Combination of *Lactobacillus* species and yeast ameliorates adverse effect of deoxynivalenol contaminated diet on immune system, gut morphology and jejunal gene expression in broiler chickens

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**ABSTRACT**

The aim of this study was to examine the effect of different levels of *Lactobacillus* species and yeast (*Saccharomyces cerevisiae*) as a toxin binder in deoxynivalenol (DON) diet on immune parameters, gut morphology and jejunal gene expression in broiler chickens. Three hundred sixty-one-day-old female broilers were assigned into nine treatments with four replicates each in a completely randomised design (3\( \times \)3 factorial arrangement) including three levels of *Lactobacillus* (0, 0.20 and 0.40 g/kg) and yeast (0, 0.75 and 1.50 g/kg) in DON contaminated diets. The results showed that DON challenged birds showed suppressed spleen relative weight and reduced white blood cell and lymphocyte percentage, while inclusion the highest level of *Lactobacillus* sp. and yeast to DON diet improved these parameters (\( p < .05 \)). Newcastle antibody titre was increased (\( p < .05 \)) by increasing the level of *Lactobacillus* sp. and yeast in DON included diet. Increasing the level of *Lactobacillus* sp. and yeast in DON diet was linearly enhanced (\( p < .05 \)) the villus height, muscular layer and absorptive surface area in ileum. Combination of 0.40 g/kg *Lactobacillus* sp. and 1.50 g/kg yeast caused an enhancement (\( p < .05 \)) in mucosa and muscular layer thicknesses of jejunum and ileum, respectively. The expressions of toll like receptor-4 and claudin-5 were down-regulated and up-regulated respectively by administration of the highest level of *Lactobacillus* sp. and yeast. In conclusion, the results indicated that dietary inclusion of the highest level of *Lactobacillus* sp. and yeast improved the spleen weight, some immunological parameters, villus height, muscular layer thickness and gene expressions in DON-challenged broilers.

**HIGHLIGHTS**

- Deoxynivalenol (DON) consumption (10 mg/kg) induce intestinal and immunological changes in broilers.
- Combination of *Lactobacillus* sp. and yeast reduce DON toxicity.
- Inclusion 0.4 g/kg *lactobacillus* and 1.5 g/kg yeast improve villus height and humoral immunity.

**Introduction**

Deoxynivalenol (DON) is the most prevalent mycotoxin in cereal grains belonging to \( \beta \)-trichothecenes, produced by *Fusarium graminearum* and *Fusarium culmorum* (Awad et al. 2010). Dietary DON contamination has been reported to cause various detrimental effects including anorexia, gastrointestinal inflammation, barrier malfunction, cellular protein kinase disturbance, immune system suppression and oxidative stress induction (Yunus et al. 2012; Wu et al. 2018; Yang et al. 2020). The DON can bind to ribosomes and activate mitogen-activated protein kinases pathway interfering with DNA and RNA synthesis and hence inhibiting protein synthesis. Tissues with high protein turnover such as intestinal tract and immune system are more susceptible to DON (Awad et al. 2010).

The gastrointestinal tract is considered as the major site for digestion and absorption of nutrients and also serves as a defensive barrier against toxins, pathogens and ingested contaminants (Yang et al. 2019). Tight junction proteins (TJPs) are included claudins, occludin and zonula occludens connecting enterocytes and...
playing an important role in paracellular pathways. The disturbance in TJPs can enhance bacterial and toxin permeability into the intestinal lumen, consequently causing immune cells activation (Van De Walle et al. 2010). Toll-like receptor (TLR-4) in the intestinal epithelium plays a fundamental role in recognising microbial agents, pathogens and natural toxins being involved in the regulation of inflammatory conditions. Furthermore, the immune system cells express TLR-4 structure to the less toxic product in birds’ gastrointestinal tract should be considered. Lactobacillus sp. (as a probiotic) is a good candidate for diminishing the adverse effect of DON in diets which can restore intestinal health through immune system stimulation and bacterial balance change (Wu et al. 2018). Several in vivo and in vitro studies have shown that Lactobacillus sp., Bacillus sp. and bifidobacteria have an ability to bind with mycotoxins in the intestinal tract and to reduce the mycotoxins bioavailability (Wan et al. 2016; Metzler-Zebeli et al., 2020). Saccharomyces cerevisiae has recently been illustrated to mitigate the harmful effects of aflatoxin exposure (Arif et al. 2020).

Based on the previous findings, Lactobacillus sp. and yeast have a positive effect on mycotoxin detoxification and thereby have protected the integrity of the intestinal morphological structure and gene expression in intestinal barrier of broilers challenged with DON (Arif et al. 2020; Yang et al. 2020). To the best of our knowledge, no study was conducted regarding the simultaneous effects of probiotic-based Lactobacillus species and Saccharomyces cerevisiae in DON contaminated diets. Also, no information is available about the effect of Lactobacillus sp. and yeast (Saccharomyces cerevisiae) combination on ameliorating the detrimental effect of DON on gene expression and immune system. Therefore, the aim of the present study was to assess the effects of Lactobacillus sp. alone or in combination with Saccharomyces cerevisiae in DON contaminated diets on growth performance, jejunal gene expression, intestinal morphology and immune system in broiler chickens.

Materials and methods

Birds and experimental diets

A total of 360 one-day-old female broiler chickens (Hubbard®) were purchased from a local commercial hatchery. All birds were housed in floor pen where wood shaving was used as a litter. The temperature was set at 32°C within 1–3 days and then was gradually reduced 3°C/wk until reached 22°C. This temperature was sustained at 22°C until the end of the experiment. Moreover, the lighting program was performed with 23L/1D and birds had free accesses to feed and water throughout the study. Birds were weighed and randomly allocated to 9 treatments with 4 replicates of 10 birds each. The experiment was carried out in a completely randomised design (3 x 3 factorial arrangement) to assay the interaction effects of 3 levels of Lactobacillus sp. (0, 0.20 and 0.40 g/kg) and yeast (0, 0.75 and 1.50 g/kg) in DON contaminated diets (Yang et al. 2017; Arif et al. 2020). Feeding

Table 1. Composition of the basal experimental diets (as-fed basis).

| Ingredients, % | Starter (1–10d) | Grower (11–22d) | Finisher (23–35d) |
|----------------|----------------|----------------|------------------|
| Maize          | 51.32          | 58.94          | 60.31            |
| Soybean Meal   | 39.15          | 32.28          | 29.82            |
| Rice           | 2.86           | 2.86           | 2.86             |
| Soybean oil    | 1.20           | 0.80           | 2.10             |
| Dicalcium phosphate | 2.50      | 2.50           | 1.91             |
| CaCO₃          | 0.90           | 0.87           | 1.10             |
| Common Salt    | 0.30           | 0.30           | 0.27             |
| Vitamin Premixᵃ | 0.25          | 0.25           | 0.25             |
| Mineral Premixᵇ | 0.25          | 0.25           | 0.25             |
| DL-Methionine  | 0.43           | 0.39           | 0.39             |
| L-Lysine HCL   | 0.35           | 0.38           | 0.38             |
| NaHCO₃         | 0.28           | 0.18           | 0.15             |
| L-Threonine    | 0.21           | 0.20           | 0.21             |

Calculated composition

| ME, Mcal/kg | 2.90 | 2.95 | 3.05 |
| Protein, %  | 22.50| 20.00| 19.00|
| Calcium, %  | 0.99 | 0.92 | 0.90 |
| Available Phosphorus, % | 0.48 | 0.44 | 0.38 |
| Lysine, %   | 1.36 | 1.23 | 1.17 |
| Met + Cys, % | 1.02 | 0.93 | 0.91 |

ᵃEach Kg of vitamin supplement contains: Vitamin A, 3600000 IU; vitamin D₃, 800000 IU; vitamin E, 7200 IU; vitamin K₃, 800 mg; vitamin B₁, 720 mg; vitamin B₂, 2640 mg; vitamin B₃, 4000 mg; vitamin B₅, 1200 mg; vitamin B₆, 1200 mg; vitamin B₉, 400 mg; vitamin B₁₂, 6 mg; biotin, 40 mg; choline chloride, 100000 mg; antioxidant, 40000 mg.
ᵇEach Kg of mineral supplement contains: Mn, 40000 mg; Zn, 33880 mg; Cu, 4000 mg; Fe, 20000 mg; Cu, 4000 mg; I, 400 mg; Se, 80 mg; choline chloride, 100000 mg. ME: Metabolizable energy.
phases were contained starter (1–10 d), grower (11–22 d) and finisher (23–35 d) during the experiment period. The basal diets were formulated to meet and exceed the minimum requirement of Hubbard strain chicken in a mash form (Table 1). Each bird was vaccinated by commercial Newcastle disease virus (NDV) vaccine via eye drop at day 17 of age. All serum samples were estimated for NDV antibody titre by hemagglutination test at day 24 of age (Bagherzadeh et al. 2012). The vaccination program was done according to the local schedule (Newcastle, avian influenza and bronchitis).

Deoxynivalenol was produced from Fusarium graminearum strain (obtained from Westerdijik Fungal Biodiversity Institute, Utrecht, the Netherlands) using fermentation of rice as a substrate. The fermented rice was autoclaved, ground and mixed and DON concentration was analysed via HPLC (Waters e 2695- Milford-MA- USA) with UV 2489 detector being set at 218 nm after clean up with an immune-affinity column (DON test HPLC columns, VICAM, Milford-MA-USA). The concentration of DON was approximately 349.93 mg/kg. Contaminated rice (2.80%) was incorporated to the basal diet providing 10 mg DON/kg feed, after that, feed samples at the beginning of starter, grower and finisher periods were taken from 8 different locations and mixed to measure the concentration of DON and other mycotoxins using HPLC (Yunus et al. 2012). The DON concentrations in starter, grower and finisher diets were 9.75 mg/kg, 10.87 mg/kg and 10.93 mg/kg, respectively. Aflatoxin (AFB1), zearalenone (ZEN) and ochratoxin were under the detection limit. Yeast (Saccharomyces cerevisiae) was purchased from Kimiazyme® (Tehran- Iran).

**Growth performance and organs weight**

Body weight and feed consumption of each pen were recorded at the end of each growth periods. Average daily gain (ADG), average feed intake (AFI) and feed conversion ratio (FCR) were measured for each phases and corrected for mortality. The mortality was less than 1% (2 chicks from 360). At the end of experiment, two birds from each replicate (8 birds/treatment) were randomly selected, weighed and sacrificed by cervical dislocation and relative weights of immune organs such as spleen and bursa of Fabricius were recorded.

**Blood samples**

At day 35 of age, two birds from each replicate (8 birds/treatments) were chosen in order to collect blood samples from wing vein. Blood samples were collected in EDTA coated glass tubes to analyse haemoglobin (Hb), haematocrit (Ht) and red blood cells (RBCs) and white blood cells (WBCs) counts. Haematocrit levels were estimated using heparinised capillary tubes after centrifugation. The WBCs and RBCs were manually counted in chambers of haemocytometer using microscope. In order to evaluate the leukocyte differential counts, blood samples were stained with Giesma in which on hundred leukocytes were tested for calculating the lymphocytes and heterophils. The heterophils to lymphocytes (H/L) ratios were measured by dividing the heterophils to lymphocytes number (Ghareeb et al. 2012).

**Intestine morphology**

Intestinal samples of two birds of each replicate were taken from duodenum, jejunum and ileum segments. Intestinal tissues were fixed in 10% formaldehyde phosphate buffer for 48 h, dehydrated, cleared and embedded in paraffin. Each paraffin block was cut as 5 μm sections by microtome (Microm GmbH, Walldorf, Germany) and placed on glass slide. Then, sections were dyed with haematoxylin and eosin and evaluated under a light microscope (Olympus, Olympus Corporation, Tokyo, Japan) in order to measure the villus height (VH), crypt depth (CD), villus width (VW) and mucosa and muscular layer thicknesses. Villus surface area (VSA) and apparent absorptive surface area (AASA) were calculated as reported by Jahanian et al. (2017) as follows:

\[
VSA = 2\pi \times \text{half of VW} \times VH
\]

\[
AASA = (3.1 \times VW + 3.2 \times VH) \times 1 - (2 \times VH)
\]

**Quantitative RT-PCR analysis of gene expression**

Three cm (in length) of middle segment of jejunum was taken and frozen in liquid nitrogen and then stored at −80 °C until the time of gene expression determination. Total RNA from jejunum was extracted using column RNA isolation kit according to the manufacturer’s instruction (Dena Zist Asia, Mashhad, Iran). The concentration and purity of extracted RNA were determined using a NanoDrop spectrometer (NanoDrop 2000, thermos Scientific, Wilmington, DE, USA) at OD of 260 and 280 nm. Afterward, 2 μg of extracted RNA was used in order to synthesise complementary DNA by MMLV (Moloney murine leukaemia virus) Reverse Transcription® kit (Biofact™ Co, Yuseong GU, Daejeon, Korea) according to the
manufacturers’ recommendation. The primers sequences for CLDN-5, MUC-2, TLR-4 and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) were obtained from the NCBI Gene Bank (Table 2). The reaction mixture for qPCR was contained 1 mL cDNA, SYBR Green Master Mix (PCR biosystems Ltd, Azetec house, London, UK), 0.50 mL of sense and anti-sense primers and sterile water which carried out in duplicate. The abundance of all samples was measured on a step one™ Real-Time PCR system (AB, Applied Bio systems, Foster, CA, USA) based on the manufactures’ instruction. Agarose gel electrophoresis of PCR and plot of melting curve obtained by the software system (REST) approved product specificity. Gene expression data were normalised by GAPDH as a housekeeper gene. The relative mRNA expression of the target genes compared to the reference gene were assessed using the $2^{-\Delta\Delta Ct}$ method (Wan et al. 2016).

**Probiotic production**

*Lactobacillus acidophilus, L. animalis, L. reuteri, L. fermentum* and *L. gallinarum* were obtained from the Laboratory of Microbiology, Faculty of Veterinary Medicine, Urmia University, Urmia, Iran. All bacteria were cultured using de Man Rogosa Sharpe (MRS; Merck, Darmstadt, Germany) broth medium and aerobically incubated at 37°C for 24–48 h (Bagherzadeh et al. 2012). At first, the obtained bacteria were confirmed using Gram staining, biochemical tests and polymerase chain reaction. Then, selective tests to check probiotic properties were implemented/administered and included low pH tolerance, antibacterial activity against *Salmonella Enteritidis* and *E. coli*, bile tolerance and antibiotic sensitivity (Kim et al. 2007).

Finally, all bacteria were cultured overnight in MRS broth at 37°C. Then, the solid phase was separated through centrifugation at 3000 × g for 30 min at 4 °C and lyophilised using freeze dryer. Lyophilised cells of *Lactobacillus* were counted using dilution method and their concentration was adjusted at 1–5 × 10⁹ cfu/g (Pizzolitto et al. 2013).

**Statistical analyses**

Data were analysed as a completely randomised design (2-way, ANOVA) by General Linear Model using the procedure of SAS (2004) software. The model has contained main effect of the three concentrations of *Lactobacillus* sp. and three levels of yeast as well as their interactions. Pen was the experimental unit. Orthogonal polynomial was applied for estimating the linear and quadratic effects of inclusion of the graded levels of *Lactobacillus* sp. and yeast in DON contaminated diets. The normality of all data was examined by univariate procedure. The non-normal data (gene expression, heterophil count and H/L ratio) were transformed by square-root ($\sqrt{x} + 0.5$). The Tukey’s test was used to compare means when significant effects ($p < .05$) were detected by analysis of variance.

**Results**

**Performance traits**

Effects of *Lactobacillus* sp. and yeast in DON contaminated diet on ADG, AFI and FCR are shown in Table 3. Neither the *Lactobacillus* sp. nor the yeast consumption could not affect the growth performance parameters during the whole experimental period ($p > .05$). Furthermore, there were no significant interactions between *Lactobacillus* sp. and yeast administrations regarding ADG, AFI and FCR during the whole experimental period.

**Immune system organs and antibody titre against NDV**

The effects of *Lactobacillus* sp. and yeast on the relative weights of immune system organs and antibody titre against NDV are summarised in Table 4. Inclusion of 0.40 g/kg *Lactobacillus* sp. to DON diet increased ($p < .05$) the relative weight of spleen compared to medium level (0.20 g/kg) of *Lactobacillus* sp. Furthermore, supplementation of *Lactobacillus* sp. at both concentrations enhanced ($p < .05$) the antibody titre against NDV. Only 1.50 g/kg yeast caused a higher ($p < .05$) bursa of Fabricius proportional weight and NDV antibody titre compared to the medium yeast concentration (0.75 g/kg). There was an

### Table 2. Gene sequence for Real-Time PCR.

| Gene  | Primer sequence | Accession number | Product size (pb) |
|-------|-----------------|-----------------|------------------|
| GAPDH | GGTGGTGCTAGGGTGTAT | K01458 | 264 |
| TLR-4 | CTCACATACCGACCTGAC | NM-001030693 | 111 |
| MUC-2 | CAACCTTATGTATGCTG | NM-00134834.1 | 187 |
| CLDN-5 | CATACCTTCTGCCTCACGC | NM-204201 | 111 |

*GAPDH: Glyceraldehyde 3-phosphate dehydrogenase; MUC-2: Mucin-2; TLR-4: Toll like receptor; CLDN-5: Claudin-5.*
interaction between *Lactobacillus* sp. and yeast regarding spleen weight. Addition of 0.40 g/kg *Lactobacillus* sp. along with yeast to DON diets caused a higher \( p < .05 \) spleen relative weight. Moreover, no interaction was observed between probiotic and yeast for serum NDV antibody titre and bursa of Fabricius relative weight \( p > .05 \). orthogonal polynomial contrast showed that addition of *Lactobacillus* sp. to DON diets caused the linear and quadratic enhancements \( p < .05 \) of spleen relative weight. In addition, increasing the level of *Lactobacillus* sp. and yeast from the lowest to the highest concentration linearly increased \( p < .05 \) NDV antibody titre, while inclusion of yeast from medium to the highest level quadratically increased \( p < .05 \) bursa of Fabricius weight.

**Hematological parameters and differential leukocyte count**

Table 5 shows that supplementation of high level of yeast enhanced \( p < .05 \) HI compared to the medium level. No effect of different dietary treatments was observed on Hb and RBCs count \( p > .05 \). Only 0.40 g/kg *Lactobacillus* sp. in DON diet resulted in enhancement \( p < .05 \) of WBCs count and lymphocyte percentage as well as heterophil count and H/L ratio reduction \( p < .05 \). Inclusion of 1.50 g/kg yeast to DON contaminated diet increased \( p < .05 \) the WBCs count. Increasing yeast supplementation to 1.50 g/kg at the highest *Lactobacillus* sp. concentration caused increments \( p < .05 \) in WBCs count and lymphocyte percentage not being observed \( p > .05 \) for the medium *Lactobacillus* sp. at different levels of yeast. Heterophil count and H/L ratio were lessened \( p < .05 \) by combination of dietary *Lactobacillus* sp. and yeast supplementation at the highest level compared to the broilers received 0.20 g/kg *Lactobacillus* sp. along with 1.50 g/kg yeast in DON contaminated diet. Linear and quadratic responses were observed for WBCs count by addition of yeast and *Lactobacillus* sp. \( p < .05 \).

**Gut morphology**

**Duodenum**

Dietary supplementation of 0.20 g/kg *Lactobacillus* sp. resulted in VH increase \( p < .05 \) at day 35 of age compared to un-supplemented diet. On the other hand, CD and VH to CD were significantly \( p < .05 \) increased and decreased by consumption of the highest level of *Lactobacillus* sp. (0.40 g/kg) respectively in DON
contaminated diet. Mucosa and muscular layer thicknesses were enhanced \((p < .05)\) as yeast concentration was increased. There was an interaction between *Lactobacillus* sp. and yeast regarding muscular layer thickness. Inclusion of 0.40 g/kg *Lactobacillus* sp. along with 0.75 g/kg yeast caused the enhancement \((p < .05)\) in muscular layer thickness. Orthogonal polynomial contrast indicated that addition of *Lactobacillus* sp. from the lowest to medium level \((0.0 – 0.75 \text{ g/kg})\) quadratically incremented \((p < .05)\) the VH, ASAA and VH:CD. Similarly, muscular layer and mucosa had linearly enhancement \((p < .05)\) by increasing yeast concentration in DON diet (Table 6).

**Jejunum**

Increasing the level of *Lactobacillus* sp. from medium \((0.20 \text{ g/kg})\) to the highest level \((0.40 \text{ g/kg})\) was quadratically enhanced \((p < .05)\) the VH, VSA and AASA. Inclusion of 0.40 g/kg *Lactobacillus* sp. to DON diet resulted in a CD enhancement \((p < .05)\) compared to DON diet. In addition, CD was elevated \((p < .05)\) by addition of 0.75 g/kg yeast. Increasing the level of yeast content up to 1.50 g/kg linearly and quadratically enhanced \((p < .05)\) the muscular layer thickness. There is no interaction between *Lactobacillus* sp. and yeast regarding VH, WV and muscular layer thickness \((p > .05)\). While, increasing *Lactobacillus* sp. supplementation to 0.40 g/kg at the highest level of yeast caused an increase \((p < .05)\) in mucosa thickness. Likewise, addition of *Lactobacillus* sp. along with yeast increased the AASA compared to the medium level of *Lactobacillus* sp. and yeast \((p < .05; \text{ Table 7})\).

**Ileum**

As shown in Table 8, an enhancement in *Lactobacillus* sp. level to 0.40 g/kg in contaminated diet resulted in the enhancements \((p < .05)\) of VH, CD, muscular layer thickness, VSA and AASA. Moreover, inclusion of 1.50 g/kg yeast in DON diet increased \((p < .05)\) the VH, VH:CD, muscular layer and mucosa thicknesses, VSA and AASA compared to DON receiving birds. There was an interaction between *Lactobacillus* sp. and yeast regarding VH, VSA and AASA. Increasing *Lactobacillus* sp. concentration at the level of 1.50 g/kg yeast caused in enhancements \((p < .05)\) in VH, VSA and AASA, but at the medium probiotic \((0.20 \text{ g/kg})\) and yeast \((0.75 \text{ g/kg})\) suppletions resulted in reduction \((p < .05)\) of VH, VSA and AASA.

### Table 6. Effects of *Lactobacillus* sp. and yeast in deoxynivalenol contaminated diets on hematological parameters in broiler chickens.

| Lactobacillus sp. (g/kg) | Haematocrit (%) | Haemoglobin (g/dl) | RBCs \((10^6/\mu l)\) | WBCs \((10^4/\mu l)\) | Lymphocyte (%) | Heterophil (%) | H/L ratio |
|--------------------------|-----------------|--------------------|---------------------|---------------------|---------------|---------------|----------|
| 0                        | 30.83           | 10.89              | 3.25                | 1.91*               | 70.79ab       | 26.50ab       | 0.39ab   |
| 0.20                     | 29.13           | 10.28              | 3.08                | 1.65b               | 69.92*        | 27.54*        | 0.40*    |
| 0.40                     | 30.44           | 10.72              | 3.05                | 1.92a               | 74.66ab       | 24.13b        | 0.33b    |
| SEM                      | 0.52            | 0.20               | 0.09                | 0.08                | 1.25          | 1.03          | 0.02     |

| Yeast (g/kg)             | Haematocrit (%) | Haemoglobin (g/dl) | RBCs \((10^6/\mu l)\) | WBCs \((10^4/\mu l)\) | Lymphocyte (%) | Heterophil (%) | H/L ratio |
|--------------------------|-----------------|--------------------|---------------------|---------------------|---------------|---------------|----------|
| 0                        | 30.35ab         | 10.66              | 3.26                | 1.69b               | 72.00         | 25.75         | 0.37     |
| 0.75                     | 28.67b          | 10.34              | 3.08                | 1.72b               | 70.50         | 27.08         | 0.39     |
| 1.50                     | 31.36b          | 10.89              | 3.04                | 2.06a               | 72.88         | 25.33         | 0.36     |
| SEM                      | 0.52            | 0.20               | 0.09                | 0.08                | 1.25          | 1.03          | 0.02     |

| Lactobacillus \times Yeast | Haematocrit (%) | Haemoglobin (g/dl) | RBCs \((10^6/\mu l)\) | WBCs \((10^4/\mu l)\) | Lymphocyte (%) | Heterophil (%) | H/L ratio |
|----------------------------|-----------------|--------------------|---------------------|---------------------|---------------|---------------|----------|
| 0 × 0                      | 32.08           | 11.33              | 3.46                | 1.55c               | 68.88b        | 27.63b        | 0.41ab   |
| 0 × 0.75                   | 28.34           | 10.35              | 3.00                | 1.94abc             | 73.00b        | 24.75b        | 0.35ab   |
| 0 × 1.50                   | 32.06           | 11.00              | 3.30                | 2.25abc             | 70.50ab       | 27.13ab       | 0.40ab   |
| 0.20 × 0                   | 29.38           | 10.40              | 3.15                | 1.88abc             | 72.00b        | 26.38ab       | 0.38ab   |
| 0.20 × 0.75                | 28.50           | 10.26              | 3.05                | 1.53c               | 68.75b        | 27.88b        | 0.42     |
| 0.20 × 1.50                | 29.50           | 10.16              | 3.04                | 1.51c               | 69.00b        | 28.38b        | 0.41*    |
| 0.40 × 0                   | 29.61           | 10.26              | 3.16                | 1.65bc              | 75.13b        | 23.25b        | 0.32b    |
| 0.40 × 0.75                | 29.18           | 10.40              | 3.20                | 1.68bc              | 69.75b        | 28.63b        | 0.41a    |
| 0.40 × 1.50                | 32.53           | 11.50              | 2.78                | 2.44a               | 79.13a        | 20.50b        | 0.26b    |
| SEM                       | 0.91            | 0.35               | 0.16                | 0.14                | 2.16          | 1.78          | 0.038    |

P-Value

| Probiotic                  | 0.062           | 0.095              | 0.233               | 0.024               | 0.021         | 0.051         | 0.033    |
|---------------------------|-----------------|--------------------|---------------------|---------------------|---------------|---------------|----------|
| Yeast                     | 0.002           | 0.167              | 0.207               | 0.004               | 0.402         | 0.425         | 0.405    |

Orthogonal Polynomials contrasts

| Linear Lactobacillus      | 0.602           | 0.555              | 0.113               | 0.976               | 0.032         | 0.102         | 0.064    |
|---------------------------|-----------------|--------------------|---------------------|---------------------|---------------|---------------|----------|
| Quadratic Lactobacillus   | 0.022           | 0.037              | 0.531               | 0.007               | 0.071         | 0.066         | 0.062    |
| Linear yeast              | 0.178           | 0.438              | 0.094               | 0.002               | 0.622         | 0.801         | 0.746    |
| Quadratic yeast           | 0.001           | 0.084              | 0.568               | 0.125               | 0.210         | 0.201         | 0.193    |

**a-c** Means in the same column with different superscripts differ significantly \((p < .05)\).

SEM: Standard error of means. RBC: Red blood cell; WBC: White blood cell; H/L ratio: Heterophil to lymphocyte.
Table 6. Effects of *Lactobacillus* sp. and yeast in deoxynivalenol contaminated diets on duodenum of broiler chickens at day 35 of age.

| Lactobacillus sp. (g/kg) | VH (µm) | VW (µm) | CD (µm) | VH:CD | Muscular layer (µm) | Mucosa (µm) | VSA (mm²) | AASA (µm²) |
|--------------------------|-------|--------|--------|-------|-------------------|----------|---------|----------|
| 0                        | 753.59b | 219.18 | 132.46b | 6.01ab | 123.94            | 30.32     | 0.52    | 1583.78  |
| 0.20                     | 876.42a | 200.32 | 139.82ab | 6.53a  | 125.78            | 31.40     | 0.56    | 1692.74  |
| 0.40                     | 815.61ab | 192.04 | 157.05ab | 5.34ab | 135.36            | 31.95     | 0.50    | 1574.04  |
| SEM                      | 26.11   | 9.06   | 6.35    | 0.27  | 4.48              | 1.13      | 0.03    | 43.85    |
| Yeast (g/kg)             |        |        |         |       |                   |          |         |          |
| 0                        | 818.18  | 209.34 | 142.91  | 6.13  | 116.16b           | 28.86b    | 0.54    | 1650.80  |
| 0.75                     | 833.46  | 200.16 | 149.73  | 5.75  | 131.48a           | 30.17b    | 0.53    | 1620.64  |
| 1.50                     | 793.98  | 202.05 | 136.69  | 5.99  | 137.43a           | 34.64a    | 0.51    | 1579.12  |
| SEM                      | 26.11   | 9.06   | 6.35    | 0.27  | 4.48              | 1.13      | 0.03    | 43.85    |

Table 7. Effects of *Lactobacillus* sp. and yeast in deoxynivalenol contaminated diets on jejunum of broiler chickens at day 35 of age.

| Lactobacillus sp. (g/kg) | VH (µm) | VW (µm) | CD (µm) | VH:CD | Muscular layer (µm) | Mucosa (µm) | VSA (mm²) | AASA (µm²) |
|--------------------------|-------|--------|--------|-------|-------------------|----------|---------|----------|
| 0                        | 2086.00b | 198.69 | 153.16b | 14.3800 | 150.32            | 30.64     | 1.30    | 3119.12ab |
| 0.20                     | 1997.28b | 190.69 | 166.63ab | 12.6329 | 152.85            | 30.47     | 1.20    | 2987.86ab |
| 0.40                     | 2189.43a | 193.91 | 175.31a | 13.0042 | 161.13            | 30.55     | 1.33    | 3228.42a |
| SEM                      | 36.79   | 6.30   | 6.40    | 0.57  | 4.67              | 1.11      | 0.05    | 48.65    |
| Yeast (g/kg)             |        |        |         |       |                   |          |         |          |
| 0                        | 2122.43b | 200.40 | 158.65b | 13.88a  | 141.20b           | 29.73     | 1.34    | 3168.15ab |
| 0.75                     | 2036.77 | 187.47 | 181.40a | 11.75b  | 163.90a           | 29.28     | 1.20    | 3142.00  |
| 1.50                     | 2113.51 | 195.42 | 155.06b | 14.39ab  | 159.21a           | 32.65     | 1.30    | 3195.90ab |
| SEM                      | 36.79   | 6.30   | 6.40    | 0.57  | 4.67              | 1.11      | 0.05    | 48.65    |

P-Value

| Lactobacillus sp. | 0.006 | 0.013 | 0.024 | 0.009 | 0.163 | 0.581 | 0.261 | 0.113 |
|------------------|-------|-------|-------|-------|-------|-------|-------|-------|
| Yeast            | 0.562 | 0.752 | 0.354 | 0.598 | 0.004 | 0.002 | 0.696 | 0.514 |
| Lactobacillus sp. × Yeast | 0.537 | 0.747 | 0.323 | 0.197 | 0.0006 | 0.149 | 0.939 | 0.942 |
| Orthogonal Polynomials contrasts | 0.009 | 0.038 | 0.008 | 0.077 | 0.077 | 0.308 | 0.674 | 0.876 |
| Linear Lactobacillus sp. | 0.006 | 0.635 | 0.528 | 0.011 | 0.483 | 0.847 | 0.114 | 0.038 |
| Quadratic Lactobacillus sp. | 0.151 | 0.747 | 0.323 | 0.197 | 0.0006 | 0.149 | 0.939 | 0.942 |
| Linear yeast     | 0.515 | 0.572 | 0.490 | 0.733 | 0.001 | 0.0006 | 0.396 | 0.252 |
| Quadratic yeast  | 0.395 | 0.620 | 0.206 | 0.341 | 0.397 | 0.255 | 0.986 | 0.916 |

SEM: Standard error of means; VH: Villus height; VW: Villus width; CD: Crypt depth; VSA: Villus surface area; AASA: Apparent absorptive surface area.
**Table 8. Effects of Lactobacillus sp. and yeast in deoxynivalenol diets on ileum of broiler chickens at day 35 of age.**

| Lactobacillus (g/kg) | VH (µm) | WV (µm) | CD (µm) | VH:CD | Muscular layer (µm) | Mucosa (µm) | VSA (mm²) | ASA (µm²) |
|----------------------|---------|---------|---------|-------|---------------------|-------------|-----------|-----------|
| 0                    | 1079.44b| 201.24  | 150.58b | 7.38  | 119.81b             | 29.405      | 0.63ab    | 1919.15b  |
| 0.20                 | 1072.75b| 186.88  | 153.25b | 7.34  | 129.40ab            | 30.236      | 0.63b     | 1866.61b  |
| 0.40                 | 1211.54a| 187.42  | 177.12a | 7.10  | 145.23a             | 31.993      | 0.71a     | 2034.84a  |
| SEM                  | 29.39   | 5.13    | 6.37    | 0.32  | 5.25                | 1.16        | 0.02      | 33.18     |
| Yeast (g/kg)         |         |         |         |       |                     |             |           |           |
| 0                    | 1064.48b| 184.52  | 156.66b | 7.03b | 112.55b             | 29.51b      | 0.61b     | 1849.1b   |
| 0.75                 | 1109.92b| 192.06  | 176.36b | 6.65b | 141.80              | 28.29b      | 0.67b     | 1927.3ab  |
| 1.50                 | 1189.33a| 198.93  | 147.92a | 8.13a | 140.09              | 33.83a      | 0.74a     | 2043.89a  |
| SEM                  | 29.39   | 5.13    | 6.37    | 0.32  | 5.25                | 1.16        | 0.02      | 33.18     |
| Lactobacillus × Yeast|         |         |         |       |                     |             |           |           |
| 0 × 0                | 1192.27abc| 189.46  | 152.52  | 7.87ab | 110.96c             | 28.21       | 0.69abc   | 2018.0b   |
| 0 × 0.75             | 1033.83bc| 203.64  | 167.91  | 6.40bc | 132.35abc            | 29.17       | 0.66abc   | 1871.85bc |
| 0 × 1.50             | 1012.21bc| 201.61  | 131.30  | 7.87bc | 116.14bc             | 30.84       | 0.67abc   | 1867.54bc |
| 0.20 × 0             | 971.09c  | 184.21  | 134.40  | 7.38bc | 104.60              | 29.19       | 0.56c     | 1736.36c  |
| 0.20 × 0.75          | 1017.02bc| 185.66  | 179.83  | 6.10bc | 149.28bc             | 28.53       | 0.59abcd  | 1795.96cd |
| 0.20 × 1.50          | 1230.13bc| 197.06  | 145.50  | 8.54bc | 134.31abc            | 32.99       | 0.73abc   | 2067.52bc |
| 0.40 × 0             | 1030.07bc| 179.92  | 183.07  | 5.84bc | 122.09bc             | 31.14       | 0.58cd    | 1793.82cd |
| 0.40 × 0.75          | 1278.90c | 186.91  | 181.34  | 7.45bc | 143.78abc            | 27.18       | 0.75abc   | 2114.09abc|
| 0.40 × 1.50          | 1325.65a | 195.43  | 166.95  | 7.99bc | 169.82a             | 37.66       | 0.81abc   | 2196.60a  |
| SEM                  | 50.90   | 8.89    | 11.02   | 0.56  | 9.09                | 2.01        | 0.04      | 57.48     |

*p*-Value

- Lactobacillus: 0.002
- Yeast: 0.013
- Lactobacillus × Yeast: <0.001

Orthogonal Polynomials contrasts

- Linear Lactobacillus: 0.002
- Quadratic Lactobacillus: 0.048
- Linear Yeast: 0.004
- Quadratic Yeast: 0.639

Inclusion of 0.40 g/kg Lactobacillus sp. along with 1.50 g/kg yeast increased (*p < 0.05*) the muscular layer thickness. Increasing Lactobacillus sp. concentration in DON diet linearly and quadratically increased (*p < 0.05*) the VH and ASA. In addition, linear and quadratic responses were observed (*p < 0.05*) in muscular layer and mucosa thicknesses by dietary yeast supplementation from 0.0 to 1.50 g/kg yeast.

**Gene expression**

The effects of Lactobacillus sp. and yeast on CLDN-5, MUC-2 and TLR-4 expressions in jejunum are presented in Table 9. Inclusion of Lactobacillus sp. and yeast to DON diets had no effect on MUC-2 and CLDN-5 expressions (*p > 0.05*). Consumption of 0.40 g/kg Lactobacillus sp. in contaminated diet caused a reduction in the TLR-4 expression compared to the medium level of Lactobacillus sp. (0.20 g/kg). No interaction was demonstrated between Lactobacillus sp. and yeast regarding MUC-2 expression (*p > 0.05*). While, there were interactions between Lactobacillus sp. and yeast for CLDN-5 and TLR-4, as addition of Lactobacillus sp. and yeast at the highest level reduced the TLR-4 expression in comparison with the medium and highest levels of Lactobacillus sp. and yeast (0.20 × 1.50 g/kg). Orthogonal polynomial contrast indicated that administration of Lactobacillus sp. to

**Table 9. Effect of Lactobacillus sp. and yeast in deoxynivalenol diets on jejunal gene expressions of broiler chickens at day 35 of age.**

| Lactobacillus sp. (g/kg) | CLDN-5 | MUC-2 | TLR-4 |
|--------------------------|--------|-------|-------|
| 0                        | 0.63   | 1.40  | 0.89ab|
| 0.2                      | 0.52   | 1.24  | 1.65a |
| 0.4                      | 0.87   | 0.65  | 0.44  |
| SEM                      | 0.12   | 0.17  | 0.38  |

*p*-Value

- Lactobacillus sp.: 0.325
- Yeast: 0.300
- Lactobacillus sp. × Yeast: 0.002

Orthogonal polynomials contrasts

- Linear Lactobacillus sp.: 0.295
- Quadratic Lactobacillus sp.: 0.281
- Linear Yeast: 0.160
- Quadratic yeast: 0.520

Means in the same column with different superscripts differ significantly (*p < 0.05*).

SEM: Standard error of means. CLDN-5: Claudin-5; MUC-2: Mucin-2; TLR-4: Toll like receptor-4.
DON dies quadratically down-regulated the TLR-4 expression. Moreover, increasing levels of yeast caused a linear enhancement in MUC-2 expression.

**Discussion**

Mycotoxins threaten animal's health, cause economic losses and hence are considered as serious problems for poultry industry. The DON as a major problem can constitute pre-harvest with occurrence being dependent on climate condition and always can be detected in poultry feedstuff (Awad et al. 2010). So, the effective approaches to control and/or reduce DON contamination are useful for food and feed processing. In this regards, some additive such as *Lactobacillus* sp. and yeast have been considered as mycotoxin detoxifiers in diet to mitigate the harmful effects of DON in intestinal tract (Anif et al. 2020; Yang et al. 2020). These studies put forward a hypothesis that simultaneous use of two mycotoxin detoxifiers might have promising effects on DON-challenged birds rather than one detoxifier.

The results of current study indicated that inclusion of *Lactobacillus* sp. and yeast in DON contaminated diet could not affect the broiler performance during the whole experimental period. There is a great inconsistency regarding performance traits. Some researchers have indicated the effectiveness of DON contamination on performance (Awad et al. 2004; Metzler-Zebeli et al. 2020). However, others have shown a reduction in body weight gain (BWG) during the grower and developer periods of turkey fed *Fusarium* contaminated grains, which inclusion of polymeric glucomanan mycotoxin adsorbent (extracted from the yeast cell wall) attenuated the adverse effect of *Fusarium* contaminated diet on BWG (Girish and Smith 2008). Sex, strain and toxin sources (natural and purified) are some factors can lead to such difference between the mentioned studies and ours. Our findings are supported by research of Chen et al. (2017) documenting that DON contaminated diets had no effect on BWG in female chickens, while dietary exposure to 2 mg/kg DON decreased the BWG of male broiler chickens versus those fed 10 mg/kg DON. It could be speculated that lack of significant effect on performance following birds’ exposure to 10 mg/kg DON from the first day of life might be due to presence of bacteria in intestinal tract which can degrade DON or detoxify DON via kidney and liver. On the other hand, DON acts as a potential inhibitor of protein synthesis which may suppress the growth via impairing the organs with high metabolic function such as intestinal tract, immune system organs or liver (Metzler-Zebeli et al. 2020). Indeed, DON contamination resulted in reductions of the spleen relative weight and antibody titre against NDV in our study. Spleen, thymus and bursa of Fabricius are the immune organs for proliferating and diversifying of T and B lymphocytes in broiler chickens (Peng et al. 2015). On the other hand, DON feeding could bring about adverse effects on immune organ. The DON has an ability to bind peptidyl transferase and inhibit the protein synthesis probably causing alterations (atrophy or enlargement) in immune organs (Peng et al. 2015). Surprisingly, combination of 0.40 g/kg *Lactobacillus* sp. and 1.50 g/kg yeast in DON diet improved the spleen relative weight which might be attributed to the stimulatory effect of *Lactobacillus* sp. and yeast supplementation. Wan et al. (2016) have reported that addition of *Lactobacillus rhamnosus* GG to contaminated diet (12 µg/g DON and 0.50 µg/g ZEN) caused an enhancement in spleen relative weight in mice. Spleen is the secondary lymphoid organ acting as a reservoir of Β-lymphocyte and filters blood from destructive erythrocytes and antigens (Metzler-Zebeli et al. 2020).

The DON feeding can cause immune-stimulatory or immune-suppressive effects depending on age, dose of exposure, distribution, metabolism and excretion of the toxins (Awad et al. 2010). Assessment of serum antibody titre against NDV and infectious bronchitis virus (IBV) after vaccination could be good criteria for evaluating the effect of DON contaminated diet on immune function (Ghareeb et al. 2012). In the present study, increasing the level of *Lactobacillus* sp. and yeast in DON contaminated diet was beneficial to reduce the adverse effect of DON on antibody titre and caused the linear increment in the NDV serum antibody titre. Enhancement of humoral immunity can promote a resistance against disease or DON toxicity. It is proven that augmentations in dietary *Lactobacillus* sp. have an ability to stimulate immunity in the intestinal tract (Yang et al. 2020). The results of our study are in accordance with previous findings of Dänicke et al. (2003) who concluding that NDV titres linearly increase in Lohmann male broilers through addition of *Fusarium* contaminated wheat (3.50–14 mg/kg DON) to basal diet. In another study, Yunus et al. (2012) have found that inclusion of 12.21 mg/kg DON in diet increases the antibody titre against NDV and reduces the IBV antibody titre during 2 and 5 weeks in Ross broiler chickens. In the present study, DON exposed birds had the lower WBCs count and lymphocyte concentration, while combination of *Lactobacillus* sp. and yeast in DON contaminated diet could increase the
number of WBCs and lymphocytes. The DON has been shown to induce immunosuppression by binding to the ribosomes and activating mitogen-activated protein kinases (MAPKs) regulating immune response pathway (Pestka 2008).

Inclusion of probiotic in DON contained diet can interact with inflammatory pathways such as P38 MAPKs. Moreover, dietary supplementation of probiotic in diet may have a stimulating effect on lymphocyte production especially B type leading to formation and improvement of humoral immunity in broilers challenged with DON (Yang et al. 2020). Furthermore, DON exposed birds appeared to have reductions in T and B lymphocytes activities and antibody titres as well as disturbance in macrophage function which are the manifestation of DON intoxication (Girish and Smith 2008). The results are in agreement with the findings of Yang et al. (2020) concluded that inclusion of 1 × 10^9 cfu/kg Lactobacillus plantarum to DON contaminated diet increased the number of peripheral blood T and B lymphocytes at day 42 in broiler chickens. Our results are also supported by findings of Swamy et al. (2004) deduced that supplementation of yeast cell wall in naturally Fusarium contaminated diet (9.50 mg/kg DON and 0.70 mg/kg ZEN) increased the WBCs and lymphocyte counts and inhibited the reduction of B-cell counts induced by contaminated diet. In addition, co-administration of Lactobacillus sp. along with yeast could ameliorate H/L ratio as better stress criteria in poultry. Ghareeb et al. (2012) have concluded that DON diet (10 mg/kg) could increase the heterophil counts and H/L ratio compared to the control.

The gastrointestinal tract is the first barrier against feed contamination and natural toxins. Any alteration in VH, CD and the VH to CD ratio can be considered as an indicator of toxins presence in feed (Ji et al. 2019). In this study, increasing the levels of Lactobacillus sp. and yeast in DON contaminated diets can ameliorate the adverse effects of DON on the intestine being more obvious in the ileum as a preferred region for bacterial colonisation. Consumption of DON contaminated diet could suppress protein synthesis through induction of epithelial cells proliferation and differentiation disturbance leading to shorter villi formation. The reduction in VH negatively affects absorptive surface area impairing the nutrient digestion and absorption (Jahanian et al. 2017). Addition of Lactobacillus sp. and yeast to DON diet improved the absorptive surface area in ileum. Accordingly, nutrient absorption increase may lead to the immunity enhancement in DON challenged birds. On the other hand, Lactobacillus supplementation has been shown to change the microbiota community composition which could act effectively to mitigate intestinal impairment (Yang et al. 2020). Increasing the level of yeast in DON diet resulted in an enhancement of mucosa thickness in duodenum and ileum. The DON diet has been shown to have a detrimental effect on mucosa thickness resulting in enhancement of enteric pathogens, toxins and antigens permeability to the epithelium increasing the bird susceptibility to diseases (Cheng et al. 2018). The results of our study are in accordance with previous findings of Yang et al. (2019) documenting that DON contaminated diet (0.50 and 1.50 mg/kg) fed rabbits show reductions in VH, CD and ileal mucosa. Wu et al. (2018) have demonstrated that addition of 1 × 10^9 cfu/kg Lactobacillus plantarum to DON contaminated diet could attenuate the harmful effects of DON on intestine restoring VH, VH to CD and expressions of CLDN-1 and occludin in broiler chickens.

The TLR-4 acts as a pathogen recognition receptor which can activate innate immunity. Activation of TLR-4 can stimulate the secretion of some inflammatory metabolites such as tumour necrosis factor-α and interleukin-1 (Jin et al. 2017). Probiotics have an ability to suppress intestinal inflammation through reduction of TLR-4 expression (Plaza-Diaz et al. 2019). According to the current findings, birds exposed to higher levels of Lactobacillus sp. along with yeast in DON contaminated diet showed a down-regulation in TLR-4 expression. Based on the Jin et al. (2017) results, inclusion of 1 kg/ton toxin binder containing yeast cell wall, clinoptilolite and bentonite to DON contaminated diet (2.60 mg/kg) down-regulated the expression of TLR-4 and up-regulated zona occludens expression in weaning piglets. Claudins have a key role in the integrity of intestinal barrier preventing the permeability of pathogens and toxins into the intestinal lumen (Yang et al. 2017). In our study, inclusion of 0.40 g/kg Lactobacillus sp. along with yeast increased the CLDN-5 expression. Similarly, Yang et al. (2017) observed that claudin expression increase in response to the probiotic administration inhibited the detrimental effects of DON diet on jejunal mucosal integrity in broiler chickens. It is presumed that consumption of probiotic could regulate/constitute expression or distribution of defensive barrier (La Fata et al. 2018). Chen et al. (2017) have deduced that addition of 2 and 5 mg/kg DON to basal diet increases the expression of CLDN-5 in female broilers, while it causes a reduction in male ones. The current experiment indicated that increasing the yeast concentration from the lowest to highest
level enhanced the MUC-2 expression. Mucin is produced by goblet cells and acts as a protective layer against harmful effects of mycotoxin contaminated diets on broiler performance, immunity status, and carcass characteristics. Animals. 10 (2):238–249.
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