Chapter 28
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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Contents

Abstract In conditions of intensive poultry farming, significant amounts of xenobiotics enter the bird's body. 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

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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 as compared with Group 1 ($p < 0.05$). In Groups 2 and 3, there was a decrease in almost all cases in the mRNA production of the *MUC2*, *OCLN* and *CLDN1* genes in intestinal tissues from 1.3 to 2.2 times as compared to the control ($p < 0.05$).

28.1 Introduction

Glyphosate is a herbicide used for weed control to improve crop yields [1]. Glyphosate molecules block the shikimate pathway in plant cells, a chain of biochemical reactions leading to the synthesis of aromatic amino acids [2]. This pathway is absent in animals and humans, which explains the widespread use of glyphosate in agriculture. However, the increasing presence of this herbicide in the environment has attracted the attention of the scientific community due to its possible hazards to non-target organisms [3]. 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 [4].

Poultry rearing systems also use significant amounts of other types of xenobiotics, including veterinary preparations and, primarily, antibiotics. The effects resulting from the combined impact of antibiotics and glyphosate can cause unpredictable changes in organisms at all levels of biological organization [5]. In addition, intestinal tissues not only play a role in the absorption and metabolism of xenobiotics, but also directly contact the microbiota that can be altered by glyphosate and antibiotics, thereby modulating gene expression [6]. 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.

The purpose of our 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.

28.2 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

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 (Leningrad Oblast), in 2023, using Ross 308 broilers aged from 1 to 40 day. 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; (3) experimental group fed BD along with the administration of glyphosate (20 mg/kg feed), as well as the antibiotics enrofloxacin and colistin methanesulfonate. Feeding and housing conditions met the requirements for the Ross 308 broiler cross [7].

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. Glyphosate concentrations in feeds were measured by enzyme-linked immunosorbent assay (ELISA; e.g.,) 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 L of water on Days 0–10. 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 L of water on Days 33–37.

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). To isolate RNA, tissues were mixed with liquid nitrogen and homogenized. Total RNA was isolated using the Aurum™ 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™ 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 28.1. The amplification mode and conditions were as follows: 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 [8–10].

The zootechnical analyses [the body weight (BW), the body weight growth (BWG), the survival rate (SR), the feed conversion rate (FCR), the coefficient of flock uniformity by BW (CV) and the European Productivity Index (EPI)] were carried out in accordance with the established recommendations [10, 11].

Table 28.1 Primers for analysis of mRNA expression in cecal tissues broiler cross Ross 308

Gene and enzyme produced	Primer sequence (5′ → 3′)
Genes associated with barrier function of the digestive system	
MUC2, mucin 2	F: CTGGCTCCTTGTGGCTCCTC R: AGCTGCATGACTGGAGACAACTG
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, glucose transporter 2	F: AGATGACAGCTCGCCTGATG R: GTCTTCAATCACCTTCTGCGG
SLC2A2, glucose transporter 2	F: GGAGAAGCACCTCACAGGAA R: CAGGCTGTAACCGTACTGGA
SLC5A1, sodium-dependent glucose transporter	F: AGCATTTCAGCATGGTGTGTCTTC R: GATGCTCCTATCTCAGGGCAGTTC
MSTN, myostatin	F: ATGCAGATCGCGGTTGATC R: GCGTTCTCTGTGGGCTGACT
TGFB1, transforming growth factor beta 4	F: CGGCCGACGATGAGTGGCTC R: CGGGGCCCATCTCACAGGGA
Gene used as reference control	
ACTB, beta actin	F: CTGTGCCCATCTATGAAGGCTA R: ATTTCTCTCTCGGCTGTGGTG

Using Microsoft Excel XP/2003 and RStudio (Version 1.1.453), 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.

28.3 Results and Discussion

We did not observe any pronounced changes in the zootechnical parameters of Ross 308 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 ($p < 0.05$) (Table 28.2). 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. A flock is considered homogeneous if the bird has BW within ±10% of the mean value. The introduction of glyphosate had a negative impact on the EPI indicator that is used in international poultry farming practice [12]. 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.

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 0.3 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

Table 28.2 Changes in zootechnical parameters of Ross 308 broiler chickens in Groups 1–3

Zootechnical characteristics	Group 1	Group 2	Group 3
Survival rate, %	98.5	96.9	100.0
BW at 7 days of age, g*	134.1 ± 1.83	141.2 ± 1.94**	134.7 ± 1.80
BW at 21 days of age, g*	756.8 ± 19.31	771.9 ± 18.00	760.7 ± 24.16
BW at 40 days of age, g*	2567.9 ± 41.92	2514.1 ± 52.32	2506.0 ± 41.45
CV, %	11	13	11
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)

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 [13]. 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 [14], may be associated with modulation of the microbiome, antioxidant system and redox balance in the intestine [14]. In broiler chickens [15, 16], the digestive microbiome composition was associated with mRNA levels of immune genes in ileal tissue. 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. 28.1).

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. 28.2a). 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) [17].

Tight junction proteins (occludin and claudin) are associated with epithelial cells and act as a barrier to prevent macromolecular translocation. 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

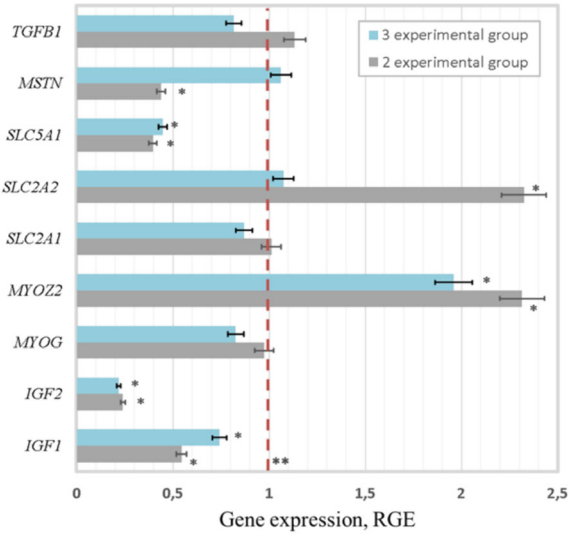

Fig. 28.1 Expression of mRNA of performance-related genes in the caecum of Ross 308 broilers. *Differences from Group 1 were statistically significant at $p < 0.05$; RGE is the relative gene expression (fold change) as compared to Group 1, in which expression was set to 1 (**dashed red line conforms to expression in control)

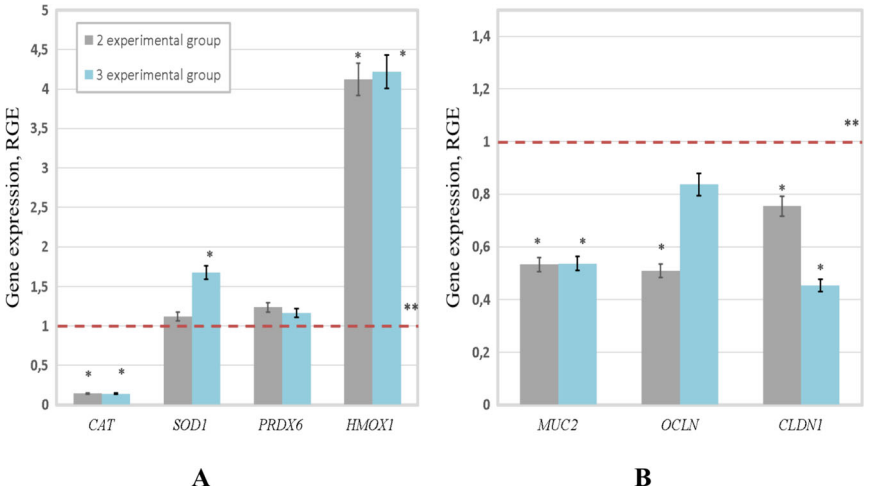

Fig. 28.2 Expression of mRNA of genes associated with antioxidant activity (**a**) and epithelial barrier function (**b**) in the cecum of Ross 308 broilers. *Differences from Group 1 were statistically significant at $p < 0.05$; RGE is the relative gene expression (fold change) as compared to Group 1, in which expression was set to 1 (**dashed red line conforms to expression in control)

tissues from 1.3 to 2.2 times as compared to control ($p < 0.05$), which may increase intestinal permeability to pathogens and toxins.

28.4 Conclusion

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.

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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References

1. Singh, S., Kumar, V., Datta, S., Wani, A.B., Dhanjal, D.S., Romero, R., Singh, J.: Glyphosate uptake, translocation, resistance emergence in crops, analytical monitoring, toxicity and degradation: a review. Environ. Chem. Lett. **18**, 663–702 (2020). https://doi.org/10.1007/s10311-020-00969-z
2. Haslam, E.: The Shikimate pathway. In: Barton, D., Kirby, G. (eds.) Biosynthesis of Natural Products Series. Butterworths, London (2014). https://doi.org/10.1016/C2013-0-04212-0
3. Martins-Gomes, C., Silva, T.L., Andreani, T., Silva, A.M.: Glyphosate versus glyphosate-based herbicides exposure: a review on their toxicity. J. Xenobiot. **12**, 3 (2022). https://doi.org/10.3390/jox12010003
4. Costas-Ferreira, C., Durán, R., Faro, L.R.F.: Toxic effects of glyphosate on the nervous system: a systematic review. Int. J. Mol. Sci. **21**, 4605 (2022). https://doi.org/10.3390/ijms23094605
5. Yildirim, E.A., Laptev, G.Y., Tiurina, D.G., Gorfunkel, E.P., Ilina, L.A., Filippova, V.A., Dubrovin, A.V., Brazhnik, E.A., Novikova, N.I., Melikidi, V.K., Kalitkina, K.A., Ponomareva, E.S., Griffin, D.K., Romanov, M.N.: Investigating adverse effects of chronic dietary exposure to herbicide glyphosate on zootechnical characteristics and clinical, biochemical and immunological blood parameters in broiler chickens. Vet. Res. Commun. **48**, 153–164 (2024). https://doi.org/10.1007/s11259-023-10195-x
6. Fu, Z.D., Selwyn, F.P., Cui, J.Y., Klaassen, C.D.: RNA-Seq profiling of intestinal expression of xenobiotic processing genes in germ-free mice. Drug Metabol. Disposit. **45**(12), 1225–1238 (2017). https://doi.org/10.1124/dmd.117.077313
7. Egorov, I.A., Manukyan, V.A., Lenkova, T.N., Okolelova, T.M., Lukashenko, V.S., Shevyakov, A.N., Ignatova, G.V., Egorova, T.V., Andrianova, E.N., Rozanov, B.L., Lysenko, M.A., Egorova, T.A., Grozina, A.A., Laptev, G.Y., Nikonov, I.N., Alexandrova, I.L., Ilina, L.A., Novikova, N.I.: Methodology for scientific and production research on poultry feeding. Molecular genetic methods for determining gut microflora. In: Fisinin, V.I. (ed.) Russian Academy of Agricultural Sciences, State Scientific Institution All-Russian Research and Technological Institute of Poultry Farming of the Russian Agricultural Academy. Sergiev Posad, Russia (2013). https://www.elibrary.ru/item.asp?id=21548916. Accessed 14 Feb 2024
8. Laptev, G.Y., Filippova, V.A., Kochish, I.I., Yildirim, E.A., Ilina, L.A., Dubrovin, A.V., Brazhnik, E.A., Novikova, N.I., Novikova, O.B., Dmitrieva, M.E., Smolensky, V.I., Surai, P.F., Griffin, D.K., Romanov, M.N.: Examination of the expression of immunity genes and bacterial profiles in the caecum of growing chickens infected with *Salmonella* Enteritidis and fed a phytobiotic. Animals **9**, 615 (2019). https://doi.org/10.3390/ani9090615
9. Laptev, G.Y., Yildirim, E.A., Ilina, L.A., Filippova, V.A., Kochish, I.I., Gorfunkel, E.P., Dubrovin, A.V., Brazhnik, E.A., Narushin, V.G., Novikova, N.I., Novikova, O.B., Smolensky, V.I., Surai, P.F., Griffin, D.K., Romanov, M.N.: Effects of essential oils-based supplement and *Salmonella* infection on gene expression, blood parameters, cecal microbiome, and egg production in laying hens. Animals **11**, 360 (2021). https://doi.org/10.3390/ani11020360
10. Halper, J., Burt, D.W., Romanov, M.N.: On reassessment of the chicken TGFB4 gene as TGFB1. Growth Fact. **22**, 121–122 (2004). https://doi.org/10.1080/08977190410001712878
11. Attia, Y., El-Kelawy, M., Al-Harthi, M., El-Shafey, A.: Impact of multienzymes dose supplemented continuously or intermittently in drinking water on growth performance, nutrient digestibility, and blood constituents of broiler chickens. Animals **10**, 375 (2020). https://doi.org/10.3390/ani10030375
12. Fisinin, V.I., Egorov, I.A., Draganov, I.F.: [*Feeding of Poultry*]. GEOTAR-Media, Moscow (2011). https://elibrary.ru/item.asp?id=19515570, https://www.google.com/search?tbm=bks&q=5970419966. Accessed 14 Feb 2024
13. Anh, N.T.L., Kunhareang, S., Duangjinda, M.: Association of chicken growth hormones and insulin-like growth factor gene polymorphisms with growth performance and carcass traits in Thai broilers. Asian Austr. J. Anim. Sci. **28**(12), 1686–1695 (2015). https://doi.org/10.5713/ajas.15.0028

14. Romanov, M.N., Grozina, A.A., Ilina, L.A., Laptev, G.Y., Yildirim, E.A., Filippova, V.A., Tyurina, D.G., Fisinin, V.I., Kochish, I.I., Griffin, D.K., Surai, P.F.: From feed regulation to regulated feeding: intestinal microbiome and performance optimization in broiler chickens in response to antibiotic and probiotic treatment. In: Life of Genomes 2022: Abstracts of the International Conference, pp. 44–45. Research Center "Regulatory Genomics", Institute of Fundamental Medicine and Biology, Kazan (Volga Region) Federal University, Kazan (2022). http://lifeofgenomes.r-genomics.com/wp-content/uploads/2022/12/Absctracts.pdf. Accessed 14 Feb 2024
15. Grozina, A.A., Ilina, L.A., Laptev, G.Y., Yildirim, E.A., Ponomareva, E.S., Filippova, V.A., Tyurina, D.G., Fisinin, V.I., Kochish, I.I., Griffin, D.K., Surai, P.F., Romanov, M.N.: Probiotics as an alternative to antibiotics in modulating the intestinal microbiota and performance of broiler chickens. J. Appl. Microbiol. **134**, lxad213 (2023). https://doi.org/10.1093/jambio/lxad213
16. Yin, Y., Lei, F., Zhu, L., Li, S., Wu, Z., Zhang, R., Gao, G.F., Zhu, B., Wang, X.: Exposure of different bacterial inocula to newborn chicken affects gut microbiota development and ileum gene expression. ISME J. **4**, 367–376 (2010). https://doi.org/10.1038/ismej.2009.128
17. Gasnier, C., Dumont, C., Benachour, N., Clair, E., Chagnon, M.C., Séralini, G.E.: Glyphosate-based herbicides are toxic and endocrine disruptors in human cell lines. Toxicology **262**, 184–191 (2009). https://doi.org/10.1016/j.tox.2009.06.006