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COMPOSITION AND METABOLIC POTENTIAL OF THE INTESTINAL MICROBIOME OF *Gallus gallus* L. BROILERS UNDER EXPERIMENTAL T-2 TOXICOSIS AS INFLUENCED BY FEED ADDITIVES

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Abstract

Mycotoxins can adversely affect the composition and function of the poultry gut microbiota, with implications for host health. The introduction of feed additives into contaminated feed is a strategy for restoring the intestinal microbiome under mycotoxicoses. This paper shows for the first time that the feed additive Zaslon 2+ effectively improves the structure and metabolic potential of the intestinal microbiome in broiler chickens with experimental T-2 mycotoxicosis. Our goal was to identify changes in the chyme microbiota and its functional annotation after 14-day exposure to T-2 toxin, artificially introduced with feed, and under the influence of the feed additive Zaslon 2+, fed alone and in combination with the proteolytic drug Axtra Pro. The experiments were carried out in the vivarium of the Federal Scientific Center ARRTPI RAS in 2021. Broiler chickens of the Smena 8 cross aged 33 days were assigned into four groups of 5 birds each. Control group I received a basal diet (BD) without T-2 toxin, group II was fed with BD added with T-2 toxin (200 μg/kg) (BD + T-2), group III — BD + T-2 + additive Zaslon 2+ (1 g/kg feed) (BIOTROF Ltd, Russia), group IV — BD + T-2 + additive Zaslon 2+ added with proteolytic preparation Axtra Pro (DuPont de Nemours, Inc., USA) (100 mg/kg feed). Zaslon 2+ contains diatomite, two *Bacillus* strains, and a mixture of natural essential oils (eucalyptus, thyme, garlic, and lemon). Feed intake averaged 150 g/day, i.e. the birds of the experimental groups received 30 μg T-2 toxin daily. At the end of the experiment, the caecum content was sampled from three broilers of each group. Total DNA was isolated from the samples using the Genomic DNA Purification Kit (Fermentas, Inc., Lithuania). The caecal bacterial community was assessed by NGS sequencing on the MiSeq platform (Illumina, Inc., USA) using primers for the V3-V4 region of the 16S rRNA gene. Bioinformatic data analysis was performed using QIIME2 ver. 2020.8 (https://docs.qiime2.org/2020.8/). The reconstruction and prediction of the functional content of the metagenome, gene families, and enzymes was carried out using the PICRUSt2 (v.2.3.0) software package (https://github.com/picrust/picrust2). MetaCyc base data (https://metacyc.org/) was used to analyze metabolic pathways and enzymes. NGS-sequencing revealed changes in biodiversity and composition of the gut microbiota at the level of phyla. I.e., in group II, the population of superphylum *Actinobacteriota* and phylum *Proteobacteria* increased 1.8 and 3.5 times, respectively ($p \le 0.05$) while the superphylum *Desulfobacterota*, on the contrary, decreased 2.2 times ($p \le 0.05$). In group IV (BD + T-2 supplemented with Zaslon 2+ and Axtra Pro), the abundance of superphylum *Actinobacteriota* and phylum *Proteobacteria* also increased compared to group I ($p \le 0.05$), while in group III (BD + T-2

supplemented with Zaslon 2+) no change occured. The members of superphylum *Verrucomicrobiota* completely disappeared in groups II and IV, while in group I they accounted for 14.1±0.8 %. In group III compared to group I, bacteria of the genus *Lactobacillus* increased ($p \leq 0.01$) from 15.9±1.32 to 30.7±1.84 %. The genus *Akkermansia* represented by the only species *A. muciniphila* sharply decreased in all groups fed T-2 toxin (groups II, III, and IV) as compared to group I ($p \leq 0.001$), up to a complete absence in groups II and IV. Pathogenic microorganisms which were absent in group I (*Enterococcus cecorum*, *Campylobacter concisus*, *Campylobacter gracilis*, *Streptococcus gordonii*, *Flavonifractor* spp.) appeared in group II. In groups III and IV, these pathogens were either absent or were present in a significantly smaller amount than in group II ($p \leq 0.05$). Gut microbial community showed differences between groups ($p \leq 0.05$) in 163 predicted metabolic pathways. When exposed to T-2 toxin (group II compared to group I, $p \leq 0.05$), there was an increase in the predicted metabolic pathways for the degradation of aromatic compounds, including xenobiotics, and amino acids and for the synthesis of coenzymes, cofactors and formation of biofilms, cell walls, spores and protective substances in cells. The feed additive Zaslon 2+ contributed to the adjustment of metabolic pathways to the level of group I. The combined use of the feed additive Zaslon 2+ and protease (group IV) had no positive effect on the potential of metabolic pathways. Thus, feed contamination with T-2 toxin has a negative impact on the composition and predicted metabolic potential of the gut microbiome of Smena 8 cross broiler chickens. In general, the effect of the feed additive Zaslon 2+ and its complex with protease was positive though the additive without the enzyme showed greater efficiency.

Keywords: mycotoxins, T-2 toxin, broilers, gut microbiome, gene expression, poultry

Mycotoxins, the compounds of the secondary metabolism in molds, are highly toxic to animals, birds and humans [1)]. Micromycetes of the genera *Aspergillus*, *Fusarium*, *Penicillium*, *Alternaria*, *Phomopsis*, *Emericella*, *Cephalosporium*, *Myrothecium*, *Trichoderma*, *Trichothecium*, *Neopetromyces*, *Byssochlamys*, *Neotyphodium* and *Claviceps* are responsible for their production. On average, 25% of the world's agricultural products and feeds are contaminated to some extent with mycotoxins [2], which causes mass poisoning of both people and farm animals in many countries [1].

T-2 toxin is one of the most dangerous mycotoxins [3]. It belongs to trichothecenes [4] produced by micromycetes of the genera *Fusarium*, *Myrothecium* and *Stachybotrys*. T-2 toxin is commonly found in grains of wheat, corn, barley, rice, soybeans, oats [5], its derivatives and compound feeds, including compound feed for poultry [6].

The main symptoms of T-2 toxicoses in poultry include hemorrhagic necrotizing ulcerative inflammation of the digestive tract with thickening of the mucous membrane, staggering gait and refusal to feed [7]. It has been noted [8] that acute intoxication in broiler chickens is manifested by symptoms of internal hemorrhage, lesions of the oral cavity and skin (necrohemorrhagic dermatitis), deterioration of the quality of feathers and disorders of the nervous system. At low doses, a significant decrease in the amount of hemoglobin in the blood of broiler chickens was observed [8]. In addition, when exposed to low doses of T-2, a decrease in total protein and cholesterol and an increase in serum uric acid and lactate dehydrogenase were noted [9, 10], which convincingly proves the toxic effect of T-2 toxin even at low doses. Pathological examinations usually reveal fatty degeneration and severe granular degeneration in the liver, kidneys, and rarely in the heart [11].

Acting as a selective permeable barrier places the intestinal mucosal epithelium at the center of interactions between the mucosal immune system and chyme, which includes normal flora, pathogens, and food toxicants [12]. It is known that mycotoxins can somehow modulate the composition of the gut microbiota, which has a detrimental effect on the health of the host [13]. When exposed to toxins, the integrity of the intestinal epithelium is disrupted, as a result of which pathogenic microorganisms penetrate into the macroorganism [14]. At the same time, the gut microbiota can metabolize mycotoxins, thereby converting them into different chemical structures with greater or lesser toxicity than the

original compounds [13]. It appears that the gut microbiome not only has a direct metabolic potential for xenobiotics, but also influences gene expression and host enzyme activity. W. Meinl et al. [15] demonstrated that the gut microbiota affects the expression of xenobiotic detoxification-associated *GSTs*, *GPX2*, *EPHXs*, and *NNAT 1* genes in colon and liver tissues of the host. Studies on the composition of the microbiome of animals and birds under the influence of mycotoxins are extremely limited [16, 17]. An analysis of changes in the predicted functional potential of the microbiome of animals, birds and humans using bioinformatic software systems such as PICRUSt2 and the like has not been previously carried out. The effect of T-2 toxin on the composition and functional potential of the gut microbiome in birds has also not been studied.

For the prevention of mycotoxicosis, sorbents are used that selectively bind toxins during digestion, preventing their absorption from the gastrointestinal tract and, consequently, reducing the toxic effect. It is known that enrichment of feed with probiotics, amino acids, lipids, enzymes has a positive effect and reduces the symptoms of T-2 toxicosis [3]. For example, the inclusion of bacterial cultures of *Lactobacillus* spp. in the diet of broiler chickens reduced the toxic effects of aflatoxin B_1, zearalenone [18] and DON [19]. H. Tozaki et al. [20] demonstrated that a promising approach for the degradation of xenobiotics could be the use of enzymes, in particular proteases.

In this work, for the first time, we established changes in the composition of the broiler microbiome under the influence of the feed additive Zaslon 2+ and the proteolytic enzyme Axtra Pro against the background of artificial contamination of feed with T-2 toxin.

Our goal was a comprehensive analysis of the effect of the 14-day exposure to T-2 toxin added to the feed, as well as the feed additive Zaslon 2+ and the proteolytic enzyme Axtra Pro, on the composition and functional potential of the chyme microbiome of the Smena 8 cross broiler chickens.

Materials and methods. The 1-day experiment was carried out in 2021 in the vivarium of the All-Russian Research and Technological Institute of Poultry Farming on broiler chickens (*Gallus gallus* L.) of the Smena 8 cross from 33 to 47 days of age in accordance with the requirements of the European Convention for the Protection of Vertebrate Animals used for experiments or for other scientific purposes (ETS No. 123, Strasbourg, 1986) [21]. Feeding and housing conditions met the requirements for broiler crosses [22]. For feeding, PK-6 compound feed (Russia) was used for broilers in the form of a scattering. Feeding and watering of the birds were not limited.

An experimental contamination of feed with T-2 toxin (2-fold excess of MPC) was performed mechanically in compliance with personnel safety requirements. We used a certified calibration standard T-2 toxin in the form of a powder with a mass fraction of the main substance of 99.7±0.3% (Romer Labs, Austria; cat. No. 10000310, LOT #S17052T). Before and after contamination, the compound feed was examined for the presence of mycotoxins by high-performance liquid chromatography with tandem mass spectrometry (HPLC-MS/MS, Agilent 1260 Infinity LC chromatographic system, Agilent Technologies, USA; mass spectrometer AB SCIEX Triple Quad™ 5500, AB Sciex, USA; Gemini® C18 110A 5 μm 150×4.6 mm reverse-phase separation column, Phenomenex, USA). A standard solution of T-2 toxin was used to build calibration graphs and as internal standards. In addition to the above, the diet of broilers practically did not contain background amounts of mycotoxins. The HPLC-MS/MS system was used to determine the background content of mycotoxins in feed. Standard solutions of mycotoxins (Romer Labs, Austria) were used in the construction of calibration graphs

and as internal standards. No aflatoxins (B_1, G_1), fumonisins (B_1, B_2, B_3), deoxynivalenol, T-2 toxin, zearalenone and ochratoxin A were found in the feed used for infection.

The birds were divided into four groups of 5 broilers each. In group I (control) broilers received the basal diet (BD) without T-2 toxin, in group II broilers received BD added with T-2 toxin (200 μg/kg), in group III BD added with T-2 toxin (200 μg/kg) and feed additive Zaslon 2+ which is a sorbent material of diatomite + two *Bacillus* spp. cultures, a mixture of of eucalyptus, thyme, garlic and lemon natural essential oils (OOO BIOTROF, Russia) (1 g/kg of feed), group IV received BD added with T-2 toxin (200 μg/kg), feed additive Zaslon 2+ (1 g/kg of feed) and enzyme preparation with proteolytic activity Axtra Pro (DuPont de Nemours, Inc., USA) (100 mg/kg of feed). Feed intake by broilers averaged 150 g/day, that is, the birds of the est groups received T-2 toxin daily with feed in the amount of 30 μg.

At the end of the experiment, the bird was decapitated and an autopsy was performed. Chyme samples were manually taken from the blind processes of the intestine from three birds from each group (30-50 g) with the maximum possible observance of aseptic conditions. The selected samples were immediately placed in sterile plastic centrifuge tubes. All samples were frozen at –20 °C and transported in dry ice for subsequent DNA isolation.

Total DNA was isolated using the Genomic DNA Purification Kit (Fermentas, Inc., Lithuania) according to the attached instructions. The method is based on selective detergent-mediated precipitation of DNA from a substrate using 1.2 M sodium chloride and chloroform solutions for cell wall lysis and DNA precipitation.

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. Forward primer is 5'-TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGCCTACGGGNGGCWGCAG-3'; reverse primer is 5'-GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGGACTACHVGGGTATCTAATCC-3'.

PCR was performed as follows: 3 min at 95 °C; 30 s at 95 °C, 30 s at 55 °C, 30 s at 72 °C (25 cycles); 5 min at 72 °C (final elongation). Sequencing was performed with Nextera® XT IndexKit library preparation reagents (Illumina, Inc., USA), Agencourt AMPure XP PCR product purification (Beckman Coulter Inc., USA), and MiSeq® ReagentKit v2 (500 cycle) (Illumina, Inc., USA). The maximum length of the resulting sequences was 2×250 bp.

Bioinformatic data analysis was performed using QIIME2 ver. 2020.8 (https://docs.qiime2.org/2020.8/). After importing sequences in the .fastq format from the sequencing instrument and creating the necessary mapping files containing the metadata of the studied files, paired read lines were aligned. The sequences were filtered for quality using the default settings. Noise sequences were filtered using the DADA2 method built into the QIIME2 package, which includes quality information in its error model, which makes the algorithm robust to lower quality sequences, while using a maximum trimming sequence length of 250 bp. (https://benjjneb.github.io/dada2/tutorial.html). To build a de novo phylogeny, multiple sequence alignment was performed using the MAFFT software package (https://mafft.cbrc.jp/al-ignment/software/), followed by masked sequence alignment to remove positions that differed significantly. The reference database Silva 138.1 (https://www.arb-silva.de/documentation/release-138.1/) was used for taxonomy analysis.

Based on the obtained table of operational taxonomic units (OTU), using the plugins of the QIIME2 software package, biodiversity indices were calculated,

and a graph of the dependence of the number of OTUs on the number of reads was plotted. In the statistical analysis of diversity indices, their additional transformation was not carried out.

The reconstruction and prediction of the functional content of the metagenome, gene families, and enzymes was carried out using the PICRUSt2 (v.2.3.0) software package (https://github.com/picrust/picrust2) (23). We worked with the program according to the recommended scenario of actions, all settings were used by default. The OTUs of each sample were arranged according to their content, from largest to smallest, and the values were converted using the logarithmic transformation of Log2. The MetaCyc database (https://metacyc.org/) was used to analyze metabolic pathways and enzymes. The predicted profiles of MetaCyc metabolic pathways were assessed by the abundance of ASV (Amplicon Sequence Variants) [24]. Data visualization and calculation of statistical indicators were performed using the Phantasus v1.11.0 web application (https://artyomovlab.wustl.edu/phantasus/), which, in addition to the main visualization and filtering methods, supports R-based methods such as like k-means clustering, principal component analysis, or differential expression analysis with the limma package.

Mathematical and statistical data processing was carried out by the method of multifactor analysis of variance (ANOVA) in Microsoft Excel XP/2003, R-Studio (Version 1.1.453) (https://rstudio.com). Results are presented as means (*M*) and standard errors of the means (±SEM). Significance of differences was established by Student's *t*-test, differences were considered statistically significant at p 0.05. Means were compared using Tuke's significant difference (HSD) test and the TukeyHSD function in the R Stats Package.

Results. When performing NGS-sequencing of the microbiome, a total of 85,121 sequenced sequences of the 16S rRNA gene were generated (with a median of reads of 7.253, min = 3.330, max = 10.859). The number of OTUs did not differ significantly ($p > 0.05$) between the experimental groups (Fig. 1).

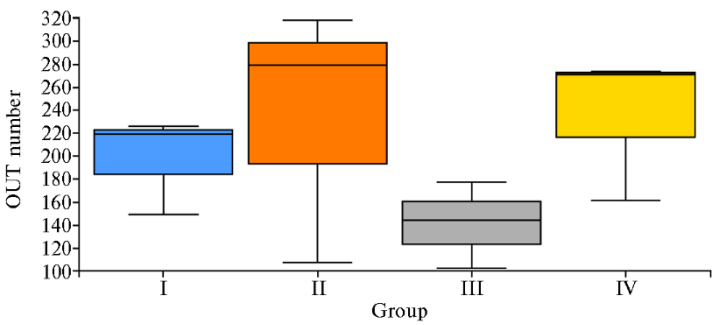

Fig. 1. The number of operational taxonomic units (OTU) **according to the results of NGS sequencing of the intestinal microbiome of broiler chickens (*Gallus gallus* L.) Smena 8 cross fed T-2 toxin, additive Zaslon 2+** (BIOTROF LLC, Russia) **and enzyme drug with proteolytic activity Axtra Pro** (DuPont de Nemours, Inc., USA) (vivarium, FSC ARRTPI RAS, 2021). For a description of the groups, see the Materials and methods section.

When compared by the Simpson index, it turned out that in group III, biodiversity decreased ($p \leq 0.05$) vs. control (group I, Fig. 2). We assume that the decrease in biodiversity is associated with the stabilization of the microbiome under the influence of the feed additive Zaslon 2+. According to J. Knol et al. [25], this can have a beneficial effect, as a large number of interacting species often tend to have a destabilizing effect on the microbiome. It has been previously shown that prebiotics, by inhibiting the growth of potentially pathogenic *Clostridium* spp. and *Salmonella* spp., contributed to the formation of more stable gut microbial communities with low biodiversity [26]. In group IV, there was an increase

($p \leq 0.05$) in biodiversity in the intestine compared to group III (see Fig. 2) which is probably associated with the introduction of a proteolytic enzyme. J.M. Lourenco et al. [27] reported that the addition of protease to the diet of broilers had an effect on both the richness and diversity of microbial populations: with the introduction of the enzyme, the number of OTUs ($p = 0.04$) and the value of the Chao1 index ($p = 0.09$) increased.

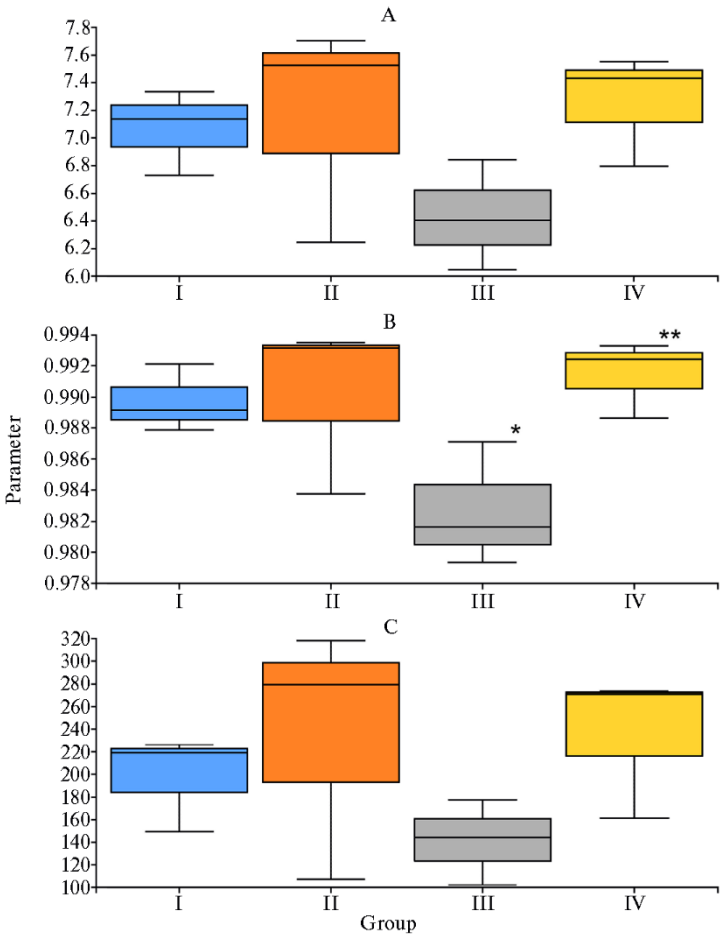

Fig. 2. Absolute values of the Shannon (A)**, Simpson** (B)**, and Chao1 (C) biodiversity indices for the gut microbiome of broiler chickens (*Gallus gallus* L.) Smena 8 cross fed T-2 toxin, additive Zaslon 2+** (BIOTROF LLC, Russia) **and enzyme drug with proteolytic activity Axtra Pro** (DuPont de Nemours, Inc., USA) (vivarium, FSC ARRTPI RAS, 2021). Calculated using plugins of QIIME2 ver. 2020.8. For a description of the groups, see the Materials and methods section.
*, ** Differences are statistically significant at $p \leq 0.05$ when compared to groups I and III.

The intestinal microbiome of birds from all groups contained 15 bacterial phyla and superphyla (Fig. 3). The phyla *Firmicutes*, *Verrucomicrobiota*, *Proteobacteria*, *Actinobacteriota*, *Bacteroidota* dominated of which the phylum *Firmicutes* was the most abundant (from 71.1±3.9 to 94.8±5.7%). The predominance of bacteria of the phylum *Firmicutes* in the poultry intestinal microbiome has been shown previously [28, 29]. An important function of *Firmicutes* is the ability to degrade complex polysaccharides with subsequent formation of short-chain fatty acids [30]. Short-chain fatty acids play an important role in host energy metabolism by promoting the growth and normal functioning of intestinal cells [31].

In the intestines of birds from group II, when T-2 toxin was introduced into the feed, bacteria of the superphylum *Actinobacteriota* and phylum *Proteobacteria*

became 1.8-fold and 3.5-fold more abundant ($p \leq 0.05$), while the bacteria of the superphylum *Desulfobacterota* decreased 2.2-fold ($p \leq 0.05$). In the intestines of broilers from group IV, the number of bacteria of the superphylum *Actinobacteriota* and the phylum *Proteobacteria* also increased compared to group I ($p \leq 0.05$), while in group III (with Zaslon 2+ feed additive), no such changes occurred.

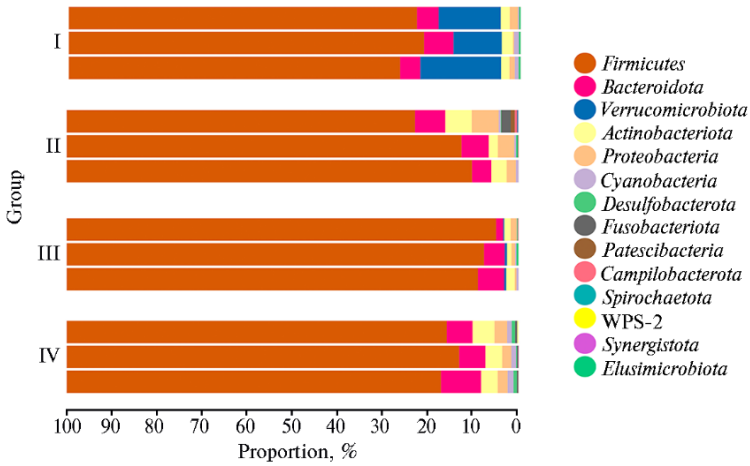

Fig. 3. The gut microbiome composition at the phyla levels (based on data of NGS sequencing of 16S rRNA gene amplicons) in broiler chickens (*Gallus gallus* L.) Smena 8 cross fed T-2 toxin, additive Zaslon 2+ (BIOTROF LLC, Russia) **and enzyme drug with proteolytic activity Axtra Pro** (DuPont de Nemours, Inc., USA) (vivarium, FSC ARRTPI RAS, 2021). For a description of the groups, see the Materials and methods section.

Interestingly, new superphyla, *Fusobacteriota* and *Spirochaetota*, appeared in the microbiome in groups II and IV (pathogens are often found among representatives of these taxa) [32, 33]. In group III, which received the feed additive Zaslon 2+, we did not identify these phyla. Particularly noteworthy is the fact that representatives of the superphylum *Verrucomicrobiota* completely disappeared in groups II and IV, while in group I, the members this superphylum was present in significant numbers (14.1±0.8%). In group III fed T-2 toxin with the Zaslon 2+ additive, their number was very low (0.3±0.03%) compared to group I ($p \leq 0.001$). It is likely that the bacteria of the superphylum *Verrucomicrobiota* are most sensitive to the T-2 toxin compared to other intestinal microbiota. It is known [34] that *Verrucomicrobiota* bacteria synthesize many glycoside hydrolases for degradation of stable polysaccharides. Therefore, a sharp decrease in their content in the intestines of birds under the influence of T-2 toxin could be associated with inhibition of fiber digestion processes.

The most abundant microorganisms among the phylum *Firmicutes* were bacteria of the genus *Lactobacillus* spp. of the *Lactobacillaceae* family (from 11.1±0.72 to 30.7±1.84% depending on the experimental group) and Clostridia_UCG-014 of the Clostridia_UCG-014 family (from 17.6±0.91 to 25.5±1.34%. Microorganisms of the phylum *Verrucomicrobiota*, bacteria of the genus *Akkermansia* of the *Akkermansiaceae* family, were also widely represented in group I (14.1±0.82%) (Fig. 4, 5). For bacteria *Lactobacillus* spp. they have also previously been shown to constitute one of the predominant groups in the gastrointestinal tract of farm birds [35, 36].

An increase in the bacteria of the genus *Lactobacillus* from 15.9±1.32 to 30.7±1.84% ($p \leq 0.01$) in the intestines of birds from group III fed feed additive Zaslon 2+ compared to group I, probably was considered positive. These microorganisms are able to produce significant amounts of lactic and acetic acids, which lower the pH values in the gastrointestinal tract [37], compete with pathogens for

nutrients and epithelial sites for adhesion [38].

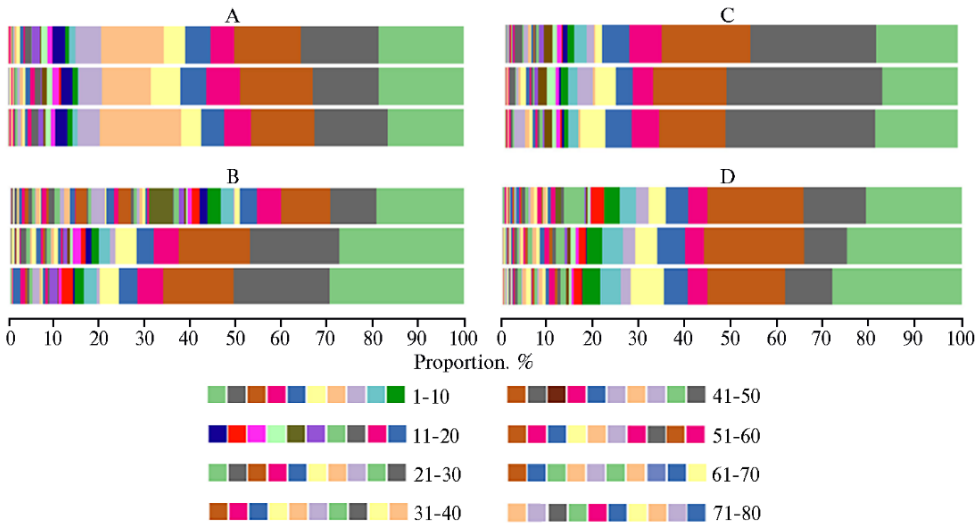

Fig. 4. The gut microbiome composition at the families levels (based on data of NGS sequencing of 16S rRNA gene amplicons) in broiler chickens (*Gallus gallus* L.) Smena 8 cross fed T-2 toxin, additive Zaslon 2+ (BIOTROF LLC, Russia) **and enzyme drug with proteolytic activity Axtra Pro** (DuPont de Nemours, Inc., USA)**:** 1-10 — Clostridia_UCG-014, *Lactobacillaceae*, *Ruminococcaceae*, *Lachnospiraceae*, *Oscillospiraceae*, *Rikenellaceae*, *Akkermansiaceae*, *Eubacterium coprostanoligenes* group, *Christensenellaceae*, RF39; 11-20 — *Erysipelatoclostridiaceae, Bifidobacteriaceae, Anaerovoracaceae, Butyricicoccaceae,* Clostridia vadinBB60 group, *Enterobacteriaceae*, *Clostridiaceae*, *Coriobacteriales*, *Gastranaerophilales*, *Peptococcaceae*; 21-30 — *Monoglobaceae*, *Erysipelotrichaceae*, *Veillonellaceae*, *Peptostreptococcaceae*, *Enterococcaceae*, *Chitinophagaceae*, *Desulfovibrionaceae*, *Prevotellaceae*, *Xanthomonadaceae*, *Pseudomonadaceae*; 31-40 — *Neisseriaceae*, *Anaerofustaceae*, *Corynebacteriaceae*, *Sphingomonadaceae*, *Actinomycetaceae*, *Leptotrichiaceae*, *Fusobacteriaceae*, *Carnobacteriaceae*, UCG-010, *Eggerthellaceae*; 41-50 — *Streptococcaceae*, *Selenomonadaceae*, *Flavobacteriaceae*, *Micrococcaceae*, *Campylobacteraceae*, *Bacillaceae*, *Saccharimonadales*, *Defluviitaleaceae*, *Xanthobacteraceae*, *Saccharimonadaceae*; 51-60 — *Izemoplasmatales*, *Oxalobacteraceae*, *Peptostreptococcales-Tissierellales*, *Spirochaetaceae*, *Propionibacteriaceae*, *Tannerellaceae*, *Gemellaceae*, *Lentimicrobiaceae*, *Atopobiaceae*, *Bacteroidaceae*; 61-70 — *Burkholderiaceae*, *Weeksellaceae*, *Pasteurellaceae*, *Porphyromonadaceae*, *Staphylococcaceae*, *Acholeplasmataceae*, *Alcaligenaceae*, JCI 0000069-P22, *Synergistaceae*, *Chloroplast*; 71-80 — *Sphingobacteriaceae*, *Cardiobacteriaceae*, *Caulobacteraceae*, *Coriobacteriaceae*, *Oscillospirales*, *Hungateiclostridiaceae*, *Mycoplasmataceae*, *Elusimicrobiaceae*, *Dysgonomonadaceae*, *Rhizobiaceae* (vivarium, FSC ARRTPI RAS, 2021). For a description of the groups, see the Materials and methods section.

The exact role of members of the genus Clostridia_UCG-014 is not completely clear due to the lack of ability to grow on laboratory media. According to C. Yang et al. [39], Clostridia_UCG-014 play a positive role for the macroorganism. The authors showed that in the human intestine against the background of ulcerative colitis and the use of sodium dextran sulfate, there was a violation of the composition of the microbiome, in particular, a decrease in the number of Clostridia_UCG-014. The use of turmeric polysaccharide led to the restoration of the microbiota composition and an increase in the content of these microorganisms. In our experiment, with introduction of T-2 toxin into the diet (group II), the abundance of Clostridia_UCG-014 increased compared to the control, which may indicate participation of Clostridia_UCG-014 in the initiation of dysbiotic disorders when the toxin was fed. Moreover, Clostridia_UCG-014 may be more resistant to T-2 toxin than other members of gut microbiota due to the ability to sporulate, high tolerance to acids and other aggressive substances [40, 41] and involvement into toxin detoxification.

Microorganisms of the genus *Akkermansia* represented by the only species *A. muciniphila* attract special attention. Their presence sharply decreased in all

groups with the introduction of T-2 toxin into the diet compared with group I ($p \leq 0.001$), up to a complete absence in groups II and IV (see Fig. 5). Decreased numbers of *A. muciniphila* in the gut can have negative consequences for poultry. The presence of *A. muciniphila* in the digestive system has been shown to be associated with a decrease in the abundance of pathogens, including *Salmonella pullorum* [42]. An increase in the proportion of *A. muciniphila* is associated with an increase in the processes of regeneration of the intestinal mucosa in chickens, which is accompanied by an increase in the number of goblet cells and an increase in mucin synthesis [43].

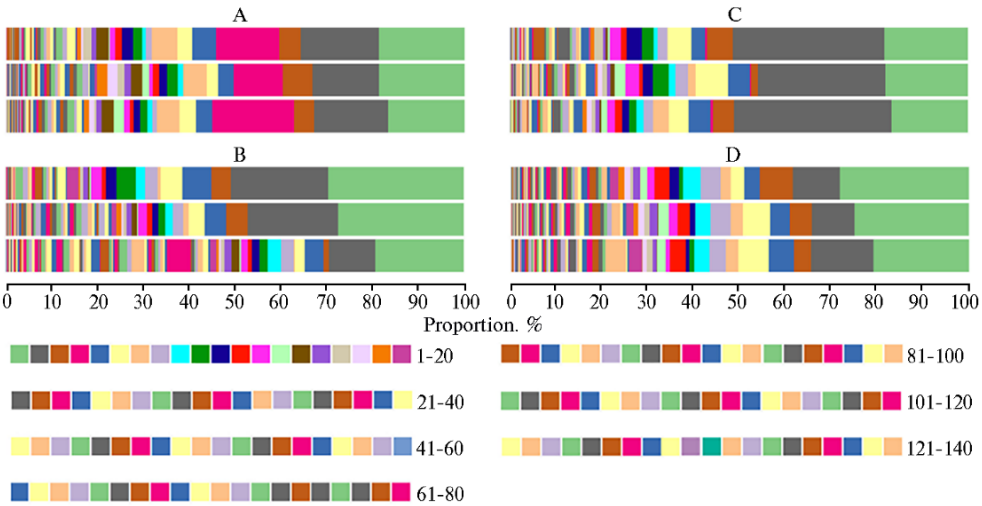

Fig. 5. The gut microbiome composition at the genera levels (based on data of NGS sequencing of 16S rRNA gene amplicons) in broiler chickens (*Gallus gallus* L.) Smena 8 cross fed T-2 toxin, additive Zaslon 2+ (BIOTROF LLC, Russia) **and enzyme drug with proteolytic activity Axtra Pro** (DuPont de Nemours, Inc., USA)**:** 1-20 — Clostridia UCG-014, *Lactobacillus*, *Alistipes*, *Akkermansia*, *Subdoligranulum*, *Faecalibacterium*, *Eubacterium coprostanoligenes* group, *Christensenellaceae* R-7 group, RF39, *Ruminococcus torques* group, UCG-005, *Fournierella*, *Erysipelatoclostridium*, *Bifidobacterium*, Clostridia vadinBB60 group, *Escherichia-Shigella, Clostridium* sensu stricto 1, *Blautia*, *Ruminococcus, Veillonella*; 21-40 — *Gastranaerophilales*, UCG-010, *Lachnoclostridium*, f. *Anaerovoracaceae* XIII AD3011 group, *Negativibacillus, Monoglobus*, *Butyricicoccus*, *Streptococcus*, *Romboutsia*, *Enterococcus,* f. *Anaerovoracaceae* XIII UCG-001, *Stenotrophomonas*, *Pseudomonas*, f. *Oscillospiraceae* V9D2013 group, *Erysipelotrichaceae*, *Eisenbergiella*, *Bilophila*, *Prevotella*, *Anaerofustis*, *Neisseria*; 41-60 — *Corynebacterium*, f. *Oscillospiraceae* NK4A214 group, *Sellimonas*, UCG-009, *Flavonifractor*, *Paludicola*, *Caproiciproducens*, *Actinomyces*, *Leptotrichia*, *Fusobacterium*, *Holdemania*, UCG-008, *Granulicatella*, *Gemella*, *Alloprevotella*, *Sphingomonas*, *Intestinimonas*, *Porphyromonas*, UC5-1-2E3, *Chryseobacterium*; 61-80 — *Ralstonia*, *Anaerotruncus*, *Gordonibacter*, *Staphylococcus*, *Anaerofilum*, *Capnocytophaga*, *Marvinbryantia*, *Haemophilus*, *Tyzzerella*, *Rothia*, *Campylobacter*, CHKCI001, *Kingella*, *Eubacterium hallii* group, *Oscillibacter*, *Saccharimonadaceae*, *Enterorhabdus*, *Anaerostipes*, *Defluviitaleaceae* UCG-011, *Gardnerella*; 81-100 — *Saccharimonadales*, *Selenomonas*, *Izemoplasmatales*, *Massilia*, *Candidatus* Soleaferrea, *Eubacterium nodatum* group, *Lautropia, Centipeda, Shuttleworthia, Treponema, Cutibacterium,* GCA-900066575, *Tannerella*, WPS-2, *Merdibacter*, *Peptococcus*, *Peptoniphilus*, *Lentimicrobium*, *Bergeyella*, *Atopobium*; 101-120 — *Aggregatibacter*, *Oscillospira*, *Frisingicoccus*, *Erysipelotrichaceae* UCG-003, *Catenibacillus*, *Vibrionimonas*, *Alcaligenes*, JGI 0000069-P22, *Johnsonella*, *Rikenellaceae* RC9 gut group, *Allorhizobium-Neorhizobium-Pararhizobium-Rhizobium*, *Megasphaera*, DTU014, *Papillibacter*, *Auricoccus-Abyssicoccus*, *Anaeroplasma*, *Bacteroides*, *Fretibacterium*, *Anaerococcus*, *Angelakisella*; 121-140 — *Tuzzerella*, *Stomatobaculum*, *Solobacterium*, *Chloroplast*, *Finegoldia*, *Lachnospira*, *Nubsella*, *Prevotellaceae* UCG-001, *Prevotellaceae* Ga6A1 group, WCHB1-41, *Cardiobacterium*, *Ezakiella*, *Filifactor*, *Hydrogenoanaerobacterium*, *Ureaplasma*, *Elusimicrobium*, *Dysgonomonas*, *Collinsella*, *Kocuria*, f. *Lachnospiraceae Incertae Sedis* (vivarium, FSC ARRTPI RAS, 2021). For a description of the groups, see the Materials and methods section.

Interestingly, in group II fed feed contaminated with T-2 toxin, pathogenic microorganisms appeared which were absent in group I. These are the *Enterococcus*

cecorum (causing gastroenteritis and diseases of the joints of birds) [44], *Campylobacter concisus* (the causative agent of gastroenteritis) [45], *Campylobacter gracili*s (gastrointestinal infections, including bacteremia which may occur primarily due to immunosuppression) [46], *Streptococcus gordonii* (infective endocarditis) [47], *Flavonifactor* spp. (bloodstream infections) [48]. This seems to be natural, since earlier Y. Li et al. [49] found that low concentrations of T-2 toxin alter the activation of Toll-like receptors, thereby reducing pathogen recognition and preventing the initiation of inflammatory immune responses against bacteria and viruses [49]. In groups III and IV, these pathogens were either absent or present in a significantly smaller amount than in group II ($p \leq 0.05$), which indicates a positive effect of the feed additive Zaslon 2+ and its complex with the enzyme on the quantitative composition of intestinal microbiota.

Interestingly, some taxa of intestinal microorganisms, mainly of the phylum *Firmicutes* which were present in birds from group I, completely disappeared in group II when food was contaminated with T-2 toxin. These were representatives of the genera *Marvinbryantia* (family *Lachnospiraceae*), UCG-008 (family *Butyricicoccaceae*), and V9D2013_group (family *Oscillospiraceae*). This probably could have a negative effect on the non-starch polysaccharides digestion and the synthesis of butyrate, since representatives of *Lachnospiraceae* and *Oscillospiraceae* belong to an important group of cellulolytic microorganisms [50, 51], while bacteria of the *Butyricicoccaceae* family are active producers of valuable butyric acid [52]. The abundance of *Eubacterium coprostanoligenes*, a representative of the *Firmicutes* phylum, also sharply decreased in group II vs. control (from 5.2 ± 0.28 to 0.68 ± 0.042 at $p \leq 0.001$) and increased vs. group I in group III ($p \leq 0.05$) when Zaslon 2+ was fed and in group IV when the enzyme + Zaslon 2+ were fed. *Eubacterium coprostanoligenes* belongs to the normoflora and produces valuable organic acids such as acetic, formic, and succinic [53].

Based on the results of reconstruction and functional annotation, we found 756 predicted metabolic pathways in the broiler gut microbial community, 163 of which showed differences ($p \leq 0.05$) between the experimental groups. These pathways were involved into protein metabolism (biosynthesis of amino acids, conversion of nitrogenous compounds), lipid metabolism (biosynthesis of lipids, oleate, palmitoleate, palmitate, stearate), carbohydrate metabolism (breakdown of complex polysaccharides such as chitin, degradation of glucose), energy metabolism (for example, Krebs cycle), into the synthesis of volatile fatty acids (in particular, propionic and butyric), nucleic acids, nucleotides and nucleosides, cofactors and coenzymes (tetrahydrofolate, acetyl-CoA, ubiquinols 7-10, heme), vitamins (biotin, thiamine diphosphate, menaquinols 6-13, dimethylmenaquinols 6, 8, 9, adenosylcobalamin), xenobiotic biodegradation, cell wall formation and spore formation (synthesis of peptidoglycan, teichoic acids), pathogenesis (synthesis of O-antigens, siderophores), biofilm formation. Of particular note is the fact that a significant number of predicted metabolic pathways that ensure the degradation of various organic substances, primarily amino acids and aromatic compounds (in particular, xenobiotics), differed between the experimental groups ($p \leq 0.05$). A similar trend was also found for metabolic pathways associated with the synthesis of cofactors and coenzymes (25 pathways), as well as vitamins (19 pathways) ($p \leq 0.05$).

When exposed to T-2 toxin (group II vs. group I), there was an increase in the following predicted metabolic pathways for the degradation of aromatic compounds, including xenobiotics: for PWY-5182 (toluene IV degradation) by 6.8 times ($p \leq 0, 05$), for PWY-5415 (degradation of catechol I) by 5.7 times ($p \leq 0.05$), for 3-HYDROXYPHENYLACETATEDEGRADATION-PWY (4-hydroxyphenylacetate degradation) by3.0 times ($p \leq 0.05$), and for PWY0-321 (degradation

of phenylacetate I) by 5.0 times ($p \leq 0.05$) (Fig. 6). The effects of probiotics [54] and bird age [55] on the metabolic potential of the gut microbiome have been previously studied. We did not find any reports on the effect of xenobiotics on the predicted metabolic pathways in the intestines of birds, animals, and humans in the available scientific literature. Nevertheless, the ability of many microorganisms to degrade various xenobiotics has long been known. Thus, the possibility of decomposition of phenylacetate, which is the main intermediate in the bacterial degradation of many aromatic compounds, was shown [56]. Microorganisms can oxidize phenylacetate under both aerobic and anaerobic conditions with the participation of the enzymes phenylacetate-CoA ligase, phenylacetyl-CoA 1,2-epoxidase, and oxygenase [57, 58]. It has also been demonstrated [59] that active processes of xenobiotic metabolism take place in the intestine with the participation of β-glucuronidase, nitroreductase, and sulfoxide reductase.

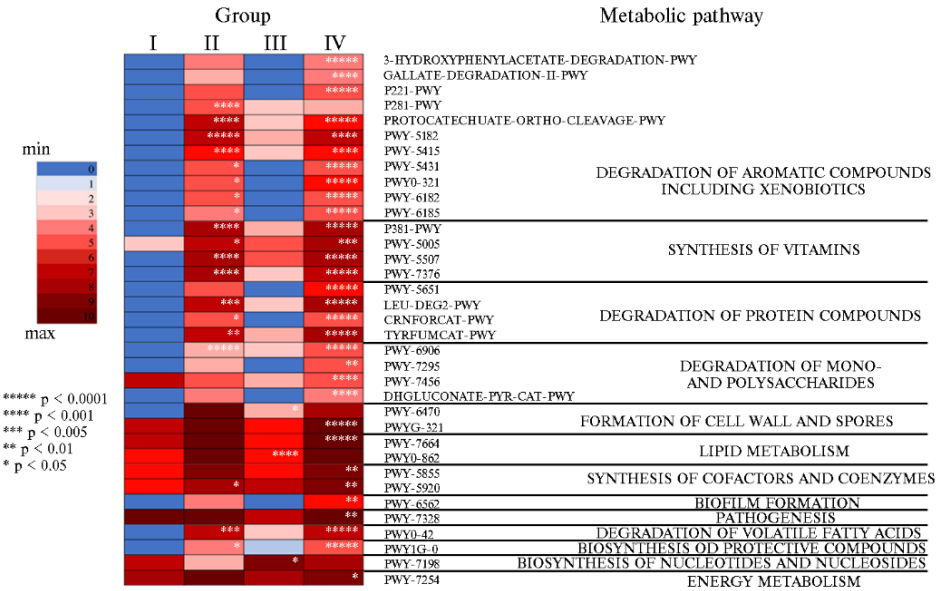

Fig. 6. Functional annotation of predicted metabolic pathways of caecum microbiome in broiler chickens (*Gallus gallus* L.) Smena 8 cross fed T-2 toxin, additive Zaslon 2+ (BIOTROF LLC, Russia) **and enzyme drug with proteolytic activity Axtra Pro** (DuPont de Nemours, Inc., USA) (vivarium, FSC ARRTPI RAS, 2021). The data were obtained using the PICRUSt2 software package (v.2.3.0). The MetaCyc database (https://metacyc.org/) was used to analyze metabolic pathways and enzymes. The scale reflects the intensity of potential metabolic pathways of the microbiome, blue is the lowest (minimum) intensity, red is the highest (maximum). For a description of the groups, see the Materials and methods section.

In our study, the Zaslon 2+ added to feed (group III) of broilers fed T-2 toxin led to a decrease in the potential of metabolic pathways for the degradation of aromatic compounds, including xenobiotics, compared with group II ($p \leq 0.05$). The activity of the PWY-5182 pathway decreased by 2.9 times, PWY-5415 by 4.1 times, 3-HYDROXYPHENYLACETATEDEGRADATION-PWY by 3.0 times, PWY0-321 by 5.0 times ($p \leq 0.05$). This effect may be associated with a decrease in the toxic load when the feed additive Zaslon 2+ is introduced into the diet. It is interesting that in group IV, when the enzyme preparation was added to the diet, there were no differences ($p > 0.05$) from group II in these metabolic pathways. That is, the introduction of protease reduced the effectiveness of the feed additive Zaslon 2+. When feed was contaminated with T-2 toxin, an increase in the potential of metabolic pathways for the degradation of such compounds important for birds as amino acids was observed ($p \leq 0.05$). In particular, in group

II vs. group I, the potential for LEU-DEG2-PWY (degradation of L-leucine I) increased 6.9-fold ($p \leq 0.05$), for PWY-5651 (degradation of L-tryptophan to semialdehyde 2-amino-3-carboxymuconate) 4.6-fold, for TYRFUMCAT-PWY (degradation of L-tyrosine I) 6.5-fold, and for CRNFORCAT-PWY (degradation of creatinine I) 6.9-fold. The enhancement of amino acid degradation pathways may be associated with the appearance of bacteria of the phylum *Fusobacteriota* in birds from groups II and IV. Members of this taxon are known to use amino acids as an energy source [60]. The Zaslon 2+ added to the diet of broilers which were fed T-2 toxin (group III) reduced ($p \leq 0.05$) the metabolic potential for amino acid degradation to the level of group I (without feed contamination with T-2 toxin). This is a positive effect, since amino acids serve as an important plastic material for protein synthesis and bird growth [61].

In addition, feed contamination with T-2 toxin (group II vs. group I) resulted in activation of the predicted metabolic pathway PWY0-42 (2-methylcitrate cycle I) leading to propionic acid (volatile fatty acid) degradation ($p \leq 0.05$). Organic acids, in particular propionic acid, are produced by representatives of the normal flora present in the intestine [62]. These substances may increase the performance of poultry by altering the pH of the digestive system and therefore altering the composition of the microbiome [63]. In particular, propionate causes acidification of the cytosol in pathogenic bacteria, dispersion of the proton-motive force, disruption of CoA homeostasis, and, in some cases, inhibition of the key enzymes of the tricarboxylic acid cycle aconitase and citrate synthase due to the formation of (2S,3S)-2-methylcitrate. In addition, organic acids are able to improve the morphology and physiology of the gastrointestinal tract [64]. However, some intestinal bacteria can degrade propionate and even use it as their sole carbon source. Of all the propionate degradation pathways, the 2-methylcitric acid cycle is the most widely used. In this pathway, the methylene group of calcium propionate is oxidized to the keto group with the formation of pyruvate, a common precursor for biosynthesis and energy generation. This pathway is well known in *Salmonella enterica enterica* serovar *Typhimurium* [65] and *E. coli* [66]. In these microorganisms, the pathway begins with the activation of propanoate to propanoyl-CoA by propionate-CoA ligase, followed by the synthesis of (2S,3S)-2-methylcitrate from propanoyl-CoA and oxaloacetate catalyzed by 2-methylcitrate synthase. Further, (2S,3S)-2-methylcitrate is dehydrated to cis-2-methylaconitate using 2-methylcitrate dehydratase, followed by rehydration to (2P,3S)-2-methylisocitrate and cleavage of the latter into pyruvate and succinate.

It also seems logical that contamination of feed with T-2 toxin led to the activation ($p \leq 0.05$) of the predicted metabolic pathways PWY-6562 (norspermidine biosynthesis) and PWY1G-0 (mycothiol biosynthesis) by 4.0 and 3.8 times, respectively. The polyamine norspermidine is known to be involved in the regulation of biofilm formation in microorganisms [67], in particular in *Vibrio cholera*, the causative agent of cholera with symptoms of diarrhea [68, 69]. Bacteria form biofilms as a survival mechanism, since such structures contribute to protection against pH extremes, osmotic stress, ultraviolet radiation, antimicrobials [70] and, in our study, probably T-2 toxin. An increase in the production of mycothiol against the background of food contamination with T-2 toxin in groups II and III compared with group I ($p \leq 0.05$) is also natural. Mycothiol is the main thiol contained in the cells of actinomycetes [71]. Probably, the activation of mycothiol production was associated with an increase ($p \leq 0.05$) in the abundance of *Actinobacteriota* superphylum bacteria in the intestines of birds from groups II and IV compared to control. In terms of functions, mycothiol is in many respects similar to glutathione, the dominant thiol in other bacteria, which is absent in actinobacteria.

This substance is involved in the detoxification of alkyl reducing agents, reactive oxygen and nitrogen species, antibiotics [72]. It also acts as a thiol buffer that has reducing properties and protects against disulfide stress [73].

Previously, it was noted [74, 75] that the reaction of birds to the introduction of proteases into diets is not always favorable. It has been shown [75] that despite an increase in amino acid digestibility in the ileum, growth and feed intake rates declined and feed conversion deteriorated. The authors [75) explained this by the difficulties in selecting the optimal dosages of proteases in each specific case. The introduction of protease at a dose of 160 mg/kg into the diet of broilers significantly reduced the activity of pancreatic trypsin. M. Mahagna et al. [76] reported that the introduction of amylase and protease preparations into the diet of sorghum and soybean meal significantly reduced the activity of its own amylase, chymotrypsin and trypsin in the intestinal contents of broilers. According to L. Liu et al. [31], proteolytic systems and selective proteolysis are considered as key regulators of tumor progression processes; proteolysis processes are directly related to the reactions of inflammation and tissue destruction.

Thus, with experimental contamination of feed with T-2 toxin (2-fold excess of MPC), in the intestines of broiler chickens, the biodiversity and composition of the microbiome changed already at the level of phyla. The abundance of bacteria of the superphylum *Actinobacteriota* and phylum *Proteobacteria* increased (by 1.8 and 3.5 times, respectively, $p \leq 0.05$), while the abundance of the superphylum *Desulfobacterota*, on the contrary, decreased (by 2.2 times, $p \leq 0.05$). With the introduction of the feed additive Zaslon 2+ into the diet in combination with the proteolytic enzyme preparation Axtra Pro, the abundance of the superphylum *Actinobacteriota* and the phylum *Proteobacteria* also increased compared to the control ($p \leq 0.05$), while the feed additive Zaslon 2+ used separately did not give such an effect. Representatives of the superphylum *Verrucomicrobiota* completely disappeared in the groups that received T-2 toxin without additives, as well as when using a complex of feed additive and enzyme, while in the control these microorganisms were present in a significant amount, 14.1±0.8%. The use of Zaslon 2+ alone increased the abundance of bacteria of the genus *Lactobacillus* vs. control from 15.9±1.32 to 30.7±1.84% ($p \leq 0.01$). With the introduction of T-2 toxin into the diet, the number of *Akkermansia muciniphila* sharply decreased ($p \leq 0.001$), and pathogenic microorganisms that were absent in the control appeared (*Enterococcus cecorum*, *Campylobacter concisus*, *Campylobacter gracilis*, *Streptococcus gordonii*, *Flavonifractor* spp.). When using a feed additive or its complex with an enzyme, these pathogens were either absent or present in a significantly smaller amount ($p \leq 0.05$). The test groups differed ($p \leq 0.05$) in 163 predicted metabolic pathways. Under the influence of T-2 toxin, metabolic pathways for the degradation of aromatic compounds (including xenobiotics), amino acids, the synthesis of coenzymes, cofactors, the formation of biofilms, cell walls, spores and protective substances increased 3.0-6.9-fold ($p \leq 0.05$) in cells. The feed additive Zaslon 2+ reduced the potential for metabolic degradation of aromatic compounds, including xenobiotics, and amino acids by 2.9-5.0 times. In general, the feed additive Zaslon 2+, as well as a complex of this additive with a proteolytic enzyme have a positive effect on the composition and potential functional activity of the intestinal microbiome in broiler chickens, and the feed additive without the enzyme is more effective.

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