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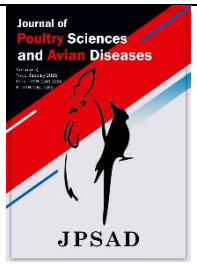

Effects of CytoGard as Drinking Water Supplement on Some Gut Health-Related Genes Expression, Inflammatory Responses, and Bone Strength Characteristics in Broiler Chickens

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A B S T R A C T

Gastrointestinal health plays a crucial role in enhancing immune function and maintaining a balanced gut microbiota in poultry. Regulation of inflammatory gene expression and upregulation of genes related to intestinal health are key factors in preserving epithelial integrity and immune efficiency. In the present study, the effects of a drinking water supplement, commercially known as CytoGard, on the expression of inflammatory and intestinal health-related genes during the rearing period of broiler chickens were evaluated. A total of 180 Ross 308 broiler chickens were randomly allocated to three experimental treatments: CytoGard, a commercial probiotic serving as a positive control, and control groups, with six replicates of 10 birds each. On day 42, ileum samples were collected to analyze the expression of pro-inflammatory genes (TNF-α and IL-1β) as well as the anti-inflammatory gene (IL-10). Additionally, the expression of gut health-related genes, including occludin, mucin-2, and immunoglobulin A, was assessed using real-time PCR. Furthermore, the mechanical properties of the tibia bone were evaluated. The results showed that CytoGard significantly reduced the expression of TNF-α and IL-1β compared to the control group (p<0.01). Moreover, the expression of intestinal health-related genes such as mucin-2 and occludin was significantly upregulated (p<0.01). The mechanical properties of tibia bone did not differ significantly among groups (p>0.05). Therefore, regular supplementation with CytoGard during the rearing period may be considered as an effective strategy to enhance poultry gut health by reducing inflammation and upregulating the expression of intestinal health-related genes.

Keywords: *Gut health, inflammatory genes, intestinal integrity, CytoGard*

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1 Introduction

The poultry industry is considered one of the most important sectors of the agricultural industry worldwide and plays a significant role in providing animal protein, ensuring food security, and generating employment. Poultry is one of the most important sources of protein globally (1).

Global poultry meat consumption has increased markedly over recent decades, making it the second most consumed type of meat worldwide. Poultry meat consumption is expected to grow by 2.4% annually until 2030 (2). Maintaining gastrointestinal health, as the most important organ system in nutrient digestion and absorption, plays a crucial role in improving feed efficiency and animal performance (3).

Various factors, including poor diet, viral, bacterial, parasitic, and fungal pathogens, among others, cause infection and inflammation in the digestive system, leading to reduced nutrient absorption, an increased feed conversion ratio (FCR), decreased weight gain, and ultimately significant economic losses (4). Multiple studies have demonstrated the beneficial effects of probiotics, such as *Lactococcus*, *Bifidobacterium*, and *Bacillus subtilis*, as nutritional supplements on regulating gut flora, enhancing nutrient absorption, boosting the immune system, and preventing and treating diseases (5, 6). One of the most significant effects of probiotic supplementation in poultry is the reduction of chronic intestinal inflammation, attributed to the production of anti-inflammatory cytokines in the intestinal tissue (7).

Mucosal interleukin-10 (IL-10), as an anti-inflammatory cytokine, plays a critical role in maintaining normal structure of the gut, promoting gut health, enhancing the expression of tight junction proteins in intestinal epithelial cells, increasing mucus production as a protective barrier, and elevating the secretion of immunoglobulin A (IgA) at the mucosal surface of the gastrointestinal tract (8, 9).

The presence of a protective mucosal layer in the intestinal epithelium plays a vital role in maintaining gastrointestinal health. The mucosal layer forms a barrier against toxins/antigens and facilitates absorption. Numerous studies have demonstrated the role of mucosal IL-10 in enhancing the integrity and strength of the intestinal epithelium (8, 10, 11).

The presence of IL-10 in the intestinal lumen suppresses the expression of pro-inflammatory genes, such as tumor necrosis factor-alpha (TNF-α), leading to the upregulation of junctional proteins (e.g., E-cadherin, occludin), thereby enhancing intestinal mucosal health. Furthermore, numerous studies have demonstrated that mucosal IL-10 promotes the intestinal immune function by increasing the population of gut macrophages and regulating IgA secretion (8, 12, 13).

Recombinant proteins, such as cytokines, are considered a novel and effective approach to modulating immunity and improving gut health (14). In this context, our previous study demonstrated that a drinking water supplement, commercially known as CytoGard (containing recombinant chicken IL-10), has valuable effects on growth performance, improves gut microbial populations, and increases intestinal microvilli length in broiler chickens during the rearing period (14).

However, the molecular mechanisms underlying the direct effects of CytoGard on the expression of TNF-α, IL-1β, IL-10, and MUC2, as related to immunity and intestinal mucosal health, remain unclear.

The present study investigated the effects of CytoGard supplementation on the expression of key pro-inflammatory, anti-inflammatory, and intestinal health-related genes in broiler chickens. In addition, we evaluated the effects of CytoGard supplementation on the mechanical properties of tibia bone. We hypothesized that water-delivered CytoGard would beneficially modulate intestinal immune and barrier-related gene expression in broilers, without affecting tibia bone strength.

2 Materials and methods

2.1 CytoGard production

CytoGard was developed in previous studies by Nika Zist Afarin, a knowledge-based company located at the Ferdowsi University of Mashhad Science and Technology Park, using recombinant DNA technology. The production method and in vitro effects of CytoGard on chicken splenocytes have been investigated in our previous study (15).

In brief, using recombinant DNA technology and genetic engineering, the chicken IL-10 coding gene was cloned into a *B. subtilis* expression vector. The positive clones were screened using specific molecular methods. The production of recombinant IL-10 was subsequently optimized in a 5-L fermentor, operated at 37°C, with the pH maintained at 7.0 and dissolved oxygen (DO) controlled at 30% saturation, using a fed-batch feeding strategy. Expression was induced at the mid-long phase (OD600=1.0) by 1% xylose and maintained for 18 h. Afterward, cultures were harvested based on OD600 and product yield. Nano-encapsulation

techniques were applied to protect the cytokine from degradation in the gastrointestinal tract. The final product was lyophilized into a stable powder form. The active ingredient was then mixed with safe diluents to prepare different doses of CytoGard. The exact dose of chicken IL-10 was determined using a specific ELISA kit (Chicken IL-10 ELISA kit, SUNLONG, China) according to the manufacturer's protocol. Furthermore, we evaluated the effects of different doses of CytoGard on body weight gain, FCR, gut microbial composition, and intestinal morphometric characteristics, with a dose of 20 ng/L demonstrating the most favorable outcomes (14).

2.2 *Experimental design and treatment groups*

The experiment was conducted at the research farm of Ferdowsi University of Mashhad. A total of 180 one-day-old Ross 308 broiler chicks, comprising both male and female birds, were purchased. Chickens were randomly assigned to three treatment groups, each consisting of six cages as experimental units, with 10 chickens per cage. Randomization was performed using a computer-generated list, ensuring equal numbers of males and females in each group. Based on previous studies, the optimal dose of CytoGard was determined to be 20 ng/L (14). The positive control group received a commercial probiotic (PrimaLac) administered in drinking water at the recommended dose: 20g per 1000 L of water from day 0 to day 20, and 60g per 1000 L of water from day 21 onward. The negative control group received drinking water without any additives. CytoGard supplementation was provided continuously from day 0 to 42. Environmental conditions, including temperature, humidity, and lighting, were adjusted according to the age of the chicks. Birds were reared under these conditions for a total of 42 days.

Diets were formulated for different growth phases: starter (days 1–10), grower (days 11–24), and finisher (days 25–42) (Table 1). Drinking water was replaced daily.

Table 1. Ingredient composition of the diet (%)

Diet components	Starter (1-10 days)	Grower (11-24 days)	Finisher (25-42 days)
Soybean	35.92	36.24	33.4
Corn	55	53.12	55.58
Oil	3	5	5.7
Di-Calcium phosphate	1.64	1.45	1.38
L-Lysine	0.32	0.34	0.13
Limestone	1.55	1.28	1.27
Salt	0.42	0.42	0.42
Mineral premix[1]	0.25	0.25	0.25
Vitamin premix[2]	0.25	0.25	0.25
D-L- Methionine	0.15	0.15	0.12
Wheat bran	1.5	1.5	1.5

[1] Mineral premix supplied the following, per kilogram of diet: Manganese sulfate 248 mg, Ferrous sulfate 125 mg, Zinc oxide 211 mg, Copper sulfate 25 mg, Calcium iodate 25 mg, Selenium 5 mg, Choline 625 mg, Antioxidant 25 mg

[2] Vitamin premix supplied the following, per kilogram of diet: Vitamin A 22500 IU, Vitamin D3 5000 IU, Vitamin E 45 IU, Vitamin B1 4 mg, Vitamin K 5 mg, Vitamin B12 0.4 mg, Pantothenic acid 24 mg, Folic acid 2.5 mg, Niacin 74 mg, Pyridoxine 7.3 mg, Biotin 0.04 mg

2.3 *Sample collection*

At the end of the experimental period (day 42), two birds from each cage, representing the average body weight of the group, were selected and slaughtered from four replicates. Ileal tissue samples were collected for gene expression analysis and immediately placed in RNA Shield solution (Dena Zist Asia, Mashhad, Iran). Samples were stored at –70°C until RNA extraction was performed.

2.4 *RNA extraction and gene expression analysis*

Total RNA was extracted from ileal tissue samples using a total RNA extraction kit (Pars Toos, Tehran, Iran) according to the manufacturer's protocol. RNA concentration was measured using a Nano Drop spectrophotometer (BioTek, Vinouski, USA). To remove genomic DNA contamination, total RNA samples were treated with 1 U DNase I (RNase-free) (Sinaclon, Tehran, Iran) at 37°C for 15min. Inactivation of the DNase was performed by adding one µL of 50 mM ethylenediaminetetraacetic acid (EDTA) at 85°C. cDNA

synthesis was performed from 1µg of RNA using a cDNA synthesis Kit (Pars Toos, Tehran, Iran). Real-time PCR reactions were carried out using RealQ Plus 2x Master Mix Green (Ampliqon, Odense, Denmark) in a Rotor-Gene Q real-time PCR cycler (Hilden, Germany).

The expression of pro-inflammatory genes TNF-α and interleukin-1 beta (IL-1β), the anti-inflammatory gene IL-10, and gut health and mucosal immune-related genes (occludin, mucin-2, and IgA) was evaluated by real-time PCR. β-actin was used as the reference gene. Standard curves were generated for all genes, and PCR efficiency (%) was calculated according to Equation 1. Table 2 lists the gene-specific primers and accession numbers of the genes used in this study.

Table 2. Gene-specific primer sequences used in the real-time PCR

Gene	Sequence primer	Ta (˚C)	PCR Product (bp)	Accession number
β-actin- F	AGATGACCCAGATCATGTTTGAGAC	59	111	NM_205518.2
β-actin-R	AGTCCATCACAATGCCAGTGG			
TNF-F	AATTTGCAGGCTGTTTCTGC	59	112	XM_046927262.1
TNF-R	TATGAAGGTGGTGCAGATGG			
IL10-F	CATGCTGCTGGGCCTGAA	62	94	NM_001004414.4
IL10-R	CGTCTCCTTGATCTGCTTGATG			
IL1β -F	GCTCTACATGTCGTGTGTGATGAG	63	80	XM_046931582.1
IL-1β -R	TGTCGATGTCCCGCATGA			
Occludin-F	GATGGACAGCATCAACGACC	62.5	142	XM_046904540.1
Occludin-R	CTTGCTTTGGTAGTCTGGGC			
Mucin-F	ATTGAAGCCAGCAATGGTGT	62	119	XM_046942297.1
Mucin-R	GCCTTGTCATCAAAGTTGCC			
IgA-F	AGCATCAGGAAGGAGACGG	62.5	160	XM_040685381.2
IgA-R	GGTTTTGTTGGGTCCACGTC			

IL10: Interleukin 10, IL-1β: Interleukin 1 beta, IgA: Immunoglobulin A

Three technical replicates were performed for each biological sample. For REST analysis, each treatment group included six biological replicates corresponding to six cages, with RNA pooled from 2–3 chicks per cage (n=6 pens per group, 10 chicks per pen). Amplification steps included 15min at 95°C (1×), followed by 30s at 95°C, 30s at annealing temperature, and 30s at 72°C (40×). Data were analyzed by REST software.

Equation 1: $(10^{-1/slple}-1) \times 100$

2.5 *Determination of the mechanical properties of the tibia bone*

At 42 days of age, one male bird from each pen (six birds per treatment) was selected using pre-randomized selection and slaughtered. Only males were chosen to minimize sex-related variation. Then, the tibia bone was carefully excised, and any adhering tissues were removed. The bones were wrapped in saline-soaked gauze, placed in sealed plastic bags, and stored at -20°C until further examination to maintain hydration. The right tibia bone was used for the strength test. After thawing, the mechanical properties of the bones were determined using a three-point bending test method. This method calculates the force required to break the bone by considering the thickness of the bone layer, as well as the diameter and length of the bone. Mechanical properties of the tibia were assessed using a Universal Testing Machine (Model H5KS, Tinius Olsen Company, Norwood, USA). The support span was set at 40% of the bone length. The bone was positioned in the anterior-posterior plane, and a perpendicular load was applied to the midpoint at a displacement rate of 5 mm/min until fracture occurred (16). The results were recorded as the force required to reach the structural failure of the tibia (N), stiffness (N/mm), and energy (J) for each bone.

2.6 *Statistical analysis*

Data were analyzed using one-way ANOVA followed by Tukey's post-hoc test for multiple comparisons. In cases of non-normal distributions, the Kruskal–Wallis test with Dunn's post-hoc correction was applied. Significant differences between groups were determined as follows: ****$p \leq 0.0001$, ***$p \leq 0.001$, **$p \leq 0.01$, and *$p \leq 0.05$.

3 Results

3.1 *Effects of CytoGard on the down-regulation of pro-inflammatory and anti-inflammatory cytokines expression*

To test whether CytoGard affected the expression of pro-inflammatory and anti-inflammatory cytokines, we evaluated the expression of TNF-α, IL-1β, and IL-10 in ileum tissues by real-time PCR analysis. Standard curves were generated for each gene, and their characteristics are presented in Table 3.

Table 3. Standard curve characteristics in Real-time PCR

Parameter/gene	β-actin	TNF-α	IL-1β	IL-10	Mucin-2	Occludin	IgA
R^2	0.999	0.995	0.987	0.999	0.997	0.996	0.994
Slope	-3.33	-3.68	-3.34	-3.28	-3.56	-3.46	-3.38
Efficiency (%)	99.5	86.6	99	100	90	94.5	97

IL10: Interleukin 10, IL-1β: Interleukin 1 beta, IgA: Immunoglobulin A

Analysis of pro-inflammatory gene expression revealed that using CytoGard as a water drinking supplement significantly reduced the expression of TNF-α and IL-1β compared to the control group (p<0.01) (Figure 1). These findings indicated the strong anti-inflammatory properties of IL-10 in suppressing pro-inflammatory cytokines. In the commercial probiotic group, TNF-α expression was reduced; however, the change was not statistically significant (p=0.18). IL-1β expression in the probiotic group showed a slight increase compared to the control, although this difference was not significant (p=0.21). Therefore, unlike CytoGard, the commercial probiotic was not effective in reducing intestinal inflammation. Expression of the anti-inflammatory cytokine IL-10 was also reduced in the CytoGard group relative to the control (p<0.1). A similar non-significant reduction was observed in the probiotic group (p<0.1).

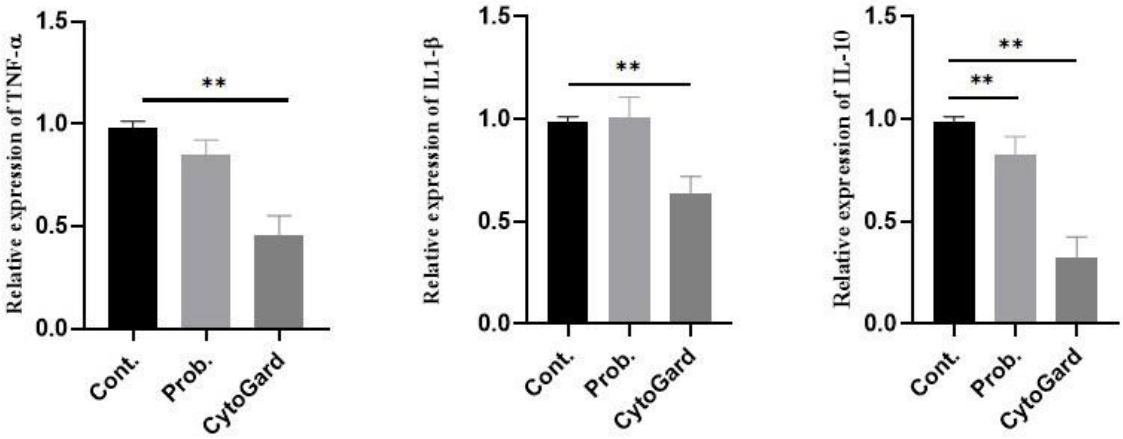

Figure 1. The expression of pro-inflammatory genes TNF-α and interleukin-1β, as well as the anti-inflammatory gene interleukin-10, in the ileal tissue on day 42 in the groups including: Cont. (control), Prob. (commercial probiotic PrimaLac), and CytoGard (20 ng/L). Data are represented as Mean ±SD. Statistical significance differences are indicated as **p≤ 0.01

3.2 *Effects of CytoGard on the upregulation of intestinal health-related gene expression*

The expression of intestinal health-related genes, including occludin, a key protein in tight junctions of epithelial cells, and mucin-2, a major component of mucus, was investigated. The results showed that the expression of occludin and mucin-2 was significantly upregulated in the CytoGard group compared to the control (p<0.01) (Figure 2).

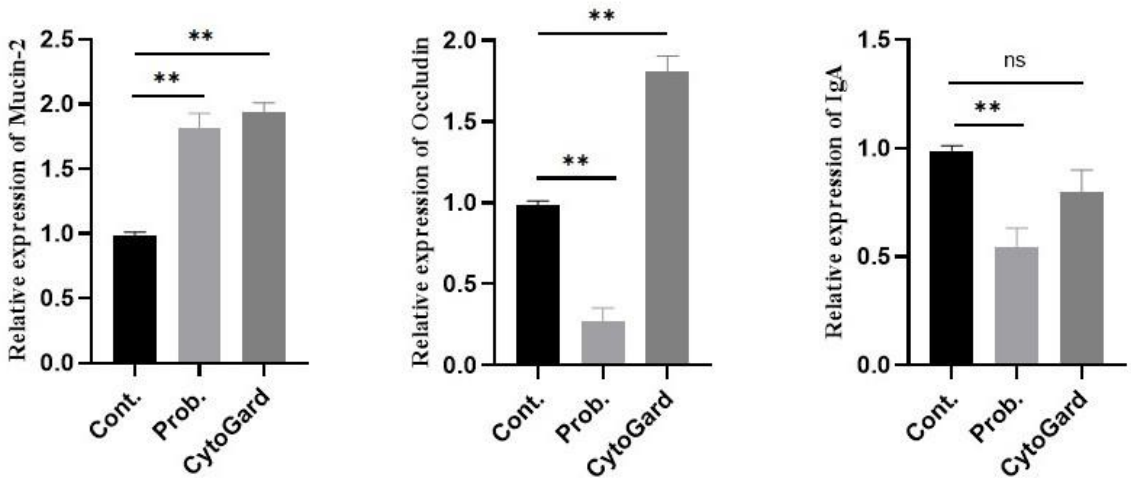

Figure 2. The expression of mucin-2, occludin, and immunoglobulin A (IgA) genes in the ileal tissue on day 42 in the groups including Cont. (control), Prob. (commercial probiotic PrimaLac), and CytoGard (20 ng/L). Data are represented as Mean ±SD. Statistical significance differences are indicated as **p≤0.01

We observed that the expression of mucin-2 increased compared to the control in the commercial probiotic group (p<0.01), whereas the expression of occludin was significantly reduced (p<0.01). The expression of IgA in the CytoGard group showed a slight decrease compared to the control, but this difference was not statistically significant. In contrast, the probiotic group exhibited a significant reduction in IgA expression relative to the control.

3.3 *Effects of CytoGard on tibia bone mechanical properties*

Evaluation of the mechanical properties of tibia bone indicated that there were no significant differences in force, stiffness, or energy among the control, commercial probiotic, and CytoGard groups (p>0.05). As shown in Table 4, the mean force required to fracture the tibia was 0.41, 0.56, and 0.37 N for the control, probiotic, and CytoGard groups, respectively, with no statistically significant differences observed. Therefore, similar to the commercial probiotic, CytoGard supplementation did not affect tibia strength.

Table 4. Mechanical properties of tibia bone

Parameter/ Group	Control	Probiotic	CytoGard
Force (N)	0.41±0.09	0.56±0.22	0.37±0.11
Stiffness (N/mm)	2.68±0.49	2.78±0.34	2.26±0.31
Energy (J)	269.4±32	293.2±82	269.28±31

Data are represented as Mean ±SD

4 Discussion

The poultry industry has continuously sought safe and scientific strategies to replace antibiotic growth promoters and improve the health of broiler flocks (17-19). In the present study, we investigated the effects of the drinking water supplement CytoGard (containing recombinant chicken interleukin-10) on ileal mRNA expression of key inflammatory and barrier markers (TNF-α, IL-1β, IL-10, MUC2, occludin, IgA) and on tibia mechanical properties, testing the hypothesis that CytoGard administration would reduce intestinal inflammation and improve gut barrier function and bone integrity in broilers. The findings of the present study demonstrated a significant reduction in the expression of the pro-inflammatory genes TNF-α and IL-1β in the CytoGard group. TNF-α and IL-1β are pro-inflammatory cytokines that are secreted in response to

pathogens, toxins, and environmental stressors (20). These molecules activate the immune system, increase intestinal epithelial permeability, disrupt tight junctions, and contribute to the development of chronic inflammation (21). The downregulation of these genes results in a reduction of chronic intestinal inflammation, preservation of tissue integrity, improved nutrient absorption, and decreased energy expenditure by the immune system. This finding is particularly important because chronic and subclinical intestinal inflammation is a highly detrimental factor in the poultry industry, leading to energy loss, reduced feed efficiency, and impairment of intestinal integrity. Studies in poultry have demonstrated that systemic inflammation is closely associated with decreased epithelial barrier integrity and reduced mucosal layer thickness (22, 23).

IL-10, an anti-inflammatory cytokine, plays a crucial role in regulating and suppressing inflammatory responses, thereby preventing tissue damage and maintaining immune system homeostasis by inhibiting the transcription factor NF-κB, a key regulator of inflammatory gene activation (24). As shown in Figure 1, CytoGard supplementation also reduced the expression of this anti-inflammatory cytokine. This concurrent reduction in pro-inflammatory genes indicates the maintenance of immune balance and a non-inflammatory intestinal state in the gut, which is desirable for animal health. Moreover, the production of IL-10 and other cytokines entails a metabolic cost, and their reduction under non-inflammatory conditions allows the body to redirect energy toward growth and production, which is particularly important in broiler rearing (8).

In the commercial probiotic group, a reduction in TNF-α expression was observed, but it was not statistically significant. This may be related to the type, dose, or duration of probiotic administration. Several studies have reported that the anti-inflammatory effects of probiotics are dependent on the species and strain used (25). IL-10 expression in the probiotic group also showed a reduction, but the magnitude of this decrease was much lower than that observed in the CytoGard group.

Furthermore, the assessment of intestinal health-related gene expression, including occludin and mucin-2, demonstrated that CytoGard supplementation is associated with up-regulation of barrier-related transcripts, suggesting potential benefits for intestinal barrier function. Occludin is a key protein in tight junctions of epithelial cells, responsible for maintaining the integrity of the intestinal barrier and preventing the translocation of pathogens and toxins from the lumen into the bloodstream. Mucin-2 is a major component of mucus, forming a protective layer over epithelial cells and helping to prevent microbial adhesion and invasion (26, 27).

Previous studies have shown that IL-10 can preserve epithelial cell junctions by reducing inflammation (28). In the probiotic group, a significant increase in mucin-2 expression was also observed, indicating the positive effect of probiotics on enhancing protective mucus secretion in the gut, a finding supported by multiple studies (29, 30). In 2024, Wasim Abbas *et al*. reported that a 14-day administration of an antibiotic cocktail led to a significant reduction in the expression of occludin and mucin-2 genes (22). Thus, prolonged use of antibiotic cocktails impaired the function of tight junctions in broilers (22, 31). In contrast, CytoGard, as a safe alternative to antibiotics, not only avoided such adverse effects but also increased the expression of occludin and mucin-2.

Expression of IgA in the CytoGard group decreased slightly compared to the control, but the reduction was not statistically significant. In contrast, the probiotic group showed a significant reduction in IgA expression relative to the control. The simultaneous increase in occludin and mucin-2 expression, along with a slight decrease in IgA, suggests that the intestine is in an actively defensive but non-inflammatory state (Figure 2).

This study is limited by the reliance on ileal mRNA expression data without complementary protein-level or functional assays. Therefore, the observed changes in gene expression should be interpreted with caution, as post-transcriptional and post-translational regulation may influence the actual biological outcomes. Additionally, the findings of this study indicated that supplementation with the commercial probiotic and CytoGard drinking solution containing IL-10 had no significant effects on mechanical bone parameters, including force, stiffness, and energy, over the 42-day rearing period. IL-10, as an anti-inflammatory cytokine, can indirectly influence bone metabolism by modulating immune responses and reducing inflammation. However, it appears that short-term supplementation under the present conditions was insufficient to induce noticeable changes in bone strength. Moreover, bone strength is influenced by complex factors such as mineral absorption and deposition, growth rate, and bird activity (32), which may have modulated the potential effects of CytoGard or probiotics.

5 Conclusion

This study clearly demonstrates that CytoGard, when used as a drinking water supplement, has considerable potential to specifically modulate the immune system and enhance intestinal integrity in broiler chickens. By reducing inflammation and strengthening the intestinal barrier, this supplement may significantly contribute to improved growth performance and reduced Feed Conversion Ratio (14). No differences in tibia mechanical properties were detected among groups. In future studies, the effect of CytoGard should be further evaluated by assessing cytokine and IgA protein levels, intestinal barrier permeability assays, as well as microbiota profiling and bone geometry–normalized testing.

Acknowledgements

None.

Conflict of Interest

The authors declare no competing interests.

Author Contributions

MH.S. designed and directed the project; MH.S. and S.H. conceived and planned the experiments. S.H., M.M.S., JK.A., H.S., and N.A. carried out the experiments. MH.S. and S.H. analyzed the data. MH.S., A.H., B.O., and A.J. contributed to the interpretation of the results. MH.S. took the lead in writing the manuscript with input from all authors. All authors discussed the results and commented on the manuscript.

Data Availability Statement

All authors are ready to provide the available data to readers upon request via email or any other communication platform.

Ethical Considerations

The animal experimental protocol was conducted with the approval of the Ethics Committee of Ferdowsi University of Mashhad (code: 528662, 1403.11.10). All procedures in the animal experiments were strictly followed by the guidelines for the care and use of laboratory animals issued by the National Institutes of Health.

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