EFFECTS OF GLYPHOSATE AND ANTIBIOTICS ON THE EXPRESSION OF GENES RELATED TO PERFORMANCE, ANTIOXIDANT PROTECTION AND HISTOLOGICAL BARRIER IN THE CECUM OF BROILERS

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Abstract. In conditions of intensive poultry farming, significant amounts of xenobiotics enter the bird's body, including glyphosate and antibiotics, however the effect this has on gene expression is currently under-studied. To investigate this, four groups of Ross 308 broiler chickens were formed: 1, control group fed the basic diet (BD); 2, experimental group fed BD supplemented with glyphosate; 3, experimental group fed BD along with combination of glyphosate and two antibiotics, enrofloxacin and colistin methanesulfonate. Analysis of the expression of genes for performance (*IGF1*, *IGF2*, *MYOG*, *MYOZ2*, *SLC2A1*, *SLC2A2*, *SLC5A1*, *MSTN* and *TGFB1*), antioxidant defense (*CAT*, *SOD1*, *PRDX6* and *HMOX1*) and histological barrier function (*MUC2*, *OCLN* and *CLDN1*) in cecal tissues of birds were carried out using quantitative RT-PCR using a DTlight thermal cycler (DNA-Technology, Russia) and the SsoAdvancedTM Universal

SYBR® Green Supermix kit (Bio-Rad, USA). The results showed that glyphosate alone (Group 2) inhibited the expression of a number of genes associated with productivity (*IGF1*, *IGF2*, *SLC5A1*, and *MSTN*) up to 4.1 times, while the expression of the *MYOZ2* and *SLC2A2* genes was stimulated up to 2.3 times as compared with Group 1 ($p < 0.05$). In Groups 2 and 3, there was a decrease in almost all cases (except for the expression of the *OCLN* gene in Group 3) in the mRNA production of the *MUC2*, *OCLN* and *CLDN1* genes (related to the synthesis of mucin, occludin and claudin) in intestinal tissues from 1.3 to 2.2 times as compared to the control ($p < 0.05$). Our results provide valuable information on the mechanisms underlying glyphosate toxicity in biological systems, as well as the levels of its interaction with the antibiotics used.

Keywords: glyphosate, antibiotics, caeca of the intestine, broilers, gene expression

1. Introduction

Glyphosate is a non-specific organophosphorus herbicide used for weed control to improve crop yields [1]. It is well estalbished that glyphosate is the most inexpensive and effective weed control technology available. The parent compound was first marketed in 1974 under the Monsanto brand name Roundup [1]. Glyphosate is the only existing herbicide whose mechanism of action is to interfere with the enzyme 5-enolpyruvyl shikimate-3-phosphate synthase (EPSPS) that, in turn, impedes the biosynthesis of aromatic amino acids through the shikimate pathway [2]. This pathway is absent in animals and humans, which explains the widespread use of glyphosate in agriculture. However, the increasing presence of glyphosate in the environment has attracted the attention of the scientific community due to its possible hazards to non-target organisms. For example, it was noted that inhibition of EPSPS by glyphosate negatively affects the life support pathways of soil microorganisms [3]. There is a growing evidence supporting direct and indirect adverse effects of heavy glyphosate application on animals [4]. The detection of

glyphosate in human urine demonstrated the penetration of this herbicide through the food chain, thereby initiating a large body of research aimed at testing the presence of glyphosate toxicity in humans [5; 6]. Glyphosate intoxication has been reported to cause cardiovascular shock, hemodynamic compromise, intravascular coagulation, myocardial infarction, and multiple organ failure [7].

Poultry rearing systems also use significant amounts of other types of xenobiotics, including veterinary preparations and, primarily, antibiotics. Antibiotics are still used for both preventive and therapeutic purposes [8]. The effects resulting from the combined impact of antibiotics and glyphosate can cause unpredictable changes in organisms at all levels of biological organization [9; 10]. Therefore, studies using the analysis of mRNA expression of intestinal tissues as an important metabolic center in terms of health effects are the first step towards analyzing the mechanisms of the effect of glyphosate and antibiotics on the body of poultry birds. In addition, intestinal tissues not only play a role in the absorption and metabolism of xenobiotics [11], but also directly contact the microbiota that can be altered by glyphosate and antibiotics, thereby modulating gene expression. On the other hand, understanding the mechanisms of mRNA expression changes could help in the development of sensitive and accurate diagnostic tools for assessing toxic effects, thereby facilitating more efficient management of the poultry industry [12].

In general, the effect of both glyphosate itself and glyphosate in combination with antibiotics on the transcriptome of such an important organ as the intestine has been poorly studied in all animals, and particualrly birds. There are only a few studies on this issue. For instance, the effect of glyphosate on the gene expression of farm animals [13] and glyphosate in combination with antibiotics on the gene expression of poultry [14] was previously shown.

The purpose of the present study was to establish changes in the expression spectrum of genes for performance, antioxidant protection, and physiological barrier function in the cecal tissues of broilers under the influence of glyphosate. The amount of 1 maximum residue limit for food products (1 MRL), i.e., at the concentration of 20 mg/kg feed [15], and glyphosate in combination with veterinary antibiotics was used.

2. Materials and Methods

The current investigation was performed in accordance with the guidelines set forth by the European Convention for the Protection of Vertebrate Animals used for Research and Other Purposes (ETS No. 123, Strasbourg, 1986). Also, it was carried out in compliance with Russian Federation ethics laws, namely Russian Federal Law No. 498-FZ on Responsible Treatment of Animals and approved by the bioethical panel of the L.K. Ernst Federal Research Center for Animal Husbandry.

The experiment was conducted in a vivarium located in the village of Fedorovskoye, Tosnensky District, Leningrad Oblast, in 2023, using Ross 308 broilers (e.g., [16]) aged from 1 day to 40 days. Three groups were selected according to the principle of analogues, with 65 animals in each group: 1, control group fed the basic diet (BD); 2, experimental group fed BD along with the introduction of glyphosate at the concentration of 20 mg/kg feed [15]; 3, experimental group fed BD along with the administration of glyphosate (20 mg/kg feed), as well as the antibiotics enrofloxacin and colistin methanesulfonate. These antibiotics are usually implemented to prevent bacterial infections. Feeding and housing conditions met the requirements for the Ross 308 broiler cross [17]. To feed birds at 1 to 4 weeks of age, complete feed PK5-1G-1101 was used, and then, until the end of the experiment, chickens were fed complete feed PK-6-G-1102. The manufacturer of both types of feed is CJSC Gatchina Feed Mill (Leningrad Oblast, Russia). Broilers of all groups were kept in three-tier cages consisting of BB-1 blocks (NPO Stimul-INK, Moscow Oblast, Russia).

For artificial contamination of the feed in experimental Groups 2 and 3, the Agrokiller drug formulation (JSC August, Moscow, Russia), which contains 500 g/l glyphosate acid in the form of an isopropylamine salt, was used. The working solution of the drug was applied to the feed using the spray method, mixing mechanically in compliance with personnel safety requirements. Glyphosate concentrations in feeds were measured by enzyme-linked immunosorbent assay (ELISA; e.g., [18]) using a Stat Fax 303+ photometer (Awareness Technology, Inc., Palm City, FL, USA) and a GLY ELISA Microtiter Plate (Eurofins Abraxis, Warminster, PA, USA). The diet of control

group broilers contained virtually no background traces of glyphosate. The antibiotic enrofloxacin was added to drinking water in the form of the Enroflon 10% solution for oral use (NPK-VIK LLC, Russia) in the amount of 0.5 ml per 1 liter of water on Days 0 to 10. The antibiotic colistin methanesulfonate was added into water in the form of the Colistin 2 Million drug (developer: Areal Medical LLC; manufacturer: AVZ-SP, Russia) in the amount of 0.25 ml per 1 liter of water on Days 33 to 37.

On the 40th day of growing, three broilers from each group were euthanized, and cecal tissues were immediately collected under the maximum possible aseptic conditions for analysis of mRNA expression. The samples were stabilized using the RNAlater reagent (Thermo Fisher Scientific, Inc., USA) and immediately sent for RNA isolation to the molecular genetic laboratory of the Research and Production Company BIOTROF+ LLC.

To isolate RNA, tissues were mixed with liquid nitrogen and homogenized. Total RNA was isolated using the Aurum^{TM} Total RNA mini kit (Bio-Rad, USA), following the manufacturer's instructions. The reverse transcription reaction was carried out to obtain cDNAs on an RNA template using iScriptTM Reverse Transcription Supermix (Bio-Rad, USA). Gene expression analysis was performed using a quantitative RT-PCR, a DTlight amplifier (DNA-Technology, Russia) and the SsoAdvanced^{TM} Universal SYBR$^{®}$ Green Supermix kit (Bio-Rad, USA) in accordance with the manufacturer's protocol. The specific PCR primers were utilized for analysis of mRNA expression as shown in Table 1. The amplification mode and conditions were as follows [19; 20]: 5 min at 95 °C (preheating); 30 s at 95 °C, 30 s at 60 °C, 30 s at 70 °C (40 cycles). Relative expression was assessed using the $2^{-\Delta\Delta CT}$ method [21].

Table 1. Primers for analysis of mRNA expression in cecal tissues broiler cross Ross 308.

Gene and protein/enzyme produced	**Primer sequence (5′→3′)**
Genes associated with barrier function of the digestive system	
MUC2, mucin 2, oligomeric mucus/gel-	F: CTGGCTCCTTGTGGCTCCTC R: AGCTGCATGACTGGAGACAACTG

forming	
OCLN, occludin	F: ACGGCAGCACCTACCTCAA R: GGGCGAAGAAGCAGATGAG
CLDN1, claudin 1	F: CATACTCCTGGGTCTGGTTGGT R: GACAGCCATCCGCATCTTCT
Genes associated with antioxidant defense	
CAT, catalase	F: ACCAAGTACTGCAAGGCGAA R: TGAGGGTTCCTCTTCTGGCT
SOD1, superoxide dismutase 1, soluble	F: CGGGCCAGTAAAGGTTACTGGAA R: TGTTGTCTCCAAATTCATGCACATG
PRDX6, peroxiredoxin 6	F: GCATCCGCTTCCACGACTTCCT R: CCGCTCATCCGGGTCCAACAT
HMOX1, heme oxygenase 1	F: GGTCCCGAATGAATGCCCTTG R: ACCGTTCTCCTGGCTCTTGG
Genes associated with productivity	
IGF1, insulin like growth factor 1	F: GCTGCCGGCCCAGAA R: ACGAACTGAAGAGCATCAACCA
IGF2, insulin like growth factor 2	F: GGCGGCAGGCACCATCA R: CCCGGCAGCAAAAAGTTCAAG
MYOG, myogenin	F: GGAGAAGCGGAGGCTGAAG R: GCAGAGTGCTGCGTTTCAGA
MYOZ2, myozenin 2	F: CAACACTCAGCAACAGAGGC R: GTATGGGCTCTCCACGATTTCT
SLC2A1, solute carrier family 1 member 1 (glucose transporter 2)	F: AGATGACAGCTCGCCTGATG R: GTCTTCAATCACCTTCTGCGG
SLC2A2, solute carrier family 2 member 2 (glucose transporter 2)	F: GGAGAAGCACCTCACAGGAA R: CAGGCTGTAACCGTACTGGA
SLC5A1, solute carrier family 5 member 1 (sodium-dependent glucose transporter)	F: AGCATTTCAGCATGGTGTGTCTTC R: GATGCTCCTATCTCAGGGCAGTTC
MSTN, myostatin	F: ATGCAGATCGCGGTTGATC R: GCGTTCTCTGTGGGCTGACT

TGFB1, transforming growth factor beta 1 (transforming growth factor beta 4; [22])	F: CGGCCGACGATGAGTGGCTC R: CGGGGCCCATCTCACAGGGA
Gene used as reference control	
ACTB, beta actin	F: CTGTGCCCATCTATGAAGGCTA R: ATTTCTCTCTCGGCTGTGGTG

The zootechnical analyses were carried out in accordance with the established recommendations [17]. Using the body weight (BW) differential between the end and the beginning of a given rearing period, body weight growth (BWG) was calculated on a weekly and individual basis. The survival rate (SR) was defined as the percentage difference between the beginning number of chicks and the number of chickens that lived to the end of the rearing process. The ratio of total feed to total BWG was used to calculate the feed conversion rate (FCR). The number (given as a percentage) of birds weighed at 35 days of age with a BW within ±15% of the average value was used to compute the coefficient of flock uniformity by BW (CV). The algorithm below was utilized to determine the European Productivity Index (EPI; [23; 24]):

$$EPI = \frac{BW \times SR}{PP \times FCR},$$

where BW stands for body weight in kilograms, SR is survival rate expressed in percentage, PP is the production time expressed in days, and FCR is feed conversion rate.

Using Microsoft Excel XP/2003 and RStudio (Version 1.1.453; [25]), multivariate analysis of variance (multi-factor ANOVA) was used to process the results mathematically and statistically. The means (M) and standard errors of the mean (± SEM) were displayed as the results. The Student's *t*-test was used to determine the significance of the differences, and a difference was deemed statistically significant at $p < 0.05$. Tukey's Significant Difference Test (HSD) and TukeyHSD function in the R Stats Package's (RDocumentation) were used to compare the means.

3. Results and Discussion

The findings of the experimental changes in zootechnical indicators for Ross 308 broiler chickens during the course of their weekly raising periods are displayed in Table 2. We did not observe any pronounced changes in the zootechnical parameters of broilers under the influence of glyphosate alone or in combination with antibiotics. In broilers at the age of 7 days, there was a slight increase in BW from 134.1±1.83 kg in Group 1 to 141.2±1.94 kg in Group 2 ($p < 0.05$). This may be due to the activation of the bird's protective reserves when glyphosate entered the body. However, noteworthy was the increase in CV to 13% in Group 2, which may have negative consequences for the effective management of the poultry industry. It is much easier to maintain a homogeneous (by BW) population than a heterogeneous one since most birds are in the same physiological state and will show similar responses to changes in feeding regimes or microclimate. A flock is considered homogeneous if the bird has BW within ±10% of the mean value [26]. The introduction of glyphosate had a negative impact on the EPI indicator that is used in international poultry farming practice [27]. The use of veterinary antibiotics for preventive purposes in Group 3 allowed not only to compensate for the negative effects of glyphosate, but also to achieve a higher result than in Group 1.

Table 2. Changes in zootechnical parameters in response to glyphosate, as well as combinations of glyphosate and antibiotics intake by Ross 308 broiler chickens in Groups 1–3

Zootechnical characteristics	**Groups**		
	1	**2**	**3**
Survival rate, %	98.5	96.9	100.0
Body weight (BW) at 1 day of age, g*	43.7±0.31	43.1±0.45	42.8±0.30
BW at 7 days of age, g*	134.1±1.83	141.2±1.94**	134.7±1.80

BW at 14 days of age, g*	349.8±6.54	366.0±9.19	352.8±9.07
BW at 21 days of age, g*	756.8±19.31	771.9±18.00	760.7±24.16
BW at 28 days of age, g*	1311.2±27.20	1324.8±32.93	1258.6±26.17
BW at 35 days of age, g*	2087.4±36.82	2044.9±35.24	2053.7±39.41
BW at 40 days of age, g*	2567.9±41.92	2514.1±52.32	2506.0±41.45
Coefficient of flock uniformity, %	11	13	11
BW of females at 40 days of age, g*	2468.7±46.45	2427.8±53.68	2404.3±43.75
BW of males at 40 days of age, g*	2698.4±65.18	2781.7±97.63	2753.1±49.91
Feed conversion rate	1.866	1.933	1.796
European Productivity Index	339	315	349

Note: *Presented as mean value (± SEM); **Significant difference as compared to Group 1 at $p < 0.05$ (as estimated by the Student's *t*-test).

It is worth noting that increasing meat productivity is the main task of broiler poultry farming that can be achieved through improving FCR and reducing waste and keeping in mind meat quality (taste, etc.), animal welfare and environmental concerns [28-33]. Growth rates are controlled by complex sets of genes [34-37]. Herewith, the intestinal mucosa functions in almost permanent contact with the feed (and the toxicants contained in it) and directly responds to signals from the intestinal environment. The impact of this interaction on the host can be enormous, and the mechanism may involve changes in gene expression [38]. As we showed by quantitative RT-PCR, glyphosate alone (Group 2) inhibited the expression of a number of genes associated with productivity (*IGF1*, *IGF2*, *SLC5A1*, and *MSTN*) up to 4.1 times, while it stimulated the expression of the *MYOZ2* and

SLC2A2 genes up to 2 .3 times as compared to Group 1 ($p < 0.05$). A similar pattern of some stimulation of the expression of some genes (*MYOZ2* by 2.0 times) associated with productivity and inhibition of others (*IGF2* and *SLC5A1* by 4.6 and 2.2 times, respectively) was observed in the group using antibiotics in combination with glyphosate (Group 3) as compared to control ($p < 0.05$). It has previously been shown that downregulation of insulin-like growth factors 1 and 2 (*IGF1* and *IGF2*) can have negative consequences for birds, especially in the case of reduced resistance or when exposed to stress factors, since these genes are known to be one of the most promising candidate genes for assessing growth performance and carcass quality in chickens [39]. However, in the group with the introduction of antibiotics (Group 3), the expression of some productivity genes was slightly different from Group 2: changes in *SLC2A2* and *MSTN* mRNA expression were not observed relative to Group 1, whereas in Group 2 there were deviations in the expression of these genes relative to the control ($p < 0.05$), which was described above. This could be related to the positive effect of antibiotics on EPI. In our opinion, the effect of antibiotics in broilers on the expression of genes, some of which act as important vitagenes [40], may be associated with modulation of the microbiome, antioxidant system and redox balance in the intestine [41-45]. Previous studies have shown that various feed antibiotics can enrich the cecum with butyrate-producing bacteria [46]. Robinson et al. [47] noted that tylosin and enramycin increased the abundance of Ruminococcaceae, while salinomycin and monensin decreased the abundance of this family. In rodents, intestinal colonization with *Bifidobacterium infantis* or *Faecalibacterium prausnitzii* stimulated the production of $Foxp3^{+}$ regulatory T-cells and interleukin-10 [48]. In broiler chickens [49], succession of the gut microbiota over time has been associated with different immune gene expression profiles in the ileum. *In vitro* human studies with monocytes and neutrophils have shown that antimicrobials can either stimulate or inhibit cytokine mRNA levels [50]. This study and several others suggest that interventions that change the quantity or quality of gut bacteria will affect gene expression in gut tissue.

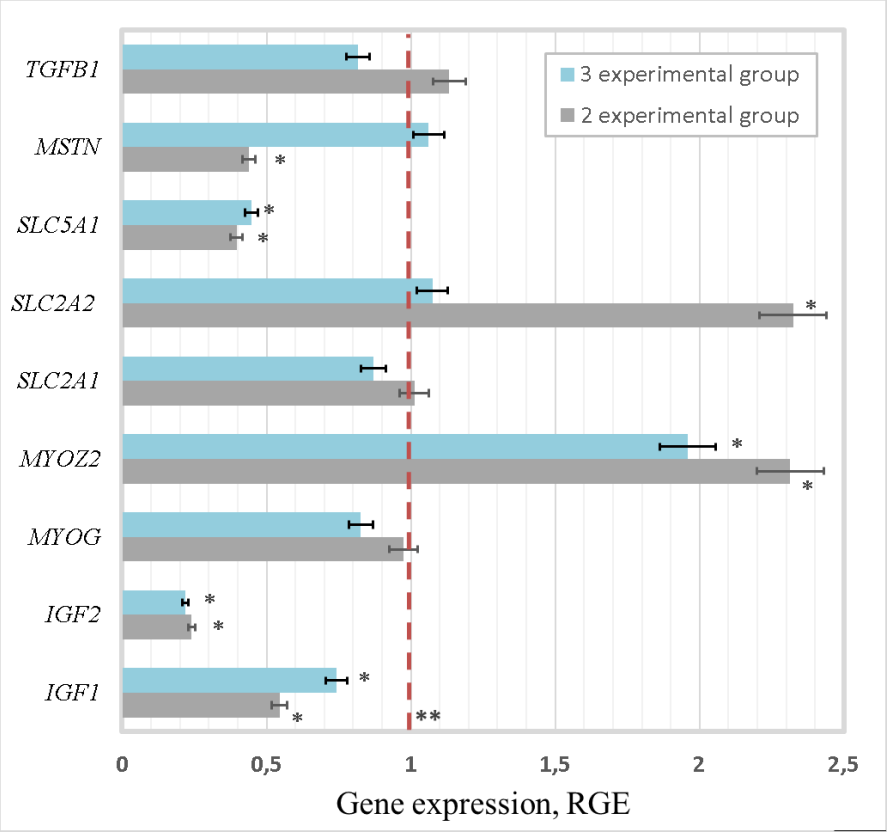

Fig. 1. Expression of mRNA of performance-related genes in the caecum of Ross 308 broilers in response to administering glyphosate and antibiotics. *Differences from Group 1 were statistically significant at $p < 0.05$; RGE is the relative gene expression (fold change) as compared to Group 1, in which expression was set to 1 (**dashed red line conforms to expression in control)

There was a pronounced stimulating effect of both glyphosate alone (Group 2) and glyphosate with antibiotics (Group 3) on the expression of the *HMOX1* gene associated with antioxidant protection, by 4.1 and 4.2 times, respectively, as compared with Group 1 ($p < 0.05$; Fig. 2A). This seems logical, since a previous study of the Roundup impact on human cell cultures revealed an increase in the activity of cytochrome P450 (CYP450), as well as other proteins associated with antioxidant protection and the function of xenobiotic detoxification [51].

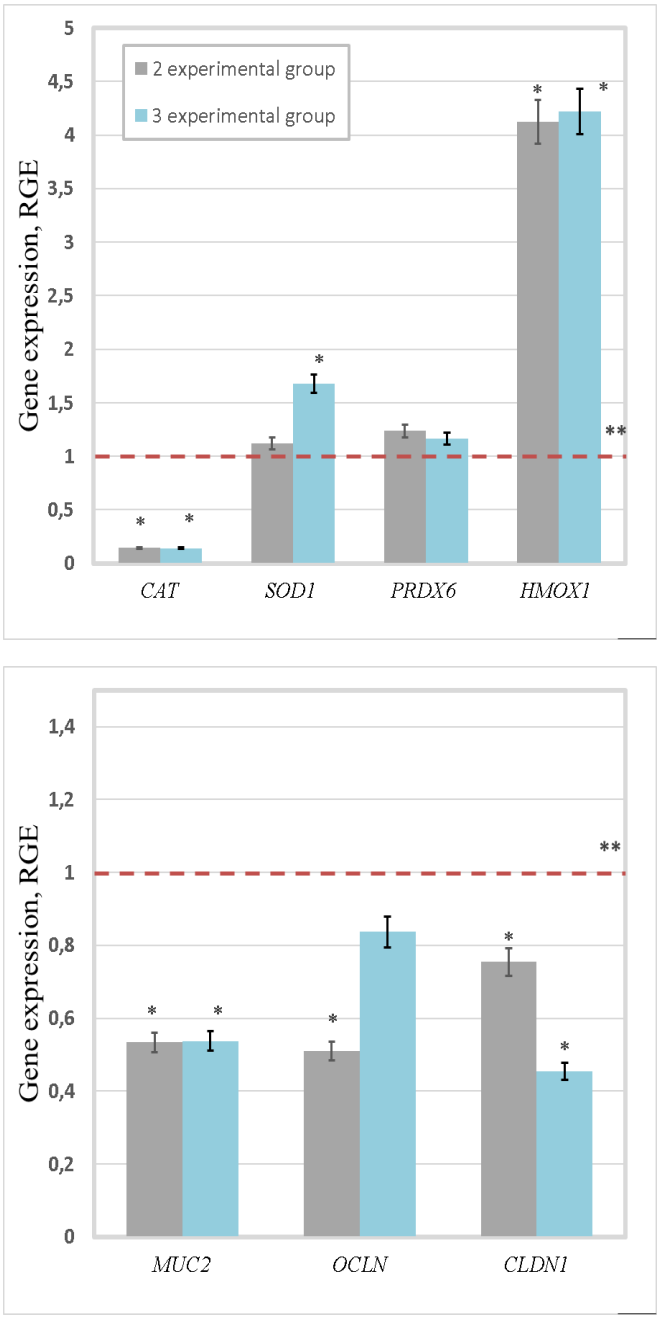

Fig. 2. Expression of mRNA of genes associated with antioxidant activity (**A**) and epithelial barrier function (**B**) in the cecum of Ross 308

broilers in response to administering glyphosate and antibiotics. *Differences from Group 1 were statistically significant at $p < 0.05$; RGE is the relative gene expression (fold change) as compared to Group 1, in which expression was set to 1 (**dashed red line conforms to expression in control)

The barrier function of the intestine is also very important for living organisms, as it is the first line of defense against pathogens and xenobiotics. Tight junction proteins (occludin and claudin) are associated with epithelial cells and act as a barrier to prevent macromolecular translocation [52]. Mucin is an intestinal mucus protein that plays an important role in protecting epithelial surfaces from pathogens by maintaining colonization by commensal bacteria, a suitable environment for digestion, and facilitating the transport of nutrients from the lumen to the underlying epithelium [53]. Our results showed that in Groups 2 and 3 there was downregulation in almost all cases (except for the expression of the *OCLN* gene in Group 3) in the mRNA of the *MUC2*, *OCLN* and *CLDN1* genes (encoding mucin, occludin and claudin, respectively) in intestinal tissues from 1.3 to 2.2 times as compared to control ($p < 0.05$), which may increase intestinal permeability to pathogens and toxins. Similarly to glyphosate impact, *Salmonella* infection also downregulated the expression of occludin and claudin genes in the ileum and jejunum of broiler chickens and decreased intestinal barrier function [54].

4. Conclusion

We observed a negative change in CV and EPI in broilers under the influence of glyphosate. The use of veterinary antibiotics for preventive purposes made it possible not only to compensate for the negative effect of glyphosate in terms of EPI, but also to achieve a higher result than in the group without additives. Changes in zootechnical parameters were preceded by deviations in the expression of some genes for performance, antioxidant protection and mucosal barrier function in the caeca. The results provide valuable information on the mechanisms underlying glyphosate toxicity in biological systems, as well as the levels of its interaction with used antibiotics. The findings could

be used to prevent adverse health effects potentially associated with exposure to toxic xenobiotics in birds, as well as animals and humans.

Since we did not observe the effect of glyphosate on changes in BW of birds, continued research in this direction is required. It may be necessary to increase the glyphosate dosage using the MRL for poultry feed rather than human food, or use additional stressors such as heat stress or a new crop grain as a component of the diet at the same or higher glyphosate dosage.

RNA biomarkers currently are recognized as useful tools for assessing the effects of pollutants on organisms. They are also potential tools for identifying the effects of xenobiotics on the body and developing responses at various levels of biological organization. Molecular biomarkers that can be developed based on our findings and future experiments have the potential to provide early detection of toxicological stress and relatively effective monitoring of flock health.

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