Insights into growth-promoting, anti-inflammatory, immunostimulant, and antibacterial activities of Toldin CRD as a novel phytobiotic in broiler chickens experimentally infected with *Mycoplasma gallisepticum*

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ABSTRACT Chronic respiratory disease (**CRD**) caused by *Mycoplasma gallisepticum* (**MG**) leads to impaired broiler growth performance and significant economic losses worldwide. The utilization of essential oils (**EO**s) as natural alternatives to antibiotics to control CRD outbreaks is not completely clarified yet. Thus, we investigated the effect of a commercial EOs mixture (toldin CRD), in comparison to tilmicosin antibiotic, on the clinical observations, growth performance, immunity, digestive enzymes, gut barrier functions, and bacterial loads in broilers experimentally infected with MG. A total of 400 one-day-old broiler chicks were assigned into four groups; negative control (**NC**), positive control (PC), tilmicosin, and toldin CRD treated groups. All groups except NC were experimentally infected with MG at 14 d of age. Our data showed that birds treated with toldin CRD showed significant enhancement in the body weight gain (**BWG**) and feed conversion ratio (**FCR**) ($P = 0.001$ each) over the whole experimental period. Likely, improved digestibility and intestinal barrier functions in the toldin CRD treated group was evidenced by the significant upregulation ($P < 0.05$) of cholecystokinin (***CCK***), alpha 2A amylase (***AMY2A***), pancreatic lipase (***PNLIP***), junctional adhesion molecule-2 (***JAM-2***), occludin, and mucin-2 (***MUC-2***) genes. Moreover, toldin CRD exhibited immunostimulant and ant-inflammatory activities via significant downregulation ($P < 0.05$) of tumor necrosis factor-alpha (***TNF-α***) and interleukin (***IL***)-6 genes, significant reduction of lysozyme (**LYZ**), myeloperoxidase (**MPO**), and nitric oxide (**NO**) levels ($P = 0.03$, 0.02, and 0.001, respectively) and significant increase in the immunoglobulin G (**IgG**) level ($P = 0.03$). Notably, immunohistochemistry and quantitative real-time polymerase chain reaction (**qPCR**) results showed prominent reductions ($P <$

Received June 30, 2022.
Accepted August 22, 2022.

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0.05) in the levels of MG antigens and MG loads in the toldin CRD treated group, which were evidenced by relieving the clinical picture of MG experimental infection. In conclusion, we recommend the utilization of toldin CRD as a potential candidate for controlling MG infection in broiler chickens.

Key words: *Mycoplasma gallisepticum*, toldin CRD, immunity, gut barrier functions, broilers

2022 Poultry Science 101:102154
https://doi.org/10.1016/j.psj.2022.102154

INTRODUCTION

Mycoplasma gallisepticum (**MG**) is one of the most significant causes of chickens' chronic respiratory disease (**CRD**) and turkeys' infectious sinusitis (Abd El-Hamid et al., 2019a). *Mycoplasma gallisepticum* infection leads to severe respiratory manifestations (dyspnea, coughing, tracheal rales, and nasal discharge), and reduction in egg production, and weight gain. Additionally, MG infection results in increased feed conversion rates, condemnation rates, medication costs, and mortality in the infected broilers, which leads to noteworthy economic losses in the poultry industry throughout the world (Garmyn et al., 2019; Awad et al., 2022).

Control programs for avian mycoplasmosis are the most practical methods to minimize the economic losses in enzootic areas (Abd El-Hamid et al., 2019a). Controlling the impact of MG infection is done via total eradication, vaccination programs, or chemotherapy (Garmyn et al., 2019). Total eradication of positive breeder flocks via test and slaughter is expensive and impractical; furthermore, vaccination programs are ineffective (Garmyn et al., 2019). Therefore, antibiotic therapy continues to be the most effective and economic method for the treatment and control of *Mycoplasma* spp. in poultry farms. Generally, MG has shown *in vivo* and *in vitro* susceptibilities to several antibiotics including quinolones, tetracyclines, and macrolides (Abd El-Hamid et al., 2019a). Macrolides, especially tilmicosin, tylosin, and tiamulin have been successfully utilized in the treatment and control of CRD associated with MG infection in poultry farms for many years worldwide (Awad et al., 2022). Moreover, tilmicosin was superior in controlling MG infection in broiler chickens (Abd El-Hamid et al., 2019a).

Recently, there is rapid development of resistant strains to the currently utilized antimicrobials (Abd El-Hamid and Bendary, 2015; Ammar et al., 2021a, 2022) due to the prolonged, excessive and uncontrolled usage of antimicrobials in humans and animals, especially in developing countries (Ammar et al., 2015, 2016a, b, 2021b, c; Abd El-Aziz et al., 2018; Abd El-Hamid et al., 2019b), which amplified the need for utilizing novel alternative antimicrobials from medicinal plants (Elmowalid et al., 2019; Ibrahim et al., 2019; Abd El-Hamid et al., 2019c) to control avian mycoplasmosis (Awad et al., 2019). Interestingly, medicinal plants such as essential oils (**EOs**), have antimicrobial, anti-inflammatory, and immunostimulant properties via minimizing the bacterial loads and modifying the expression of virulence and pro- and anti-inflammatory cytokines-related genes (Abd El-Hamid et al., 2021; Bendary et al., 2021). Therefore, they are utilized in herbal medicine as effective and safe promising natural alternatives to antimicrobials for the treatment of different diseases in recent years (Ammar et al., 2021d). Moreover, EOs can be used as feed additives in broilers' nutrition as they improve chicken feed efficiency parameters, growth performance, and meat quality via strengthening the intestinal integrity and mucosal barriers of broiler chickens, which in turn enhance their digestibility (Farahat et al., 2021; Ibrahim et al., 2021b; Aljazzar et al., 2022). Of note, a recent study reported that toldin CRD, which consists of a mixture of EOs such as propolis, ginger, oregano, licorice, anise oils, curcumin, aliumcepa, and cinnamon was more effective than the EOs of clove, cinnamon, and cumin in controlling MG infection in broiler chickens (Abd El-Hamid et al., 2019a). However, the *in vivo* effectiveness of toldin CRD against MG experimental infection in broiler chickens did not be investigated yet.

To the best of our knowledge, few researchers described the *in vivo* effectiveness of tilmicosin and EOs mixture against MG infection in broiler chickens. Thus, this study aimed to investigate, for the first time, the *in vivo* effectiveness of a mixture of essential oils (toldin CRD) in comparison to tilmicosin antibiotic against MG experimental infection in broiler chickens considering their activities on the growth performance, digestive enzymes, gut barrier functions, and cytokines-related genes expression, MG loads, immunohistochemical changes, and immunological hematological parameters.

MATERIALS AND METHODS

Ethical Approval

All experimental procedures were conducted in compliance with the regulations and guidelines approved by the Institutional Animal Care and Use Committee (IACUC), Faculty of Veterinary Medicine, Zagazig University, Egypt under the reference number (ZU-IACUC/2022).

Tilmicosin and Toldin CRD

Tilmicosin antibiotic was purchased from the Elanco Animal Health Company, Geneva, Switzerland as a phosphate form containing 250 mg/mL and it is dispensed as a water-soluble powder. Moreover, toldin CRD (B.NO, 00101) is a prophylactic and therapeutic compound for CRD complex in chickens and it contains many active natural compounds including propolis (50 mL/L), tulsi (40 mL/L), licorice (30 mL/L), curcumin

(55 mL/L), allicin (30 mL/L), cinnamon (60 mL/L), ginger (35 mL/L), and aliumcepa (40 mL/L) oils. It was kindly supplied by Dawa International for Pharmaceutical Industries, Cairo, Egypt.

Mycoplasma Gallisepticum *Field Strain*

The virulent field MG strain utilized in the present experimental trial was previously isolated from broiler chickens with respiratory manifestations according to a previous research study of one of the co-authors (Awad et al., 2019). *Mycoplasma gallisepticum* strain was cultivated onto pleuro pneumonia like organism (**PPLO**) broth and agar media (Cat. No. CM0403 and CM0401; Oxoid, Basingstoke, Hampshire, UK) supplemented with 20% heat-inactivated horse serum (General Egyptian Organization for Biological Products and Vaccines, Giza, Egypt) under humid microaerophilic conditions in 10% CO_2 incubator at 37°C for 3 to 5 d. The concentration of MG inoculum utilized in the current experimental trial was approximately 10^9 color changing units (**CCU**)/mL (Awad et al., 2019, 2022). *Mycoplasma gallisepticum* stain was evidenced to harbor *crm, gap,* and *mgc2* virulence genes by PCR investigations. Moreover, this strain was evidenced to be resistant to enrofloxacin and lincomycin antibiotics. These antimicrobial resistances and virulence profiles were utilized as re-isolation markers (Awad et al., 2019, 2022).

Birds, Experimental Design, and Feeding Regime

A total of 400 one-day-old broiler chicks (Ross 308) purchased from a local commercial poultry hatchery were utilized for a 39-d experiment. Birds were weighed individually and assigned randomly into four groups in floor pens (100 chicks in each group) and each group consisted of 5 replicates/20 birds each. The first two chicks' groups were served as negative control (NC, noninfected and nontreated) and positive control (PC, infected with MG strain and nontreated). At 14 d of age, the chicks in the other 2 treatment groups were experimentally infected with MG field strain and at 22 d of age, they were treated for five successive days in drinking water with tilmicosin (1 mL/3 L water) and toldin CRD (1.5 mL/ 1.5 L water), respectively. Of note, all birds in PC and other treatment groups were inoculated intratracheally with 200 μL of 10^9 CCU/mL of the MG inoculum at 14 d of age, while the NC group was kept noninfected. The bacterial infection was checked via observing the characteristic clinical picture, mortality, and postmortem lesions of the sacrificed chicks besides re-isolation and identification of MG strain from trachea, lung, and air sac samples of all infected groups through conventional, serological, and molecular techniques. Furthermore, re-examining the antimicrobial susceptibility pattern and virulence gene profile of the MG strain was done to ascertain that the re-isolated strain corresponded to the infecting one. All broiler chicks were vaccinated intraocularly with Hitchiner B1 + IB vaccine (CEVA-Phylaxia, Budapest, Hungry) on day 3, LaSota NDV vaccine (CEVA-Phylaxia, Budapest, Hungry) on day 7, IBD vaccine (CEVA-Phylaxia, Budapest, Hungry) on day 10 and LaSota NDV vaccine at 21 d of age. The coccidiostat-free and antibiotic-free, in mash form, diets for starter (1−10 d), grower (11−20 d), and finisher (21−39 d) periods were prepared following the guidelines of Ross broiler nutrition specifications handbook (Aviagen, 2018) as presented in Table 1. All birds were fed the basal diet and allowed admission to feed and

Table 1. Components and chemical contents of the control experimental diet.

Ingredient (%)	Starter (1−10 d)	Grower (11−20 d)	Finisher (21−39 d)
Soybean meal (48)	34.40	30.80	25.90
Soybean oil	1.80	3.00	4.20
Yellow corn	59.00	62.00	66.20
L-Lysine HCL (Lysin, 78%)	0.35	0.35	0.33
DL-Methionine (Methionine, 99%)	0.25	0.25	0.17
Choline chloride	0.20	0.20	0.20
Ca carbonate	1.20	1.20	1.1
Dicalcium phosphate	1.50	1.50	1.2
Common salt	0.30	0.30	0.30
Anti-mycotoxin	0.10	0.10	0.10
Premix*	0.30	0.30	0.30
Calculated content			
Lysine (%)	1.43	1.33	1.19
Methionine (%)	0.58	0.56	0.44
Ca (%)	1.11	1.16	0.95
Available P (%)	0.52	0.50	0.43
EE (%)	4.34	5.58	6.80
CF (%)	2.64	2.57	2.48
CP (%)	23.11	21.60	19.62
ME (Kcal/Kg)	3019	3117	3240

Ca, calcium; CF, crude fiber; CP, crude protein; EE, ether extract; ME, metabolizable energy; P, phosphorus.

*The mineral premix supplied/kilogram of diets: Fe, 80 mg; Cu, 7.6 mg; I, 1.1 mg; Se, 0.6 mg; biotin, 0.05 mg; Zn, 69 mg; Mn, 100 mg; choline chloride, 350 mg; vitamin A (from retinyl acetate), 6000 IU; vitamin K (menadione sodium bisulphate), 1.2 mg; vitamin D3 (cholecalciferol), 1,000 IU; vitamin B1, 2.3 mg; vitamin B6, 4 mg; vitamin B11,1.5 mg; vitamin B12, 0.017 mg; vitamin B2, 12 mg; vitamin B3, 13 mg and vitamin B5, 80 mg.

drinking water ad libitum during a 39-d experimental period. The Chemical analysis (ether extract, crude fiber, crude protein, and moisture) of all feed constituents and diets was carried out following the guidelines of the Association of Official Analytical Chemists (AOAC) (AOAC, 2012).

Clinical Observations and Monitoring the Growth Performance of Experimental Birds

Birds in all groups were dialy examined for recording the clinical signs, gross lesions and mortality percentages throughout the experiment. The average body weight (**BW**) and daily feed intake (**FI**) were recorded for calculating the feed conversion ratio (**FCR**) and cumulative body weight gain (**BWG**) over the whole experimental period (1−39 d) as previously pronounced (Al-Khalaifah et al., 2020; Ibrahim et al., 2020a, 2021d; e).

Sampling

After the end of the treatment period (28 d of age), blood specimens collected aseptically from the wing vein of broiler chicks were utilized for serum separation for analysis of some immunological parameters. Additionally, the pancreatic, intestinal, and splenic tissues were collected aseptically, washed with sterile phosphate buffer saline (PBS; Cat. No. BR0014; Oxoid, Basingstoke, Hampshire, UK), and utilized for subsequent analysis of the expression levels of genes encoding digestive enzymes, tight junction proteins (**TJP**s), gut barrier functions and cytokines by reverse transcription-quantitative polymerase chain reaction (**RT-qPCR**) technique. At 7- and 14-d postinfection (dpi), 5 birds from each replicate were sacrificed, and then splenic and tracheal samples from each bird were aseptically removed and utilized for subsequent immunohistochemistry and quantification of MG populations by quantitative real-time PCR (qPCR) technique.

Analysis of Non-Specific Immunological Parameters

Determination of lysozyme (**LYZ**), myeloperoxidase (**MPO**), and nitric oxide (**NO**) activities were carried out utilizing commercial kits (Jiancheng Biotechnology Institute, Nanjing, China). Additionally, enzyme-linked immunosorbent assay (**ELISA**) kits were utilized for spectrophotometric analysis of serum immunoglobulin G (**IgG**) (Cusabio Biotech Co. Ltd., China) as pronounced previously (Abd El-Hamid et al., 2021).

Gene Expression Analysis via Reverse Transcription-Quantitative PCR Technique

Pancreatic and intestinal samples were utilized for detecting the mRNA expression levels of genes encoding digestive enzymes [pancreatic lipase (***PNLIP***), cholecystokinin (***CCK***), and alpha 2A amylase (***AMY2A***)], tight junction proteins [occludin and junctional adhesion molecule-2 (***JAM-2***)] and gut barrier functions [mucin-2 (***MUC-2***)]. Moreover, splenic tissues were used for investigating the mRNA expression levels of genes encoding cytokines [interleukin (***IL***)-6 and tumor necrosis factor-alpha (***TNF-α***)]. Extraction of total RNA was done via the QIAamp RNeasy Mini kit (Cat. No. 51304; Qiagen, Hilden, Germany) as endorsed by the instructions of the manufacturer. The extracted RNA concentration was detected at 260 nm and the clarity of RNA was detected by Spectrostar NanoDropTM 2000 spectrophotometer (Cat. No. ND-2000; Thermo Fisher, CA). The Stratagene MX3005P real-time PCR machine (Cat. No. PF1457N; Thermo Fisher, CA) was utilized for one-step RT-qPCR amplification, in triplicates, via a QuantiTect SYBR Green RT-PCR Kit (Cat. No. 204243; Qiagen, Hilden, Germany) according to the protocol of the manufacturer. All PCR amplifications were verified via melting curve analysis. The glyceraldehyde 3-phosphate dehydrogenase (***GAPDH***) gene was used as an endogenous control to normalize the transcripts' expression levels. The sequences of the primers utilized in RT-qPCR assays are presented in Table 2. The $2^{-\Delta\Delta Ct}$ method was used to assess the relative mRNA expression outcomes of the examined genes (Livak and Schmittgen, 2001).

Quantification of Mycoplasma Gallisepticum *DNA Copies Via Quantitative Real-Time PCR Technique*

The QIAamp DNA Mini Kit (Cat. No. 51304; Qiagen, Hilden, Germany) was utilized for DNA extraction from the tracheal samples following the recommendations of the manufacturer. Absolute quantification of MG populations was carried out in the Stratagene MX3005P RT-PCR recognition system (Cat. No. PF1457N; Thermo Fisher, CA), in triplicate, using the QuantiTect SYBR Green PCR Master Mix (Cat. No. 204143; Qiagen, Hilden, Germany) according to the instructions of the manufacturer. The sequence of the primer used in the qPCR assay targeting *mgc2* gene of MG is as follows: F-CGCAATTTGGTCCTAATCCCCAACA and R-TAAACCCACCTCCAGCTTTATTTCC (Awad et al., 2022). The standard calibration curves for qPCR were generated via 10-fold serial dilutions of the DNA samples extracted from pure MG cultures. The target genomic DNA copies were determined and MG quantities were expressed as $\log_{10}$ colony forming units (CFU)/gram of the samples.

Immunohistochemical Analyses

The splenic tissues were collected from slaughtered chickens (n = 5 from each group) for immunohistochemical analyses as previously pronounced (Kalyuzhny, 2016). Briefly, fixation of the splenic tissues was carried

Table 2. Primer sequences of target and reference genes utilized in the reverse transcription quantitative PCR technique.

Specificity/target gene	Primer sequence (5′-3′)	Accession no.
Digestive enzymes		
PNLIP	F: GCATCTGGGAAG↓GAACTAGGG R: TGAACCACAAGCATAGCCCA	NM_001277382.1
CCK	F-AGGTTCCACTGGGAGGTTCT R-CGCCTGCTGTTCTTTAGGAG	XM_015281332.1
AMY2A	F-CGGAGTG↓GATGTTAACGACTGG R-ATGTTCGCAGACCCAGTCATTG	NM_001001473.2
Tight junction proteins and gut barrier functions		
Occludin	F-ACGGCAAAGCCAACATCTAC R-ATCCGCCACGTTCTTCAC	XM_031604121.1
JAM-2	F-AGACAG GAACAGGCAGTGCT R-TCCAATCCCATTTGA GGCTA	XM_031556661.1
MUC-2	F-AAACAACGGCCATGTTTCAT R- GTGTGACACTGGTGTGCTGA	NM_001318434
Cytokines		
IL-6	F: AGGACGAGATGTGCAAGAAGTTC R: TTGGGCAGGTTGAGGTTGTT	NM_204628.1
TNF-α	F: CGTTTGGGAGTGGGCTTTAA R: GCTGATGGCAGAGGCAGAA	NM_204267.1
House keeping		
GAPDH	F: GGTGGTGCTAAGCGTGTTA R: CCCTCCACAATGCCAA`	NM205518

AMY2A, alpha 2A amylase; *CCK*, cholecystokinin; *GAPDH*, glyceraldehyde 3-phosphate dehydrogenase; *IL*, interleukin; *JAM-2*, junctional adhesion molecule-2; *MUC-2*, mucin-2; *PNLIP*, pancreatic lipase; *TNF-α*, tumor necrosis factor-alpha.

out in 10% formalin solution (Cat. No. HT501128; Sigma-Aldrich, St. Louis, MO). After that, the examined tissues were dehydrated in absolute ethanol (Cat. No. 493511; Sigma-Aldrich, St. Louis, MO), cleared in xylene (Cat. No. 534056; Sigma-Aldrich, St. Louis, MO), impregnated in paraffin wax, (Cat. No. 327212; Sigma-Aldrich, St. Louis, MO), and cut at thin sections (5 mm thicknesses) using a microtome. Then, these tissue sections were deparaffinized in xylene and rehydrated through a series of decreasing alcohol solutions and finally dipped in a jar containing distilled water for 5 min and then in 3% H_2O_2 methanol solution (Cat. No. H1009; Sigma-Aldrich, St. Louis, MO) for 10 min to block the endogenous peroxidase activity. After that, these tissue sections were processed for staining with a primary antibody against chicken MG (Cat. No. ab35156, R&D Systems, Minneapolis, MN) and secondary HRP-labeled anti-chicken IgG H&L (Cat. No. ab205718, Abcam, Waltham, MA). Staining with DAB brown was utilized to visualize the bacterial antigen microscopically.

Statistical Analysis

All results were analyzed via the SPSS Inc. software version 26 (IBM Corp., Armonk, NY). The normality and homogeneity among the experimental groups were determined utilizing Shapiro−Wilk's and Levene's tests, respectively. Variations among the results of experimental groups were determined as the standard error of the mean (**SEM**) and ANOVA and Tukey's tests were employed to evaluate the significant variations between the mean values. When the *P*-value was lower than 0.05, statistically significant variations were considered. All graphs were prepared via the GraphPad Prism software Version 8 (San Diego).

RESULTS

Clinical Observations and Growth Performance Parameters

There were no respiratory signs observed in broiler chickens of the NC group throughout the whole experimental period. Meanwhile, the clinical signs in the form of sneezing, coughing, and tracheal rales were observed in chickens of the PC group after 1 week of MG experimental infection. Moreover, there were only 2 birds died in the PC group due to severe air sacculitis, but no mortalities were recorded in NC and the other two treated groups. The gross lesions of the freshly died and sacrificed birds in the PC group were catarrhal exudate in the tracheal lumen and air sacculitis with sometimes foamy exudate in the air sacs. Interestingly, toldin CRD and tilmicosin treatments used in the present study succeeded in improving the previous clinical picture of the birds in the treated groups.

The experimental infection with MG strain significantly decreased BW and BWG (1,967.4 and 1,927 g/bird, respectively) ($P = 0.001$ each) in the PC group concerning the NC group (2,522 and 2,478 g/bird, respectively) (Table 3). Additionally, the MG experimental infection significantly amplified FI and FCR (4,109 g/bird and 2.13 , respectively) ($P = 0.001$ each) in the PC group unlike the NC group (3,642.7 g/bird and 1.47 , respectively) (Table 3). Furthermore, toldin CRD and tilmicosin treated groups showed significant enhancement in the BWG (2,431 and 2,414 g/bird, respectively) and FCR (1.59 and 1.53 , respectively) in comparison with the PC group (1,927 g/bird and 2.13 , respectively). Of note, toldin CRD and tilmicosin treated groups exhibited significant ($P = 0.001$) improvement in all parameters when compared with the

Table 3. Effects of toldin CRD and tilmicosin treatments on the growth performance of broilers experimentally infected with *Mycoplasma gallisepticum* over 39-d experimental period.

	Experimental group				*P*-value	SEM
	PC	NC	Tilmicosin	Toldin CRD		
Initial BW, g/bird	40.80	42.40	43.2	42.8	0.99	0.71
Allover growth performance paramters						
BW, g/bird	1967.4[c]	2522[a]	2457.52[b]	2474[b]	0.001	21.54
BWG, g/bird	1927[c]	2478[a]	2414[b]	2431[b]	0.001	18.63
FI, g/bird	4109[a]	3642.7[c]	3684[c]	3876[b]	0.001	29.78
FCR	2.13[a]	1.47[c]	1.53[bc]	1.59[b]	0.001	0.05

BW, final body weight; BWG, body weight gain; FCR, cumulative feed conversion ratio; FI, total feed intake; PC (positive control), birds fed a basal diet and experimentally infected with *M. gallisepticum* at 14 d of age; NC (negative control), birds fed a basal diet; SEM, standard error of the mean.
[a-c]Means with different superscripts within the same row differ significantly ($P < 0.05$).

PC group with no significant differences between both treatments except for FI.

Nonspecific Immunological Parameters

Table 4 summarizes the serum nonspecific immunological parameters in broiler chickens at 28 d of age. Serum activities of LYZ, MPO, and NO were significantly elevated in the PC group (166.28 U/mL, 47.75 U/L, and 13.40 μmol/L) unlike the NC group (92.35 U/mL, 23.69 U/L, and 2.69 μmol/L), respectively. Notably, treatment with toldin CRD and tilmicosin significantly reduced the LYZ (126.35 and 124.36 U/mL), MPO (38.66 and 37.26 U/L), and NO (3.54 and 3.22 μmol/L) activities ($P = 0.03$, 0.02, and 0.001) unlike the PC group (166.28 U/mL, 47.75 U/L, and 13.40 μmol/L), respectively with no significant variations among tilmicosin and toldin CRD treated groups. Higher significant ($P = 0.03$) serum IgG level was detected in chicks' groups treated with toldin CRD and tilmicosin (34.69 and 33.9 mg/dL, respectively) compared to the PC group (30.35 mg/dL). Moreover, the group treated with toldin CRD restored the IgG level (34.69 mg/dL) to be the same as those in the NC group (34.26 mg/dL) with no significant variations among both groups.

Gene Expression Analysis by RT-qPCR Assay

The outcomes of the expression analysis of genes encoding digestive enzymes are presented in Figure 1. At 28 d of age, the relative gene expression levels of *AMY2A* and *PNLIP* were significantly ($P < 0.05$) upregulated in the toldin CRD treated group (0.96 and 0.85-fold), followed by the tilmicosin treated group (0.85 and 0.76-fold) concerning the PC group (0.69 and 0.52-fold), respectively with no significant variation in the expression level of *AMY2A* gene among NC and toldin CRD treated groups. Additionally, the highest upregulation in the expression level of the *CCK* gene was detected in the tilmicosin treated group (0.91-fold), followed by the toldin CRD treated group (0.89-fold) in comparison with the PC group (0.81-fold).

The expression levels of genes encoding TJP and gut barrier functions are illustrated in Figure 2. Notably, regardless of the MG experimental infection, toldin CRD treatment enhanced the barrier functions as evidenced by the highest prominent ($P < 0.05$) transcription levels of *JAM-2* (1.15-fold), occludin (1.22-fold), and *MUC-2* (1.25-fold) genes concerning the PC group (0.54, 0.22 and 0.64-fold, respectively) at 28 d of age.

The outcomes of the expression analysis of cytokines-related genes are presented in Figure 3. At 28 d of age, the significant ($P < 0.05$) downregulation in the expression level of the *IL-6* gene was detected in the toldin CRD (1.2-fold), followed by tilmicosin (1.23-fold) treated groups concerning the PC group (1.55-fold) with no significant variations between both treated groups. Additionally, the *TNF-α* relative gene expression levels were significantly ($P < 0.05$) downregulated in the tilmicosin treated group (1.11-fold), followed by the toldin CRD treated group (1.15-fold) in comparison with the PC group (1.34-fold) and there were no significant

Table 4. Efficacy of tilmicosin and toldin CRD treatments on the serum nonspecific immunological parameters of broiler chickens experimentally infected with *Mycoplasma gallisepticum*.

Experimental group	LYZ (U/mL)	MPO (U/L)	NO (μmol/L)	IgG (mg/dL)
PC	166.28[a]	47.75[a]	13.40[a]	30.35[c]
NC	92.35[c]	23.69[c]	2.69[c]	34.26[a]
Tilmicosin	124.36[b]	37.26[b]	3.22[bc]	33.9[b]
Toldin CRD	126.35[b]	38.66[b]	3.54[b]	34.69[a]
P value	0.03	0.02	0.001	0.03
SEM	0.85	0.16	0.17	0.19

IgG, immunoglobulin G; LYZ, lysozyme; MPO, myeloperoxidase; NC (negative control), birds fed basal diet; NO, nitric oxide; PC (positive control), birds fed basal diet and experimentally infected with *M. gallisepticum* at 14 d of age; SEM, standard error of the mean. [a-c]
[a-c]Means with various superscripts in the same row vary significantly ($P < 0.05$).

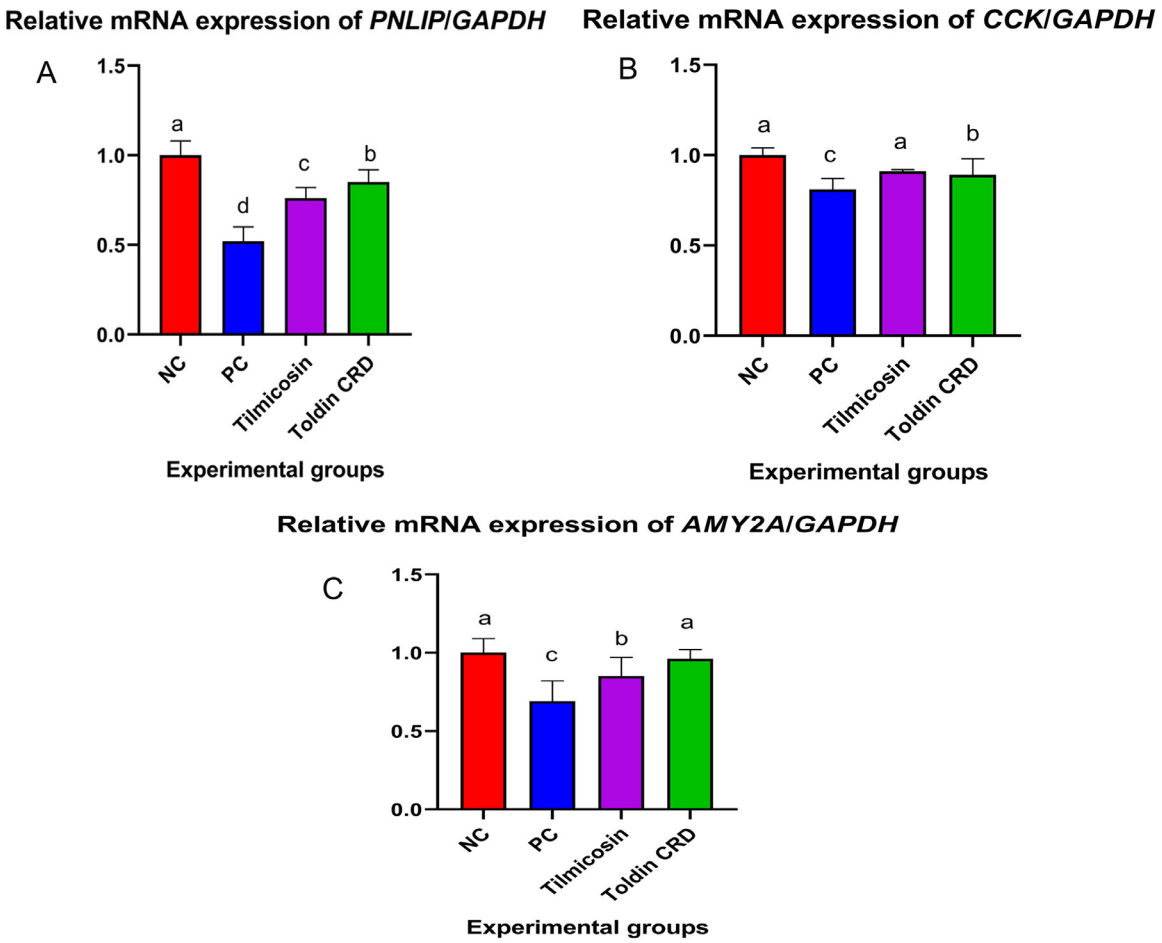

Figure 1. Transcript levels of pancreatic lipase (*PNLIP;* **A**), cholecystokinin (*CCK*; **B**) and alpha 2A amylase (*AMY2A;* **C**) genes in the pancreatic samples of experimental broiler chickens as determined via RT-qPCR assay at 28 days of age. NC (negative control): birds fed basal diet and were not experimentally infected; PC (positive control): birds fed basal diet and were experimentally infected with *M. gallisepticum*; tilmicosin: birds fed basal diet and treated with tilmicosin; toldin CRD: birds fed basal diet and treated with toldin CRD. All groups except the NC group were experimentally infected with *M. gallisepticum* at 14 d of age. Results are expressed as means ± standard error of the mean (SEM, error bars). [a-d]Means of columns with various letters indicate a statistically significant difference ($P < 0.05$).

variations among NC and tilmicosin and toldin CRD treated groups.

Quantification of Mycoplasma *gallisepticum DNA Copies*

The quantification results of MG in the trachea of experimentally infected birds are shown in Figure 4. At 7 and 14 dpi, the most marked minimization in MG loads was observed in birds treated with tilmicosin (5.44 and 3.12 $\log_{10}$ CFU/g, respectively), followed by toldin CRD (6.97 and 4.56 $\log_{10}$ CFU/g, respectively) treated groups in comparison with the PC group (8.56 and 5.59 $\log_{10}$ CFU/g, respectively). Of note, there were significant ($P < 0.05$) variations in the $\log_{10}$ copies of MG populations in the trachea of birds treated with toldin CRD and tilmicosin at 7 and 14 dpi.

Immunohistochemical Findings

The immunohistochemical findings of MG antigens in the splenic tissues of experimental birds are shown in Figure 5. *Mycoplasma gallisepticum* antigens were observed by localization of granular brown pigments with various intensities in the examined tissues. Birds in the NC group showed no positive results (no brown staining); meanwhile, the tissues of birds in the PC group revealed the most intense staining at 7 dpi, followed by 14 dpi. The most marked ($P < 0.05$) minimization in the level of MG antigens (light staining) was observed in birds treated with toldin CRD, followed by tilmicosin treated group (moderate staining) in comparison with the PC group at both intervals.

DISCUSSION

In the present study, treatment with a mixture of many active natural compounds (toldin CRD) was able to modulate the immune responses of broiler chickens to overcome the experimental infection with MG strain and improve their growth parameters. Interestingly, supplementing toldin CRD for broiler chickens in drinking water post experimental infection with MG improved their overall growth rate and restored their

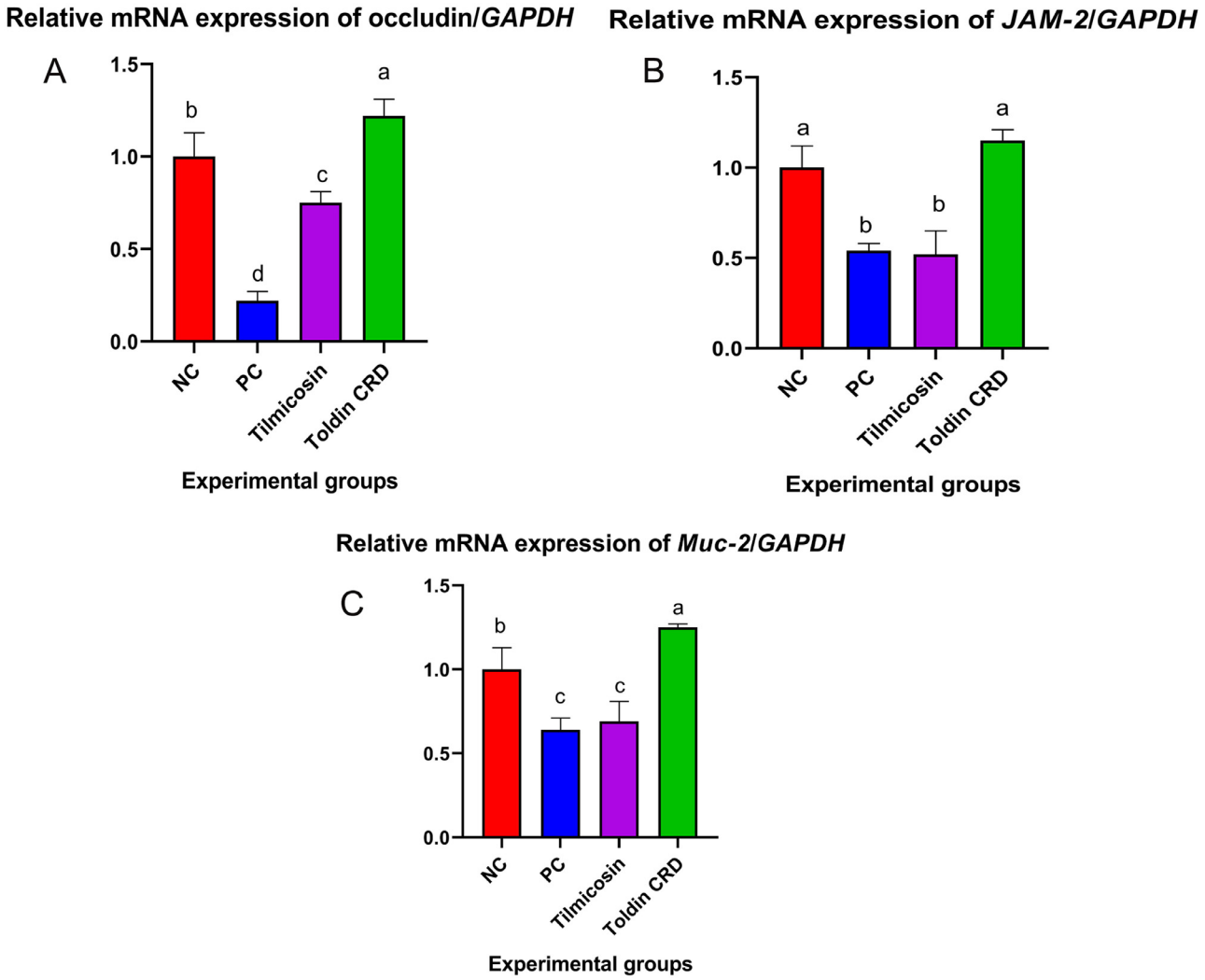

Figure 2. Relative mRNA expression levels of occludin (**A**), junctional adhesion molecule-2 (*JAM-2*; **B**) and mucin-2 (*MUC-2*; **C**) genes in experimental broiler chickens as estimated by RT-qPCR assay at 28 d of age. NC (negative control): birds fed basal diet and were not experimentally infected; PC (positive control): birds fed basal diet and were experimentally infected with *M. gallisepticum*; tilmicosin: birds fed basal diet and treated with tilmicosin; toldin CRD: birds fed basal diet and treated with toldin CRD. All groups except the NC group were experimentally infected with *M. gallisepticum* at 14 d of age. Results are expressed as means ± standard error of the mean (SEM, error bars). [a–d]Means of columns with various letters show statistically significant differences ($P < 0.05$).

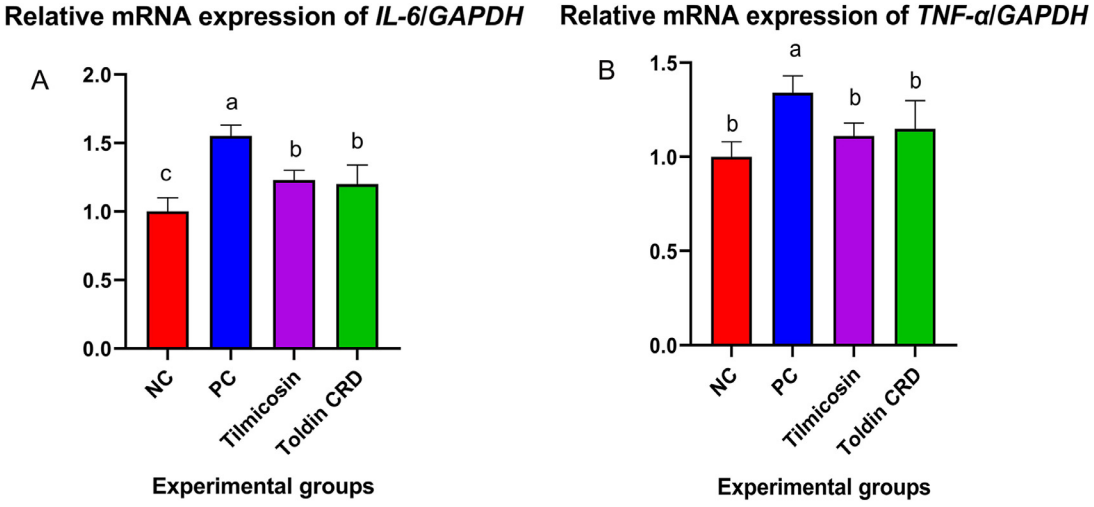

Figure 3. Relative mRNA expression levels of interleukin-6 (*IL-6*; **A**) and tumor necrosis factor-alpha (*TNF-α*; **B**) in the splenic tissues of experimental broiler chickens at 28 d of age. NC (negative control): birds fed basal diet and were not experimentally infected; PC (positive control): birds fed basal diet and were experimentally infected with *M. gallisepticum*; tilmicosin: birds fed basal diet and treated with tilmicosin; toldin CRD: birds fed basal diet and treated with toldin CRD. All groups except the NC group were experimentally infected with *M. gallisepticum* at 14 d of age. Error bars represent the standard error of the mean (SEM). [a–c]Means of columns with various letters indicate a statistically significant difference ($P < 0.05$).

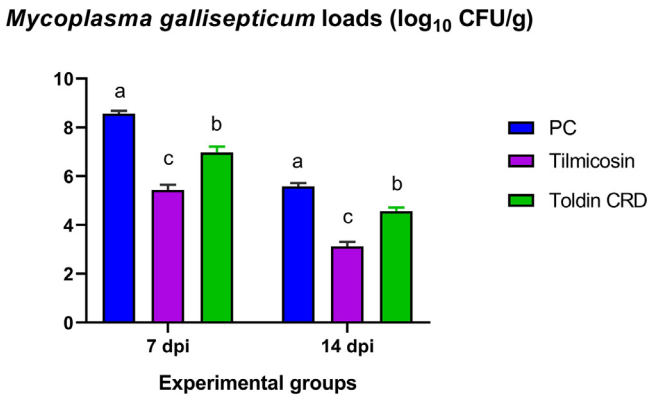

Figure 4. Quantification of tracheal *M. gallisepticum* loads ($\log_{10}$ CFU) in response to treatment with tilmicosin and toldin CRD at 7- and 14-days post infection (dpi) as detected by qPCR assay. PC (positive control): birds fed basal diet and were experimentally infected with *M. gallisepticum*; tilmicosin: birds fed the basal diet and treated with tilmicosin; toldin CRD: birds fed the basal diet and treated with toldin CRD. All groups were experimentally infected with *M. gallisepticum* at 14 d of age. Error bars represent the standard error of the mean (SEM). [a-c]Means of columns with various letters indicate a statistically significant difference ($P < 0.05$).

minimized body weight, and reduced FCR compared to the PC group. In agreement with our results, previous studies stated that medicinal plants had improved the mean body weights (Müştak et al., 2015) and FCR (Barbour et al., 2008; Müştak et al., 2015) in broilers infected with MG; however, the efficacy of toldin CRD on the growth performance of broiler chickens experimentally infected with MG strain did not be studied until now. Moreover, previous studies stated that dietary inclusion of various levels of plant-active principles was able to improve the growth performance variables in broiler chickens after experimental infection concerning the PC group (Ibrahim et al., 2020b, 2021a). Similarly, a recent study reported that EOs supplementation in drinking water improved the BWG and FCR in broiler chickens experimentally infected with *S.* Enteritidis, which indicates that drinking water is a successful route of administration for EOs (Bendary et al., 2021). The EOs' positive effects on growth performance might be correlated to the improved digestibility of nutrients via endogenous enzymes stimulation, regulation of the intestinal microflora, the antimicrobial characteristics of their bioactive components in addition to their ability to enhance the appetite and improve the immune responses (Aljazzar et al., 2022; Ibrahim et al., 2022). However, the mode of action of EOs' extracts is not defined yet and it might differ due to various sources, forms, and structures of the used active compounds (Ibrahim et al., 2021a).

Blood immunological variables are crucial markers, which provide good information on the birds' general health (Ibrahim et al., 2021c). Of note, microbial infections induce systemic inflammatory reactions that are fundamental issues in persuading stresses on immune functions, which impair the chick's performance and threaten its general health condition (Ibrahim et al., 2021b, c). Essential oils can improve the immune response of broiler chickens that might be involved in improving gut health, intestinal microbiota, and chicken resistance against bacterial infections. The bactericidal characteristics of macrophages, monocyte, and neutrophils have been correlated to the action of LYZ, NO, and MPO, which are useful in the protective immune responses to eradicate the infecting bacteria (Ibrahim et al., 2021b). The MPO, LYZ, and NO, which are considered important inflammatory reaction indicators, are mainly produced by phagocytic cells and bacterial infection could increase their levels (Ibrahim et al., 2021b). In the current work, decreased levels of LYZ, NO, and MPO in the toldin CRD treated group at 28 d of age clarify its potential role in decreasing the bacterial harmful effects, which could be attributed to

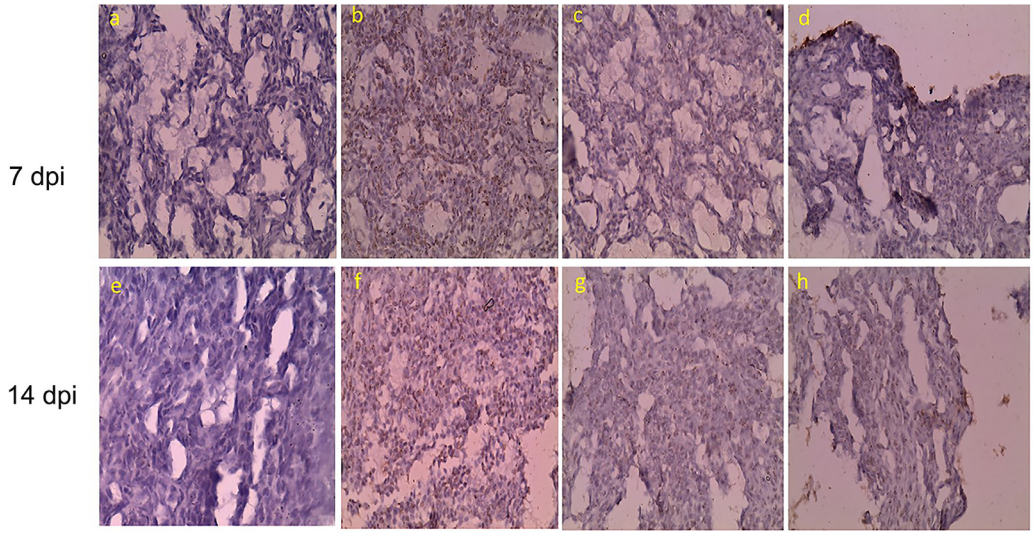

Figure 5. Immunohistochemical detection of *M. gallisepticum* antigens in the splenic tissues of birds in different experimental groups at 7 (a, b, c, and d) and 14 (e, f, g, and h) days post infection (dpi). a and e: negative control, birds fed basal diet and were not experimentally infected; b and f: positive control, birds fed basal diet and were experimentally infected with *M. gallisepticum*; c and g: birds fed the basal diet and treated with toldin CRD; d and h: birds fed the basal diet and treated with tilmicosin. All groups except the NC group were experimentally infected with *M. gallisepticum*.

the anti-inflammatory effects of toldin CRD. These findings are in concurrence with a recent study, which reported that medicinal plants had anti-inflammatory activities through their positive effects on humoral nonspecific immunity via minimizing the MPO and LYZ activities and also NO, which is the main mediator of oxidative tissue injury and host defense in *Clostridium perfringens* infected broilers (Ibrahim et al., 2021b); however, the anti-inflammatory activities of toldin CRD in broiler chicks experimentally infected with MG strain did not be explored until now. Interestingly, the immunoglobulins such as IgG are among the most important immune indicators investigated after supplementing EOs enriched diets, and elevations in their levels might result from a strong innate immune response, which plays an effective role in inhibiting bacterial infection (Abd El-Hamid et al., 2021; Ibrahim et al., 2021a). Herein, at 28 d of age (after the treatment end), elevated levels of IgG were observed in birds treated with toldin CRD with no significant variations between NC and toldin CRD treated groups suggesting its immunostimulant activity. In agreement with our findings, a previous study reported that medicinal plants had immunostimulant activities by elevating the level of immunoglobulins in fish infected with *Streptococcus agalactiae* (Abd El-Hamid et al., 2021); however, the immunostimulant activities of toldin CRD in broiler chickens experimentally infected with MG strain did not be investigated yet.

Recent studies reported that medicinal plants have improved effects on broiler digestion at the molecular levels (Ibrahim et al., 2021b; a). In line with the enhanced growth rate, BWG, and FCR in the toldin CRD treated group, the expression levels of digestive enzymes encoding genes (*CCK, AMY2A,* and *PNLIP*) were also increased. In agreement with our findings, previous studies reported that EOs had enhanced effects on the growth performance of broiler chickens, utilization of nutrients, and gut microbial flora (Ibrahim et al., 2021b; a, 2022). Additionally, it has been stated that phytochemicals can improve the gene expression levels of the intestinal mucosa and trigger digestive enzymes for enhancing feed utilization and digestibility in broiler chickens (Ibrahim et al., 2021a, 2022). However, upregulating the expression levels of digestive enzymes encoding genes following toldin CRD treatment in MG experimentally infected broilers was not studied till now.

The gut mucosal barrier has a significant role in water, electrolytes, and nutrient absorption in addition to protecting the gastrointestinal tract from enteric bacterial invasion and avoiding the proinflammatory molecules leakage via the gut mucosa to the circulatory system (Ibrahim et al., 2021a). The gut barrier is regulated via tight junction proteins, which comprise multiple unique proteins such as JAM, occludin, claudins-1, and zona occludens-1 and those are vital for creating an intact physical barrier among the gastrointestinal epithelial cells (Ibrahim et al., 2021b). Disruption of the TJP secretion and formation usually occurs in the pathogenesis of several inflammatory diseases. This disturbance in TJP might cause a reduction in the absorption of nutrients, increasing the luminal antigens permeability, translocation of the bacteria, continuous inflammation, and tissue injury (Ibrahim et al., 2021a; b). Of note, mucin considers the gut's first line of defense, and improvement of its production might help in avoiding bacterial invasion and toxin production (Ibrahim et al., 2022). Meanwhile, downregulation of the expression level of the *MUC-2* gene, which controls the mucin secretion in the infected untreated group could be attributed to stimulating gastrointestinal inflammation. Moreover, inflammatory lesions could decrease the goblet cells that produce the mucin, stop the gut mucosa regeneration and increase translocation of the bacteria and inflammation of the gastrointestinal tract (Ibrahim et al., 2020b). A previous study reported that EOs improved gastrointestinal integrity and the gut mucosal barrier (Ibrahim et al., 2022). In line with the enhanced growth rate, BWG, and FCR in the toldin CRD treated group, the expression levels of TJPs and gut barrier functions encoding genes (*JAM*, occludin, and *MUC-2*) were greatly upregulated, which indicates its potential role in restoring the gut barrier function following the experimental infection with MG strain. In agreement with our findings, upregulation of genes encoding TJPs and enhancement of gut barrier functions were detected in EOs treated broiler chickens, which were infected with *Clostridium perfringens* (Ibrahim et al., 2021a).

Cytokines play fundamental regulatory roles in the intestinal tract`s inflammatory response. When the microorganisms invade the epithelial cells of the intestinal tract, the intestinal immune cells are stimulated to secrete cytokines, which play significant roles in the immune responses of the host cells against pathogens (Aljazzar et al., 2022). *TNF-α* is a fundamental proinflammatory cytokine, which regulates the immune response of the host cells against pathogens through proliferating and differentiating the immune cells; however, the over secretion of proinflammatory cytokines for a long time may lead to damage in the intestinal tract (Ibrahim et al., 2022). Additionally, *IL-6* and *TNF-α* are stimulating an inflammatory response via recruiting neutrophils and macrophages, which are considered antimicrobial cells (Aljazzar et al., 2022). Herein, at 28 d of age, the gene expression levels of the splenic proinflammatory cytokines (*TNF-α* and *IL-6*) were increased in untreated birds that were experimentally infected with MG. In line with the decreased serum levels of LYZ, NO, and MPO and increased IgG level in the toldin CRD treated group, the gene expression levels of the proinflammatory cytokines encoding genes (*IL-6* and TNF-α) were significantly decreased, which might counteract the inflammatory response resulting from MG experimental infection indicating their immunostimulant effects. This might be attributed to the EOs' role in enhancing the nonspecific immune response of the host cells through the nonspecific killing of fungi, tumor cells, parasites, and bacteria with a consequence of decreasing

the pathogens' loads (Aljazzar et al., 2022; Ibrahim et al., 2022). In agreement with our findings, previous studies reported that EOs had immunostimulant activities by downregulating the *IL-6* (Ibrahim et al., 2021a) and *TNF-α* (Aljazzar et al., 2022) genes expression levels in broiler chickens because of their regulatory activities. Moreover, another recent study stated a downregulation in the expression of *TNF-α* gene in broiler chicks experimentally infected with *S.* Enteritidis after supplementing EOs in the drinking water, which indicates their immunostimulant activities (Bendary et al., 2021), but, the activities of toldin CRD in broiler chicks experimentally infected with MG strain did not be investigated yet.

Mycoplasma gallisepticum can colonize the respiratory tract of broiler chickens causing CRD and major economic losses worldwide (Abd El-Hamid et al., 2019a; Awad et al., 2022). In the current study, quantitative analysis of MG loads in the tracheal tissues of experimentally infected birds showed that treatment with toldin CRD significantly decreased MG populations at 7 and 14 dpi compared to the PC group. Additionally, in parallel with the previous findings, immunohistochemical examination showed significant decreases in the MG antigens (light staining) in the examined splenic tissues of birds in the toldin CRD treatment group at 7 and 14 dpi with MG strain, which was evidenced by the improvement in the clinical picture of the experimental infection. Our findings are close to those previously illustrated in another recent research in Egypt, where toldin CRD possessed robust *in vitro* antimycoplasmal activities against MG field isolates (Abd El-Hamid et al., 2019a). The robust antimycoplasmal activity of toldin CRD could be explained by the presence of many active compounds, which are strong bacteriolytic agents such as propolis, ginger, oregano, licorice, anise oils, curcumin, aliumcepa, and cinnamon. Similarly, a recent study reported that EOs significantly minimized *C. jejuni* populations in the ceca (Aljazzar et al., 2022) of experimentally infected broiler chicks concerning the PC group. Moreover, another recent study stated a minimization in cecal *S.* Enteritidis loads in infected broiler chicks after supplementing EOs in the drinking water, which indicates their antibacterial activities (Bendary et al., 2021); however, the efficacy of toldin CRD on MG loads and MG antigens in the tissues of broiler chickens experimentally infected with MG strain did not be investigated yet.

CONCLUSION

From our interesting findings, we concluded that toldin CRD exhibited growth-promoting, anti-inflammatory, immunostimulant, and antibacterial effects and improved digestive enzymes, gut barrier functions, and TJPs in broiler chickens experimentally infected with MG field strain. Therefore, we recommend the utilization of toldin CRD as an alternative to antimicrobials for the treatment and control of MG infection in broiler chickens.

ACKNOWLEDGMENTS

The authors would like to thank Taif University research supporting project number (TURSP- 2020/71) Taif University, Taif, Saudi Arabia. Also, we want to extend our thanks to Faculty of Veterinary Medicine, Zagazig University, Egypt and Animal Health Research Institute, Egypt for supporting our research.

Funding: This research was funded by Taif University research supporting project number TURSP-2020/71, Taif University, Taif, Saudi Arabia.

DISCLOSURES

The authors declare no conflict of interest.

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