with immunity and the formation of adaptive potential occurring in the bursa and liver), the bird's genotype interacts with the environment, which ensures resistance to diseases and changes in conditions feeding, etc. Ultimately, this affects productivity and reproduction rates. A number of studies have determined the functional activity of genes in the organs and tissues of broilers of various genotypes [6], but the expression of genes associated with immunity and adaptation in the tissues of the bursa and liver of the lines of the new cross Smena 9 has not been studied previously.

There are also indications that the host genetic background may also influence the variability of some species in the gut microbiome [12]. The chicken gut microbiome includes a huge taxonomic diversity of species as well as the functional potential of their genomes and is known to have a significant impact on the health and productivity of animals and birds [13, 14]. There are also works demonstrating differences in the composition of the microbiome and its predicted functions in animals depending on gender [15]. However, an analysis of changes in the predicted functional potential of the microbiome of animals and birds depending on the genotype using bioinformatics software systems, such as PICRUSt2 and the like, has not previously been carried out.

In previous studies, we studied gene expression in the tissues of the pancreas and intestinal epithelium in broilers of the Smena 8 cross with experimental T-2 toxicosis [16], using T-RFLP (terminal restriction fragment length polymorphism) we analyzed the composition of the intestinal microbiota in two lines of meat chickens B5 (Cornish breed) and B9 (Plymouth Rock breed) [17]. Using NGS sequencing and bioinformatics methods, we studied the composition of the microbiome and its predicted metabolic potential in Smena 8 broilers exposed to T-2 toxin and the use of feed additives [18]. In chickens of different lines of the new Smena 9 cross, the composition and predicted functions of the intestinal microbiota and gene expression have not been previously studied.

As in mammals, in birds males are characterized by a higher growth rate than females. In part, sex differences in growth rate may be due to differences between sexes in the microbiome of the digestive system, since its composition has a significant effect on the digestion, absorption and metabolism of nutrients in the host, and is also closely related to its immune system and health. A number of researchers have observed a pronounced manifestation of sexual dimorphism in the composition of microbiomes in animals [19], as well as in the pattern of gene expression [20], which suggests conducting similar studies in birds.

Finding connections between a host's genetic background, its microbiome composition, and gene expression levels may help uncover new biological mechanisms associated with high productivity and reproductive function, as well as contribute to the development of more efficient and therefore sustainable poultry production systems [21].

In this study, we for the first time identified in hens and roosters of the parent stock, the lines CM5 and CM9 of the new cross Smena 9 differences

associated with genotype and sex in the expression of some immunity genes and genes associated with adaptive potential, as well as in the composition and pro-predicted functional potential of the microbiome.

The purpose of our study was to assess differences in the expression levels of a set of immunity genes in the bursa and genes associated with adaptive potential in the liver, as well as the composition and functions of the microbiome in the caeca of the intestine in hens and roosters of the parent flock of different cross lines Smena 9, namely the paternal line CM5 of the Cornish breed and the maternal line CM9 of the Plymouth Rock breed, which have genetic differences in growth rate, feed efficiency and reproductive function.

Materials and methods. Experiments were carried out in the vivarium of the SGC Zagorskoe EPH (Moscow Province, 2022) on two lines of parent stock of chickens (*Gallus gallus* L.) of the domestic cross Smena 9 selection of the SGC Smena (Moscow Province): on the paternal line CM5 of the Cornish breed and the maternal line CM9 of the Plymouth Rock breed. Experimental conditions complied with the requirements of the European Convention for the Protection of Vertebrate Animals Used for Experimental or Other Scientific Purposes (ETS No. 123, Strasbourg, 1986) [22]. Feeding and maintenance regimes met the requirements for cross-breeding [23] and were identical for all experimental birds. At the age of 30 weeks, the birds were assignrd to 4 groups, each of 5 birds with a similar live weight. Group I was hens of the paternal line CM5 of the Cornish breed, group II was roosters of the paternal line of the CM5 of the Cornish breed, group III was hens of the maternal line of the CM9 of Plymouth Rock breed, group IV was roosters of the maternal line of the CM9 of Plymouth Rock breed. The bird was decapitated at 39 weeks of age and necropsied.

After decapitation, bursa and liver tissues were collected for gene expression analysis. The samples were immediately stabilized using the RNAlater reagent (Thermo Fisher Scientific, Inc., USA) and immediately sent to the laboratory (BIOTROF+ LLC) for RNA isolation.

Gene expression analysis was performed using quantitative reverse transcription PCR (RT-qPCR). Tissue samples were homogenized after adding liquid nitrogen. Total RNA was isolated using the Aurum™ Total RNA Mini Kit (Bio-Rad, USA) following the manufacturer's instructions. The reverse transcription reaction to obtain cDNA on an RNA template was carried out using the iScript™ Reverse Transcription Supermix kit (Bio-Rad, USA) [24].

To analyze the expression of mRNA in the liver and bursa, specific primers were selected [25]. As a reference control, primers were used to amplify the housekeeping gene encoding the ACTB beta-actin protein: F - 5′-CTGTGCCCATC-TATGAAGGCTA-3′, R - 5′-ATTTCTCTCTCGGCTGTGGTG-3′. Amplification was carried out using SsoAdvanced™ Universal SYBR® Green Supermix (Bio-Rad, USA) in accordance with the manufacturer's protocol [26] (DTlight detection amplifier, NPO DNA-Technology, Russia). The amplification mode and conditions for the analysis of liver and bursa tissues were as follows: 5 min at 95 °C (pre-denaturation); 30 s at 95 °C, 30 s at 60 °C, 30 s at 70 °C (40 cycles) [27]. The relative expression level was assessed using the $2^{-\Delta\Delta CT}$ method [28].

To analyze the composition of the microbiome, at the end of the experiment, chyme samples were manually taken from the cecum of the intestines of three birds from each group, observing aseptic conditions as much as possible. The collected samples were immediately placed into sterile plastic centrifuge tubes. All samples were frozen at –20 °C and transported in dry ice to the laboratory (BIOTROF+ LLC) for DNA extraction.

Total DNA for analysis of the composition of the intestinal microbiome was isolated using the Genomic DNA Purification Kit (Thermo Fisher Scientific,

Inc., USA) according to the attached instructions using a method based on selective detergent-mediated precipitation of DNA from the substrate using solutions of 1.2 M sodium chloride and chloroform to lyse cell walls and precipitate DNA.

The cecum bacterial community composition was assessed using next generation sequencing (NGS) on the MiSeq platform (Illumina, Inc., USA) with primers for V3-V4 region of the 16S rRNA gene. The forward primer was 5′-TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGCCTACGGGGNGGCWGCAG-3′, the reverse primer 5′-GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGGACTACHVGGGTATCTAATCC-3′; the PCR protocol 3 min at 95 °C; 30 s at 95°C, 30 s at 55°C, 30 s at 72 °C (necessary to lengthen the sequence) (25 cycles); 5 min at 72 °C (final elongation). Sequencing was carried out with reagents for library preparation Nextera® XT IndexKit (Illumina, Inc., USA), for purification of PCR products Agencourt AMPure XP (Beckman Coulter, Inc., USA) and for sequencing MiSeq® ReagentKit v.2 (500 cycle) (Illumina, Inc., USA) were used. The maximum length of the obtained sequences was 2×250 bp.

Bioinformatics data analysis was performed using QIIME2 v.2020.8 software (https://docs.qiime2.org/2020.8/). After importing the sequences in .fastq format from the sequencing instrument and creating the necessary mapping files containing metadata of the files being studied, paired strings of reads were aligned. Next, the sequences were filtered by quality using default settings. Filtering of noise sequences was carried out using the DADA2 method built into the QIIME2 package, which includes quality information in its error model, providing the algorithm robust to sequences of lower quality. A maximum trimming sequence length was 250 bp (https://benjjneb.github.io/dada2/tutorial.html). To construct a de novo phylogeny, multiple sequence alignment was performed using the MAFFT software package (https://mafft.cbrc.jp/alignment/software/), followed by masked sequence alignment to remove positions that were significantly different. The Silva 138.1 reference database (https://www.arb-silva.de/documentation/release-138.1/) was applied for taxonomy analysis.

Based on the resulting table of operational taxonomic units (OTUs), using plugins of the QIIME2 software package, biodiversity indices were calculated, and a graph of the dependence of the OTUs number on the number of reads was constructed. When statistically analyzing diversity indices, no additional transformation was performed.

Reconstruction and prediction of the functional content of the metagenome, gene families, and enzymes was carried out (the PICRUSt2 v.2.3.0 software package, https://github.com/picrust/picrust2). We worked with the program according to the recommended action scenario, all settings were used by default. The OTUs of each sample were ranked according to their abundance, from highest to lowest, and the values were transformed using the Log2 logarithmic transformation. MetaCyc database (https://metacyc.org/) was used to analyze metabolic pathways and enzymes. Predicted MetaCyc metabolic pathway profiles were assessed by ASV (Amplicon Sequence Variants) abundance. Data and calculation of statistical indicators were visualized using the Phantasus v.1.11.0 web application (https://artyomovlab.wustl.edu/phantasus/), which, in addition to basic visualization and filtering methods, supports methods based on R such as k-means clustering, principal component analysis, or differential expression analysis with the limma package.

Mathematical and statistical data processing was carried out using the method of multivariate analysis of variance (ANOVA) in Microsoft Excel XP/2003, R-Studio v.1.1.453 (https://rstudio.com). The results are presented as means (*M*) and standard errors of the means (±SEM). The significance of differences was determined using

Student's *t*-test; differences were considered statistically significant at p ≤ 0.05. Means were compared using Tukey's honestly significant difference (HSD) test and the TukeyHSD function in the R Stats Package.

Results. The primers used to analyze the expression of the studied genes are presented in Table 1.

1. Primers used to study gene expression in chickens and roosters (*Gallus gallus* L.) of CM5 and CM9 lines of the new meat cross Smena 9 (vivarium of the SGC Zagorskoe EPH, Moscow Province, 2022)

Gene, its product	Nucleotide sequence (5′→3′)
Genes associated with adaptive potential (in the liver)	
CAT1, cationic amino acid transporter 1	F: ACCAAGTACTGCAAGGCGAA, R: TGAGGGTTCCTCTTCTGGCT
HSF1, heat shock protein transcription factor 1	F: CAGGGAAGCAGTTGGTTCACTACACG, R: CCTTGGGTTTGGGTTGCTCAGTC
HSF2, heat shock protein transcription factor 2	F: CGCTGCTCGCATTCCT, R: TGTGGCCTCACTTGCTTCT
SOD, superoxide dismutase	F: CGGGCCAGTAAAGGTTACTGGAA, R: TGTTGTCTCCAAATTCATGCACATG
Gpx1, glutathione peroxidase 1	F: GCATCCGCTTCCACGACTTCCT, R: CCGCTCATCCGGGTCCAACAT
HO-1, heme oxygenase 1	F: GGTCCCGAATGAATGCCCTTG, R: ACCGTTCTCCTGGCTCTTGG
Immunity-related genes (in bursa of Fabricius)	
IL8, interleukin 8	F: GGAAGAGAGGTGTGCTTGGA, R: TAACATGAGGCACCGATGTG
IRF7, interferon regulatory factor 7	F: ATCCCTTGGAAGCACAACGCC, R: CTGAGGCAACCGCGTAGACCTT
PTGS2, prostaglandin endoperoxide synthase	F: TCGAGATCACACTTGATTGACA, R: TTTGTGCCTTGTGGGTCAG
AvBD1, β-defensin 1	F: CCGTTTCTGTCACCGTCA, R: CCTTTGCTAAAAATCCCTTC
AvBD2, β-defensin 2	F: GCACTCCAGGTTTCTCCA, R: GGCGTCCGACTTTGATTA
AvBD9 (*Gal9*), β-defensin 9	F: AACACCGTCAGGCATCTTCACA, R: CGTCTTCTTGGCTGTAAGCTGGA
AvBD10 (*Gal10*), β-defensin 10	F: GCTCTTCGCTGTTCTCCTCT, R: CCAGAGATGGTGAAGGTG
Casp6, caspase 6	F: CAGAGGAGACAAGTGCCAGA, R: CCAGGAGCCGTTTACAGTTT

Figure 1 shows expression of genes associated with adaptive potential in the liver tissues of hens and roosters of the CM5 and CM9 lines of the Smena 9 cross. Noteworthy is the increase in the expression of the *HSF1* and *HSF2* genes in the paternal Cornish CM5 roosters (group II) compared to groups I, III and IV (p ≤ 0.05). The difference in the expression level of the *HSF1* and *HSF2* genes with the maternal Plymouth Rock CM9 roosters (IV group) was 68 and 218%, respectively (p ≤ 0.05). The *HSF1* gene, an important paralogue of which is *HSF2*, encodes a stress-inducible transcription factor and plays a central role in the activation of the heat shock response, which leads to the expression of a large class of molecular chaperones, heat shock proteins (HSPs), that protect cells from damage [29]. Previously, a team of scientists [30] analyzed the expression of the *HSF1*, *HSF3*, *HSP70* and *HSP90* genes in two local Brazilian chicken breeds (Peloco and Caneluda) and a commercial broiler line Cobb 500 in response to heat stress (39±1 °C). It was found out that the expression levels of some heat shock genes (*HSP70* and *HSP90*) during heat stress varied significantly between breeds. Increased gene expression was detected in local breeds compared to Cobb 500 cross, which was associated with behavioral responses and productivity under heat stress.

The main selection characteristics of the Cornish CM5 paternal line are live weight, muscularity of the chest and legs [3]. It is known that in most cases, phenotypic differences between individuals are caused by genetic changes, and

these genetic differences are closely related to gene expression and function [31]. As in other species, body weight in chickens is a polygenic trait and can be influenced by variants at many loci. In addition, in poultry, especially broilers, body weight is subject to direct selection [32]. In mice, body weight has been reported to be a quantitative trait driven by regulatory variation at the *Glypican 3* locus [33] the effect of which is expressed through changes in gene expression in liver tissue. By analogy, genetic variants that affect body mass in birds may indirectly affect, for example, resistance to heat stress. In addition, the liver, in the tissues of which we observed changes in the expression of the *HSF1* and *HSF2* genes, is a key organ involved in the metabolism of carbohydrates, proteins and fats; it produces and breaks down hormones. These facts indicate that changes in the expression of the *HSF1* and *HSF2* genes may be associated with different levels of productivity in the studied lines. Interestingly, these genes also play a role in the regulation of lifespan [34] and, accordingly, may be valuable as a marker associated with productive longevity in poultry.

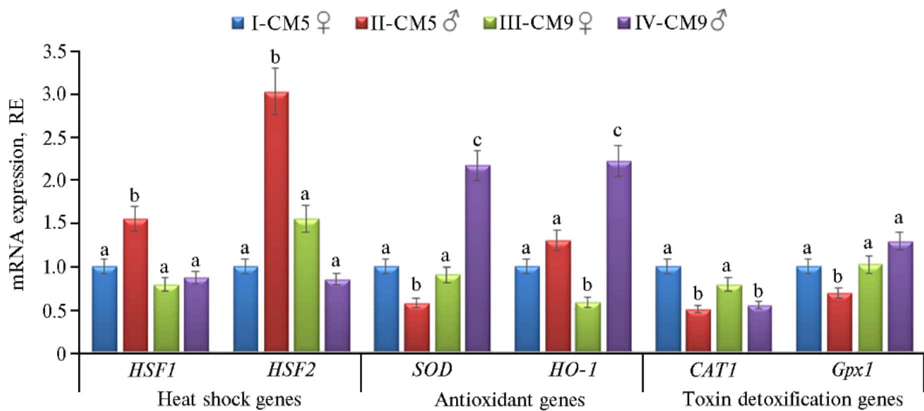

Fig. 1. **Expression of genes associated with adaptive potential in the liver of chickens and roosters (*Gallus gallus* L.) of the cross Smena 9 CM5 and CM9 lines** (vivarium of the SGC Zagorskoe EPH, Moscow Province, 2022). All groups were kept under identical conditions and received identical diets (data for 39 wk of age). RE is the fold change in the expression level compared to that in group I taken as 1; a-c — differences between values without a common letter designation are statistically significant at $p \leq 0.05$. Results are presented as the mean with standard error of the mean (*M*±SEM) for mRNA expression.

In addition, an increase in the expression of the *SOD* and *HO-1* genes was observed (see Fig. 1) in maternal Plymouth Rock CM9 roosters from group IV of the *SOD* and *HO-1* genes with paternal Cornish CM5 roosters (group II) was 160 and 92%, respectively ($p \leq 0.05$). The *SOD* and *HO-1* genes belong to the antioxidant enzyme genes. The superoxide dismutase (SOD) catalyzes the dismutation of superoxide into oxygen and hydrogen peroxide and thus plays a critical role in the antioxidant defense of virtually all cells that are somehow in contact with oxygen [35]. The main function of HO-1 is to catabolize heme to produce biliverdin, free iron, and carbon monoxide. Under stress conditions, the activity of heme oxygenase-1 can increase more than 10 times. Heme oxygenase and heme degradation products exhibit pronounced cytoprotective properties [36]. As noted above, the main selective characteristics of the line of the maternal form CM9 (Plymouth Rock), in contrast to the paternal line CM5 (Cornish), are egg production, egg weight, chick yield, timing of puberty, and viability [4]). Among the various nutrients in the mother's diet that can significantly influence embryonic development and chick viability in the early post-hatching period, natural antioxidants are hypothesized to be critical. The accumulation of endogenous antioxidants in the egg and embryonic tissues appears to serve as a major adaptive mechanism

for protection against oxidative stress experienced at hatching, with SOD as a key element of the antioxidant network playing a major role here [37]. In our opinion, this adaptive mechanism may be related to different levels of expression of the antioxidant genes *SOD* and *HO-1*.

It is worth noting that differences between the sexes occurred in the level of gene expression in the liver of birds within the studied lines. Thus, the expression of the *HSF1* and *HSF2* genes in the CM5 line was 1.6 and 3.0 times higher, respectively, in roosters compared to hens ($p \le 0.05$). In the CM9 line, the expression of the *SOD* and *HO-1* genes in roosters was higher ($p \le 0.05$) than in hens. Therefore, in poultry liver, the expression profile of genes associated with adaptive potential varies depending on sex. Given the role of the liver in energy balance and the difference between males and females in body size and physiology, the findings seem logical. Wide sexual dimorphism in gene expression in the liver has previously been demonstrated in mice [20]. It has been shown [38] that approximately 72% of the genes functionally active in mouse liver are sexually dimorphic in expression.

Data from the analysis of the expression of genes associated with immunity in the bursa of chickens and roosters of different lines of the Smena 9 cross are shown in Figure 2. There was a significant activation of the expression of genes for antimicrobial peptides and pro-inflammatory genes in roosters of the CM9 line (group IV) compared to roosters and hens of the CM5 line (groups I and II, $p \le 0.05$). Thus, compared to group II, the expression of the *AvBD2*, *AvBD9*, *AvBD10*, *IL8* and *PTGS2* genes increased 7.6, 5.3, 2.1, 6.3 and 1.5 times ($p \le 0.05$), respectively. Expression of the *AvBD2* and *PTGS2* genes was also increased in hens of the CM9 line compared to hens of the CM5 line ($p \le 0.05$). These data are in accordance with the results presented above of increased expression of the *HO-1* gene in CM9 line roosters compared to CM5 line roosters.

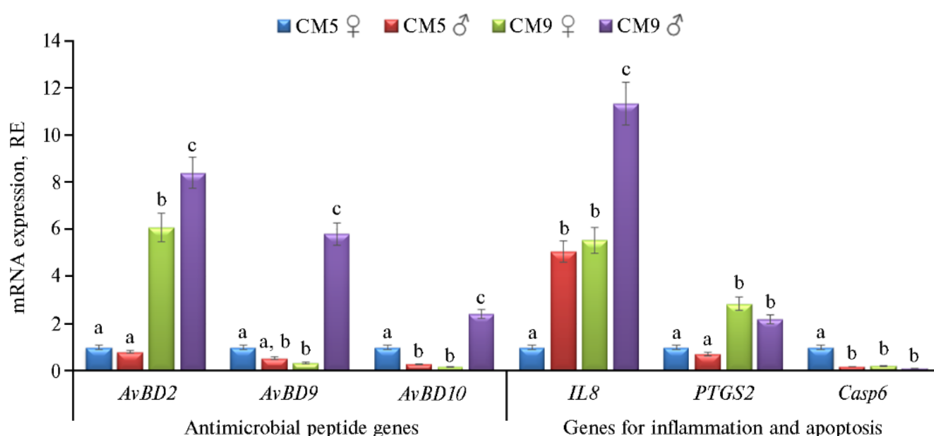

Fig. 2. Expression of genes associated with immunity in the bursa of chickens and roosters (*Gallus gallus* L.) of the cross Smena 9 lines CM5 and CM9 (vivarium of the SGC Zagorskoe EPH, Moscow Province, 2022). All groups were kept under identical conditions and received identical diets (data for 39 wk of age). RE is the fold change in the expression level compared to that in group I taken as 1; a-c — differences between values without a common letter designation are statistically significant at $p \le 0.05$. Results are presented as the mean with standard error of the mean (*M*±SEM) for mRNA expression.

It is known that induction of HO-1 by cells provides a reduced intensity of oxidative processes and also stimulates the production of anti-inflammatory cytokines [36]. AvBD2, AvBD9, and AvBD10 are defensins that have antimicrobial activity against a variety of pathogens, including gram-negative and gram-positive bacteria, viruses, and fungi [39]. In birds, 14 β-defensins have been identified, from

AvBD1 to AvBD14 [40]. Antimicrobial activity of AvBD2, AvBD3, AvBD4, AvBD6, AvBD7, AvBD11 and AvBD13 proteins against *Escherichia coli* has been reported [41]. Proinflammatory cytokines, such as interleukins, play important roles in immunomodulation and inflammation [42]. IL-8 is a chemokine that recruits leukocytes [43]. The activation of proinflammatory cytokines is closely related to the expression of the *PTGS2* gene, since cytokines are able to induce transcription of this gene [44]. The *PTGS2* gene is associated with the synthesis of prostaglandin endoperoxide synthase (cyclooxygenase 2), which catalyzes the oxidative conversion of arachidonic acid to prostaglandin. Prostaglandin is subsequently metabolized to various biologically active metabolites, prostacyclin and thromboxane A2, participating in both local and systemic inflammatory responses [45]. A higher level of expression of immunity genes in bursa tissues in the CM9 line, in contrast to the CM5 line, may be associated with improved reproductive qualities. Defense against infection and reproduction are key traits throughout an individual's life, and selection must therefore ensure optimal regulation of both processes [46]. The consequences of damage from infection with pathogens caused by immunopathologies can lead to deterioration in reproductive qualities. That is, the reproductive health of birds depends on an immune response that can prevent the development of the disease by blocking the penetration of pathogens. However, C.H. Chao and Y.P. Lee [47], in contrast, showed that in Taiwanese chickens, high plasma β-globulin levels were genetically associated with low fertility.

Changes in host gene expression, which are directly related to host metabolism, can influence both the composition of the gut microbiome and the predicted functional profile of microbial communities, with a possible counter-effect (influence of microbiota on expression) [48]. Given the identified differences in gene expression in the liver and bursa of poultry, we further studied the composition and function of the microbiota in hens and roosters of the CM5 and CM9 lines.

In our study, NGS sequencing of the microbiome of the intestinal cecum in birds of the CM5 and CM9 lines generated a total of 109,690 sequenced 16S rRNA gene sequences with a median (*Me*) of reads of 9,601 (min = 4013; max = 13541) (Fig. 3).

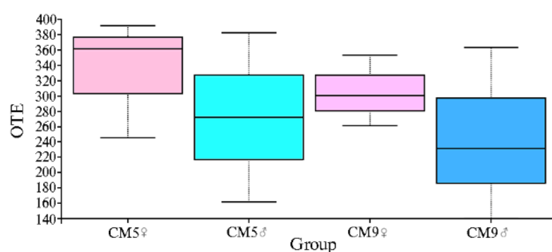

Fig. 3. The number of operational taxonomic units (OTUs) based on NGS sequencing of the intestinal microbiome of chickens and roosters (*Gallus gallus* L.) of cross Smena 9 lines CM5 and CM9 (the vivarium of the SGC Zagorskoe EPH", Moscow region, 2022). All groups were kept under identical conditions and received identical diets (data for 39 wk of age).

When comparing the Chao1, Shannon and Simpson biodiversity indices (Fig. 4), we were unable to identify significant differences between the groups.

The intestinal microbiome of birds from all groups contained 15 bacterial phyla and superphyla (Fig. 5). Of these taxons, *Bacteroidota*, *Bacillota* and *Verrucomicrobiota* dominated, with the phylum *Bacillota* being the most numerous (from 48.7±8.5 to 54.0±13.0%). The dominance of the phylum *Bacillota* bacteria in the poultry gut microbiome was previously reported by other researchers [49, 50].

At the phylum level, the main difference between the CM9 line and the CM5 line in terms of microbiome composition was the presence of bacteria of the superphylum *Elusimicrobiota* in the cecum of the intestines of hens and roosters (groups III and IV) (0.32±0.11 and 0.49±0.19%, respectively; $p \leq 0.05$). These microorganisms were not found in the intestines of roosters of the CM5 line (group

II), while in the intestines of hens of the CM5 line (group I), their content averaged 0.04±0.01%. It is possible that bacteria from the superphylum *Elusimicrobiota* may be associated with a phenotype of improved egg production and reproductive performance. Representatives of this phylum are known to be permanent inhabitants of the intestines of the beetle Pachnoda ephippiata, which feeds mainly on humus, and its intestinal chyme contains high concentrations of glucose, peptides and amino acids [51]. Members of the superphylum *Elusimicrobiota* are thought to promote better digestion in *P. ephippiata* [52].

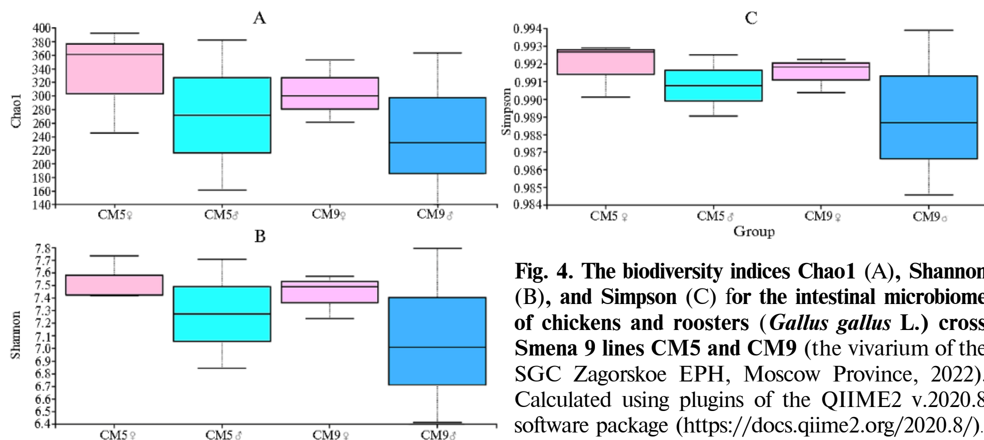

Fig. 4. The biodiversity indices Chao1 (A), **Shannon** (B), **and Simpson** (C) **for the intestinal microbiome of chickens and roosters** (***Gallus gallus* L.) cross Smena 9 lines CM5 and CM9** (the vivarium of the SGC Zagorskoe EPH, Moscow Province, 2022). Calculated using plugins of the QIIME2 v.2020.8 software package (https://docs.qiime2.org/2020.8/).

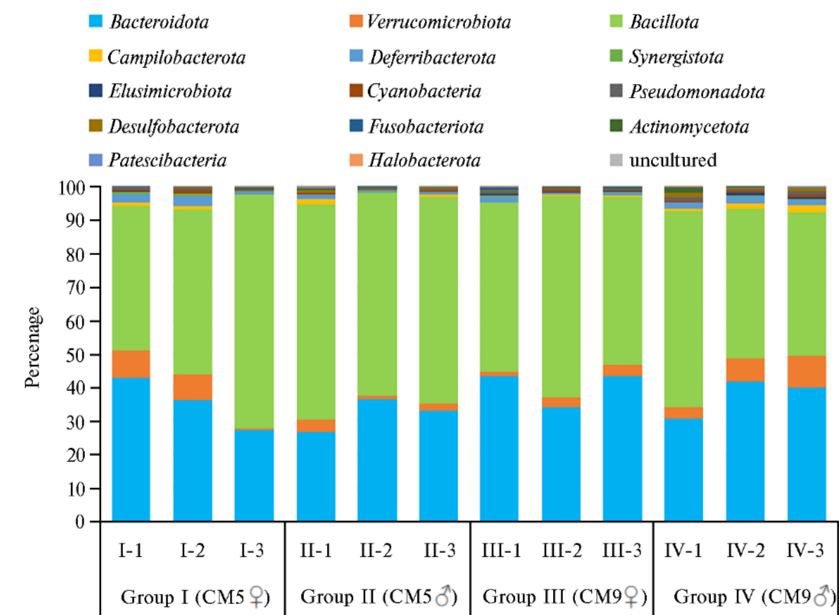

Fig. 5. The intestinal microbiome composition in chickens and roosters (*Gallus gallus* L.) of the Smena 9 cross CM5 and CM9 lines at the level of bacterial phyla (based on NGS sequencing of 16S rRNA gene amplicons; the vivarium of the SGC Zagorskoye EPH, Moscow Provine, 2022).

At the level of bacterial genera, differences between groups ($p \le 0.05$) were found in 25 genera (Fig. 6), in some of which the representation varied depending on the genotype of the bird, and in some on its sex. Thus, in roosters of the CM5 line (group II) compared to roosters of the CM9 line (group IV), in the cecum, the abundance of the genera *Barnesiella* (superphylum *Bacteroidota*), Clostridia_UCG-014 and *Frisingicoccus* (phylum *Bacillota*) was higher ($p \le 0.05$) 17.2-fold, 2.0-fold and 4.9-fold, respectively. In addition, in hens and roosters of the

CM5 line (groups I and II) compared to hens and roosters of the CM9 line (groups III and IV), the abundance of the genus *Colidextribacter* (phylum *Bacillota*) in the intestine was 4.7 and 7.5 times higher ($p \leq 0.05$).

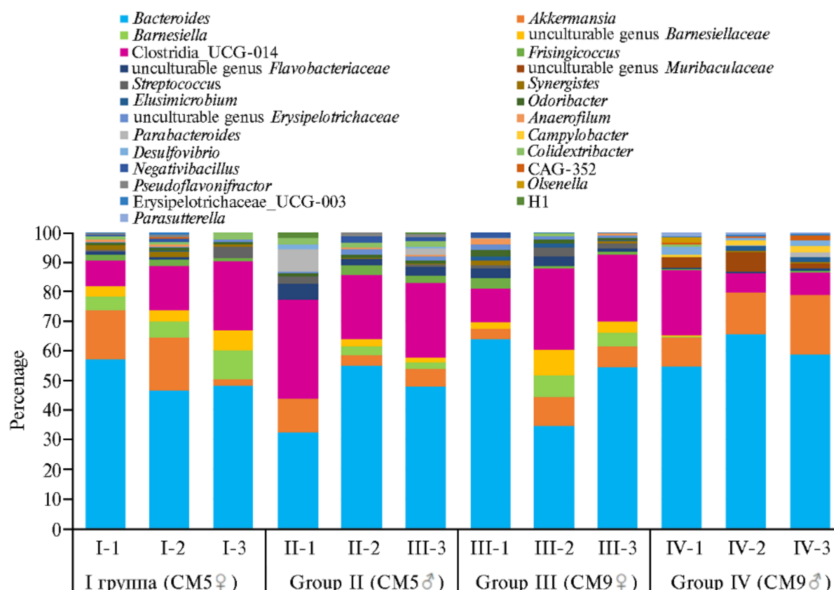

Fig. 6. The intestinal microbiome composition in chickens and roosters (*Gallus gallus* L.) of the Smena 9 cross CM5 and CM9 lines at the level of bacterial genera (based on NGS sequencing of 16S rRNA gene amplicons; the vivarium of the SGC Zagorskoye EPH, Moscow Provine, 2022).

Representatives of the genus *Pseudoflavonifractor* (phylum *Bacillota*) were completely absent in the intestines of hens and roosters of the CM9 line (groups III and IV), while in the chyme of hens and roosters of the CM5 line (groups I and II) these microorganisms accounted for 0.22±0.09 and 0.26±0.05%, respectively. It is likely that representatives of these genera most of which belong to the phylum *Bacillota* may be associated with the phenotype of higher meat productivity observed in the CM5 line compared to the CM9 line. The fact is that an important function of *Bacillota* is the ability to decompose complex polysaccharides with the subsequent formation of short-chain fatty acids [53] for the host's energy metabolism (and, as a result, in the formation of productivity). These substrates promote the growth and normal functioning of intestinal cells which is important for nutrient absorption [54]. Representatives of *Barnesiella* spp. superphylum *Bacteroidota* are involved in regulation of the intestinal microbiota composition, limiting the proliferation of oxygen-tolerant pathogens, in particular those carrying genes for multiple antibiotic resistance, which can affect the health and productivity of the host [55]. Previously, using the example of two lines of chickens, it was shown that a representative of the phylum *Bacillota*, the genus *Lactobacillus*, plays a key role in increasing live weight [12]. In our opinion, the host genotype can affect the structure of the microbial community with the help of genotypically determined factors (these include the composition of the secretion of the glands of the intestinal mucosa, features of peristalsis, modification of the surface of epithelial cells), and as was shown in our study, through changes in gene expression.

Interestingly, at the genus level, there were also differences between sexes ($p \leq 0.05$) in the composition of the intestinal microbiota. Thus, members of the genus *Desulfovibrio* (superphylum *Desulfovibrionia*) were present in the intestines of roosters of both the CM5 and CM9 lines (groups II and IV) in the amount of 0.25 ± 0.08 and 0.73±5.6%. At the same time, these microorganisms were not found in the intestines of chickens of both lines (groups I and III). The abundance

of representatives of the genera *Barnesiella* (superphylum *Bacteroidia*) and *Synergistes* (superphylum *Synergistia*), on the contrary, decreased (p ≤ 0.05) in roosters of the lines CM5 and CM9 (groups II and IV) compared to chickens (groups I and III). Previously, it was also shown in chickens [56] that the composition of the cecal microbiota differed depending on sex: in cockerels, an enrichment of the intestinal microbiota with representatives of *Bacteroidetes* was noted, and in chickens, an increase in the number of clostridia and Shigella was noted. When trying to identify the reasons for such changes in mice, it turned out that a decrease in testosterone levels during castration of animals during puberty eliminated sex differences in the composition of the intestinal microbiota in adults. This indicates the importance of pubertal testosterone in the formation of sexually dimorphic microbial communities that persist in males into adulthood [57]. However, the mechanism by which testosterone influences the composition of such communities is currently not understood.

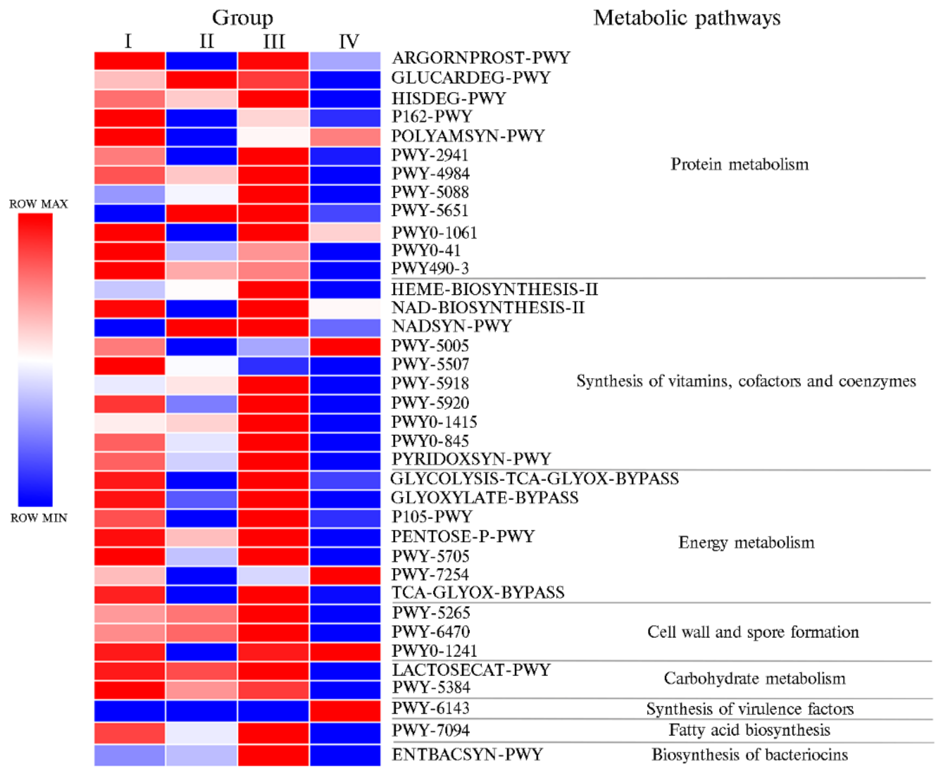

FIg. 7. Functional annotation of predicted metabolic pathways in the cecum microbiome of chickens and roosters (*Gallus gallus* L.) of the cross Smena 9 lines CM5 and CM9 (based on NGS sequencing of 16S rRNA gene amplicons; the vivarium of the SGC Zagorskoye EPH, Moscow Province, 2022). The data were obtained using the PICRUSt2 v.2.3.0 software package (https://github.com/picrust/picrust2). To analyze metabolic pathways and enzymes, the MetaCyc database (https://metacyc.org/) was used. The scale reflects the intensity of potential metabolic pathways of the microbiome: blue color is the lowest (minimum) intensity, red is the highest (maximum). For a description of the groups, see the Materials and methods section.

Based on the results of bioinformatics reconstruction and functional annotation of NGS sequencing data in the intestinal microbial community of the studied birds, we found 357 predicted metabolic pathways, 65 of which showed differences (p ≤ 0.05) between experimental groups (Fig. 7). These pathways related to protein metabolism (biosynthesis and breakdown of amino acids, transformation of nitrogenous compounds), carbohydrate metabolism (biosynthesis and degradation of sugars), energy metabolism (Krebs cycle, glycolysis, glyoxylate

cycle), to the synthesis of fatty acids, vitamins, cofactors and coenzymes (biotin, adenosylcobalamin, pyridoxal phosphate, heme), to the formation of a cell wall and sporulation (synthesis of peptidoglycan, ADP-L-glycero-beta-D-manno-heptose), to the synthesis of virulence factors (pseudamic acid), bacteriocins. The majority of predicted metabolic pathways that differed between experimental groups ($p \le 0.05$) were associated with protein and energy metabolism, as well as the synthesis of vitamins, cofactors and coenzymes. The most significant ($p \le 0.05$) quantitative differences between lines CM5 and CM9 in predicted metabolic pathways concerned the formation of cell walls and bacterial spores (up to 4.9-fold when comparing groups II and IV), protein metabolism (up to 3.4-fold) multiples for groups II and IV), synthesis of adenosylcobalamin (3.2 times when comparing groups I and III). It is likely that the increased activity of these metabolic pathways in the CM5 line may be associated with high meat productivity. This seems natural, since the main component in poultry feed is protein, the hydrolysis products of which are absorbed from the intestines into the blood and used by the body for plastic purposes. Due to the intensification of protein metabolism, additionally synthesized proteins can be directed to the construction of new tissues in a growing organism. The stimulating effect of adenosylcobalamin on poultry meat productivity has also long been established [58].

Within the CM9 line, we identified significant differences ($p \le 0.05$) in a number of predicted metabolic pathways between chickens (group III) and roosters (group IV). The activity of the putative biological functions of the microbial community, affecting the metabolism of proteins, carbohydrates, energy metabolism, biosynthesis of fatty acids, bacteriocins, vitamins, cofactors and coenzymes, the formation of cell walls and bacterial spores, was higher in chickens (group III) than in roosters (group IV) (differences up to 5.9-fold). Moreover, in roosters of the CM9 line, compared with chickens (group IV vs. group III), the synthesis pathway of CMP-pseudamic acid (PWY-6143), a pathogen virulence factor, was increased 5.8 times ($p \le 0.05$) [59]. Previously, similar studies in mice revealed that the activity of predicted fatty acid and lipid metabolic pathways was more common in males than in females [15]. The increase in protein metabolism observed in our study in hens of the CM5 line compared to the CM9 line may be associated with an increase in the abundance of representatives of the genus *Synergistes* in the intestine (their main function is the utilization of amino acids) [60]. The enhancement of the CMP-pseudamic acid synthesis pathway in CM9 roosters compared to hens may be influenced by a decrease in the number of bacteria of the genus *Barnesiella*, which, as already noted, limits the proliferation of oxygen-resistant pathogens [55]. An increase in the pathogenicity of the intestinal microbiota in CM9 roosters could induce the expression of genes for antimicrobial peptides and IL8 in the bursa.

So, on the example of the new cross Smena 9 paternal line Cornish CM5 and maternal line Plymouth Rock CM9 with different phenotypes (patterns) in meat productivity and reproductive efficiency [3-5], we demonstrated a fairly high dependence of the transcriptional activity of genes associated in hens with adaptive potential and immunity [35, 39] on genotype and sex. The data obtained may be valuable for identifying new candidate genes affecting meat productivity and egg production. We also obtained results demonstrating the relationship between the quantitative composition of the microbiota and its predicted metabolic pathways with the genotype and sex of the bird. Genotype- and sex-specific changes in gene expression, as well as in the structure and function of the intestinal microbiome, may form the basis of physiological adaptations of the macroorganism to various conditions. We believe that the mechanism that determines differences in the composition of the intestinal microbiota depending on sex and genotype in birds

of the CM5 and CM9 lines of the Smena 9 cross may lie in the subtle harmonization by the transcriptional activity of genes of adaptation and immunity, on the one hand, and, apparently, hormonal background, nutritional needs and provision of nutrients and energy, on the other hand. Understanding these relationships is important for maintaining health, improving reproductive performance and enhancing poultry productivity, and further research is required to establish cause-and-effect relationships between them. In particular, it is of interest to compare the expression of genes for egg and meat productivity in the CM5 and CM9 lines in combination with an analysis of the composition and functional annotation of the intestinal microbiota.

Thus, we assessed differences in the expression of the spectrum of immunity genes and genes associated with adaptive potential, respectively, in the bursa and liver, and determined the composition and functions of the microbiome of the intestinal cecum in hens and roosters of the CM5 and CM9 lines of the new cross Smena 9 (parent stock). The lines differ in live weight gain and egg production. The results showed an increase in the transcriptional activity of the *HSF1* and *HSF2* genes in CM5 line roosters (group II) compared to groups I, III and IV (maternal line CM5 hens of the Cornish breed, maternal line CM9 hens of the Plymouth Rock breed, maternal line CM9 roosters of the Plymouth Rock breed, respectively). The difference in the expression of the *HSF1* and *HSF2* genes compared to roosters of the CM9 line (group IV) was 68 and 218%, respectively. The expression of the *HSF1* and *HSF2* genes in the CM5 line in roosters (group II) was 1.6 and 3.0 times higher compared to hens (group I). There was a significant activation of the expression of genes for antimicrobial peptides and pro-inflammatory genes in roosters of the CM9 line (group IV) compared to roosters and hens of the CM5 line (groups I and II). Thus, compared to group II, the expression of the *AvBD2*, *AvBD9*, *AvBD10*, *IL8* and *PTGS2* genes increased by 7.6, 5.3, 2.1, 6.3, and 1.5 times, respectively. NGS sequencing of the microbiome of the cecum in hens and roosters of the CM9 line (groups III and IV) identified bacteria of the superphylum *Elusimicrobiota* (0.32±0.11 and 0.49±0.19%, respectively). These microorganisms were not found in the intestines of roosters of the CM5 line (group II), and in hens of the CM5 line (group I) their share averaged 0.04±0.01%. Differences were found between the groups in 25 bacterial genera. In some genera, the abundance varied depending on the genotype, in some on the sex of the birds. Thus, in roosters of the CM5 line (group II), the abundance of the genera *Barnesiella*, Clostridia_UCG-014 and *Frisingicoccus* in the cecum was higher (17.2, 2.0 and 4.9 times, respectively) than in roosters of the CM9 line (group IV). Representatives of the genus *Desulfovibrio* were present in the intestines of roosters of both lines CM5 and line CM9 (groups II and IV, 0.25±0.08 and 0.73±5.6%), but in the intestines of hens of both lines (groups I and III), these microorganisms were not detected. Based on the results of bioinformatics reconstruction and functional annotation of NGS sequencing data, we identified 357 predicted metabolic pathways in the intestinal microbial community of the studied birds, 65 of which showed differences between groups. Genotype- and sex-specific modulations in gene expression, as well as in the structure and function of the intestinal microbiome, may provide adaptation to changing conditions.

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