Animal Industry and Technology (축산기술과 산업) TITLE PAGE

Upload this completed form to website with submission

ARTICLE INFORMATION	Fill in information in each box below
Article Type	Research article
Article Title (English; within 20 words without abbreviations)	Effect of dietary levels of arginine on mycotoxin contaminated feed on laying hens
Article Title (Korean; English paper can be omitted)	
Running Title (English; within 10 words)	Effect of arginine on mycotoxin contaminated feed on laying hens
Author (English)	Se young Yoon, Habeeb Tajudeen, Jun Young Mun, Abdolreza Hosseindoust, Sang Sik Lee, Priscilla Neves-Silvestre, and Jin Soo Kim
Affiliation (English)	Department of Animal Industry Convergence, Kangwon National University, Chuncheon, 24341, Republic of Korea.
Author (Korean; English paper can be omitted)	
Affiliation (Korean; English paper can be omitted)	
ORCID (for more information, please visit https://orcid.org)	Se Young Yoon (https://orcid.org/0000-0001-6601-8201) Habeeb Tajudeen (https://orcid.org/0000-0002-5623-3175) Jun Young Mun (https://orcid.org/0000-0001-6071-8020) Abdolreza Hosseindoust (https://orcid.org/0000-0001-9191-0613) Sang Sik Lee (https://orcid.org/0009-0003-2171-8388) Priscilla Neves Silvestre (https://orcid.org/0009-0007-0473-2603) Jin Soo Kim (https://orcid.org/0000-0002-9518-7917)
Competing interests	No potential conflict of interest relevant to this article was reported.
Funding sources State funding sources (grants, funding sources, equipment, and supplies). Include name and number of grant if available.	Not applicable.
Acknowledgements	The authors appreciate Daesang Corporation for supplying us the arginine used in the study. We also thank the National Institute of Agricultural Sciences for supplying the mycotoxin.
Availability of data and material	Upon reasonable request, the datasets of this study can be available from the corresponding author.
Authors' contributions Please specify the authors' role using this form.	Conceptualization: S.Y, Yoon, J.Y. Mun, H. Tajudeen Data curation: J.Y. Mun, H. Tajudeen Formal analysis: A. Hosseindoust, P. Neves-Silvestre

	Methodology: J.S. Kim, S.Y, Yoon Software: S.S Lee, A. Hosseindoust Validation: J.S. Kim Investigation: H. Tajudeen, J.Y. Mun Writing - original draft: H. Tajudeen, J.Y. Mun Writing - review & editing: H. Tajudeen, J.Y. Mun, S.Y, Yoon, A. Hosseindoust, P. Neves-Silvestre, S.S. Lee, J.S. Kim
Ethics approval and consent to participate	This experiment was carried out at the Kangwon National University Teaching and Research farm with the ethical code (KW-220413-1) from the Animal Experimental Ethics Committee.

CORRESPONDING AUTHOR CONTACT INFORMATION

For the corresponding author (responsible for correspondence, proofreading, and reprints)	**Fill in information in each box below**
First name, middle initial, last name	Jin Soo Kim
Email address – this is where your proofs will be sent	kjs896@kangwon.ac.kr
Secondary Email address	
Address	Room 201 and 202, Department of Animal Industry Convergence, Kangwon National University.
Cell phone number	+82-102566-5961
Office phone number	+82-102566-5961
Fax number	

Abstract

A total of 240 Hy-Line Brown laying hens with similar body weights of 1.89± 0.12 kg at 24 weeks old were randomly divided into 4 treatments with 6 replicates, and 10-layer hens per replicate. The treatments are (1) 1.1% arginine (Arg) without toxin (Arg1 / CON). (2) 1.1% Arg with a toxin (Arg2), (3) 1.2% Arg with a toxin (Arg3), (4) 1.3% Arg with a toxin (Arg4). In the results, there was a tendency ($p=0.063$) towards higher egg mass in Arg1, Arg3 and Arg4 compared with Arg2. There was also a tendency ($p=0.092$) towards higher yolk color in Arg1, Arg3 and Arg4 compared with Arg2. A tendency ($p=0.053$) towards lower liver weight was observed in Arg1, Arg3 and Arg4 compared with Arg2. A tendency ($p=0.063$) towards higher villus height in the duodenum was observed in ARG1, ARG3 and Arg4 compared with Arg2. Interleukin (IL)-6 was higher ($p<0.05$) in Arg2, Arg3 and Arg4 compared with Arg1, while the interferon (IFN)-γ was higher ($p<0.05$) in Arg2 compared with Arg4 and Arg1 in the liver. The toll-like receptor (TLR)-4 and tumor necrosis factor α were higher ($p<0.05$) in Arg2 compared with Arg3, Arg4, and Arg1. In the muscle, the IL4 was higher ($p<0.05$) in Arg2, Arg3 and Arg4 compared with Arg1. The IL6 was higher ($p<0.05$) in the Arg2 and Arg4 compared with Arg1. The IFNγ was higher ($p<0.05$) in Arg2 and Arg3 compared with Arg1, and TLR4 was higher ($p<0.05$) in Arg3 compared with Arg1. In the jejunum, Toll-like receptor 4 was higher ($p<0.05$) in Arg2 compared with Arg3 and Arg1. The incorporation of Arg in diets of laying hens at 1.1% without mycotoxin, as well as 1.2% and 1.3% in mycotoxin-contaminated diets, could improve the performance and health of laying hens.

Keywords

Aflatoxin, amino acid, egg production, mycotoxin, laying hens.

Introduction

Mycotoxin, a harmful secondary metabolite produced by fungi such as aspergillus, penicillium, and fusarium, are pervasive contaminants in animal feed, posing significant risks to poultry health and productivity [1]. According to Rodrigues and Naehrer [2], the majority of feeds supplied to farm animals generally contain higher level of mycotoxins compared to human diets, as human food undergoes more extensive refining and processing, which helps reduce mycotoxin contamination. Hence, among other environmental contaminants, mycotoxins are susceptible to exposure in poultry [3]. Mycotoxins are difficult to identify, particularly in agricultural areas with climates that support the growth of the fungus producing them [4]. The farm animals most vulnerable to the effects of mycotoxins include pigs and poultry. Laying hens in particular are highly susceptible to various disease outbreak during the laying period as intensive egg production can weaken their immune system [5]. For this reason, a substantial amount of research on the toxicity of mycotoxin has been conducted on these animals [6].

Globally, it is reported that about 25-50% of poultry feed ingredients especially cereal is being contaminated by mycotoxins with 5-10% of that contamination becoming perpetual resulting in major economic loss [7]. Mycotoxin also has the potential to trigger both acute and long-term consequences including carcinogenic, estrogenic, immunosuppressive, and mutagenic effects which in the long run lead to the deterioration of liver, kidney, poor performance, and reproductive disorders in animals [7-8].

The majority of research on mitigating mycotoxicosis has concentrated on techniques for the physical and chemical breakdown of mycotoxin [9], and the use of adsorbents and Anti-mycotoxins mixed in the feed to absorb, eliminate, or bio-transform mycotoxins, thus lowering the harmful effects of the toxins [10]. Numerous agents have also been investigated, including those derived from organic acids and yeasts, especially *Saccharomyces cerevisiae*, whose cell wall is made up of a large capacity for adsorption of mycotoxins. These strategies, however, are not always successful against chemically different mycotoxins, as demonstrated by in vivo investigations [9].

A recent approach in mycotoxin mitigation is the dietary inclusion of functional amino acids (AA), particularly arginine (Arg), which plays a crucial role in enhancing immune function, improving antioxidant status, and supporting organ health [11-12]. Despite previous studies on Arg role in reproduction and growth performance, its potential as a detoxifying agent against mycotoxin-induced damage in laying hens remains underexplored. The novelty of this study lies in evaluating Arg ability to mitigate mycotoxin-induced stress in laying hens by modulating inflammatory responses, preserving organ integrity, and enhancing overall performance.

The detoxification potential of Arg is primarily mediated through multiple physiological pathways. First, Arg serves as a precursor for nitric oxide synthesis, which is crucial for hepatic detoxification processes and immune modulation [13-14]. Nitric oxide enhances the breakdown and clearance of toxic compounds while regulating oxidative stress and inflammation [13, 15]. Additionally, Arg supports the synthesis of polyamines, which play a vital role in cellular repair and intestinal mucosal integrity, thereby counteracting mycotoxin-induced intestinal damage [16]. Furthermore, dietary Arg supplementation at 1.36% in feed could affect production performance and health of laying hens by promoting hepatic protein synthesis and antioxidant enzyme activity, mitigating the adverse effects of mycotoxins on liver health [17].

Therefore, the present research aimed to investigate the impact of dietary Arg levels on laying performance, egg quality, relative organ weight, intestinal morphology, and immune-related cytokine expression in laying hens fed a mixed-mycotoxin-contaminated diet.

Materials and Methods

Birds, experimental designs, and feeds

This project had a total of 240 Hy-Line Brown with similar body weights of 1.89± 0.12 kg at 24 weeks old. The hens were randomly assigned to 4 treatment groups, comprising 6 replicates, and 10-layer hens per replicate for 8 weeks. Group 1 was supplemented with 1.1% Arg without toxin (Arg1 / CON). Group 2 was supplemented with 1.1% Arg with a toxin (Arg2), group 3 was supplemented with 1.2% arginine

with toxin (Arg3), and group 4 was supplemented with 1.3% Arg with a toxin (Arg4). The toxin made from two species of fungi (*Aspergillus flavus* and *Aspergillus parasiticus*) were found to produce mycotoxins at the following concentrations: 392 µg/kg (ppb) of aflatoxin B1, 290 µg/kg (ppb) of aflatoxin G1, 158 µg/kg (ppb) of ochratoxin A, 136 µg/kg (ppb) of aflatoxin B2, and 0.73 µg/kg (ppb) of deoxynivalenol. The laying hens were housed in a three-dimensional pen with 16 hours of daily lighting from a clean 7 W LED bulb (Overdrive, L10NA19DIM 3000 K; White) and good ventilation at a temperature and relative humidity of 22-24°C, and 60-70%, respectively. The pens were enriched in accordance with the European Union's standards for the protection of laying hens [18]. Each pen included a feed trough (12 cm), a perch (15 cm long; providing 15 cm/hen), a nest, and a claw-shortening device. Additionally, each pen had two nipple drinkers (Big Dutchman AG, Vechta-Calveslage, Germany) that were tested on a regular basis, with a total area of 6,400 cm2 (or 1,225 cm2/hen). Feeds and water were supplied ad libitum, and the experiment's nutritional needs were determined to be either greater than or equal to the nutrient requirements listed in Table 2, which correspond to the Hy-Line Brown commercial management guide and provided in mash form. Cleaning and other management procedures were done twice a day between 9:30 am and 4:30 pm. Experimental environment was precisely controlled during the whole period.

Mycotoxin source

The toxins were distributed by the National Institute of Agricultural Sciences, and cultured in cornmeal according to the method suggested by Muller et al. [19]. The concentration of mycotoxins in the cultured corn meal was analyzed and quantified by HPLC according to the method of Moturi et al. [1]. A mixed-mycotoxin model was used to reflect field conditions, where feed ingredients are often contaminated with multiple mycotoxins rather than a single toxin. Aflatoxins and ochratoxin A were selected for their hepatotoxic, nephrotoxic, and immunomodulatory effects, whereas deoxynivalenol was included as a Fusarium-derived toxin associated with reduced feed intake, intestinal dysfunction, and immune alteration. The cultured mycotoxins with volumes showed in Table 1 were top-dressed at the time of feeding. The mycotoxin levels in the feed were set to align with the upper limit permitted by the Korean Control of Livestock and Fish Feed Act (Act No. 19752, Enforcement on October 24, 2023) and the

104 standard and criteria of Feed (Notification of the Ministry of Agriculture, Food and Rural Development
105 (MAFRA), No. 2024-04, Enforcement on 4 Apr 2024), ensuring that while they may impact laying
106 hen's productivity, they do not cause mortality.

107 *Laying performance and egg quality*

108 Laying performance such as the hen-day-egg-production (HDEP), average egg weight (AEW),
109 egg mass (EM), average daily feed intake (ADFI), and feed conversion ratio (FCR) were measured
110 weekly. The HDEP was calculated by dividing the total daily egg output by the number of live hens
111 during the time, multiplied by 100. The AEW was evaluated using an egg multi-tester (Tohoku Rhythm
112 Co. Tokyo, Japan), and egg mass (EM) was derived via % HDEP × AEW. The feed consumption was
113 recorded daily to calculate ADFI, and the FCR was determined using ADFI / EM.

114 At every 7th day of the experiment (within 25-32 weeks), 20 non-cracked eggs per treatment
115 were chosen based on their average weight to evaluate egg quality. Egg quality (external and internal)
116 such as shell color, yolk color, albumin height, and Haugh unit (HU) were evaluated using the egg
117 multi-tester (Tohoku Rhythm Co. Tokyo, Japan). The shell thicknesses were measured after drying the
118 eggshells, and the membrane removed. The thickness of each shell was then measured using eggshell
119 thickness gauge NFN-380 (Fujihara Industry co., Ltd. Tokyo, Japan) by targeting the egg's equator,
120 pointed end, and blunt end). Shell breaking strength was evaluated using a model II eggshell force gauge
121 (Robotmation, Tokyo, Japan) [5].

122 *Relative organ weight*

123 At the end of the experiment (56 days of age), 192 randomly selected hens (8 hens/replicate) were
124 weighed, humanely euthanized, and samples such as the heart, liver, spleen, and kidney were harvested,
125 coded, weighed, and analyzed by blinding method to avoid prejudice to dietary treatment. The organ
126 weight was then analyzed as

127 $$Relative\ organ\ weight = \frac{Weight\ of\ organ\ (g)}{Body\ weight\ (g)} \times 100\%$$

128

Intestinal morphology

Three cross-sections from each intestinal tissue were collected from all the euthanized birds (8 hens/replicate) using a paraffin embedding technique after staining with azure A and eosin [20]. For each cross-section, 10 complete, correctly aligned crypt-villus units were selected in triplicate. Villus height (VH) was determined as the distance from the apex of the villus to the villus-crypt transition, whereas crypt depth (CD) was evaluated as the depth of the mucosal invagination between adjacent villi. Image processing and analysis were performed using Optimus software version 6.5 (Media Cybergenetics, North Reading, MA), with measurements taken in 10-µm increments for all morphological parameters (VH and CD).

Relative expression of interleukin-6, interferon-γ, toll-like receptor-4, and tumor necrosis factor-α in the liver, muscle, and jejunum

Samples from the euthanized laying hens (8 hens/replicate) were further prepared for ribonucleic acid (RNA) extraction and gene expression analysis. A 100g total RNA was extracted from the lateral lobe of the liver, 100g muscle from the longissimus dorsi, and 100g jejunum from the center of the jejunum subsection with the aid of a Trizol reagent (Invitrogen, Carlsbad, CA, USA) following the manufacturer's direction. The concentration and purity of the recovered RNA were determined using a spectrophotometer, with A260/280 ratios ranging from 1.8 to 2.0. The amplification efficiency of all primer pairs was evaluated using standard curves generated from serial dilutions of pooled cDNA, and the efficiencies were verified to be within the acceptable average of 97.6%. The relative mRNA expression levels of the target genes were calculated using the $2^{-\Delta\Delta Ct}$ method. The expression levels were normalized to the β-actin, which showed stable expression across all treatment groups. The isolated RNA was diluted to 1 µg/µL and the cDNA formation was achieved using the Improm-II Reverse transcription system (Promega, Fitchburg, MA, USA). An aggregate of 20 µL reaction was achieved by adding 10 µL SYBR Premix Ex Taq, 0.8 µL of forward and reverse primer (10 µM), 0.4 µL ROX Reference Dye II (50×), 2.0 µL cDNA template, and 6 µL dd H2O, with beta-actin (β-actin)

as the housekeeping gene as shown in Table 3 [21]. The final polymerized chain reaction (PCR) was conducted using Mx3000P real-time PCR system (Stratagen, Redmond, WA, USA), and the delta-delta approach was used to express the outcomes as a relative expression for interleukin-4 (IL4), interleukin-6 (IL6), interferon-γ (IFNγ), toll-like receptor-4 (TLR4), and tumor necrosis factor-α (TNFα) with cycling condition of 30 s at 95 ˚C, 40 cycles of denaturation step at 95 ˚C for 3 s, 60 ˚C annealing step for 34 s and a 72 ˚C extension step for 15 s [22].

Statistical analyses

Following the confirmation of data normality (Shapiro-Wilk test) and variance homogeneity (Levene's test), statistical evaluations were carried out using the General Linear Model (GLM) procedure in SAS software according to a completely randomized experimental layout. When assumptions were not met, data were appropriately transformed or analyzed using non-parametric methods. Individual pens containing the laying hens per treatment were considered the experimental unit for all parameters. The statistical model used for the analysis was:

$$Yij=\mu+Ti+eij$$

where: Yij is the observed response variable, μ is the overall mean, Ti is the fixed effect of treatment (Arg1, Arg2, Arg3, Arg4), eij is the residual error term. Differences were considered statistically significant at $p < 0.05$, and trends were discussed when $p < 0.10$, using Tukey's Honestly Significant Difference test for mean separation.

Results

Laying performance

The Effects of dietary Arg supplementation on the laying performance of hens fed a mold-contaminated diet are shown in Table 4. There was no significant difference in HDEP, AEW, ADFI, and FCR. However, there was a tendency ($p=0.063$) towards higher EM as the ARG inclusion level increased in

Arg3 and Arg4, as well as in the in-laying hens supplemented with the Arg without mold contamination (Arg1) compared with Arg2.

Egg quality

The effects of dietary Arg supplementation on the egg quality of hens fed a mold-contaminated diet are shown in Table 5. There was no significant difference in the external quality such as shell color, shell thickness, and shell breaking strength, and the internal quality such as albumin height, and HU. However, there was a tendency ($p=0.092$) towards higher yolk color in Arg1, Arg3 and Arg4 compared with Arg2.

Relative organ weight

The effects of dietary Arg supplementation on the relative organ weight of hens fed a mold-contaminated diet are shown in Table 6. There was no significant difference in the relative weight of the heart, liver, spleen, and kidney. However, there was a tendency ($p=0.053$) towards lower liver weight in the non-toxic diets Arg1 and the mold-contaminated diets with higher levels of Arg (Arg3 and Arg4) compared with the mold-contaminated diet with lower Arg (Arg2).

Intestinal morphology

The effects of dietary Arg supplementation on intestinal morphology in hens fed a mold-contaminated diet are shown in Table 7. There was no significant difference in the VH in the duodenum, jejunum, and ileum. There was also no significant difference in crypt depth in the duodenum, jejunum, and ileum. Hens fed the ARG1 diet tended to have higher villus height in the duodenum compared to those fed the AGR2 diet ($P = 0.063$).

Relative gene expression in the liver, muscle, and jejunum

The relative expression of IL4, IL6, IFNγ, TLR4, and TNFα in the liver of laying hens are shown in Fig1. There was no significant difference in IL4 across all treatments in the liver. However, IL6 was higher ($p<0.05$) in Arg2, Arg3 and Arg4 compared with Arg1. The IFNγ was higher ($p<0.05$) in Arg2 compared with Arg4 and Arg1. The TLR4 and TNFα were higher ($p<0.05$) in Arg2 compared with Arg3,

Arg4, and Arg1. Fig. 2 shows the relative expression of IL4, IL6, IFNγ, TLR4, and TNFα in the muscle of laying hens. The IL4 was higher ($p<0.05$) in Arg2, Arg3 and Arg4 compared with Arg1. The IL6 was higher ($p<0.05$) in the Arg2 and Arg4 compared with Arg1. The IFNγ was higher ($p<0.05$) in Arg2 and Arg3 compared with Arg1. The TLR4 was higher ($p<0.05$) in Arg3 compared with Arg1, and there was no significant difference in TNFα across all treatments. Fig. 3 shows the relative expression of IL4, IL6, IFNγ, TLR4, and TNFα in the jejunum of laying hens. There was no significant difference in IL4, IL6, IFNγ, and TNFα across all treatments. However, TLR4 was higher ($p<0.05$) in Arg2 compared with Arg3 and Arg1.

Discussions

The EM was lower in Arg2 compared with Arg1, Arg3, and Arg4. A possible explanation for this is the negative effect of mycotoxins on laying performance as previously reported by Lee et al.; Dazuk et al. [23-24]. On comparing these parameters with diets containing higher levels of dietary Arg with mycotoxin (Arg3, Arg4) and diets supplemented with Arg without mycotoxin (Arg1), better laying performance and egg qualities were observed. In its capacity as a vital essential AA, Arg plays a key role in layer performance. Our result shares similarities with the study by Silva et al. [25] which states that dietary Arg incorporated at 1.36% showed the highest laying rate. They estimated that Arg could impact ovarian follicles and the ovaries to enhance the secretion of luteinizing hormone leading to higher performance in hen. The lower yolk color observed in Arg2 can be related to the prevalence of aflatoxins in this treatment limiting the synthesis of bile acid. Consequently, this results in lesser absorption of carotenoid pigments affecting the yolk color [24]. Although the laying performance and egg qualities were numerically higher in Arg1, Arg3 and Arg4, the results were not significantly different. This suggests that while Arg may contribute to performance recovery in laying hens, it might not be sufficient to counteract mycotoxin-induced suppression, potentially due to persistent oxidative stress and impaired metabolism [26-27]. Contrary to this, a prior study has demonstrated higher growth performance when AA and peptide were supplemented to ameliorate the effect of mycotoxins in chicken, mice and pigs [28-29]. We presumed this could have resulted from the synergistic effect of

multiple peptides with each having an added impact. Our study lacked the bolstering advantage as Arg was the only supplemented AA. While the multi-mycotoxin model used in this study closely mimics natural feed contamination in practical poultry production, it introduces certain complexities in interpreting the results. The co-occurrence of aflatoxins, ochratoxin A, and DON likely induced additive or synergistic toxicological effects, making it challenging to delineate the individual contribution of each mycotoxin to the observed physiological damages. Furthermore, a key limitation of our experimental design is the absence of a treatment group supplemented with high levels of Arg under non-contaminated conditions. Consequently, it is difficult to clearly separate the specific mycotoxin-mitigating effects of Arg from its general nutritional benefits, or to determine whether Arg preferentially counteracted a single dominant toxin or the complex interactions within the mixture. Future studies employing a full factorial design (mycotoxin exposure × Arg level) in both contaminated and normal environments are warranted to fully elucidate these underlying mechanisms.

When laying hen consumes mycotoxins, organ damage occurs concurrently with the body's detoxification and elimination processes. The liver is principally responsible for the detoxification of mycotoxins [30], and the accumulation of the detoxified residue is chiefly stored in the liver, kidney, and other tissues, including the muscles and reproductive organs including the eggs [31-32], effectuating oedema and liver enlargement [33]. Comparably, CHU et al. [34] noted that hepatic activities were disrupted due to an upsurge in liver weight and fat deposition in the liver. In this experiment, there was a tendency ($p=0.053$) towards lower liver weight in the non-toxic diets (Arg1) and the mold-contaminated diets with higher levels of L-arginine (Arg3 and Arg4) compared with the mold-contaminated diet with lower L-arginine (Arg2) having higher liver weight. The higher amount of Arg incorporation may have suppressed the harmful impact of mycotoxin, and strengthened the liver's hepatic function, tentatively preventing oedema accounting for the lower liver weight. This result suggests that Arg protects immune function and reduces liver damage in layer hen.

Aside from the liver, the small intestine and immune systems of monogastric are the next acutely affected tissues by mycotoxin infestation as they are important cells with higher protein yield [23], and to achieve optimum digestion and absorption of nutrients, the mucosa of small

intestine encompassing the CD and VH must have a typical framework [35]. Our study showed a tendency (p=0.063) towards higher VH in the duodenum in the non-toxic diet supplemented with Arg (Arg1), and in the mold-contaminated diets with higher levels of Arg (Arg3 and Arg4) compared with lower Arg (Arg2). A similar report by Yu et al. [36] demonstrated higher VH in laying hens fed Arg-supplemented diets at levels 1.44% above the NRC recommendation. As a metabolic precursor for polyamines, Arg acts as a trophic agent that enhances intestinal mucosal development by promoting mitotic activity within the villus-crypt region [36]. This stimulation of villus cell proliferation suggests an improvement in the intestinal absorptive capacity, which is particularly beneficial under conditions that challenge gut integrity, such as mycotoxin exposure [16].

Pro-inflammatory cytokines such as IL4, IL6, IFNγ, TNFα, and TLR4, are affiliated with immunological conditions in poultry, and one of the immediate cytokines synthesized when the immune system is compromised by mycotoxin [37]. As earlier explained that liver plays the front role in protecting other organs such as the gut against mycotoxin distribution via the muscle, its genetic expression surges in the liver and muscle [38]. In our report, Arg supplementation, particularly Arg3 and Arg4 appeared to reduce key inflammatory cytokine and immune-related markers in the liver and muscle tissues than Arg2. We presumed this could be due to the immune system's defense mechanism triggered by the elevated level of Arg which can influence cytokine expression patterns. In line with our study, Ghareeb et al. [39]; Shastak et al. [40] observed a spike in TNFα and IL-2 mRNA in chicken administered diet compromised with mycotoxin. The digestive tract is a host to high expression levels of the receptor TLR4. It serves as the gastroinnate immune system's initial barrier to immunity essential for the transcription and translation of inflammatory mediators [41-43]. Our experiment showed higher TLR4 in Arg2 compared with Arg3 and Arg1 in the jejunum of laying hens. This could potentially emerge as a consequence of the toxicity inducing the overexpression of TLR4. Our finding suggests that elevated levels of Arg may alleviate the effects of mycotoxin toxicity by modifying immune responses in laying hens.

Conclusion

This study reveals that the incorporation of higher dietary Arg showed a tendency for increased EM and yolk color. This suggests that supplementing Arg in diets of laying hens at 1.1% without mycotoxin, as well as 1.2% and 1.3% in mycotoxin-contaminated diets could lower the liver injury incited by mycotoxin poisoning in feeds. Moreover, higher Arg levels markedly suppressed liver cytokine TNFα, TLR4, and IFNγ shielding both the liver and immune system. This ameliorated the well-being of mycotoxin-debilitated laying hens.

REFERENCES

[1] Moturi J, Yoon S, Hosseindoust A, Ha S, Tajudeen H, Mun J, Sa S, Kim J. Effects of dietary arginine in ameliorating the deleterious effects induced by mycotoxins on growth, immune system, body organs in growing pigs. J Anim Sci Technol. 2022; 64:727. https://doi.org/10.5187%2Fjast.2022.e54

[2] Rodrigues I, Naehrer K. A three-year survey on the worldwide occurrence of mycotoxins in feedstuffs and feed. Toxins. 2012; 4:663-675.https://doi.org/10.3390/toxins4090663

[3] Yu J, Pedroso IR. Mycotoxins in cereal-based products and their impacts on the health of humans, livestock animals and pets. Toxins. 2023; 28; 15:480. https://doi.org/10.3390/toxins15080480

[4] Hassan RA, Farouk ZM, Abou-Shehema B, El-Shafey A, Attia YA, Alhotan RA, Addeo NF, Bovera F, de Oliveira MC. Investigating the Potential of Silymarin And/Or Spirulina Platensis to Attenuate the Deleterious Consequences of Aflatoxin Contamination in Broilers' feeds. J Anim Plant Sci. 2024; 31:34. https://doi.org/10.36899/JAPS.2024.5.0799

[5] Tajudeen H, Mun J, Ha S, Hosseindoust A, Lee S, Kim J. Effect of wild ginseng on the laying performance, egg quality, cytokine expression, ginsenoside concentration, and microflora quantity of laying hens. J Anim Sci Technol. 2023;65(2):351. https://doi.org/10.5187/jast.2022.e108

[6] Yang C, Song G, Lim W. Effects of mycotoxin-contaminated feed on farm animals. J Hazard Mater. 2020; 389:122087. https://doi.org/10.1016/j.jhazmat.2020.122087

[7] Haque MA, Wang Y, Shen Z, Li X, Saleemi MK, He C. Mycotoxin contamination and control strategy in human, domestic animal and poultry: A review. Microb pathog. 2020; 142:104095. https://doi.org/10.1016/j.micpath.2020.104095

[8] D'mello JF, Placinta CM, Macdonald AM. Fusarium mycotoxins: a review of global implications for animal health, welfare and productivity. Anim Feed Sci Technol. 1999; 80:183-205. https://doi.org/10.1016/S0377-8401(99)00059-0.

[9] Liu YL, Meng GQ, Wang HR, Zhu HL, Hou YQ, Wang WJ, Ding BY. Effect of three mycotoxin adsorbents on growth performance, nutrient retention and meat quality in broilers fed on mould-contaminated feed. Br Poult Sci. 2011; 52:255-63. https://doi.org/10.1080/00071668.2011.559453

[10] Carao ÁC, Burbarelli MF, Polycarpo GD, Santos AR, Albuquerque RD, Oliveira CA. Métodos físicos e químicos de detoxificação de aflatoxinas e redução da contaminação fúngica na cadeia produtiva avícola. Ciência Rural. 2014; 44:699-705.https://doi.org/10.1590/S0103-84782014000400021.

[11] Wu G, Bazer FW, Davis TA, Kim SW, Li P, Marc Rhoads J, Carey Satterfield M, Smith SB, Spencer TE, Yin Y. Arginine metabolism and nutrition in growth, health and disease. Amino acids. 2009; 37:153-68. https://doi.org/10.1007/s00726-008-0210-y

[12] Attia YA, Hassan RA, Tag El-Din AE, Abou-Shehema BM. Effect of ascorbic acid or increasing metabolizable energy level with or without supplementation of some essential amino acids on productive and physiological traits of slow-growing chicks exposed to chronic heat stress. J Anim Physiol Anim Nutr. 2011; 95:744-55. https://doi.org/10.1111/j.1439-0396.2010.01104.x

[13] Nikolic J, Stojanovic I, Pavlovic R, Sokolovic D, Bjelakovic G, Beninati S. The role of L-arginine in toxic liver failure: interrelation of arginase, polyamine catabolic enzymes and nitric oxide synthase. Amino acids. 2007; 32:127-31. https://doi.org/10.1007/s00726-006-0309-y

[14] Hu S, Li X, Rezaei R, Meininger CJ, McNeal CJ, Wu G. Safety of long-term dietary supplementation with L-arginine in pigs. Amino Acids. 2015; 47:925-36.

https://doi.org/10.1007/s00726-015-1921-5

[15] Wu L, Wang W, Yao K, Zhou T, Yin J, Li T, Yang L, He L, Yang X, Zhang H, Wang Q. Effects of dietary arginine and glutamine on alleviating the impairment induced by deoxynivalenol stress and immune relevant cytokines in growing pigs. PLoS One. 2013;8:e69502.

https://doi.org/10.1371/journal.pone.0069502

[16] Murakami AE, Fernandes JI, Hernandes L, Santos TC. Effects of starter diet supplementation with arginine on broiler production performance and on small intestine morphometry. Pesqui Vet Bras. 2012; 32:259-266. https://doi.org/10.1590/S0100-736X2012000300014

[17] Duan X, Li F, Mou S, Feng J, Liu P, Xu L. Effects of dietary L-arginine on laying performance and anti-oxidant capacity of broiler breeder hens, eggs, and offspring during the late laying period. Poult Sci. 2015; 94:2938-2943.https://doi.org/10.3382/ps/pev283

[18] Directive EU. Council Directive 99/74/EC of 19 July 1999 laying down minimum standards for the protection of laying hens. Official journal of the European Communities. 1999; 203:53-7.

[19] Muller FM, Seidler M, Beauvais A. Aspergillus fumigatus biofilms in the clinical setting. Med Mycol. 2011;49(sup1): S96-100.https://doi.org/10.3109/13693786.2010.502190

[20] Lee SH, Hosseindoust A, Laxman Ingale S, Rathi PC, Yoon SY, Choi JW, Kim JS. Thermostable xylanase derived from Trichoderma citrinoviride increases growth performance and non-starch polysaccharide degradation in broiler chickens. Br Poult Sci. 2020; 61:57-62. https://doi.org/10.1080/00071668.2019.1673316

[21] Hosseindoust A, Ha S, Lokhande A, Mun J, Kim YI, Kim J. The targeted anti-Salmonella bacteriophage attenuated the inflammatory response of laying hens challenged with Salmonella Gallinarum. Poult Sci. 2023; 102:102296. https://doi.org/10.1016/j.psj.2022.102296

[22] Yoon SY, Sa SJ, Cho ES, Ko HS, Choi JW, Kim JS. Effects of zinc oxide and arginine on the intestinal microbiota and immune status of weaned pigs subjected to high ambient temperature. Animals. 2020; 10:1537. https://doi.org/10.3390/ani10091537

[23] Lee JT, Jessen KA, Beltran R, Starkl V, Schatzmayr G, Borutova R, Caldwell DJ. Effects of mycotoxin-contaminated diets and deactivating compound in laying hens: 2. Effects on white shell egg quality and characteristics. Poult Sci. 2012; 91:2096-2104.https://doi.org/10.3382/ps.2012-02137

[24] Dazuk V, Boiago MM, Rolim G, Paravisi A, Copetti PM, Bissacotti BF, Morsch VM, Vedovatto M, Gazoni FL, Matte F, Gloria EM. Laying hens fed mycotoxin-contaminated feed produced by Fusarium fungi (T-2 toxin and fumonisin B1) and Saccharomyces cerevisiae lysate: Impacts on poultry health, productive efficiency, and egg quality. Microb Pathog. 2020; 149:104517.

https://doi.org/10.1016/j.micpath.2020.104517.

[25] Silva LM, Murakami AE, Fernandes JI, Dalla Rosa D, Urgnani JF. Effects of dietary arginine supplementation on broiler breeder egg production and hatchability. Braz J of Poult Sci. 2012; 14:267-73. https://doi.org/10.1590/S1516-635X2012000400006

[26] Kulcsár S, Kövesi B, Balogh K, Zándoki E, Ancsin Z, Márta BE, Mézes M. Effects of Fusarium mycotoxin exposure on lipid peroxidation and glutathione redox system in the liver of laying hens. Antioxidants; 2021; 10:1313. https://doi.org/10.3390/antiox10081313

[27] Zhu F, Zhu L, Xu J, Wang Y, Wang Y. Effects of moldy corn on the performance, antioxidant capacity, immune function, metabolism and residues of mycotoxins in eggs, muscle, and edible viscera of laying hens. Poult Sci. 2023; 102:102502. https://doi.org/10.1016/j.psj.2023.102502

[28] Belokrylov GA, Popova OY, Sorochinskaya EI. Detoxication of benzene and aflatoxin B1 with amino acid and peptide preparations in mice and chicken. Bull Exp Biol Med. 1999; 128: 1020-1022. https://doi.org/10.1007/BF02433195.

[29] MEZES, M., BALOGH, K., TOTH, K, 2010: Preventive and therapeutic methods against the toxic effects of mycotoxins-A review. Acta Vet. Hung. 58, 1-17. https://doi.org/10.1556/avet.58.2010.1.1

[30] Kovesi B, Kulcsár S, Ancsin Z, Erdélyi M, Zándoki E, Gömbös P, Balogh K, Mézes M. Multi-Fusarium mycotoxin exposure activates Nrf2 and Ahr pathway in the liver of laying hens. Toxicol Lett. 2024; 391:55-61. https://doi.org/10.1016/j.toxlet.2023.12.006

[31] Maresca M, Fantini J. Some food-associated mycotoxins as potential risk factors in humans predisposed to chronic intestinal inflammatory diseases. Toxicon. 2010; 56:282-294. https://doi.org/10.1016/j.toxicon.2010.04.016

[32] Stoev SD. Natural feed additives and bioactive supplements versus chemical additives as a safe and practical approach to combat foodborne mycotoxicoses. Front Nutr. 202419; 11:1335779.

https://doi.org/10.3389/fnut.2024.1335779

[33] Raj J, Farkaš H, Jakovčević Z, Vasiljević M, Kumar R, Asrani RK. Effects of supplemented multicomponent mycotoxin detoxifying agent in laying hens fed aflatoxin B1 and T2-toxin contaminated feeds. Poult Sci. 2023;102(8): 102795.https://doi.org/10.1016/j.psj.2023.102795

[34] Chu Y, Zheng Y, Li Y, Gui S, Zhao J, Zhao Y, Chen X. Dietary supplementation of magnolol alleviates fatty liver hemorrhage syndrome in postpeak Xinhua laying hens via regulation of liver lipid metabolism. Poult Sci. 2024; 103:103378. https://doi.org/10.1016/j.psj.2023.103378.

[35] Yao K, Yin Y, Li X, Xi P, Wang J, Lei J, Hou Y, Wu G. Alpha-ketoglutarate inhibits glutamine degradation and enhances protein synthesis in intestinal porcine epithelial cells. Amino acids. 2012; 42:2491-2500. https://doi.org/10.1007/s00726-011-1060-6

[36] Yu J, Yang H, Wang Z, Dai H, Xu L, Ling C. Effects of arginine on the growth performance, hormones, digestive organ development and intestinal morphology in the early growth stage of layer chickens. Ital J Anim Sci. 2018; 17:1077-1082. https://doi.org/10.1080/1828051X.2018.1434692

[37] Mehrzad J, Hosseinkhani S, Malvandi AM. Human microglial cells undergo proapoptotic induction and inflammatory activation upon in vitro exposure to a naturally occurring level of aflatoxin B1. Neuroimmunomodulation. 2018; 25:176-83. https://doi.org/10.1159/000493528

[38] Okasha H, Song B, Song Z. Hidden hazards revealed: Mycotoxins and their masked forms in poultry. Toxins. 2024; 16:137. https://doi.org/10.3390/toxins16030137

406 [39] Ghareeb K, Awad WA, Soodoi C, Sasgary S, Strasser A, Böhm J. Effects of feed contaminant
407 deoxynivalenol on plasma cytokines and mRNA expression of immune genes in the intestine of broiler
408 chickens. PloS One. 2013; 8: e71492.https://doi.org/10.1371/journal.pone.0071492

409 [40] Shastak Y, Pelletier W. Delving into vitamin A supplementation in poultry nutrition: current
410 knowledge, functional effects, and practical implications. World's Poult Sci J. 2024; 80:109-31.
411 https://doi.org/10.1080/00439339.2023.2250327

412 [41] Attia YA, Böhmer BM, Roth-Maier DA. Responses of broiler chicks raised under constant
413 relatively high ambient temperature to enzymes, amino acid supplementations, or a high-nutrient diet.
414 Eur Poultry Sci. 2006; 1: 70:80-91. https://doi.org/10.1016/S0003-9098(25)00569-7

415 [42] Attia YA, Addeo NF, Bovera F, Abdallah AA, Alhotan RA, Abd El-Hamid AE, Elnaggar AS, Zakey
416 RM. Arginine: lysine ratio influences on performance, egg quality, haematology, biochemistry,
417 antioxidant status and immunity of dual-purpose breeding hens exposed to cyclic heat stress. Ital J Anim
418 Sci. 2024; 23:200-14. https://doi.org/10.1080/1828051X.2023.2301444

419 [43] Hu X, Yuan X, Zhang G, Song H, Ji P, Guo Y, Liu Z, Tian Y, Shen R, Wang D. The intestinal
420 epithelial macrophage-crypt stem cell axis plays a crucial role in regulating and maintaining intestinal
421 homeostasis. Life Sci. 2024; 344:122452. https://doi.org/10.1016/j.lfs.2024.122452

Table 1. Volume of Mycotoxin

Items	Volume
Aflatoxins B1 (µg/kg) (ppb)	392
Aflatoxins G1 (µg /kg) (ppb)	290
Ochratoxin A (µg /kg) (ppb)	158
Aflatoxins B2 (µg /kg) (ppb)	136
Deoxynivalenol (mg/kg)	0.73
Aflatoxins G2 (µg /kg) (ppb)	-
Fumonisin B (1)	-
Tricothecene-2	-

- (Not detected)

Table 2. Formula and chemical composition of basal diets (as-fed basis)

Items	Basal diet
Ingredient (%)	
Corn	62.39
Soybean meal dehulled	17.40
Rapeseed meal	4.00
Corn gluten meal (HP)	3.70
Soy Oil	1.00
Choline-Liquid (50%)	0.05
$_{L}$-Lysine 78%	0.062
$_{DL}$-Methionine (98%)	0.07
Limestone (75% coarse: 25% fine)	10.70
Salt	0.35
NaHCO3	0.06
Vitamin premix[1]	0.11
Mineral premix[2]	0.10
Total	100.00
Calculated chemical composition	
Metabolizable energy (MJ/kg)	11.50
Crude protein	17.00
Crude fat	3.39
Crude fiber	2.40
Ash	12.67
Calcium	4.19
Phosphorus	0.40
Sodium	0.16
Chloride	0.26
Lysine	0.81
Methionine	0.37
Methionine + cysteine	0.67
Threonine	0.63
Tryptophan	0.17
Arginine	1.00
Analyzed chemical composition	
Gross energy (MJ/kg)	15.93
Crude protein	17.22
Crude fat	3.58
Calcium	4.35
Phosphorus	0.41
Arginine	1.07
Lysine	0.83
Methionine	0.40

[1]Supplied per kilogram of diet: 16,000 IU vitamin A, 3,000 IU vitamin D_3, 40 IU vitamin E, 5.0 mg vitamin K_3, 5.0 mg vitamin B_1, 20 mg vitamin B_2, 4 mg vitamin B_6, 0.08 mg vitamin B_{12}, 40 mg pantothenic acid, 75 mg niacin, 0.15 mg biotin, 0.65 mg folic acid.

[2]Supplied per kilogram of diet: 45 mg Fe, 0.25 mg Co, 50 mg Cu, 15 mg Mn, 25 mg Zn, 0.35 mg I, 0.13 mg Se.

Table 3. Primer information

Relative gene expressed	Primer sequence	Accession No./Gen Bank
IL4	F: TGTGCCCACGCTGTGCTTACA R: CTTGTGGCAGTGCTGGCTCTCC	AJ621249.1
IL6	F: AGAAATCCCTCCTCGCCAAT R: AAATAGCGAACGGCCCTCA	NM_204628.1
IFNγ	F: CTGAAGAACTGGACAGAGAG R: CACCAGCTTCTGTAAGATGC	X99774
TLR4	F: GTCTCTCCTTCCTTACCTGCTGTTC R: AGGAGGAGAAAGACAGGGTAGGTG	NM_001030693.1
TNFα	F: CCCCCAGAAGGAAGAGTTTC R: CGGGCTTATCTGAGGTTTGA	JF831365.1
β. Actin	F: CAACACAGTGCTGTCTGGTGGTA R: ATCGTACTCCTGCTTGCTGATCC	X00182.1

Abbreviations: IL (interleukin); IFN (interferon); TLR (toll like receptor); TNF (tumour necrosis factor); β. Actin (beta-actin)

Table 4. Effects of dietary L-arginine supplementation on laying performance of layers fed a m old-contaminated diet[1]

Item	Non-toxin	Toxin with Arg			SEM	p-value
	Arg1.1	Arg2	Arg3	Arg4		
Hen day egg production (%)	85.62	82.29	84.83	83.85	1.247	0.301
Average egg weight (g/egg)	61.61	60.66	60.94	61.60	0.539	0.516
Egg mass (g/egg/bird)	52.77	50.02	51.72	51.66	0.673	0.063
Feed intake (g/day/bird)	101.94	98.69	99.56	98.18	1.372	0.249
Feed conversion ratio	1.947	1.987	2.022	1.973	0.041	0.617

SEM, standard error of mean; 1.1% arginine without toxin (Arg1 / CON); 1.1% Arg with a toxin (Arg 2); 1.2% arginine with toxin (Arg3); 1.3% Arg with toxin (Arg4)

[1]Data represent means based on 8 hens per replicate

Table 5. Effects of dietary L-arginine supplementation on egg quality of layers fed a mold contaminated diet[1]

Item	Non-toxin Arg1.1	Toxin with Arg			SEM	p-value
		Arg2	Arg3	Arg4		
External						
Shell color	14.05	13.88	14.03	13.92	0.165	0.852
Shell thickness (mm)	0.39	0.40	0.40	0.39	0.013	0.280
Shell breaking strength (kgf)	4.25	4.21	4.22	4.20	0.142	0.996
Internal						
Yolk color	7.46	7.05	7.42	7.10	0.131	0.092
Albumin height (mm)	7.20	7.02	7.11	7.14	0.174	0.888
Haugh unit	82.98	81.25	81.97	82.95	1.292	0.743

SEM, standard error of mean; 1.1% arginine without toxin (Arg1 / CON); 1.1% Arg with a toxin (Arg2) ; 1.2% arginine with toxin (Arg3); 1.3% Arg with toxin (Arg4)

[1]Data represent means based on 8 hens per replicate

Table 6. Effects of dietary L-arginine supplementation on relative organ weight (g/100g of BW) of layers fed a mold-contaminated diet[1]

Item	Non-toxin	Toxin with Arg			SEM	p-value
	Arg1.1	Arg2	Arg3	Arg4		
Heart	0.55	0.56	0.50	0.52	0.018	0.314
Liver	2.01	2.38	2.04	2.03	0.104	0.053
Spleen	0.14	0.16	0.13	0.15	0.013	0.470
Kidney	0.20	0.21	0.20	0.19	0.019	0.883

SEM, standard error of mean; 1.1% arginine without toxin (Arg1 / CON); 1.1% Arg with a toxin (Arg 2); 1.2% arginine with toxin (Arg3); 1.3% Arg with toxin (Arg4)

[1]Data represent means based on 8 hens per replicate

Table 7. Effects of dietary L-arginine supplementation on intestinal morphology in layers fed a mold contaminated diet[1]

Items	Non-toxin Arg1.1	Toxin with Arg			SEM	p-value
		Arg2	Arg3	Arg4		
Villus height (㎛)						
Duodenum	1121	1033	1092	1084	21.882	0.063
Jejunum	720	704	710	709	25.711	0.976
Ileum	496	468	502	494	15.503	0.446
Crypt depth (㎛)						
Duodenum	173	171	188	176	12.936	0.784
Jejunum	112	104	107	113	8.383	0.854
Ileum	84	80	83	81	4.091	0.897

SEM, standard error of mean; 1.1% arginine without toxin (Arg1 / CON); 1.1% Arg with a toxin (Arg2); 1.2% arginine with toxin (Arg3); 1.3% Arg with toxin (Arg4)

[1]Data represent means based on 8 hens per replicate

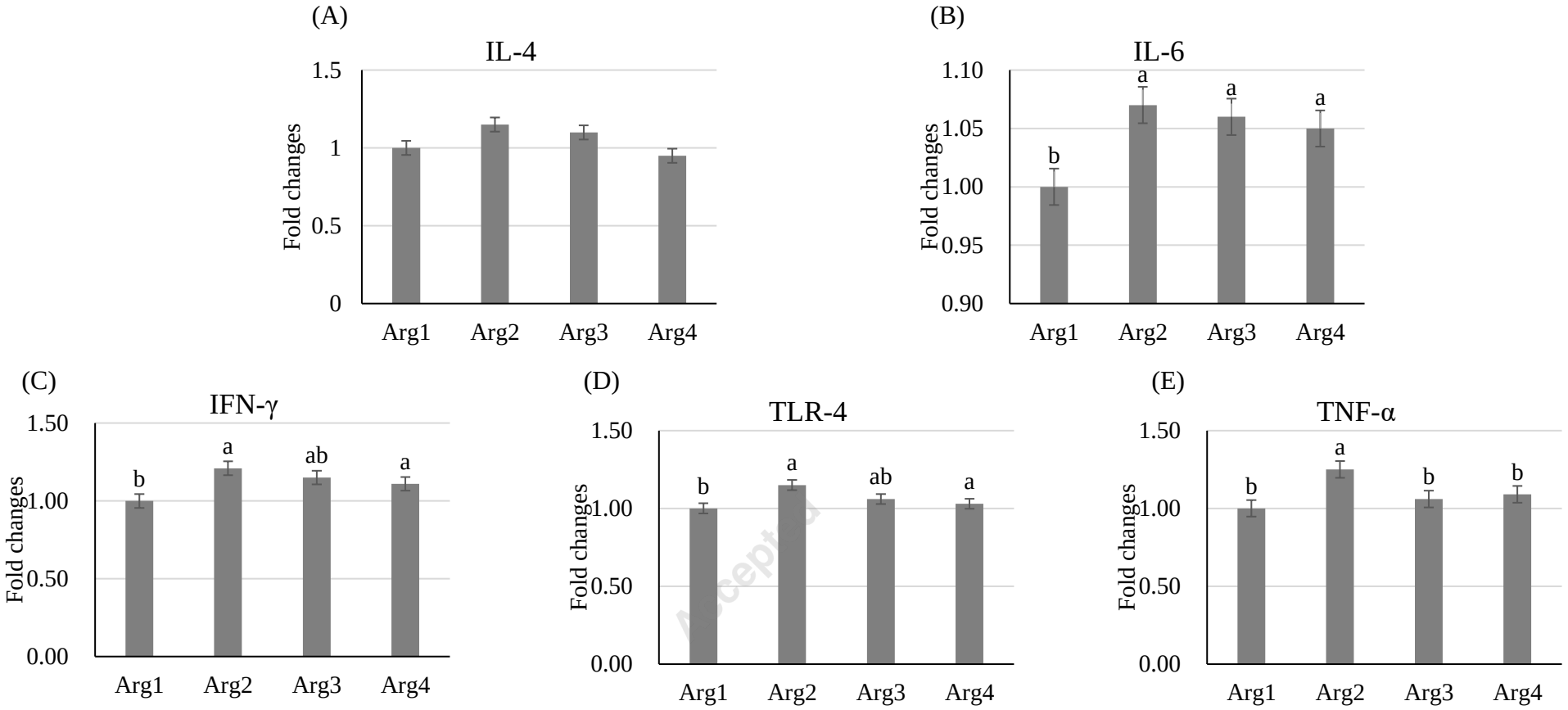

Figure 1. Relative expression of IL-4, IL-6, IFNγ, TLR4 and TNFα in liver of laying hens.

Abbreviations: IL, Interleukin; IFN, Interferon; TLR, Toll like receptor; TNF, Tumor necrosis factor.
1.1% arginine without toxin (Arg1/CON); 1.1% Arg with a toxin (Arg2); 1.2% arginine with toxin (Arg3); 1.3% Arg with toxin (Arg4).
Data represent means based on 8 hens per replicate
Bars with different letters (a~b) differ significantly across all 4 treatment groups ($P < 0.05$).

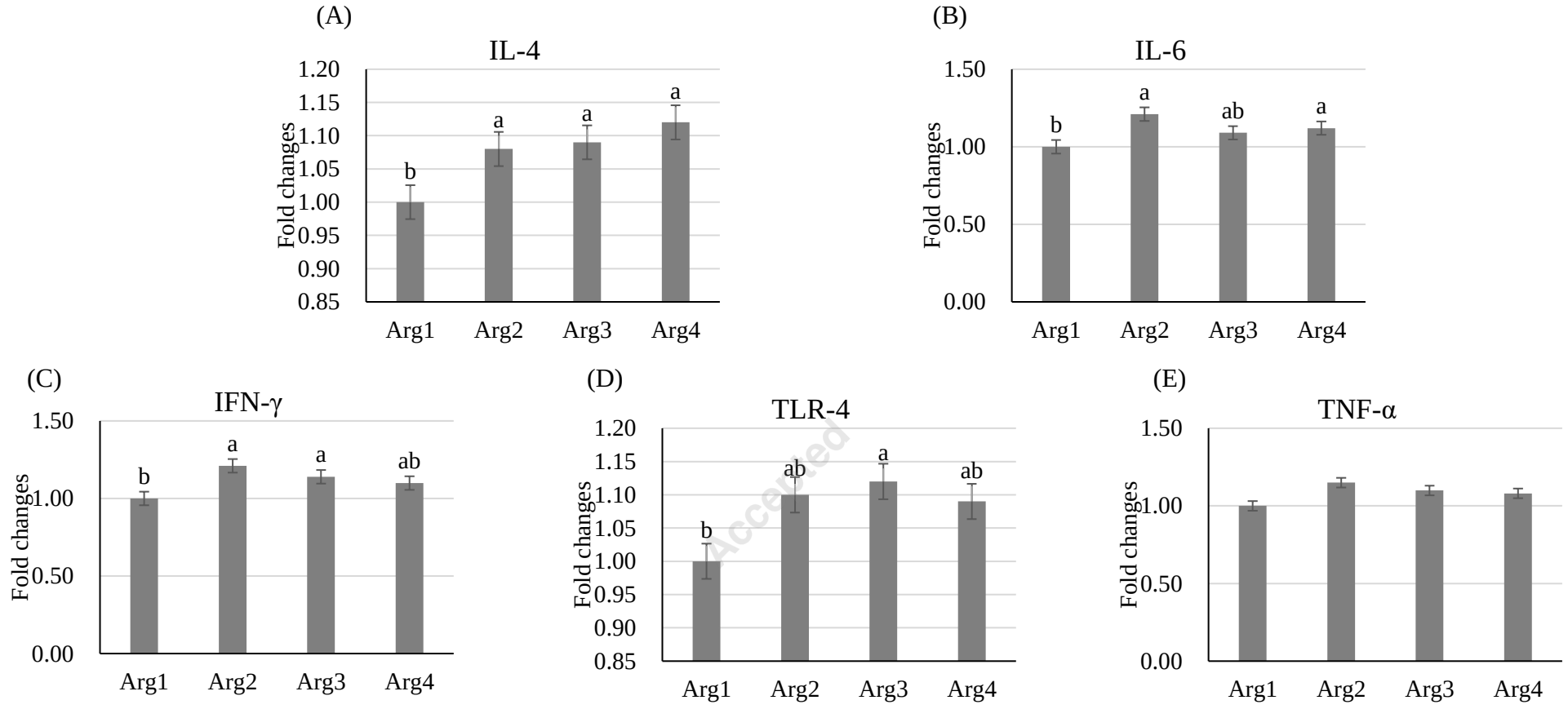

Figure 2. Relative expression of IL-4, IL-6, IFNγ, TLR4 and TNFα in muscle of laying hens.

Abbreviations: IL, Interleukin; IFN, Interferon; TLR, Toll like receptor; TNF, Tumor necrosis factor.
1.1% arginine without toxin (Arg1/CON); 1.1% Arg with a toxin (Arg2); 1.2% arginine with toxin (Arg3); 1.3% Arg with toxin (Arg4).
Data represent means based on 8 hens per replicate.
Bars with different letters (a~b) differ significantly across all 4 treatment groups ($P < 0.05$).

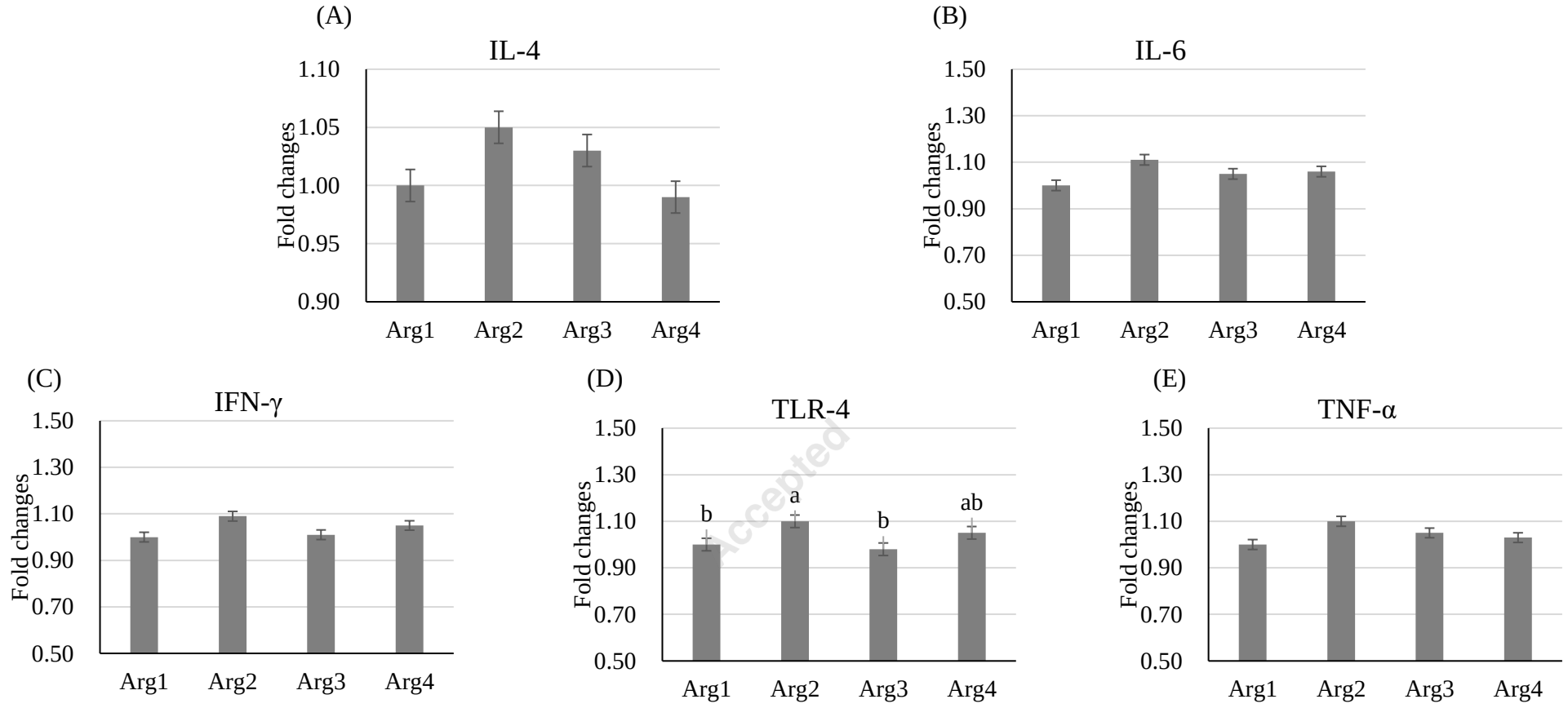

Figure 3. Relative expression of IL-4, IL-6, IFNγ, TLR4 and TNFα in jejunum of laying hens.

Abbreviations: IL, Interleukin; IFN, Interferon; LR, Toll like receptor; TNF, Tumor necrosis factor.
1.1% arginine without toxin (Arg1/CON); 1.1% Arg with a toxin (Arg2); 1.2% arginine with toxin (Arg3); 1.3% Arg with toxin (Arg4).
[1]Data represent means based on 8 hens per replicate
Bars with different letters (a~b) differ significantly across all 4 treatment groups ($P < 0.05$).