P-ISSN: 2304-3075; E-ISSN: 2305-4360
International Journal of Veterinary Science
www.ijvets.com; editor@ijvets.com

Research Article https://doi.org/10.47278/journal.ijvs/2025.124

Characteristics of Proinflammatory Cytokines Expression in Rabbits with Mixed Mycotoxicosis on the Background of Prophylactic Agents Application

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Article History: 25-165 Received: 06-Jul-25 Revised: 11-Sep-25 Accepted: 25-Sep-25 Online First: 21-Oct-25

ABSTRACT

In this article, the molecular mechanism of the combined action of T-2 toxin, aflatoxin B1, and zearalenone was studied, which we hope will serve as a new direction and fundamental basis for the development of an effective prophylactic complex. The experiments were conducted on 128 Gray Giant rabbits, both males and females, which were divided into eight groups. Group 1 served as a biological control and received a normal diet without toxins or treatment, while group 2 acted as a toxic control (compound feed with the addition of mycotoxins). Groups 3 to 5 were given diets containing mycotoxins along with prophylactic complexes No. 1, 2, and 3, respectively. Groups 6 to 8 received diets without mycotoxin but supplemented with prophylactic complexes No. 1, 2, and 3. To induce mycotoxicoses, the animals received crystalline mycotoxins with the main diet for 21 days: T-2 toxin (1.2mg/kg), aflatoxin B1 (0.3mg/kg), and zearalenone (1.7mg/kg). As a means of protection, three recipes of prophylactic complexes based on sorbents, probiotic bacteria, antioxidants, and hepatoprotectors were used. Expression of proinflammatory cytokine mRNA (IL-1β, IL-6, IFN-γ) was determined by quantitative real-time PCR in spleen, thymus, jejunum, and liver samples extracted at the end of the experimental period. Gene expression was assessed by the number of cDNA copies in the studied material, taking into account the normalization of the indicator relative to the number of DNA copies in the same sample. It was found that the associated effect of mycotoxins caused an increased expression of proinflammatory cytokine genes at mRNA level in the spleen (expression of IL-1β, IL-6, and IFN-γ by 2.8, 2.2, and 1.5 times compared to the biological control group), thymus (45.0%, 82% and 1.12 times) and liver (61.0, 59.3 and 31.8%), and suppressed expression in the jejunum (decrease by 42.8%, 25.2% and 52.0% relative to the biological control group). All the differences are statistically significant in the Mann–Whitney Test (α=0.05) with P-criterion correction using Bonferroni's Multiple Comparison Correction. The proposed complexes modulated the production of proinflammatory cytokines. However, the most pronounced immunomodulatory potential was shown by the prophylactic complex based on halloysite, silymarin, β-glucans, and methionine (a significant increase was noted only in the spleen in relation to IL-1β and in the liver in relation to IL-1β and IL-6 by 20.3% and 23.1%). The data obtained indicate that this prophylactic complex is promising and requires further study as a means of preventing mixed mycotoxicoses in productive animals.

Keywords: Mycotoxins, Inflammation, Cytokines, Mycotoxicosis, Prophylaxis, Rabbits

INTRODUCTION

Mycotoxins are toxic metabolites of microscopic fungi of the *Aspergillus*, *Fusarium*, *Penicillium*, and *Claviceps* genera. Of the wide variety of natural toxic substances, the most dangerous for productive animals are T-2 toxin, aflatoxin B1, and zearalenone (Awuchi et al. 2021; Tsouloufi 2024). T-2 toxin inhibits protein synthesis, causing predominant damage to the hematopoietic organs, digestive, immune, nervous and reproductive systems (Yang et al. 2020; Janik et al. 2021). Aflatoxin B1 is one of the most highly toxic types of aflatoxins, has hepatoxic,

Cite This Article as: Tarasova EY, Khammadov NI, Kashevarov GS, Tanaseva SA, Ermolaeva OK, Erokhondina MA, Efimova MA, Semenov EI, Smolentsev SY, Matrosova LE and Sachivkina NP, 2026. Characteristics of proinflammatory cytokines expression in rabbits with mixed mycotoxicosis on the background of prophylactic agents application. International Journal of Veterinary Science 15(1): 194-202. https://doi.org/10.47278/journal.ijvs/2025.124

immunosuppressive, carcinogenic, mutagenic, and teratogenic effects (Benkerroum 2020; Wang et al. 2023a). Zearalenone is a xenoestrogen that has a negative effect on the reproductive system (Rai et al. 2020). The pathogenesis of mycotoxicosis is determined by the fact that mycotoxins are powerful inhibitors of protein synthesis that cause damage to actively dividing cells of the spleen, gastrointestinal lymphoid tissue, thymus, and bone marrow in animals (Ülger et al. 2020).

Mycotoxins are called "hidden killers" due to the fact that they are odorless and do not change the taste of food and feed. This makes them especially dangerous from the point of view of veterinary medicine. Because most micromycetes produce several mycotoxins simultaneously, the occurrence of mixed mycotoxicoses with an aggravation of the clinical picture is a common case in practice (Tolosa et al. 2021; Xu et al. 2022a; Yang et al. 2024).

Since aflatoxins were discovered in the early 1960s, many studies have focused on understanding the pathogenesis of mycotoxicoses (Pickova et al. 2021). These studies have also aimed to develop preventive measures. However, the effect of high-dose combinations of mycotoxins on the cytokine profile, which is a key indicator of immune system function, has not been sufficiently clarified. A group of cytokines (interferons, interleukins, chemokines, tumor necrosis factors, and colony-stimulating growth factors) plays an important role in the regulation of the inflammatory response and the protection of the body (Sun et al. 2023).

In order to prevent the negative impact of mycotoxins, a number of control strategies have been developed, mainly prophylactics (Kihal et al. 2022; Oguz et al. 2022; Kemboi et al. 2023). Prophylactics or a prophylactic complex is a nutritional or therapeutic formulation and a combination of numerous biologically active ingredients (Puri et al. 2022). Prophylactics have synergistic effects and protect the body from harmful impacts (Doğan et al. 2022). Prophylactics have also been designed to enhance immunity, protect vital organs, neutralize toxins, maintain gut microbiota balance, reduce oxidative stress and improve metabolism and regeneration (Lázaro et al. 2024; Islam et al. 2025; Yue et al. 2025).

Furthermore, a special place is occupied by research on the creation of effective drugs of complex action that can suppress or reduce absorption, stimulate the elimination of mycotoxins, or change the mechanism of their action. The research aimed to assess the expression of proinflammatory cytokines in the internal organs of rabbits with mixed mycotoxicosis on the background of prophylactic complexes application and without it.

Research objectives were to:

- Determine the metabolic activity due to the effect of mycotoxins on the animal body;
- Develop the amplification modes (PCR and reverse transcription PCR) of markers of proinflammatory cytokine expression in rabbits;
- Determine the induction of proinflammatory cytokines in the thymus, spleen, liver and jejunum in terms of combined mycotoxicosis;

Evaluate the protective potential of three different prophylactic complexes.

MATERIALS AND METHODS

Experimental animal and design

The experiments were conducted on 128 two-month-old rabbits (males and females of the Grey Giant breed), divided into 8 groups according to the principle of analogues.

The pelleted compound feed concentrate PZK-94-1 for fattening young rabbits at the age of 50 days and older was used for feeding (wheat bran, sunflower meal, barley grain waste with husks, barley, alfalfa grass meal, beet pulp, oats, limestone flour, granule fixative, table salt, defluorinated phosphate, organic acids, vitamin and mineral premix, amino acid, antioxidant). The main diet contained 17.3% crude protein and 13.0% crude fiber with a moisture level of 14.0%. The feed also contained 0.8% calcium, 0.6% phosphorus, 0.64% lysine, and 0.56% methionine with cysteine. The energy value (metabolisable energy) of the feed was 230kcal/100g.

Mycotoxin exposure

The animals were kept in individual wire cages, without bedding, with constant access to drinking water and compound feed. In the experiment, the animals were given Sigma crystalline mycotoxins in their diet every day for 21 days: T-2 toxin (1.2mg/kg), aflatoxin B1 (0.3mg/kg) and zearalenone (1.7mg/kg), which significantly exceeded the permissible levels proposed by the draft Technical Regulations of the Customs Union (T-2 toxin – 0.1mg/kg with the recommended standard of 0.05mg/kg, aflatoxin B1 – 0.02mg/kg with the recommended standard of 0.01mg/kg, zearalenone – 1mg/kg with the recommended standard of 0.5mg/kg).

Prophylactic treatment

As preventive measures, enterosorbents of plant and mineral origin, hepatoprotectors, antioxidants, and probiotic bacteria were used at a dose of 0.25% of the diet.

Three recipes of prophylactic complexes were developed:

- No. 1 (β-glucans, silymarin, vitamin E, vitamin C and levamisole);
- No. 2 (bentonite, succinic acid, methyl uracil, vitamin A, *Bifidobacteria*, and *Lactobacilli*);
- No. 3 (halloysite, methionine, β-glucans, silymarin).

Experimental design is presented in Table 1.

Table 1: Experimental design outlining treatment groups and interventions

Group	The main diet (PZK-94-1)	Addition of mycotoxins	Addition of prophylactic complex
1 – BC	+	-	-
2 – TC	+	+	-
3 – TC+PC1	+	+	Complex No. 1
4 – TC+PC2	+	+	Complex No. 2
5 – TC+PC3	+	+	Complex No. 3
6 – BC+PC1	+	-	Complex No. 1
7 – BC+PC2	+	-	Complex No. 2
8 – BC+PC3	+	-	Complex No. 3

*BC: Biological control; TC: Toxic control; PC: Prophylactic complex; +: Present; –: Absent

Nucleic acid extraction and reverse transcription

The nucleotide sequences of the required genetic

markers were determined by searching the resource of NCBI (National Center for Biotechnology Information) databases. The occurrence of the analyzed nucleotide sequences in the genetic code of various organisms was determined using the "*nBLAST*" software utility. The design of the nucleotide sequences of primers and probes was performed using the "*VectorNTI*" 9.1.0 program (Invitrogen Corporation). To determine the quantitative data during the amplification of genetic markers, a control nucleotide sequence was designed, including the nucleotide sequences of all detected cytokines.

For DNA/RNA extraction, the commercial "*RiboPrep*" kit (Federal Budgetary Scientific Institution "Central Scientific Research Institute of Epidemiology") was used after processing tissue pieces on a "*FastPrep-24*" homogenizer using the lysis matrix A. Nucleic acids were extracted according to the user guide. Lysis of DNA residues in the RNA solution after nucleic acid extraction was performed by adding the DNase enzyme to the preparation of extracted nucleic acids. Reverse transcription of total RNA was carried out by adding the MMLV-riveratase transcriptase enzyme to the main mixture for PCR, 0.3μL of reverse transcriptase per sample ("Eurogen" JSC).

Real-time PCR analysis

The efficiency of the obtained oligonucleotides and positive controls was tested using the real-time PCR method according to the method presented below. The composition of the PCR mixture was calculated for one sample (10-fold PCR buffer ("Eurogen" JSC) – 1.5μL; 10mM nucleoside triphosphate mixture ("Eurogen" JSC) – 0.35μL; 10pM solutions of forward and reverse primers, fluorescently labeled oligonucleotide probes to the required markers, 0.5μL of each oligonucleotide; Taq polymerase 5units/μL – 0.5μL; deionized water – 6.15μL; DNA solution – 5μL. The total volume of the reaction mixture was 15μL. PCR program: the first cycle 95°C – 5min; the second cycle 95°C – 15sec; 60°C – 30sec (at this temperature, fluorescence was detected in the R6G, Rox, and Cy3 channels); the second cycle was repeated 40 times.

Reverse transcription real-time PCR reactions of the samples under study were performed similarly to the efficiency test, with the addition of a cycle with reverse transcription.

Amplification conditions were as follows: 37°C for 30min (in the presence of reverse transcription), 95°C for 5min, 40 cycles of 95°C for 15s, 60°C for 30s, 72°C for 5s.

The specific PCR components were based on the genetic sequence of rabbit interleukins and interferon. Oligonucleotides used for the amplification of rabbit gamma interferon and interleukins are presented in Table 2. Design of oligonucleotides was carried out on the basis of the corresponding RNA sequence (gamma interferon – GeneBank ID: EF589141.1, interleukin 1 beta – GeneBank ID: KT216047.1, interleukin 6 – GeneBank ID: DQ680161.1).

Gene expression normalization

To determine the quantitative parameters of the amplification reaction of genetic markers of rabbit interferon gamma and interleukins, plasmid DNA was used (sequences are also presented in Table 2).

PCR and reverse transcription PCR were performed using a C1000 detection amplifier with a CFX 96 optical unit from "Bio-Rad". The expression of mRNA of proinflammatory cytokines was determined by real-time PCR in samples of spleen, thymus, jejunum, and liver, extracted at the end of the experimental period (preliminary placed into tubes with the "IntactRNA" fixative to stabilize RNA in the samples) and frozen (at -70°C). Primers, probes for PCR, and nucleotide sequences of genetic markers of interferon gamma and interleukins were synthesized by Eurogen JSC.

Normalization of the amount of nucleic acids was carried out based on the results of amplification of the chromosomal DNA of rabbit interleukin 1β, since this nucleotide sequence (intron) has almost the same number of nucleotides as the RNA sequence (exon), which makes it possible to amplify the intron. This allows us to determine the initial level of DNA in each sample and normalize the reaction results relative to this parameter.

Gene expression was assessed by the number of cDNA copies in the studied material.

Table 2: Nucleotides used for the amplification of rabbit gamma interferon and interleukins

Cytokine	Nucleotide*	Sequence
Rabbit gamma interferon	INFG F	CCTCATCTTGGGTTCTTACGGCTGT
	INFG R	GGTCCACCATTTGCCACATCTGA
	INFG P	Cy5-TGCCAGGACACACTAACCAGAGAAACAGAACAC-BHQ3
	Plasmid DNA	CCTCATCTTGGGTTCTTACGGCTGTCGCAAACGCGATTCAGCAGTGCCAGGACA
		CACTAACCAGAGAAACAGAACACTGTCCAGACCCTTGCTAATGCAACTGCTCA
		GATGTGGCAAATGGTGGACCGGTACAGCATGTGGCTAAAGGCGT
Rabbit interleukin 1 beta	Rabbit IL1B F	CAGAAATCCTTGGTGTTGTCTGGCA
	Rabbit IL1B R	AAACTCATGGAGAACACCACTTGTTGG
	Rabbit IL1B P	R6G-AAAGCTCTCCACCTCAATGCAGAGAATCTGAA-BHQ2
	Plasmid DNA	CAGAAATCCTTGGTGTTGTCTGGCATGGTGCTGAAGAACATCCAACACCTAAA
Rabbit Interleukin 6		GCTCTCCACCTCAATGCAGAGAATCTGAAGCCACCCTCAAGCCAGCCGTTCCAA
		CAAGTGGTGTTCTCCATGAGTTTAAAACCAGTGGCTGAAGACGACCA
	Rabbit IL6 F	TGGTGCTGAAGAACATCCAACACCT
	Rabbit IL6 R	TGGTCGTCTTCAGCCACTGGTTTT
	Rabbit IL6 P	Rox-AAGCCACCCTCAAGCCAGCCGTT-BHQ2

* F: Forward primer; R: Reverse primer; P: Probe; INFG: Interferon gamma; IL1B: Interleukin 1 beta; IL6: Interleukin 6; Cy5-, R6G, Rox: Fluorescent reporter dyes; BHQ2, BHQ3: Black hole quench dyes.

The ratio of the amount of RNA of the analyzed marker to the amount of chromosomal DNA in the same sample (the marker of which was the amplification of the intron of the IL-1β gene of rabbits) was applied as a method of normalization of the amplification results for assessing the level of expression. To determine the number of detected genetic markers, quantitative PCR was performed for each genetic marker with indication of the marker DNA copies in test tubes (eight standards with tenfold dilutions of DNA were used, the minimum dilution corresponds to the sensitivity threshold of the method - one copy of DNA in 1μL) according to a standard for constructing a calibration graph using the amplifier software. Normalization (standard units) was expressed in an indicator obtained by dividing the conditional amount of RNA by the conditional amount of DNA.

Statistical analysis

The following descriptive statistics were calculated when processing digital data: arithmetic mean±SD. The statistical significance of intergroup differences was assessed using the Mann–Whitney test (α=0.05) with P-criterion correction using Bonferroni's Multiple Comparison Correction. Statistica 6.0 and MS Excel were used for data processing.

RESULTS AND DISCUSSION

The ability of protein synthesis inhibitors to significantly enhance and prolong the normally transient mitogenic gene induction has been defined as "superinduction". Cytokine superinduction by protein synthesis inhibitors involves transcriptional and/or post-transcriptional mechanisms and is closely linked to the activation of NF-kB (a transcription factor involved in the regulation of a large number of cellular genes, including cytokines). Moreover, cytokine superinduction may be a key mechanism by which mycotoxins mediate toxicity and immunological effects. Inflammation and oxidative stress are closely linked, and all pathways that generate inflammatory mediators (e.g., adhesion molecules, interleukins, etc.) are induced by oxidative stress. Based on accumulated evidence, increased production of IL-1β, IL-6, and TNF-α enhances local inflammation and promotes tissue damage induced by pathological stimuli (Zhang et al. 2019).

In previous studies, we assessed the effect of various prophylactic complexes on the clinical status, hemato-biochemical blood parameters, and the structure of internal organs of animals exposed to mycotoxins (Tarasova et al. 2024a, b). However, little is known about the effect of combined exposure to mycotoxins on the expression of inflammatory markers in animals.

To gain insight into the mechanisms of the combined action of T-2 toxin, zearalenone, and aflatoxin B1 and to better assess the therapeutic mechanism upon co-administration, we assessed the expression levels of some cytokines involved in inflammation. To determine the metabolic activity due to the effect of mycotoxins on the animal body, genetic markers of proinflammatory cytokines such as IL-1β, IL-6 and IFN-ɣ were selected. Numerous *in vivo* and *in vitro* studies have demonstrated the ability of mycotoxins to modulate immune function. Studies of Cai et al. (2020), Li et al. (2022), Furian et al. (2022), Xu et al. (2022b), Sun et al. (2022) and Shandilya et al. (2023) revealed that mycotoxins either suppress or stimulate lymphocyte proliferation, body resistance, cell-mediated immunity, and humoral immune function depending on the dose and frequency of exposure.

Data on the mechanisms of genotoxicity of combined mycotoxicoses are limited. A group of researchers found that chronic exposure of young pigs to deoxynivalenol and zearalenone significantly reduced the expression of genes involved in the inflammatory response in the liver (Reddy et al. 2018). Genome-wide gene expression analysis showed that deoxynivalenol and zearalenone exposure reduced the expression of most differentially expressed genes associated with inflammatory cytokines (IL10RB, CXCL9), proliferation (IGF1, MFSD2A, IGFBP2, LIPG, and SIK1), and other immune response networks (PILRB, SLA-1, SLA-3, SLA-5, SLA-7, CLDN4, NNMT, TRHDE, UBD, HIST1H2B, and SAA), indicating impaired immune processes in the liver. Increased expression of cytokines (TNF-α, IL-1β, IFN-γ, IL-6 and IL-10) was found in the intestine of pigs after long-term exposure to deoxynivalenol and fumonisin (Bracarense et al. 2012).

The thymus and spleen were chosen to determine the induction of proinflammatory cytokines because they are primary (central) and secondary (peripheral) lymphoid organs that play a central role in the inflammatory response and the development of acquired immunity.

The comparison of cytokine induction in the spleen, thymus, and jejunum is justified by the fact that these organs represent systemic and mucosal immune compartments. In addition, IgA production is primarily a mucosal response. The small intestine and the intestinal cell layer are the first organs exposed to mycotoxins and microorganisms after oral contamination.

With normal structure and functional integrity, the intestinal epithelium forms a physical and immunological barrier that prevents pathogens and toxins from entering the body. Intestinal epithelial cells participate in protecting the mucous membrane from bacteria, as well as in regulating the homeostasis of the mucous tissue by secreting various agents with antimicrobial and regulatory properties (cytokines, chemokines, defensin, immunoglobulins, etc.). Consumption of feed contaminated with mycotoxins leads to disruption of the integrity of the epithelium (changes in tight junctions of proteins), increased permeability of the intestinal wall, inflammation, apoptosis, inhibition of cell proliferation and activation of immunoglobulin synthesis (Jia et al. 2020; Zhang et al. 2022a, b; Wang et al. 2022; Sarker et al. 2023; Ruan et al. 2023; Malczak et al. 2025).

The effect of the developed prophylactic complexes on the expression of mRNA of rabbit spleen genes associated with inflammation in mycotoxicosis is presented in Table 3.

Rabbits fed with mycotoxin-contaminated feed had higher ($P<0.001$) expression of IL-1β, IL-6, and IFN-γ mRNA. Addition of prophylactic complexes to the feed showed a positive protective effect on the expression of IL-1β, IL-6 and IFN-γ mRNA, which was most pronounced in group 5.

Expression of IL-1β, IL-6, and IFN-γ genes in the spleen of rabbits fed with T-2 toxin, zearalenone, and aflatoxin B1 increased by 2.8, 2.2, and 1.5 times compared

to the biological control group. Whereas in the third and fourth groups, the increase in gene expression occurred by 110.0% (P<0.01), 54.6% (P<0.01), 21.6% (P<0.05), and 140.0% (P<0.01), 34.5% (P<0.01), 32.0% (P<0.01), respectively. In the fifth group of rabbits, a significant increase was noted only in relation to IL-1β (P<0.05).

Table 3: Values of mRNA expression of proinflammatory cytokines in the spleen of rabbits

Groups	IL-1β	IL-6	IFN-ɣ
BC	1.00±0.08	1.10±0.21	1.25±0.20
TC	2.80±0.16***	2.4±0.37***	1.91±0.29***
TC+PC1	2.10±0.25**	1.7±0.16**	1.52±0.15*
TC+PC2	2.40±0.31**	1.48±0.09**	1.69±0.25**
TC+PC3	1.50±0.17*	1.25±0.20	1.39±0.29
BC+PC1	1.08±0.20	1.2±0.27	1.2±0.16
BC+PC2	1.00±0.13	1.00±0.09	1.15±0.16
BC+PC3	1.10±0.16	1.19±0.19	1.3±0.09

BC: Biological control; TC: Toxic control; PC: Prophylactic complex; IL=Interleukins, IFN=Interferons.* P<0.05. ** P<0.01. *** P<0.001 when compared with the biological control group

Similar effects on spleen cytokines were observed in other farm and laboratory animals. Zearalenone increased IL-2 and IFN-γ levels and decreased IL-6 gene expression in stimulated spleen lymphocytes of poultry (Wang et al. 2012). Mycotoxin exposure significantly increased proinflammatory cytokine gene expression in the thymus of rabbits, but this effect was less pronounced, especially with respect to IFN-γ (Table 4).

Table 4: Values of mRNA expression of proinflammatory cytokines in the thymus of rabbits

Groups	IL-1β	IL-6	IFN-ɣ
BC	1.20±0.14	1.00±0.11	1.15±0.14
TC	1.74±0.18***	1.82±0.20***	2.44±0.16**
TC+PC1	1.70±0.12**	1.62±0.18**	1.28±0.1*
TC+PC2	1.50±0.16**	1.64±0.14**	1.22±0.13
TC+PC3	1.31±0.18	1.28±0.16	1.20±0.18
BC+PC1	1.24±0.12	1.05±0.12	1.12±0.16
BC+PC2	1.20±0.14	1.12±0.18	1.25±0.14
BC+PC3	1.28±0.12	1.09±0.10	1.20±0.08

BC: Biological control; TC: Toxic control; PC: Prophylactic complex; IL=Interleukins, IFN=Interferons. * P<0.05. ** P<0.01. *** P<0.001 when compared with the biological control group

The increase in the expression of IL-1β, IL-6, and IFN-ɣ in the second group was 45.0%, 82% and 1.12 times (P<0.001). In the rabbits of the fifth group, the expression of proinflammatory cytokines did not differ significantly from the biological control group. In the third and fourth groups, the level of cytokines was increased by 41.7% (P<0.01), 62.0% (P<0.01), 11.3% (P<0.05), and 25.0% (P<0.01), 64.0% (P<0.01), 6.1%, respectively, in relation to IL-1β, IL-6, and IFN-γ. Cytokine mRNA production was also detected in the liver, the main organ of mycotoxin metabolism, which is an immunoreactive organ containing a large number of resident macrophages, which constitute up to 15% of all liver cells (Guo et al. 2021). Mycotoxin exposure also increased the expression of proinflammatory cytokine genes in the liver of rabbits (Table 5).

Expression of IL-1β, IL-6, and IFN-γ genes in the liver of rabbits treated with T-2 toxin, zearalenone, and aflatoxin B1 increased by 61.0, 59.3, and 31.8% (P<0.001) compared to the biological control group. Whereas in the third and fourth groups, gene expression increased by 39.0% (P<0.001), 33.0% (P<0.001), 23.5% (P<0.01) and 43.9% (P<0.01), 42.9% (P<0.01), 14.1% (P<0.05), respectively. In the fifth group of rabbits, a significant increase was noted only in relation to IL-1β and IL-6 by 20.3%, and 23.1 (P<0.05).

Table 5: Values of mRNA expression of proinflammatory cytokines in the liver of rabbits

Groups	IL-1β	IL-6	IFN-ɣ
BC	0.82±0.14	0.91±0.16	0.85±0.18
TC	1.32±0.08***	1.45±0.12***	1.12±0.14***
TC+PC1	1.14±0.18***	1.21±0.18**	1.05±0.10**
TC+PC2	1.18±0.22***	1.30±0.14***	0.97±0.08*
TC+PC3	0.99±0.10*	1.12±0.18*	0.90±0.16
BC+PC1	0.80±0.17	1.02±0.22	0.87±0.14
BC+PC2	0.88±0.14	0.98±0.16	0.90±0.22
BC+PC3	0.90±0.08	0.94±0.12	0.86±0.18

BC: Biological control; TC: Toxic control; PC: Prophylactic complex; IL=Interleukins, IFN=Interferons. * P<0.05. ** P<0.01. *** P<0.001 when compared with the biological control group

Enhanced mRNA induction of proinflammatory cytokines in the liver confirms the hepatotoxicity of mycotoxin co-exposure and potentiation of the initial induction into further activation of the cytokine network (Lu et al. 2022; Ruan et al. 2022).

The results obtained by Pistol et al. (2015) showed that zearalenone exposure lead to a significant reduction in the levels of proinflammatory (tumor necrosis factor, IL-8, IL-1b, IL-6 and IFN-g) and anti-inflammatory cytokines in the liver of young pigs, both at the gene expression and protein levels, which correlates with a decrease in the levels of other inflammatory mediators.

We found that co-administration of oral T-2 toxin, zearalenone, and aflatoxin B1 at high doses increased the expression of proinflammatory cytokine mRNA in the spleen, thymus, and liver of rabbits. The observed differences in the analyzed effects from previous studies could be due to the different toxicities of the mycotoxins studied, their combined action, high doses, and animal species. The effects of mycotoxins on cytokine mRNA profiles in the spleen, thymus, and liver differed from those in the jejunum (Table 6).

Table 6: Values of mRNA expression of proinflammatory cytokines in the jejunum of rabbits

Groups	IL-1β	IL-6	IFN-ɣ
BC	1.12±0.19	1.07±0.14	1.02±0.22
TC	0.64±0.12***	0.80±0.22**	0.49±0.18***
TC+PC1	0.79±0.20**	0.94±0.18	0.67±0.12**
TC+PC2	0.87±0.22**	0.85±0.14**	0.74±0.04**
TC+PC3	1.05±0.08	1.00±0.08	0.90±0.14
BC+PC1	1.19±0.16	1.12±0.18	1.08±0.08
BC+PC2	1.15±0.12	1.09±0.18	1.15±0.16
BC+PC3	1.10±0.18	1.11±0.16	1.06±0.06

BC: Biological control; TC: Toxic control; PC: Prophylactic complex; IL=Interleukins, IFN=Interferons. * P<0.05. ** P<0.01. *** P<0.001 when compared with the biological control group

Mycotoxin intoxication caused a sharp decrease in the synthesis of IL-1β, IL-6, and IFN-γ by 42.8% (P<0.001), 25.2% (P<0.01), and 52.0% (P<0.001) relative to the biological control group. In the rabbits of the fifth group, the expression of proinflammatory cytokines did not differ significantly from the biological control group. In the third

and fourth groups, the level of cytokines was increased by 29.4% (P<0.01), 12.1%, 34.3% (P<0.01) and 22.3% (P<0.01), 20.5% (P<0.01), 27.4% (P<0.01), respectively, in relation to IL-1β, IL-6 and IFN-γ.

The immunotoxicity of mycotoxins in relation to the intestine has long attracted the attention of researchers. The function of the intestinal immunological barrier primarily depends on immunoglobulins, proteins of the complement system, and cytokines (Gao et al. 2020; 2024).

Immunoglobulins such as IgM, IgG, and secretory IgA (secreted by the lamina propria of the intestinal mucosa) are the major humoral immune components and effector molecules on the intestinal mucosal surface. The complement system is an integral part of the innate immunity in the body and is associated with the initiation of adaptive immune responses. Mycotoxins have been shown to impair humoral and cellular immunity in the intestinal mucosa (Lai et al. 2022). Wang et al. (2023b) showed that aflatoxin B1 impaired intestinal immune function by suppressing macrophages, especially M2 macrophages, and Paneth cells secreting antimicrobial peptides. T-2 toxin exposure exerted immunosuppressive effects by suppressing immunoglobulin secretion and decreasing the level of components of the complement system in serum (Wang et al. 2024; Stone-Lawrence, 2025).

Wang et al. (2018) showed that aflatoxin B1 reduced T cell subsets, cytokine expression, IgA + cell numbers, and immunoglobulin expression in the small intestine of broilers, thereby impairing adaptive immunity.

Other researchers suggested that aflatoxin B1 may negatively affect the innate immunity of the small intestine of chickens by suppressing TLR2-2, TLR4, and TLR7 mRNA levels. Activation of NF-κB in various cell types was triggered by the downstream TLR2 and TLR4 signaling pathway, resulting in the release of various cytokines (Anderson 2000; Medzhitov and Janeway 2000).

Previous experiments have shown that aflatoxin B1 (0.6mg/kg) in broiler diets reduced the expression of cytokines (IL-2, IL-4, IL-6, IL-10, IL-17, IFN-γ, and TNF-α mRNA) in the small intestine, and also caused a decrease of goblet cells (which deliver luminal antigen to CD103+ dendritic cells in the small intestine) and the mucins secreted by them (He et al. 2014). A number of studies have been devoted to the effect of zearalenone on the intestinal inflammatory response (Guan et al. 2023). Taranu et al. (2015) showed that zearalenone increased the expression of certain cytokines involved in inflammation in the intestine (TNF-α, IL-1β, IL-6, IL-8, MCP-1, IL-12p40, CCL20) or responsible for the activation of immune cells (IL-10, IL-18). The results of Marin et al. (2015) showed that zearalenone leaded to an increase in the synthesis of IL-8 and IL-10 in the intestine. The studies of Li et al. (2023) found that AFB1 increased inflammation by altering the levels of inflammatory cytokines TNF-α, IL-1β, IL-6, and IL-8, and disrupted the intestinal mucosal barrier by affecting claudin-3, occludin and MUC2.

Thus, the suppressed expression of cytokines in the jejunum observed in our study is consistent with literature data and indicates that the conjoint content of mycotoxins in feed leads to a disruption of the immune function of the intestinal mucosa, namely, suppression of the immune response, and the use of developed prophylactic agents mitigated this effect.

Conclusion

In the present study, long-term exposure of rabbits to high doses of mycotoxins (T-2 toxin, zearalenone, deoxynivalenol) caused a decrease in the proinflammatory cytokine expression in the jejunum and activation in the thymus, and spleen. The absence of reliable differences compared to the biological control group was observed only when using a prophylactic complex based on halloysite, milk thistle meal, and methionine. The other two complexes showed significant deviations compared to both the toxic and the biological control group. When using prophylactic complexes with the main diet, the expression did not differ from that in the control group.

DECLARATIONS

Funding: This study did not get any financial support from any organization

Acknowledgment: The data will be available upon request to the corresponding author.

Conflict of Interest: The authors declare that there is no known conflict of interest associated with this publication.

Data Availability: The data will be available upon request to the corresponding authors.

Ethics Statement: All animal studies have been approved by the relevant ethics committee or institutional supervisory board and have been conducted in accordance with the ethical standards outlined in the 1964 Helsinki Declaration and its subsequent amendments. The Scientific Research Council of the Federal Center for Toxicological, Radiation and Biological Safety (Kazan, Russia) approved the experiment's protocols vide number 11 (28 February 2023). Animals were reared and treated per the EU Directions 2010/63/EU.

Author's Contribution: Evgenya Tarasova, Nail Khammadov developed a scheme for conducting experiments, determined the expression of mRNA of proinflammatory cytokines using quantitative real-time PCR in samples of the spleen, thymus, jejunum, and liver, analyzed the data, and drew conclusions. Gleb Kashevarov performed statistical processing of the data. Svetlana Tanaseva, Olga Ermolaeva and Marina Erokhondina conducted an experiment on animals. Marina Efimova and Eduard Semenov conducted an analysis of literary data. Sergey Smolentsev and Lilia Matrosova wrote an article based on the data received. The final draft manuscript was revised by all authors. All authors edited, read, and approved the final manuscript.

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