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Effects of glyphosate, antibiotics and an anticoccidial drug on pancreatic gene expression and blood physiology in broilers

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Abstract: Drugs and pesticide residues in broiler feed can compromise the therapeutic and production benefits of antibiotic application and affect gene expression. In this study, we analyzed the expression of 13 key pancreatic genes and blood physiology parameters after administering the following: one maximum residue limit of herbicide glyphosate (GLY), two antibiotics (ANT), and one anticoccidial drug (AD). A total of 260 Ross 308 broilers aged 1 to 40 days were divided into the following four groups of 65 birds each: (1) control group fed the main diet (MD), and three experimental groups fed MD supplemented with (2) GLY, (3) GLY+ANT (enrofloxacin and colistin methanesulfonate) and (4) GLY+AD (ammonium maduramicin). The results showed that the addition of GLY, GLY+ANT and GLY+AD caused significant changes in the expression of several genes of physiological and economic importance. In particular, genes related to inflammation and apoptosis (*IL6*, *PTGS2* and *CASP6*) were downregulated by up to 115.4 times, and those related to antioxidant protection (*CAT*, *SOD1* and *PRDX6*) by up to 69.4 times, compared to controls. There was also a significant decline in the values of immunological characteristics in the blood serum observed in the experimental groups and certain changes in gene expression were concordant with changes in the functioning of the pancreas and blood. The changes revealed in gene expression and levels of blood indices in response to GLY, ANT and AD provide insights into the possible mechanisms of action of these agents at the molecular level. Specifically, these changes may be indicative of physiological mechanisms to overcome the negative effects of GLY, GLY+ANT and GLY+AD in broilers.

Key words: Glyphosate; Antibiotics; Anticoccidial drug; Pancreas; broilers; Gene expression; Blood indices

1 Introduction

Pesticides and antibiotics (ANT) are often indispensable for modern agricultural production (Dayan et al., 2019). However, these substances are often found in food and animal feed, prompting public concern (Klümper and Qaim, 2014; Cattani et al., 2017; Duke, 2018). Glyphosate (GLY), or N-(phosphonomethyl) glycine, is the most widely used herbicide in the world (Dayan et al., 2019; Maggi et al., 2020). A competitive inhibitor of the shikimate pathway, it mediates the biosynthesis of aromatic amino acids in plants and microorganisms (Cattani et al., 2017; Duke, 2018).

In recent years, GLY use has accounted for about 60% of the global non-selective herbicide

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market due to the development of GLY-resistant crops (Klümper and Qaim, 2014). GLY was classified as relatively non-toxic by the United States Environmental Protection Agency (Martínez et al., 2018). However, based on recent research on the health effects of GLY residues (reviewed by Costas-Ferreira et al., 2022), the International Agency for Research on Cancer of the World Health Organization classified GLY in 2015 as probably carcinogenic to humans. There is now growing evidence that GLY has cytotoxic and genotoxic effects, increases oxidative stress, and disrupts the estrogen pathway and some brain functions. It is also believed to be correlated with some types of cancer (Peillex and Pelletier, 2020).

The pancreas poses a particular challenge to study due to the complex morphogenetic processes underlying the development of this important organ (Slack, 1995). The effects of GLY on its function and histoarchitecture have been poorly explored in animals. However, a few studies have demonstrated severe negative effects of GLY on the pancreas and blood parameters in rats (Tizhe et al., 2014, 2018).

At present, research into the effects of pesticide on the health of animals, including poultry, focuses mainly on the consequences of their isolated exposure (e.g., Ma and Li, 2015). However, the combined effects of pesticides, ANT and other xenobiotics may lead to more negative effects (Haiser and Turnbaugh, 2013; Štefanac et al., 2021). ANT and anticoccidials (AD; chemicals and ionophores) are widely used together, alone or in combination with other drugs in programs for preventing bacterial infections and coccidiosis (Noack et al., 2019). Furthermore, ANT use is known to affect the gut microbiota by reducing its abundance, altering community structure, and reducing bacterial diversity (Romanov et al., 2022b; Grozina et al., 2023). The gut microbiota, in turn, can influence drug metabolism and host toxicity. Thus, ANT-induced changes to the gut microbiota (Banerjee et al., 2018; Choi et al., 2018) may have an impact on how xenobiotics are chemically transformed within the host body.

The development of pathologies associated with GLY may be due to both direct deleterious changes and disturbances in the expression of various genes and in the composition of the blood and its functions. Understanding how these changes are regulated or mediated is critical for enhancing poultry production and protecting human health. To control pathogens and coccidiosis, ANT and AD programs are widely practiced in commercial poultry production (Chapman et al., 2010).

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The main goal of the broiler industry is to enhance meat production, which implies as full as possible realization of the genetic potential of fattened birds (Patreva et al., 2010; Attia et al., 2014, 2020a,b; Streltsov and Ryabicheva, 2018). Growth rates are controlled by complex sets of genes. When rearing broilers, the intestinal mucosa is in almost constant contact with the feed and toxicants contained in it, responding directly to signals from the intestinal environment. The impact of this interaction on the host can be enormous, from changes in gene expression to regulation of metabolism and body weight (Clemente et al., 2012). In our preliminary studies (Laptev et al., 2023; Yildirim et al., 2023), we observed negative effects of GLY on microbiome composition, zootechnical characteristics and clinical, biochemical, and immunological blood parameters in broiler chickens. We also established that, under the influence of GLY+ANT (enrofloxacin, colistin and florfenicol) in the broiler cecum, there was a pronounced upregulation of antimicrobial and antiviral defense (*AvBD9*, *AvBD10* and *IRF7*) and proinflammatory genes (*IL6*, *IL8L2* and *PTGS2*) (Tyurina et al., 2022).

The aim of the present study was to examine the effects of xenobiotics on the expression spectrum of important genes and on the indices of blood physiology in broiler chickens. As the tested xenobiotics, GLY in the amount of one maximum residue limit (MRL) for food products, two ANT (enrofloxacin and colistin methanesulfonate), and one AD (ammonium maduramicin) were administered via feed and drinking water.

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2 Materials and methods

2.1 Animals, experimental design, and treatments

The experiment was conducted in the BIOTROF+ Ltd vivarium located in the village of Fedorovskoye, Tosnensky District, Leningrad Oblast, Russia in 2023 using broiler chickens [*Gallus gallus* (Linnaeus, 1758); Genus *Gallus*: urn:lsid:zoobank.org:act:B0DA321D-F372-4CB2-8C85-162ED143D295] aged 1 to 40 days from the widespread Ross 308 cross (e.g., Bondarenko et al., 2013). There were four groups of 65 birds each, totaling 260 broilers. Group I consisted of control animals fed the main diet (MD); experimental Group II was fed MD with the addition of GLY; experimental Group III was fed MD with the addition of GLY and two veterinary ANT, enrofloxacin and colistin methanesulfonate (GLY+ANT); and experimental Group IV was fed MD with GLY and AD, ammonium maduramicin (GLY+AD). Housing and dietary conditions met the management guidelines and requirements for broilers of this cross according to Aviagen (2002, 2014), Fisinin et al. (2011) and Egorov et al. (2013). Chickens from the control and experimental groups were housed in three-tiered cages made of BB-1 blocks (NPO Stimul-INK, Moscow Oblast, Russia). For feeding birds from 1 to 4 weeks old, the complete mixed fodder PK5-1G-1101 (CJSC Gatchinsky Feedmill, Leningrad Oblast, Russia) was used. Thereafter, i.e., from Day 28 to Day 40, broilers were fed the complete mixed fodder PK-6-G-1102 (CJSC Gatchinsky Feedmill) (see details in Supplementary Table S1). The birds also received an additional vitamin and mineral supplement (see details in Supplementary Table S2).

The Agrokiller formulation (CJSC Avgust, Moscow, Russia), containing 500 g/L GLY acid (isopropylamine salt), was used in this experiment as a GLY source. In accordance with the

manufacturer's instructions for use, the drug contained 50% glyphosate and 18% surfactant. An Agrokiller working solution was created for this purpose and applied to mixed feed using the spraying method to a final concentration of 1 MRL, or 20 ppm (SanPiN, 2021). Mechanical mixing was done while keeping in mind worker safety regulations. The enzyme-linked immunosorbent assay (ELISA; e.g., Tereshchenko and Ryabinin, 2009) was used to measure the GLY concentration in the feedstuffs after it had been applied. The purity of the experiment was demonstrated by the near complete lack of background residues of GLY in the broiler meal. The Stat Fax 303+ strip immunoassay (Awareness Technology, Inc., Palm City, FL, USA) and the GLY ELISA Microtiter Plate test kit (Eurofins Abraxis, Warminster, PA, USA) were used for the ELISA analysis of the GLY concentration in the feed and nutrient media. The test relies on a direct competitive immunoassay reaction between GLY available in a sample and an enzyme labeled with GLY for binding rabbit ANT to GLY and goat antibodies to rabbit immobilized in microtiter plates. The immunoassay was carried out in microplate wells, and the amount of GLY present in the samples was determined by the strength of the color signal produced by the solution.

The ANT enrofloxacin was added to drinking water using Enroflon (10% solution for oral use; NPK-VIK LLC, Moscow, Russia) at 0.5 ml/L water on Days 0 to 10. According to the manufacturer's instructions, the drug contained 10% enrofloxacin. The ANT colistin methanesulfonate was added to the water in the form of the Colistin 2 Million preparation (developed by Areal Medical LLC and manufactured by AVZ-SP, Moscow, Russia) at 0.25 ml/L water on Days 33 to 37. In accordance with the manufacturer's instructions, 1 ml Colistin 2 Million contained colistin sulfate as an active ingredient (2,000,000 IU). During the administration period, the birds received only water containing these two ANT. Working solutions of Enroflon (10%) and Colistin 2 Million were prepared immediately before adding to the drinking system and were not stored. To prepare a 10% working solution of Enroflon, 1 ml of the drug was dissolved in 200 ml of water (stock solution) and thoroughly mixed for at least 5 min. Then, the volume of water was brought to 2 L and mixed thoroughly again. The working solution was added to the drinking system. To prepare 10 L of the Colistin 2 Million working solution, 2.5 ml of the drug was diluted in 200 ml of water, mixing thoroughly; afterwards, the volume of water was brought to 5 L and stirred, and then to 10 L and stirred. The resultant solution was added to the drinking system twice a day. Until the chickens were 35 days old, the AD ammonium maduramicin was added at 500 g/t feed, evenly mixing it directly with the feed and avoiding clumping of the drug. The working mixture was pre-prepared at a rate of 5 g of the drug and 100 g feed; after that, 900 g feed was added and mixed thoroughly, and then the resultant mixture was added to 9 kg feed and also mixed thoroughly. According to the manufacturer's instructions, the drug contained 1% monoglycoside polyether ionophore (maduramicin) in the form of ammonium salt.

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2.2 Gene expression analysis

At the conclusion of the experiment, three pancreatic tissue samples were collected from each control and experimental group to ascertain gene expression. The samples were stabilized with the RNAlater reagent (Thermo Fisher Scientific, Inc., Waltham, MA, USA) and submitted

right away to the OOO BIOTROF+ lab for RNA isolation. After combining with liquid nitrogen, the tissues were homogenized. The Aurum^{TM} Total RNA mini kit (Bio-Rad, Hercules, CA, USA) was used to isolate total RNA in accordance with the manufacturer's instructions. Using iScriptTM Reverse Transcription Supermix (Bio-Rad, USA), the reverse transcription procedure was carried out to produce cDNA from the RNA template (e.g., Zeka et al., 2016). Quantitative real-time PCR (qRT-PCR) was used to analyze gene expression (e.g., Meza Cerda et al., 2020). Appropriate primers specific to 13 key genes (Table 1) were chosen for expression analysis.

Table 1 Forward (F) and reverse (R) primers for qRT-PCR analysis of mRNA expression of 13 key genes

Gene (Synonym), Gene Product	NCBI Reference Sequence	Primer Sequence (5'→3')
Genes related to inflammation and apoptosis		
IL6, interleukin 6	NM_204628.2	F: AGGACGAGATGTGCAAGAAGTTC R: TTGGGCAGGTTGAGGTTGTT
IL8L2 (*IL8*), interleukin 8-like 2	NM_205498.2	F: GGAAGAGAGGTGTGCTTGGA R: TAACATGAGGCACCGATGTG
PTGS2, prostaglandin-endoperoxide synthase 2	NM_001167719.2, NM_001167718.2	F: TCGAGATCACACTTGATTGACA R: TTTGTGCCTTGTGGGTCAG
CASP6, caspase 6	NM_204726.2, NM_001396146.1, NM_001396147.1, NM_001396148.1	F: CAGAGGAGACAAGTGCCAGA R: CCAGGAGCCGTTTACAGTTT
Genes associated with resistance to viruses and bacteria		
AvBD9 (*GAL9*), avian beta-defensin 9	NM_001001611.3	F: AACACCGTCAGGCATCTTCACA R: CGTCTTCTTGGCTGTAAGCTGGA
AvBD10 (*GAL10*), avian beta-defensin 10	NM_001001609.3	F: GCTCTTCGCTGTTCTCCTCT R: CCAGAGATGGTGAAGGTG
Genes related to productivity		
IGF1, insulin like growth factor 1	NM_001004384.3	F: GCTGCCGGCCCAGAA R: ACGAACTGAAGAGCATCAACCA
MYOG, myogenin	NM_204184.2	F: GGAGAAGCGGAGGCTGAAG R: GCAGAGTGCTGCGTTTCAGA
Genes associated with antioxidant defense		
CAT, catalase	NM_001031215.2	F: ACCAAGTACTGCAAGGCGAA R: TGAGGGTTCCTCTTCTGGCT
SOD1 (*SOD*), superoxide dismutase 1, soluble	NM_205064.2	F: CGGGCCAGTAAAGGTTACTGGAA R: TGTTGTCTCCAAATTCATGCACATG
PRDX6, peroxiredoxin 6	NM_001039329.3	F: GCATCCGCTTCCACGACTTCCT R: CCGCTCATCCGGGTCCAACAT
HMOX1 (*HO-1*), heme oxygenase 1	NM_205344.2	F: GGTCCCGAATGAATGCCCTTG R: ACCGTTCTCCTGGCTCTTGG
Gene associated with resistance to toxins and drugs		
GSTA3, glutathione S-transferase alpha 3	NM_001001777.2	F: TACATCGCAGGGAAATACA R: GGAGAGAAAGGAAACACCA

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As a reference control, the housekeeping gene for beta actin (*ACTB*) was amplified using the following primers: forward, 5'-CTGTGCCCATCTATGAAGGCTA-3', and reverse, 5'-ATTTCTCTCTCGGCTGTGGTG-3' (Yue et al., 2010). Following the manufacturer's instructions, the reaction was performed using a SsoAdvancedTM Universal SYBR® Green Supermix kit (Bio-Rad) and a DTlight amplifier (DNA-Technology LLC, Moscow, Russia). Conditions and the mode of amplification were as follows (Laptev et al., 2019, 2021): 5 min at 95 ºC (preheating); 30 s at 95 ºC, 30 s at 60 ºC, and 30 s at 70 ºC (40 cycles). The $2^{-\Delta\Delta CT}$ method was used to evaluate relative expression (Livak and Schmittgen, 2001).

2.3 Blood serum analyses

Blood samples from the broilers were examined at 7, 14 and 40 days of raising in a laboratory within the Department of Biochemistry and Physiology, St. Petersburg State Academy of Veterinary Medicine, Russia. At each age stage, three samples were collected from each control and experimental group. Laboratory analyses were conducted using standard procedures. In a counting chamber, the quantity of blood erythrocytes was determined using Goryaev's grid in compliance with the widely recognized procedure outlined elsewhere (Tietz, 1997). A counter chamber equipped with Goryaev's grid was also used to calculate the white blood cell count (WBC; Tietz, 1997). May-Grünwald fixation solution and Romanowsky azure-eosin dye were used to stain blood smears to create blood leukograms using microscopy (Tietz, 1997). The hemoglobin cyanide technique was used to assess serum hemoglobin levels (Tietz, 1997). A heat-inactivated *Staphylococcus aureus* culture (strain 209) was used to determine phagocytosis values under a microscope, and an optical turbidity standard was applied to standardize the results (Menshikov, 1997). By using a culture of *Mycobacterium lisodecticus* and altering the temperature mode of the chicken blood serum lysis reaction, the photoelectrocolometric method developed by Dorofeichuk (reviewed in Sadovnikov et al., 2009) was used to assess lysozyme activity (LA). By adopting the Michel Teffer method modified by Smirnova and Kuzmina (as discussed in Sadovnikov et al., 2009), serum bactericidal activity (SBA) was calculated as the percentage of *E. coli* lysis. According to Kostyna (1983), the discrete precipitation method was used to define the immunoglobulin classes. The proportion of leucocytes engaged in phagocytosis to all neutrophil leucocytes enumerated was used to identify phagocytic activity (PA; Karput, 1993). The average number of bacteria that had been phagocytosed in a single leucocyte was used to compute the phagocytic index (PI) that characterizes the degree of phagocytosis (Kolyakov, 1990). The phagocytic number (PN) was represented by the average number of microorganisms that one leucocyte could absorb (Kolyakov, 1990). By using commercial kits (SPF Abris+, St. Petersburg, Russia) and the methods detailed elsewhere (e.g., Nikolaienko et al., 2011), the concentrations of total protein, albumin, globulin, creatinine, bilirubin, and uric acid were quantified, along with the activity of enzymes such as amylase (AMY), alkaline phosphatase (ALP), alanine aminotransferase (ALAT), and aspartate aminotransferase (ASAT).

2.4 Statistical analyses

Mathematical and statistical processing of the data was executed using multivariate analysis of variance (multi-factor ANOVA) in Microsoft Excel XP/2003 and RStudio (Version 1.1.453;

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RStudio Team, 2018). Means (M), and standard errors of the mean (± SEM) are shown as resulting values. The Student's t-test was performed to validate the significance of differences, with $p<0.05$ being the threshold for statistical significance. Tukey's Significant Difference Test (HSD) and TukeyHSD function in the R Stats Package (RDocumentation, n.d.) were used to compare the means.

3 Results

3.1 Changes in gene expression in the pancreas

A summary of key gene expression patterns in broiler pancreatic tissue is shown as a heat map in Fig. 1. Feed including GLY, GLY+ANT or GLY+AD (Groups II–IV) reduced the mRNA expression of genes associated with inflammation and apoptosis (*IL6*, *PTGS2* and *CASP6*) by up to 115.4 times, and that of genes associated with antioxidant protection (*CAT*, *SOD1* and *PRDX6*) by up to 69.4 times, compared to Group I ($p<0.05$).

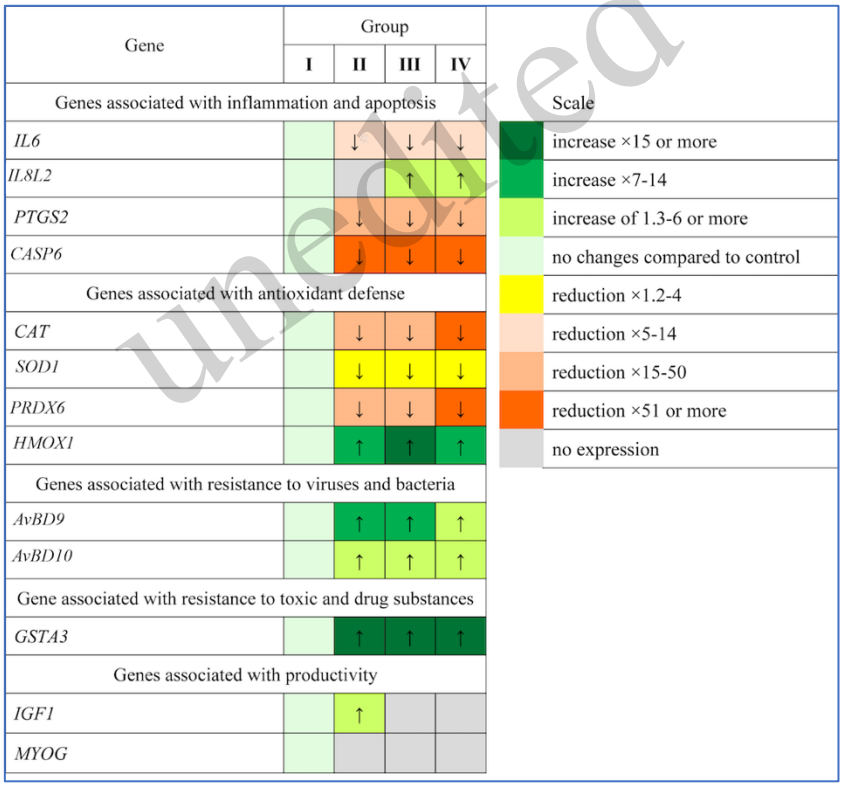

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Fig. 1 Heat map of the expression of 13 key genes in pancreatic tissues of Ross 308 broiler chickens (Groups I–IV)
Squares with arrows pointing down or up indicate a significant difference compared with Group I (at $p<0.05$): ↑, upregulation; ↓, downregulation

In general, the most pronounced changes in the expression of these genes were observed in Group IV ($p<0.05$). As for the proinflammatory gene *IL8L2*, there was complete inhibition of its expression in Group II and, in contrast, an elevation in its mRNA synthesis in Groups III and IV, compared to Group I ($p<0.05$). An induction of the *HMOX1* gene expression associated with

antioxidant protection was also noted in Groups II–IV compared to Group I ($p<0.05$). In addition, there was a marked activation of the expression (up to 29.9 times) of the *GSTA3* gene associated with resistance to drugs and toxic substances, as well as that of the *AvBD9* and *AvBD10* genes related to resistance to pathogens (up to 14.9 times in experimental Groups II–IV compared to Group I, $p<0.05$).

3.2 Clinical and biochemical blood parameters

The results of the clinical and biochemical blood tests are presented in Supplementary Table S3. The use of GLY (Group II) and GLY+AD (Group IV) had an adverse effect on the hemoglobin level and the color indicator of the broiler blood ($p<0.05$). A significant decline in these indicators was detected at the age of 7 and/or 14 days ($p<0.05$). The hemoglobin content also deviated significantly from the reference values. When using the concomitant complexes GLY+ANT (Group III) and GLY+AD (Group IV), the blood color index and hemoglobin values were restored to the level of the control group ($p<0.05$) in almost all cases (excluding the variant at the age of 14 days in Group IV). The values of AMY, ALP, ALAT and ASAT increased throughout the entire period of bird growth in Groups II–IV in many cases, compared to Group I ($p<0.05$; Supplementary Table S4). In our experiment, AMY levels at the age of 40 days and ASAT at the age of 7 days in Groups III and IV decreased compared to Group II ($p<0.05$). In addition, there was no elevation in AMY, ALP, ALAT and ASAT in Groups III and IV compared to Group II ($p<0.05$).

When assessing biochemical blood parameters characterizing the metabolic state, a significant reduction in the level of total protein in the blood serum of birds was revealed on days 7, 14 and 40 in all experimental groups compared to the control Group I ($p<0.05$; Supplementary Table S5). The albumin concentrations in all experimental groups fed GLY, GLY+ANT and GLY+AD were also significantly lower than those of Group I at the same ages ($p<0.05$). For instance, the absolute values of this indicator on day 7 were 18% lower in Group II, 24% lower in Group III, and 28% lower in Group IV, compared to those of Group I ($p<0.05$). On day 14, the reductions relative to the control were 30% in Group II, 17% in Group III, and 16% in Group IV. On day 40, these dynamics were similar: the albumen concentration was 34% lower in Group II, 27% lower in Group III, and 29% lower in Group IV. There were no significant differences in the concentrations of α-globulins in birds at 7 days of age. At the age of 14 days, there was a sharp growth in the concentration of α-globulins by 1.7 times in Group II relative to Group I ($p<0.05$). This trend was also observed in birds at 40 days of age, when a significant rise in this protein fraction was characteristic of all three experimental groups ($p<0.05$). In particular, the values of this indicator were 1.7 times higher in Group II, 1.6 times in Group III, and 1.4 times in Group IV, relative to Group I at the respective age ($p<0.05$). An analysis of the β-globulin fraction values revealed an increase at 7 and 14 days of age in all experimental groups compared to the control group ($p<0.05$). In contrast, the values of the γ-globulin fraction dropped in all experimental groups at all ages compared to Group I ($p<0.05$).

3.3 Immunological blood tests

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When assessing the state of the immune system, it was revealed that the innate immunity indicators of blood serum, PA, PI, PN, SBA and LA, were in some cases lower in chickens of Groups II, III and IV than in those in Group I (p<0.05), and was especially pronounced at the age of 40 days. Specifically, there was a 12% decrease in BA in birds at 14 days in Group II and 10% decrease in each of Groups III and IV relative to Group I (p<0.05). In 40-day-old broilers, these dynamics of BA were similar, showing reductions of 14% in Group II, 15% in Group III, and 13% in Group IV compared with Group I (p<0.05; Fig. 2). At 14 days, LA was reduced relative to the control by 26% in Group II, 28% in Group III, and 25% in Group IV (p<0.05). In 40-day-old chicks, these dynamics in LA were similar, with reductions of 22% in Group II, 14% in Group III, and 16% in Group IV compared with the control (p<0.05).

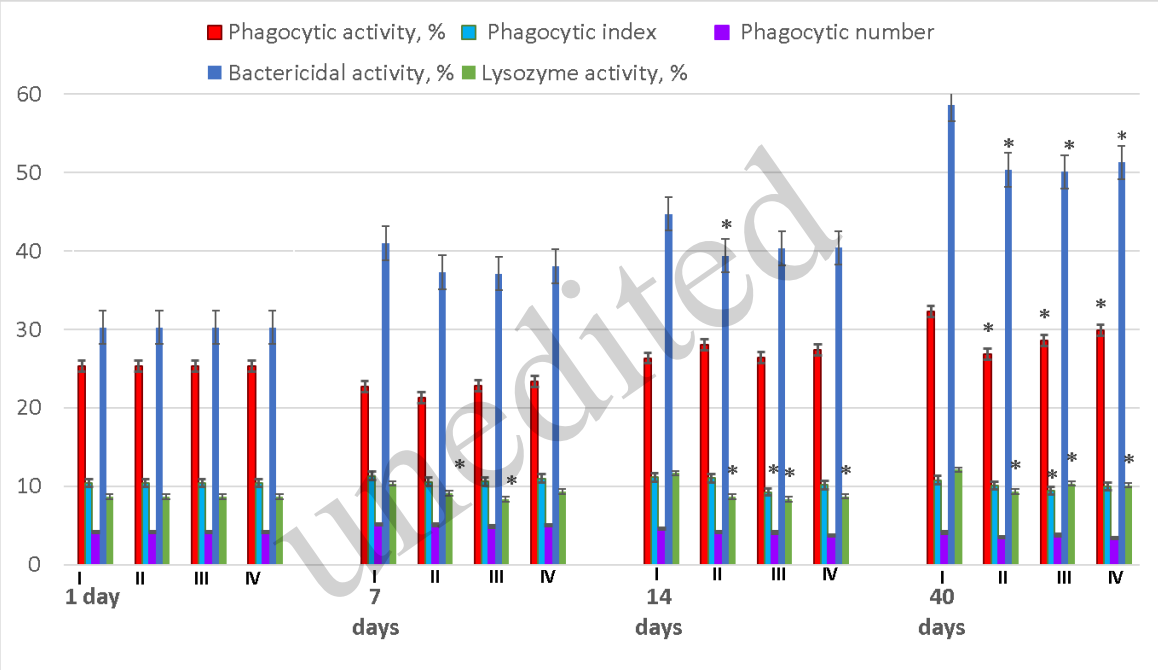

Fig. 2 Immunological blood indicators in response to the intake of GLY (Group II), GLY+ANT (Group III) and GLY+AD (Group IV) in the Ross 308 broiler chickens relative to the control Group I

* Significant difference in comparison with Group I (at p<0.05). Results are presented as mean values (± SEM)

4 Discussion

4.1 GLY effects on gene expression in the pancreas

In this study, we established a significant inhibitory effect of GLY, GLY+ANT and GLY+AD on the mRNA expression of the *IL6*, *PTGS2* and *CASP6* genes associated with inflammation and apoptosis in the pancreatic tissue. This can have negative consequences for the broiler body. The interaction of pathogens with toll-like receptors, whose genes were downregulated in the cecum in our GLY experiment (Laptev et al., unpublished), leads to the secretion of interleukins IL1, IL6 and IL8 on the surface of macrophages (Zhang et al., 2012). IL6 is a protein involved in the recruitment and control of cells of both innate and adaptive immune systems. Such cytokines are essential for a successful host immune response to pathogens (Morikawa et al., 1996; Kaiser et al.,

2000; Reato et al., 2004). Therefore, downregulation of *IL6* may lead to reduced short-term protection against infection or injury, and may reduce the proliferation and differentiation of leukocytes that destroy pathogens (Rodes et al., 2013). On the other hand, in our present experiment, induction of *IL8L2* expression was noted under the influence of GLY, GLY+ANT and GLY+AD. As a significant neutrophil activator, the chemokine IL8 plays an important role in the inflammatory cascade (Palomino and Marti, 2015). Previously, rats treated with Roundup® at elevated concentrations showed increased levels of the proinflammatory cytokines IL1β, TNFα and IL6, as well as C-reactive protein in the liver and adipose tissue (Pandey et al., 2019).

The observed downregulation of *CASP6* under the influence of GLY, GLY+ANT and GLY+AD could also have negative effects. In caspase-dependent apoptosis, the initiation of apoptotic pathways ultimately leads to the activation of caspases (cysteine proteases) that function as conventional cell death effector molecules (Degterev et al., 2003; Fig. 3). Apoptosis has played an important role throughout evolution in the control of various physiological and pathological processes (Hengartner, 2000). It is known, for example, that a characteristic feature of cancers, including pancreatic carcinoma (Duong et al., 2017), is disruption of the normal balance between proliferation and cell death, which is critical for maintaining tissue homeostasis (Evan et al., 2001). A decline in apoptosis processes is associated with the formation of tumors (Lowe and Lin, 2000). Defects in apoptotic programs may contribute to the primary or acquired resistance of pancreatic carcinoma to treatment protocols used at present in medicine. Thus, the response of cancer cells to current treatment approaches is largely determined by their ability to undergo cell death in response to cytotoxic stimuli (Johnstone et al., 2002). In this respect, our findings may also raise concerns regarding the health of people who inevitably consume products containing GLY residues. In contrast, Gao et al. (2022a) reported a significant increase in the expression level of the Caspase-3 gene due to exposure of white carp hepatocytes (L8824 cells) to 3 mg/ml triazine herbicide (atrazine) for 24 h. The change in gene expression ultimately led to hepatocyte death (Gao et al., 2022b).

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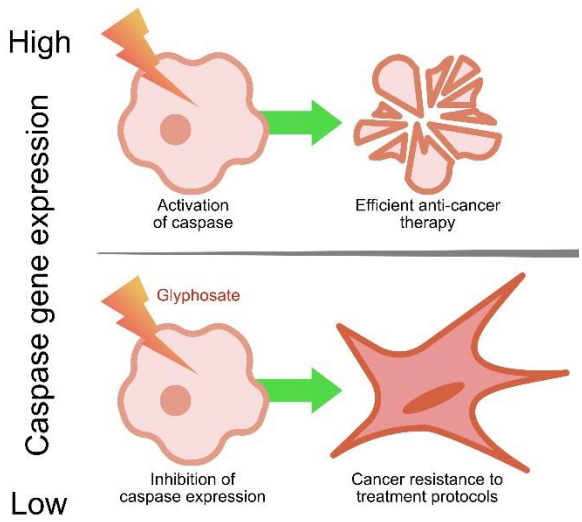

Fig. 3 Suggested scheme for the influence of glyphosate residues on the processes of apoptosis and the effectiveness of antitumor therapy (as inferred from the results of this experiment)

The concept of an antioxidant defense cascade in poultry was previously developed and validated (Surai, 2002; Surai, 2006). It was proposed that there are three main levels of antioxidant defense in the cell. The first level involves three main antioxidant enzymes, namely SOD1, glutathione peroxidase (GSH-Px) and CAT, that are responsible for detoxification of radicals at the very beginning of their formation (Fig. 4). Ognjanović et al. (2008) previously noted that chronic cadmium poisoning reduced SOD1 and GSH-Px activity in the rat liver and kidney. Liu et al. (2018) also found a reduction in antioxidant activity markers (CAT, SOD1 and GSH-Px) upon exposure to high concentrations of cadmium. Therefore, the downregulation of the *CAT*, *SOD1* and *PRDX6* genes associated with antioxidant protection established here due to GLY, GLY+ANT and GLY+AD treatments suggest a toxic effect of the studied xenobiotics on broilers.

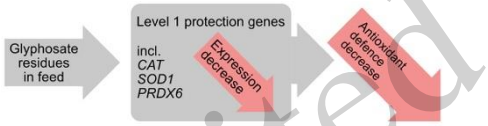

Fig. 4 The effect of glyphosate residues on the antioxidant defense of broilers (as suggested by the results of this experiment)

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The observed undesirable enhancement or inhibition of the expression of some genes under the influence of AD supplementation in the presence of GLY may indicate a heightening in the bioavailability of GLY. Greater bioavailability implies a higher risk of increased herbicide amounts entering the bloodstream, tissues and organs. It was also previously found that the bioavailability of triazine herbicides increased in rats after 3 and 7 days of ampicillin administration and was associated with declined hepatic detoxification efficiency (Zhan et al., 2018). Our data (Laptev et al., unpublished) also suggest that GLY may negatively influence the expression of the *TGFB1* gene, a member of the family of multiple multifunctional cytokines previously erroneously called "TGFB4" (Halper et al., 2004) and associated with growth processes, immunity (Karaffová et al., 2015) and fertility (Rostami et al., 2020).

4.2 GLY and changes in blood serum

Considering the impact of GLY on blood physiology, the use of GLY and GLY+AD had an adverse effect on the hemoglobin level in the broiler blood. This may indicate dehydration of the body as a result of intoxication and the negative effect of GLY on the hematopoietic system. With continued chronic exposure to GLY, this could lead to adverse effects in terms of weight gain. An elevation in AMY, ALP, ALAT and ASAT in the blood serum in all experimental groups suggests a negative effect of GLY on the functioning of the liver and pancreas. Xenobiotics are known to cause oxidative stress in the body and may contribute to the inhibition of endothelial synthase of nitric oxide (NO), also known as an important bioindicator of metabolism, myogenesis and growth in chick embryos (Romanov et al., 2022a; Kochish et al., 2023a,b). This can also reduce

the basal endogenous synthesis of NO in blood vessels (Vleeming et al., 2002). NO is involved in the induction of the synthesis of pancreatic secretion stimulants in the liver (Wu et al., 2004). Elevated serum AMY is a biochemical abnormality associated with poisoning with certain xenobiotics and may be caused by excessive parasympathetic stimulation of the pancreas (Dagli and Shaikh, 1983). The negative effect of GLY on the functioning of the pancreas (Tizhe et al., 2018) and liver (Manne et al., 2018) was noted previously. However, our analysis of biochemical parameters suggests there was a certain reduction in the negative effects of GLY on the functioning of the liver and pancreas when using ANT and AD. Nevertheless, ANT intake is often associated with drug-induced liver injury (Park et al., 2021). Despite this, simultaneous exposure of the body to multiple chemicals can cause interactions through pharmacokinetic modulation (Yoo et al., 2016). The main pathways of pharmacokinetic interactions are modulation of the production of enzymes that metabolize xenobiotics, their efflux pumps, as well as changes in the intestinal microbiota and subsequent changes in the metabolism of xenobiotics mediated by microbial enzymes. Thus, if GLY undergoes biotransformation by intestinal microbiota, altered by the influence of ANT and AD, its level of absorption in the gastrointestinal tract may be changed. This may lead to modulation of the GLY effect. By comparison, Zhang et al. (2018) found a significant increase of ASAT and ALAT concentrations in broiler chickens whose diet was supplemented with the pesticide thiram. Recently, Wang et al. (2023) showed a negative effect of this pesticide on the content of various metabolites in the liver. However, Kammon et al. (2011) did not find any significant changes in hemoglobin concentration in broiler chickens at the age of 24 or 45 days in response to chlorpyrifos administration at a dose of 0.8 mg/kg body weight.

Note that a drop in the level of total protein in the blood serum was detected on days 7, 14 and 40 in all experimental groups. These changes may be associated with impaired protein synthesis in the liver and/or diminished absorption of amino acids from the gastrointestinal tract (Ballmer, 2001). At the same time, the protein metabolism factor is very important for broiler chickens due to the extremely rapid gain of body weight in a relatively short fattening time. The albumin concentrations in birds of all experimental groups fed GLY, GLY+ANT and GLY+AD was also lower than those of birds in the control group, while α-globulins increased sharply. Because albumins are the main transport proteins of the body, their reduced values in the experimental groups may cause birds to experience disorders caused by disruption of the transport of various endogenous and exogenous substances carried by albumins (Levitt and Levitt, 2016). α-globulins are represented by proteins of the acute phase of inflammation. A sharp rise in the proteins of this fraction indicates the development of an acute inflammatory process in poultry (Cray et al., 2009). Previously, an increase in these proteins was also demonstrated when the body was exposed to various stressors (Pradeep, 2014).

Our analysis of the state of the immune system revealed experimental birds fed GLY, GLY+ANT and GLY+AD showed a decrease in PA, PI, PN, BA and LA, suggesting inhibition of the nonspecific components of immunity. Previously, a study in carp showed that the immunoglobulin M was reduced following exposure to GLY (Ma et al., 2015). In contrast, Kreutz et al. (2014) unexpectedly observed an increase in immunoglobulin concentration in silver catfish exposed to GLY and then infected with an inactivated form of *Aeromonas hydrophila*. This is

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consistent with our data (Laptev et al., unpublished) showing a downregulation of *TLR2* and *TLR4* in broilers exposed to GLY. These two genes are members of the toll-like receptor family, which is a highly conserved group of proteins involved in the detection of pathogens, as well as the initiation and regulation of innate and adaptive immune responses (Krishnan et al., 2007; Temperley et al., 2008). Exposure to triazine herbicides was shown to cause oxidative stress, alter antioxidant systems and lead to DNA damage (Santos et al., 2015; Adedosu et al., 2017; Lovaković et al., 2017). We previously showed that GLY suppressed the expression of antimicrobial and antiviral genes in broilers (Tyurina et al., 2022). In European flounder exposed to low concentrations of GLY and other herbicides, an elevation in the expression of the C1 inhibitor precursor, a key negative regulator of the complement system, was observed after a 62-day contamination (Evrard et al., 2010). Ma et al. (2015) showed a downregulation of the C3 component gene as well as significant damage to the kidneys (the main immune organ in fish) in common carp after 3 and 7 days of sublethal exposure to GLY, demonstrating that GLY can indeed suppress the complement pathway, with LA also being inhibited. Also, addition of the imidacloprid insecticide to the diet of broiler chickens reduced the body's immune response, including LA (Eleiwa et al., 2023).

Collectively, our findings and other published data on gene expression in the pancreas and on indices of blood physiology, biochemistry and immunity provide more pervasive insights into the responses of broilers to GLY, ANT and AD. After all, these data indirectly reflect their state of health, including the functioning of the pancreas (e.g., through association with the level of AMY). Alterations in the blood serum immunity indicators overlapped with the affected expression of immune genes, including proinflammatory genes, while changes of other blood indices fell within the norm.

Our findings may be of concern due to possible adverse effects on humans who consume products containing GLY residues. Inhibition of apoptosis gene expression may indicate the potential for carcinogenic health risks of GLY for consumers. In our opinion, regular monitoring of GLY content in food products is necessary, as well as a number of other preventive measures, to mitigate the impact of this herbicide on consumer health.

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5 Conclusions

In conclusion, the results of the current study provide new insights into exogenous factors influencing the physiology, immune status and gene expression of broilers. The development of pathologies associated with GLY and drugs used in the poultry industry may be due to both direct pathological changes and disturbances in the expression of various genes. These may then be related to changes in the pancreas and blood, warranting further research in this direction. Understanding how these changes are regulated or mediated is critical to improving poultry production. A mixture of xenobiotics, even at low non-lethal doses, may pose an increased threat due to the potential for synergistic effects between the components. Since we observed an effect of GLY on gene expression, this requires further research that may include a higher dosage of GLY using 1 MRL for poultry feed rather than for human food.

Data availability

The original contributions presented in the study are included in the article/Supplementary Material, further inquiries can be directed to the corresponding author.

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Author contributions

Conceptualization, G.Yu.L., L.A.I., V.A.F. and E.A.Y.; methodology, L.A.I. and V.A.F.; software, E.S.P. and E.A.B.; validation, E.P.G.; formal analysis, E.S.P. and E.P.G.; investigation, E.P.G., V.Kh.M., A.S.D., K.A.K. and V.A.Z.; resources, D.G.T. and N.I.N.; data curation, V.Kh.M., D.G.T. and M.N.R.; writing—original draft preparation, E.A.Y. and M.N.R.; writing—review and editing, E.A.Y., M.N.R. and D.K.G.; visualization, A.V.D., E.A.Y. and M.N.R.; supervision, G.Yu.L. and D.K.G.; project administration, G.Yu.L. and N.I.N.; funding acquisition, G.Yu.L. All authors have read and agreed to the published version of the manuscript.

Compliance with ethics guidelines

The study was carried out in compliance 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). The research procedure was approved by the Bioethical Commission of the L.K. Ernst Federal Research Center for Animal Husbandry (Protocol No. 2021-02/1, dated February 1, 2021), and was conducted in compliance with Russian Federation ethics laws as stated in Russian Federal Law No. 498-FZ on Responsible Treatment of Animals.

Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Supplementary information:

Table S1–S5

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