Influence of Lacto-Immuno-Vital on growth performance and gene expression of IgA, MUC-2, and growth factor IGF-2 in the jejunum of broiler chickens

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ABSTRACT The effects of Lacto-Immuno-Vital synbiotic preparation on gene expression of IgA, MUC-2, and growth factor IGF-2 in the jejunum and on BW gain in broiler chickens were studied. A flock of 64,400 1-day-old Hybrid ROSS 308 chickens was inducted in the 42-day experiment. The chickens were divided into 2 equally size groups in separate halls. The chickens in the experimental (**E**) group received 500 g of Lacto-Immuno-Vital in 1,000 L of drinking water. The preparation was administered daily from the first day (day 1) to day 7 of the experiment. From day 7 to day 22, it was given in pulsed manner (every third day) at a dose of 300 g in 1,000 L of drinking water. The broiler chickens in the E group gained more weight ($P < 0.001$) compared with control from day 10 to day 42. Death of animals during feeding period was 1,078 chickens in the E group compared with 1,115 dead chickens in the control group. Feed conversion ratio was 1.61 kg of supplemented diet/kg of BW in the E group compare with 1.67 kg of non-supplemented diet/kg of BW in control. The relative expression of IgA gene in the jejunum was upregulated on day 22 in the E group compared with control ($P < 0.05$), whereas relative expression of MUC-2 gene was upregulated in the E group compared with control on day 8 and day 22 ($P < 0.05$; $P < 0.001$). Similarly, relative expression of IGF-2 gene was upregulated in the E group compared with control on both samplings ($P < 0.01$). The composition of Lacto-Immuno-Vital synbiotic preparation showed beneficial effects on growth performance, feed conversion ratio, morbidity, mortality, and selected parameters of mucosal immunity in the chicken jejunum.

Key words: synbiotic preparation, growth factor, mucin, Iga, broiler

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INTRODUCTION

Worldwide consumption of poultry meat has increased both in developed and in developing countries. Chicken meat is still popular because of its high-quality protein content and relatively low prices compared with other types of meat (Beski et al., 2015).

In today's consumer-oriented world, it is very important to produce healthy and safe animal products. In this context, the healthy and properly functional gastrointestinal tract of animals forms the basis for safe food production. Animal metabolism is a complex process, which is also regulated by the presence of both host and commensal intestinal microbiota. In the small intestine, the mucosal surface is particularly exposed to pathogens and is therefore covered with a loosely attached mucus layer. MUC-2 gene is a major component of the loose mucus layer secreted by goblet cells, limiting microbial adherence and regulating growth (Butler, 2015). IgA antibodies are among the most important humoral immune factors present on mucosal surfaces, where in addition to protecting against absorption of mucosal antigens, they play a strategic role in inhibiting inflammatory effects (Herich, 2017). Moreover, in the dynamic environment of the developing chicken intestine, growth factors represent important mediators of gastrointestinal repair, with key roles in

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cellular processes such as proliferation, differentiation, migration, and survival (Rowland et al., 2013). Similarly, intestinal development is also modified by insulin-like growth factors (**IGF**). It has been shown that IGF-2 is involved in mechanisms that control the differentiation of the intestinal epithelium (Georgiev et al., 2003). In addition, IGF-2 plays an essential role in the growth process of skeletal muscle and the growth plate of developing bone. Even in developing endochondral bones, chondrocyte proliferation is absolutely dependent on IGF signaling (Kawai and Rosen, 2012).

To improve the quality of chicken meat, alternative substances are increasingly used including probiotics and β-glucans. Specific probiotic strains can improve animal growth by modulating the intestinal microbiota as well as the secretion of IgA and mucin. Likewise, β-glucans modulate the intestinal morphology by increasing the number of mucin-producing goblet cells, as well as cells expressing secretory IgA (**sIgA**) with increased sIgA in the intestinal lumen. At the same time, they reduce bacterial translocation to various other organs (Anwar et al., 2017).

Several studies focusing on the relationship between the gut microbiota and immunology have emphasized the importance of using synbiotics to promote farm animal health. On the other hand, most studies investigating the effects of synbiotics concentrate on humans (Markowiak and Śliżewska, 2017). In general, a synbiotic is defined as a combination of prebiotics and probiotics, which synergically support gastrointestinal health by improving survival and adherence of live microbial dietary supplements (Yari and Hekmatdoost, 2019). Lacto-Immuno-Vital is a synbiotic preparation that improves conditions for the development of beneficial microbiota, thereby enhancing mucosal immunity in the intestine. Although the effects of synbiotics have been clarified, important information regarding their influence on chicken health is still incomplete. The aim of this study was therefore to evaluate the effects of Lacto-Immuno-Vital synbiotic preparation on selected parameters of mucosal immunity (IgA, MUC-2) and growth factor IGF-2 in the jejunum and on BW gain in broiler chickens.

MATERIAL AND METHODS

Experimental Design

The experiment was conducted in a commercial broiler chicken fattening farm, and the birds were handled and sacrificed in a humane manner. A flock of 64,400 1-day-old Hybrid ROSS 308 chickens were inducted in the 42-day experiment. The chickens were divided into 2 equal groups in separate halls. The chickens in the experimental (**E**) group received 500 g of Lacto-Immuno-Vital (Hajduvet Kft., Hungary) in 1,000 L of drinking water. Lacto-Immuno-Vital was administered daily from the first day (day 1) to day 7 of the experiment. From day 7 to day 22, it was given in a pulsed manner (every third day) at a dose of 300 g in 1,000 L of drinking water. The composition of Lacto-Immuno-Vital is shown in Table 1. The control (**C**) group received only the standard diet (see Table 2). Groups of 60 chickens randomly selected in each hall were weighed at 1, 5, 10, 16, 20, 26, 30, and 35 d of age (Table 3). For analyses, 16 chickens from each group (E, C) were taken from the halls. The sampling day were set at day 8 and day 22 of the experiment. The chickens were euthanized with an intra-abdominal injection of xylazine (Rometar 2%; SPOFA, Czech Republic) and ketamine (Narkamon 5%; SPOFA, Czech Republic) at doses of 0.7 mL/kg BW. Samples from the caudal part of the jejunum were collected during necropsy.

Table 1. Composition of Lacto-Immuno-Vital.

Probiotic strain	Cfu/g
Enterococcus faecium (CECT 4515)	10×10^9
Bacillus amyloliquefaciens (CECT 5940)	10×10^9
Mannan oligosaccharide	12%
β-glucan (*Saccharomyces cerevisiae*)	12%
Microbial protein	10%

Homogenization of Jejunum Samples and Isolation of Total RNA

Jejunum tissue samples were cut into 20-mg pieces, immediately placed in RNA Later solution (Qiagen, UK), and stored at −70°C before RNA purification, as described in the study by Karaffová et al. (2019).

Relative Expression of IgA, MUC-2, and IGF-2 Genes in Quantitative Real-Time PCR

The mRNA levels of IgA, MUC-2, and IGF-2 were determined. In addition, mRNA relative expression of the reference gene, coding glyceraldehyde-3-phosphate dehydrogenase, was determined based on stability of expression using BestKeeper software. The primer sequences used for quantitative real-time PCR are listed in Table 4. All primer sets allowed DNA amplification efficiencies between 94 and 100%.

Amplification and detection of specific products were performed using the CFX 96 RT system (Bio-Rad) and Maxima SYBR Green qPCR Master Mix (Thermo Scientific). Subsequent quantitative real-time PCR to detect relative expression of mRNA selected parameters was based on 36 cycles performed with initial denaturation at 94°C for 3 min, followed by denaturation at 93°C for 45 s. The optimal annealing temperature and time for each primer are shown in Table 4, and there was an elongation step at 72°C for 10 min. A melting curve from 50°C to 95°C with readings at every 0.5°C was produced for each individual quantitative real-time PCR plate. Analysis was performed after every run to ensure a single amplified product for each reaction. All real-time PCR reactions were performed in duplicate, and mean values of the duplicates were used for subsequent analysis. We also confirmed that the efficiency of amplification of each target gene was essentially 100% in the exponential phase of the reaction,

Table 2. Composition of feed mixtures.

Components	Starter Day 1–Day 10	Grower I Day 11–Day 17	Grower II Day 18–Day 22
Corn %	42.77	43.31	46.14
Soya extracted scrap %	25.0	24.0	23.2
Wheat %	20.0	20.0	16.0
Full-fat soya	7.0	7.0	6.0
Sunflower meal %	0	0	1.5
Rapeseed scrap %	0	0	1.5
Fodder lime %	1.21	1.12	0.91
Monocalcium phosphate %	1.17	0.76	0.64
Plant oil %	0.6	1.7	2.1
Premix %	0.5	0.5	0.5
Methionine %	0.36	0.33	0.30
Lysine %	0.30	0.25	0.24
Sodium bicarbonate %	0.25	0.25	0.20
Threonine %	0.16	0.10	0.10
Salt	0.16	0.17	0.17
Lupro-Cid nal %	0.30	0.30	0.30
FRA LeciMax dry %	0.05	0.05	0.05
l valine %	0.05	0.07	0.01
Anticoccidials	Maxiban G160 50 mg/kg	Maxiban G160 50 mg/kg	Sacox 70 mg/kg
Myco fix select	0.08%	0.08%	0.08%
Declared values			
Dry mass %	87.83	87.91	87.95
ns %	20.33	19.80	19.47
Fat %	4.09	5.18	5.93
Dietary fiber %	2.65	2.62	3.08
Ash %	5.46	4.80	4.46
ME_n (mj.kg)	12.53	12.90	13.04
Lysine %	1.27	1.20	1.19
Methionine %	0.64	0.61	0.59
Met + lys %	0.99	0.95	0.93
Threonine %	0.88	0.81	0.83
Tryptophan %	0.23	0.22	0.22
Valine %	0.95	0.94	0.87
Ca %	0.79	0.68	0.59
P total %	0.65	0.55	0.53
Sodium %	0.15	0.15	0.16
Mg %	0.14	0.14	0.14
Zn (mg/kg)	125.27	124.90	123.99

where the quantification cycle (**Cq**) was calculated. The Cq values of the genes studied were normalized to the average Cq value of the reference gene ($^{\Delta}$Cq), and the relative expression of each gene was calculated mathematically as $2^{-\Delta Cq}$.

Collection of Jejunum Samples for ELISA

During necropsy, jejunal segments were taken from the intestine at the same site in each chicken. Length of intestinal segments reached approximately 3 cm. Small pieces of intestinal loops were washed and prepared for determination of sIgA content as well MUC-2 production and secretion. Syringes were filled with an optimal volume (5 mL per each sample) of warm flushing solution (1 M tris/glycine buffer with 0.25% Tween 20, pH 7; Sigma-Aldrich). Then, a needle was inserted into one end of each intestinal loop, and by emptying the syringe in several pulses, the whole intestinal content was flushed out. The complete luminal

Table 3. Effect of Lacto-Immuno-Vital on the weight of broiler chickens depending on age.

Day of experiment	Control group (means + SD)	Experimental group (means + SD)
1 d	34.35 ± 0.16	34.44 ± 0.19
5 d	118.60 ± 0.17	119.60 ± 0.36
10 d	275.02 ± 0.25	285.42 ± 0.13*
16 d	589.56 ± 0.36	592.36 ± 0.30*
20 d	906.43 ± 0.15	910.17 ± 0.59*
26 d	1,443.09 ± 0.37	1,447.18 ± 0.28*
30 d	1,742.86 ± 1.97	1,791.35 ± 4.27*
35 d	1,980.38 ± 1.07	2,060.74 ± 1.46*
42 d	2,599.15 ± 2.94	2,709.93 ± 1.91*

*Means with superscripts are significantly different ($P < 0.001$).

Table 4. List of primers used in qRT-PCR for target gene mRNA detection in chickens.

Primer	Sequence 5′–3′	Annealing temperature/time	References
IgA Fw	GTCACCGTCACCTGGACTACA	59°C/30 s	Lammers et al., 2010
IgA Rev	ACCGATGGTCTCCTTCACATC		
MUC-2 Fw	GCTGATTGTCACTCACGCCTT	54°C/1 min	Smirnov et al., 2006
MUC-2 Rev	ATCTGCCTGAATCACAGGTGC		
IGF-2 Fw	CTCTGCTGGAAACCTACTGT	55°C/30 s	Mudroňová et al., 2018
IGF-2 Rev	GAGTACTTGGCATGAGATGG		
GAPDH Fw	CCTGCATCTGCCCATTT	59°C/30 s	De Boever et al., 2008
GAPDH Rev	GGCACGCCATCACTATC		

Abbreviation: qRT-PCR, quantitative real-time PCR.

brush-lined epithelial wall was flushed, and the content was emptied into 20-mL-volume test tubes. The jejunal flushes were centrifuged at 12,000 × *g* for 5 min (Hettich Rotina 75 420R Centrifuge DJB Labcare, UK), and the supernatants from each sample were used for ELISA (Husáková et al., 2015).

Detection of sIgA With Enzyme–Antibody Conjugate

To determine sIgA content in the jejunal flushes, we used a chicken IgA ELISA kit (Kamiya Biomedical Company). A 96-well microtiter plate was coated with affinity purified anti-chicken IgA antibody. Under laboratory conditions, the volume on each microtiter plate was incubated (22°C, 20 min), and subsequently, the content was aspirated and washed 3 times with solution, following the ELISA kit instructions. Determination of sIgA content was previously described by Karaffová et al. (2015).

Determination of Total MUC-2 by ELISA

For detection and determination of total MUC-2, we used a chicken MUC-2 ELISA kit (Kamiya Biomedical Company). For detection, 96-well microtiter plates were coated with affinity purified anti-chicken MUC-2 antibody. The plates were incubated, then washed and filled with 50 µL substrate solution in each well. The detected samples were diluted 1:5 in PBS with pH between 7.0 and 7.2 and added in 100-µL doses into predesignated wells in duplicates. Mixtures of balance solution in 10 µL and 50 µL of conjugate bound with horseradish peroxidase in stabilizing buffer were added into the plate wells, then incubated at 37°C for 1 h. Determination of total MUC-2 was previously described (Karaffová et al., 2019).

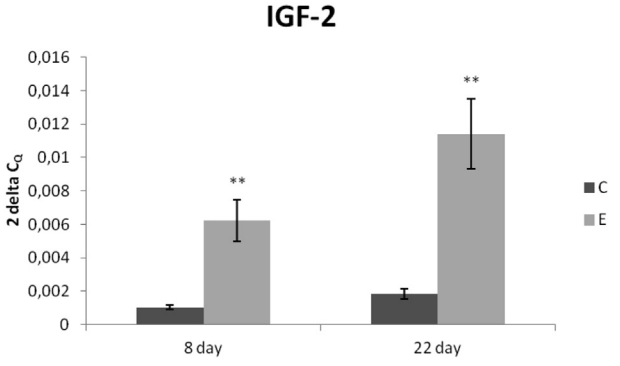

Figure 1. Relative expression of IGF-2 gene in the jejunum of chickens fed with Lacto-Immuno-Vital. Results at each time point are the median of $2^{-\Delta Cq}$. Superscripts indicate significant differences between the control and experimental groups. $^{**}P < 0.01$. Abbreviations: C, control group; E, experimental group; IGF, insulin-like growth factor.

Statistical Analysis

Statistical analysis of data was performed using *t* test in Minitab 16 software (SC & C Partner, Brno, Czech Republic). Differences between the mean values for the groups were considered statistically significant at $P < 0.05$, $P < 0.01$, and $P < 0.001$. Values are given as means ±SD.

RESULTS

Measurements of average weight and relative expression of the IGF-2 gene in the jejunum were used to evaluate the effect of Lacto-Immuno-Vital on growth performance.

The E group of chickens in the hall fed the diet supplemented with Lacto-Immuno-Vital demonstrated higher average weight ($P < 0.001$) from day 10 to day 42 of the experiment (Table 3) compared to the C broilers in the other hall. As for the observed mortality, 1,078 chickens in the E group died during the feeding period compared with 1,115 chickens in the C group. Similarly, lower number of chickens because of crawling (dwarfism—428; locomotor system—202) was found in the E group compared with the C group (dwarfism—456; locomotor system—212). Feed conversion ratio was 1.61 kg of supplemented diet/kg of BW in the E group compared with 1.67 kg of nonsupplemented diet/kg of BW in the C group. Relative expression of IGF-2 gene was markedly upregulated in the E group ($P < 0.01$) compared with the C group, on both samplings (Figure 1).

To evaluate the effect of Lacto-Immno-Vital on mucosal protection, measurements of the relative expression for MUC-2 and IgA genes in the jejunum and concentration of MUC-2 and sIgA in intestinal flush were performed.

Relative expression of MUC-2 gene showed significant upregulation in the E group when compared with the C group in both samplings ($P < 0.05$; $P < 0.001$) (Figure 2).

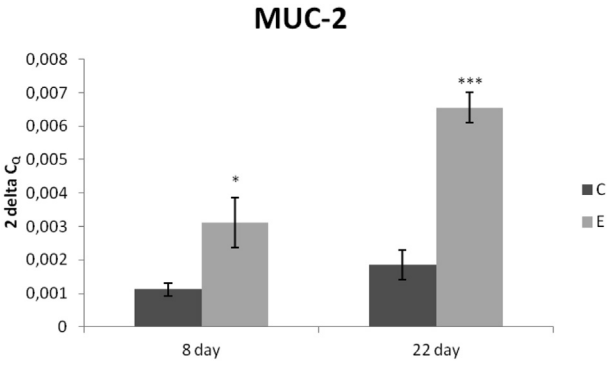

Figure 2. Relative expression of MUC-2 gene in the jejunum of chickens fed with Lacto-Immuno-Vital. Results at each time point are the median of $2^{-\Delta Cq}$. Superscripts indicate significant differences between the control and experimental groups. $^{*}P < 0.05$; $^{***}P < 0.001$. Abbreviations: C, control group; E, experimental group; MUC-2, mucin 2.

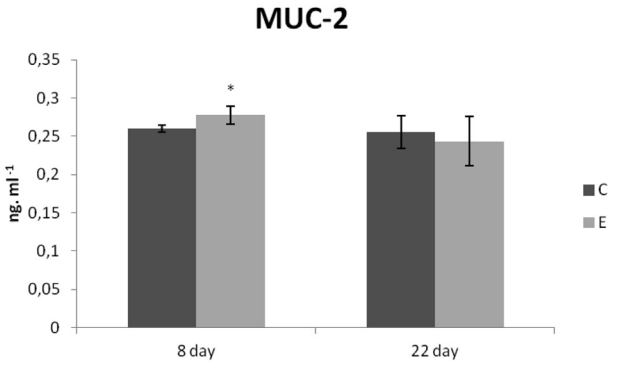

Figure 4. Mucin 2 concentrations (ng/mL) in the jejunum of chickens fed with Lacto-Immuno-Vital. Superscripts indicate significant differences between the control and experimental groups. $^{*}P < 0.05$. Abbreviations: C, control group; E, experimental group; MUC-2, mucin 2.

However, the relative expression of IgA gene in the jejunum was upregulated in the C group compared with the E group on day 8 of the experiment ($P < 0.05$). The opposite result was recorded on day 22, when gene expression was upregulated in the E group ($P < 0.05$) compared with the C group(Figure 3).

Concentration of MUC-2 (ng/mL) in the intestinal flush from the jejunum was increased in the E group compared with the C group ($P < 0.05$) on day 8. Interestingly, MUC-2 concentration was almost the same in both groups on day 22 of the experiment (Figure 4). Similarly, concentration of sIgA (ng/mL) in the intestinal flush from the jejunum was very resembled in both groups on both samplings (Figure 5).

DISCUSSION

Probiotics and combinations of probiotics and prebiotics (synbiotics) have been introduced as an alternative to antibiotics and growth promoters in poultry production. The use of synbiotics could be a promising option.

Data on the average BW of the broiler chickens showed improved growth performance. In our experiment, Lacto-Immuno-Vital was administered from the first day. The first wk after hatching is crucial for broilers' pectoralis major muscle development (Halevy et al., 2000; Žitňan et al., 2019). Malnutrition or enteral infection during this period can have irreversible negative effects on growth performance (Dina and Hams, 2016). Moreover, preventive early application of *Enterococcus faecium* has been shown to decrease cecal pathogenic microorganisms, promoting the development of the small intestine and its protective barrier (Herich et al., 2010; Ševčíková et al., 2016) and stimulating innate and acquired immune responses (Levkut et al., 2012; Dina and Hams, 2016). After 7 d of our experiment, the Lacto-Immuno-Vital dosing frequency was reduced. Levkut et al. (2009) demonstrated antimicrobial effects of *E. faecium* against pathogens on day 7 after continuous administration of the probiotic bacteria. Similarly, our previous results showed that 21 d of feeding with *E. faecium* had protective effect on the immune response in chickens (Levkut et al., 2012). However, the economic cost of long-term synbiotic administration played an important role in our experiment. This prompted us to stop the diet supplementation with Lacto-Immuno-Vital on day 23 of the present experiment and then to check for permanent improvement in the chickens' growth performance and health status. In our trial, the weight gain increased by 110.78 g for chickens in the experimental group on day 42 of the experiment. Beneficial effect of Lacto-Immuno-Vital

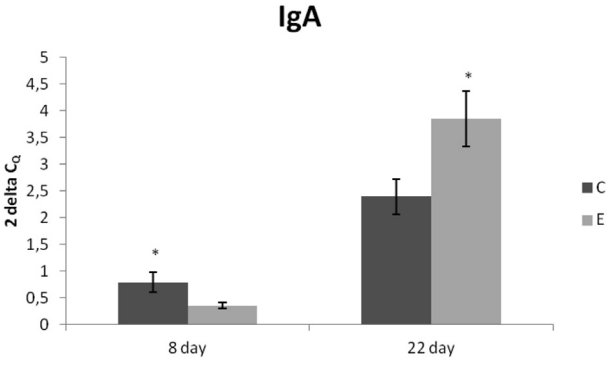

Figure 3. Relative expression of IgA gene in the jejunum of chickens fed with Lacto-Immuno-Vital. Results at each time point are the median of $2^{-\Delta Cq}$. Superscripts indicate significant differences between the control and experimental groups. $^{*}P < 0.05$. Abbreviations: C, control group; E, experimental group.

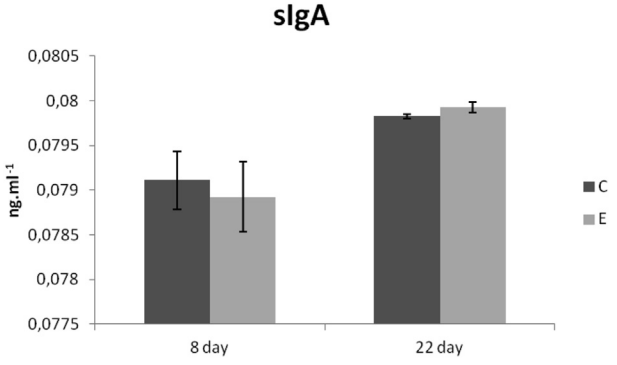

Figure 5. sIgA concentrations (ng/mL) in jejunum of chickens fed with Lacto-Immuno-Vital. Abbreviations: C, control group; E, experimental group; sIgA, secretory Ig A.

was demonstrated also on feed conversion ratio (increased 3.6%), morbidity (decreased 6%), and mortality (decreased 3.4%).

Several studies have observed the stimulating effect of *Bacillus amyloliquefaciens* alone on the average daily weight gain in chickens (Ahmed et al., 2014; Lei et al., 2015). *E. faecium* has been shown to support gut villi development and thereby affect the capacity for digestion and absorption in a positive way (Herich et al., 2010; Ševčíková et al., 2016). Similarly, Mallo et al. (2010) reported that addition of *E. faecium* CECT4515 (10^6 cfu/g) improved intestinal microbiota balance by increasing the number of *Lactobacillus* and reducing the number of coliforms in the ileum, cecum, and faeces, thus promoting the growth of weaned piglets. However, several studies have shown no significant effect on feed conversion and thus on the growth of broiler chickens when fed a diet supplemented with *B. amyloliquefaciens* alone (Wizna et al., 2009; Jerzsele et al., 2012). Moreover, the effect of the combination of *B. amyloliquefaciens* CECT5940 and *E. faecium* CECT4515 on broiler chickens has not been fully clarified so far.

Supplementation of Lacto-Immuno-Vital in the broiler diet in the present experiment increased relative expression of IGF-2 in the chicken jejunum on day 8 and day 22 (sampling day). It is known that IGF are essential for the growth and development of muscle (Fu et al., 2015). Furthermore, IGF contribute to maintaining the satellite cell niche by reducing depletion (Chakravarthy et al., 2000) and inhibiting the degradation of myofibers derived from chick embryonic myoblasts (Janeczko and Etlinger, 1984).

The basic protection of the mucous membranes is mediated by mucin produced by goblet cells, which is either localized on the cell membrane or secreted into the lumen to form a mucosal layer. Mucus is necessary for ensuring of hydration and physical protection and also serves as a reservoir for antimicrobial molecules (Robbe-Masselot et al., 2008). The gel forming MUC-2 provides not only nutrients but also attachment sites for host bacteria, and it can contribute to the selection of species-specific intestinal microbiota (Johansson et al., 2011).

The results of the present study demonstrate that a broiler diet supplemented with Lacto-Immuno-Vital stimulates the gene expression of MUC-2, total IgA, as well as secretion of MUC-2 in the jejunum of broiler chickens even on the eighth day of their age. The influence of *E. faecium* EF55 on the dynamics of intestinal mucin production in birds infected with *Salmonella* Enteritidis was previously demonstrated by Levkut et al. (2012). Similarly, in a recent study, Luan et al. (2019) reported that treatment with *B. amyloliquefaciens* CECT5940 upregulated gene expression of MUC-2 on the mucosal surface of the respiratory tract in broilers.

Two of the main components of Lacto-immuno-vital are the gram-positive strains *B. amyloliquefaciens* CECT5940, which has been shown to increase modified IgG and IgA levels in the serum of broilers (Ahmed et al., 2014), as well as *E. faecium* CECT4515, which has a positive effect on growth and feed intake of broiler chickens (Sanchez et al., 2007). In addition, the preparation includes an extract from the yeast strain *Saccharomyces cerevisiae*, which contains a large amount of peptides, a mannan oligosaccharide and a β-glucan, which binds pathogenic microorganisms, inhibits their attachment to cells,and increases the length of intestinal villi. Moreover, β-glucans increase the gene expression of tight junction proteins, thereby ensuring integrity of the intestinal wall in chickens (Anwar et al., 2017). In mice, dendritic cell uptake of *B. amyloliquefaciens* SQR9 alone induced the expression of cytokines and secretion of sIgA (Huang et al., 2016). Secretory IgA produced by IgA^+ plasma cells is transported to the lumen of the mucosal layer by epithelial cells, where it protects the epithelium against colonization by pathogens (Macpherson et al., 2008).

Despite these findings, diet supplementation with synbiotic preparation in our case had no significant effect on the concentration of sIgA in the jejunum of the E group. On the other hand, there was no infection, and the broilers remained in good condition. An alternative explanation could involve the combination of different strains, the concentration, or interactions between the strains used. In any case, there are only a few studies about the influence of *B. amyloliquefaciens* or *E. faecium* alone on the parameters of mucosal immunity in chickens.

CONCLUSION

Based on our results, it can be said that Lacto-Immuno-Vital improved growth performance of broilers during the experiment and decreased morbidity and mortality of chickens. Similarly, Lacto-Immuno-Vital increased feed conversion ratio. Moreover, gene expression of IgA, MUC-2, and IGF-2 gene and secretion of MUC-2 in the jejunum were increased in a nonchallenging model.

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DISCLOSURES

No potential conflict of interest was reported by the authors.

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