Research Note: Immunocompetent cells in blood and intestine after administration of Lacto-Immuno-Vital in drinking water of broiler chickens

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ABSTRACT The understanding of the synbiotics' impact on the host is incomplete. To improve the knowledge, we study the effect of Lacto-Immuno-Vital synbiotic preparation in chickens on local and systemic immune response by evaluation of immunocompetent cells in the peripheral blood and jejunal mucosa. Hematological method was used for determination of white blood cell count, and flow cytometry for measurement the functions of phagocytes and subpopulation of lymphocytes (CD3, CD4, CD8, IgM, and IgA). Cell Qest programme (Germany) was used for analysing of data obtained from flow cytometer and GraphPad Prism version 4.0 for comparison by paired *t* test between control and experimental groups. The experiment was conducted in a commercial broiler chicken fattening farm, the birds were handled and sacrificed in a humane manner. A flock of 64,400 one-day-old Hybrid ROSS 308 chickens were included in the 42-d experiment. The chickens were randomly divided into 2 equal groups, experimental and control, and each group of chickens was housed in a different hall while maintaining the same conditions. The chickens in the experimental group (Lactovital) 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 (D1) to D7 of the experiment. From D 7 to D 22 it was given in a pulsed manner (every third day) at a dose of 300 g in 1,000 L of drinking water. Control group received only the standard diet. For immune analyses 6 randomly chosen chickens from experimental and control group were taken from the halls. The sampling days were set at D 8 and D 22 of the experiment. Samples of peripheral blood were collected from vena subclavia. The chickens were euthanized and whole jejunum was taken during necropsy into Hanks ice solution (pH 7.2−7.3). Administration of Lacto-Immuno-Vital in drinking water of non-stressed broilers during fattening period in commercial production increased phagocytic activity and phagocytic index. The number of IgA+ and CD8+ cells in lamina propria of intestine was decreased in chickens fed diet supplemented with Lacto-Immuno-Vital in drinking water. We suggest that increased phagocytic activity and decreased number of immunocompetent cells in mucosa of intestine was caused by improved systemic and local immune system function.

Key words: broiler, synbiotic preparation, peripheral blood, intestine, immunocompetent cells

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INTRODUCTION

Increase pressure to reduce antibiotic use in food animal production forces producers to look for an alternative approach to decrease the effects of intestinal pathogens (Wu et al., 2019). In this regard, it has previously been reported that early supplementation of beneficial microorganism in the gastrointestinal tract of broiler chicks helps to stabilize intestinal ecosystem (Wu et al., 2019; Levkut et al., 2020). Probiotics are live, nonpathogenic microorganisms, known to have a positive effect on the host by beneficially modifying gut microbiota and modulating the immune system (Cox and Dalloul, 2015).

A combination approach that is probiotic and prebiotic (synbiotics) is most accepted practice in modern poultry production (Awad et al., 2009). Lacto-Immuno-Vital is a synbiotic preparation which consists of probiotics (*Enterococcus faecium, Bacillus amyloliquefaciens*) and prebiotics (mannan oligosaccharide, β-glucan of *Saccharomyces cerevisiae,* and microbial protein) in

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order to improve conditions for the development of beneficial microbiota in intestine (Levkut Jr. et al., 2020).

Preparation includes *Enterococci* belonging to the lactic acid bacteria which are important in foods due to their involvement in food spoilage and fermentation, as well as their utilisation as probiotics in humans and food animals. *Enterococcus faecium* was also approved by the FDA and EU for animal feed supplementation (Franz et al., 2011). Previous poultry experiments have demonstrated that supplementation of *Enterococcus faecium* in diet improved growth performance and modulated composition of intestinal microbiota (Capcarová et al., 2010; Wu et al., 2019).

Bacillus amyloliquefaciens, other component of preparation, is a key player for the production of a variety of extracellular enzymes including phytase, α-amylases, cellulase metalloproteases and proteases that could enhance digestibility and absorption of nutrients (Lee et al., 2008; Jha et al., 2020). β-glucans, also produced by *B. amyloliquefaciens*, have been receiving increasing attention as potential alternatives due to their immunomodulatory ability without negatively affecting performance of the birds (Cox et al., 2010). β-glucans can act as prebiotics, were described as carbohydrates that are not digestible by most monogastric animals and stimulate growth of commensal bacteria in the GI tract, thus contributing to gut health and overall well-being of the animals (Gibson and Roberfroid, 1995). In vitro studies have shown that β-glucans can enhance the functional activity of macrophages as well as activate antimicrobial activity of mononuclear cells and neutrophils (Qureshi, 2003; Williams, 1997; Tzianabos, 2000). With the desire to find alternatives to antibiotics in poultry production, there has been recently proliferation of research dealing with the effect of β-glucans on avian immune system (Jacob and Pescatore, 2014, 2017; Anwar et al., 2017, Revajová et al., 2018).

Mannan oligosaccharide, other component of Lacto-Immuno-Vital, is an indigestible short chain polymer and a well-known supplement for increasing the life quality of livestock (Nopvichai et al., 2019). It can enhance the immunity as well as metabolic, the stress response, and improves the intestinal morphology in poultry (Cheled-Shoval et al., 2014).

Several papers (Awad et al., 2009; Levkut et al., 2020) reported that synbiotic product has beneficial effects on broiler performance parameters including average daily BW gain and FCR. For the present there is lack of information about influence of synbiotic on immune system. This prompt us to study immune response of immunocompetent cells in blood, intraepithelial (**IEL**) and lamina propria lymphocytes (**LPL**) of jejunum in nonstressed broilers in commercial poultry production after administration of synbiotic Lacto-Immuno Vital preparation in drinking water.

MATERIAL AND METHODS

Experimental Design

The study conducted in a commercial broiler chicken fattening farm is an integral part and expansion of knowledge summarized in previous paper "Influence of Lacto-Immuno-Vital on growth performance and gene expression, MUC-2, and growth factor IGF-2 in the jejunum in broiler chickens" (Levkut et al. 2020).

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%

A flock of 64,400 one-day-old Hybrid ROSS 308 chickens were included in the 42-d experiment. The chickens were divided into 2 equal groups in separate halls. The chickens in the experimental (Lactovital) group received 500 g of Lacto-Immuno-Vital (Hajduvet Kft., Hungary) in 1,000 L of drinking water. The composition of Lacto-Immuno-Vital is shown in Table 1. Lacto-Immuno-Vital was administered daily from the first day (D 1) to D 7 of the experiment. From D 7 to D 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. Control group received only the standard diet (Table 2). The chickens were provided with feed and water ad libitum. For immune analyses 6 randomly chosen chickens of each group (Lactovital, Control) were taken from the halls. The sampling days were set at D 8 and D 22 of the experiment. The chickens were euthanized with an intraabdominal injection of xylazine (Rometar 2%, SPOFA, Czech Republic) and ketamine (Narkamon 5%, SPOFA, Czech Republic) at doses of 0.7 mL/kg body weight. Samples from peripheral blood and whole jejunum were collected during necropsy. One ml of peripheral blood was taken from brachial wing vein into Heparin (20 IU. mL^{-1} PBS, Zentiva, CZ) and stored at laboratory temperature. Jejunum were cut behind the duodenal loop, the mesentery was cut off and intestine placed into ice-cold buffered Hanks solution was stored at 4°C for next procedure.

White Blood Cell Count and Phagocytic Assay

Total leukocytes were counted in a haemocytometer using Fried-Lukačová solution (475 μL of solution plus 25 μL of blood). White blood cell count determination was done on blood smears stained with Hemacolor (Merck, Germany) by light microscopy. Total numbers of different subtypes of white blood cells was calculated by the formula: total leukocyte count × proportion of differential cells counted (%)/100 (Levkut et al., 2009).

The function of polymorphonuclear cells was assessed by flow cytometry (FACScan, BD Germany) using whole heparinised whole blood and a commercial PHAGOTEST kit (ORPEGEN Pharma, Germany). The Phagotest kit contains fluorescein (FITC)-labelled

Table 2. Composition of feed mixtures.

Components	StarterD 1−D 10	Grower ID 11− D 17	Grower IID 18 − D 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
Fatt %	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

ME_n is calculated value.

Vitamin and mineral premix: vitamin A 12,500 IU/kg, vitamin D3 4,000 IU/kg, vitamin E 80.00 mg/kg, Cu 15.00 mg/kg, vitamin D/25 cholekalciferol 1,000 IU/kg, Jod 1.00 mg/kg, Mn 50.00 mg/kg, Zn 90.00 mg/kg, Fe 40.00 mg/kg, Se 30.00 mg/kg

opsonised bacteria (E. Coli-FITC), and necessary reagents to measure the overall percentage of granulocytes which ingest one or more bacteria per cell. Procedure of staining and measuring was specified in the company protocol.

Flow Cytometry Procedure

Peripheral blood lymphocytes were separated by Histopaque gradient sedimentation (1.077 g/mL, Sigma-Aldrich, Germany) according to Boyum (1974). Mouse anti-chicken monoclonal antibodies (MoAbs) for CD3, CD4, CD8 (T-cells), IgA and IgM (B-cells) labeled with FITC (Southern Biotech, Birmingham, AL, USA; Cat. No.: CD3 8200-02; CD4 8210-02; CD8 8390-02; IgA 8330-02; IgM 8310-02) were used for immunophenotyping of lymphocytes by direct immunofluorescent method. The control antibody, polyclonal goat-anti mouse FITC-conjugated immunoglobulin $F(ab')_2$ fragment (Dako, Denmark) was used at a working dilution of 1:50 with phosphate buffered saline (**PBS**).

Jejunal lymphocytes (IEL, LPL) were isolated and purified by the modified method of Solano-Aguilar et al. (2000). Briefly, removed jejunum was placed into an ice-cold buffered Hanks solution (HBSS, pH 7.2−7.3), cut longitudinally and lengthwise into 0.5 cm pieces followed by washing three times. The intestine was placed into 50 mL conical plastic tubes (Falcon, BD, Germany) containing warmed (37°C) 5 mM dithiotreitol (HSS-DTT) for removing of mucin in thermostat during 15 min. The supernatant was discarded and gut fragments were rinsed twice in the cold HBSS, followed by incubation of fragments in warmed (37°C) 0.1 mM EDTA-HBSS for 1 h. EDTA released IEL into harvested supernatant. Then the intestine was incubated with 30 mL RPMI-1640 (Sigma, Germany) to remove previous medium during 15 min at 37°C in thermostat. The supernatant was discarded and the gut fragments were incubated in RPMI-1640 with collagenase type I (15 mg/60 mL RPMI; Sigma-Aldrich Chemie Gmbh, Steinheim, Germany) for 1 h at 37°C. Collagenase released LPL into medium. Five minutes shaking intervals was used during all incubations. The harvested supernatants with IEL and LPL were filtered and immediately centrifuged at 600 *g* for 10 min followed by twice rinse at 250 *g* for 10 min after resuspention with PBS

(Bucková and Revajová, 2014). Isolated lymphocytes were stored in PBS at 4°C to the immunophenotypization and measuring by flow cytometry.

Procedure for flow cytometry started by adjusting the concentration of bloody and intestinal lymphocytes to $10^6/50$ μL PBS and labelling with MoAbs was done. After 15 min incubation of cells in dark at room temperature the rince in PBS and centrifugation 5 min at 110 g followed. The lymphocytes were resuspended in 0.2 mL of PBS with 0.1% paraformaldehyde and storage at 4°C to the measurement by flow cytometry.

FACScan cytometer and Cell Quest Program (Becton Dickinson, Germany) were used to measure and analyse labeled cells. Gates were drawn around cells and the fluorescence data collected on at least 10,000 lymphocytes were analysed by 2-parameter dot-plot histogram. The results were expressed as the relative percentage of the lymphocyte subpopulation which was positive for the specific monoclonal antibodies. Counting of absolute number of bloody lymphocytes was done: absolute lymphocyte count × relative percentage of subpopulation/100. Procedure for flow cytometry was previously described by Levkut Jr. et al. (2019).

Statistical Analysis

Statistical analysis of obtained data was done by two-paired t test using GraphPad Software, statistical version 4.0, (San Diego, CA), USA. The differences between the mean values for the groups of control (n = 6) and experimental (n = 6) chickens were considered significant when $P < 0.05$. Values were expressed as means ± standard deviation (SD).

RESULTS AND DISCUSSION

In previously, presented results of our trial (Levkut et al., 2020), supplementation with synbiotics was found to promote growth performance and improve the feed conversion and intestinal morphology in noninfected birds.

In this second part, the results of the same trial, performed by leukocytic responses in the peripheral blood and intestine are presented. White blood cell numbers showed that inclusion of synbiotics did not lead to the significant change in absolute count of total leukocytes, lymphocytes, heterophils, eosinophils, and monocytes in chickens fed diet supplemented Lacto-Immuno-Vital in drinking water compared to control chickens (Table 3). Similarly, the absolute number of lymphocytes was not changed at $CD3^+$, $CD4^+$, $CD8^+$, IgA+, and IgM^+ subpopulation levels in the Lactovital group compared to control in the peripheral blood of chickens (Table 4). Wu et al. (2019) observed slight increase of $CD8^+$ T cells in peripheral blood of nonstressed broilers. On the other hand, our previous results demonstrated increase of lymphocytes in peripheral blood and tendency to increase CD3, CD4, CD8, and IgM positive cells in peripheral blood in chickens challenged with *Salmonella* Enteritidis

Table 3. White blood cell counts in peripheral blood (absolute number – $G.L^{-1}$; mean ± SD).

Parameter	Sampling(d)	Controlgroup	Lactovitalgroup	P values
Leukocytes	8	7.00 ± 2.00	6.40 ± 2.40	0.55
	22	6.59 ± 3.01	7.37 ± 3.38	0.76
Lymphocytes	8	5.28 ± 1.21	4.83 ± 1.64	0.52
	22	3.92 ± 1.66	4.37 ± 1.52	0.71
Heterophils	8	1.40 ± 0.75	1.34 ± 0.82	0.96
	22	2.44 ± 1.55	2.72 ± 1.68	0.82
Eosinophils	8	0.14 ± 0.10	0.08 ± 0.03	0.29
	22	0.12.± 0.07	0.14 ±.0.10	0.84
Monocytes	8	0.18 ± 0.12	0.14 ± 0.07	0.58
	22	0.11 ± 0.04	0.14 ± 0.09	0.50

Table 4. Subpopulations of lymphocytes in peripheral blood (absolute number – $G.L^{-1}$; mean ± SD).

Subpopulation	Sampling(d)	Controlgroup	Lactovitalgroup	P values
CD3	8	0.37 ± 0.11	0.28 ± 0.08	0.20
	22	2.92 ± 1.28	3.44 ± 1.34	0.62
CD4	8	0.35 ± 0.08	0.33 ± 0.10	0.81
	22	2.23 ± 0.97	2.72 ± 0.94	0.54
CD8	8	0.41 ± 0.17	0.34 ± 0.13	0.21
	22	0.83 ± 0.41	1.08 ± 0.58	0.53
IgM	8	0.20 ± 0.10	0.18 ± 0.04	0.45
	22	0.23 ± 0.14	0.24 ± 0.09	0.92
IgA	8	0.22 ± 0.06	0.20 ± 0.07	0.19
	22	0.55 ± 0.25	0.76 ± 0.44	0.43

(Levkut et al., 2012). Li et al. (2015) concluded that *B. amyloliquefaciens* could partially alleviate the compromised growth performance and immune status of broilers under immune stress at early age.

Examination of the same cell subpopulations in jejunum revealed only a weak tendency for intraepithelial lymphocytes to increase at the first sampling in the Lactovital group when compared to controls (Table 5). It was interesting that number of IgA^+ cells isolated from lamina propria of jejunum was lower ($P < 0.05$) at d 8 in the Lactovital group compared to control. On the other hand, $CD8^+$ cells decreased ($P < 0.05$) at d 22 of the experiment in Lactovital group compared to controls (Table 6). Probiotic bacteria can induce beneficial effects by producing antimicrobial substances (Strompfová et al., 2003) that limit growth and survival of pathogenic microbes. Additionally, the benefits include an enhanced epithelial barrier, increased adhesion of profitable bacteria to the intestinal mucosa, and

Table 5. Subpopulations of intraepithelial lymphocytes (IEL) in the jejunum (relative percentage; mean ± SD).

Subpopulation	Sampling(d)	Controlgroup	Lactovitalgroup	P values
CD3	8	26.10 ± 9.99	36.60 ± 3.83	0.19
	22	76.68 ± 11.52	76.05 ± 8.64	0.89
CD4	8	4.34 ± 1.92	4.50 ± 1.28	0.88
	22	12.84 ± 3.11	9.40 ± 2.50	0.34
CD8	8	54.13 ± 10.30	63.76 ± 12.81	0.45
	22	78.26 ± 9.78	78.74 ± 5.03	0.99
IgM	8	2.38 ± 0.77	2.70 ± 0.39	0.54
	22	5.85 ± 2.68	4.71 ± 0.67	0.48
IgA	8	11.67 ± 5.98	12.91 ± 1.98	0.71
	22	8.14 ± 3.63	8.33 ± 2.52	0.91

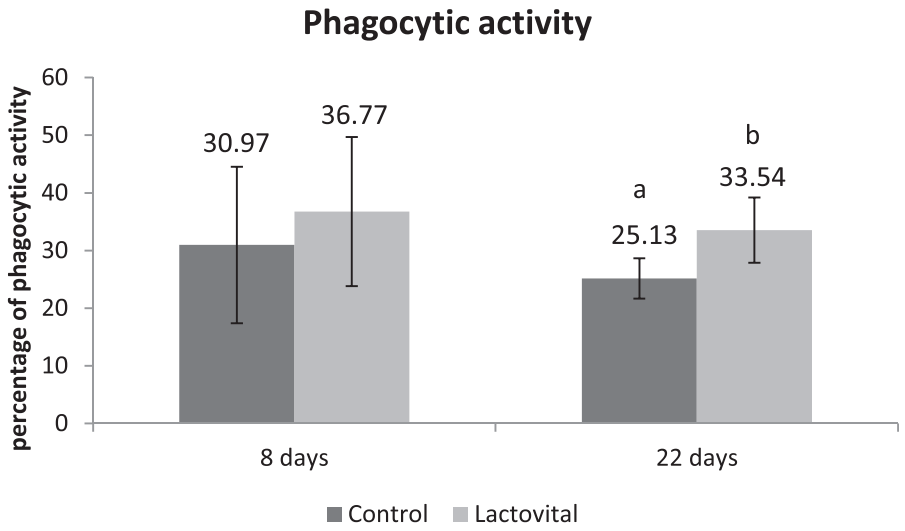

Figure 1. Phagocytic activity of polymorphonuclear cells (percentage; mean ± SD; $^{ab}P < 0.05$).

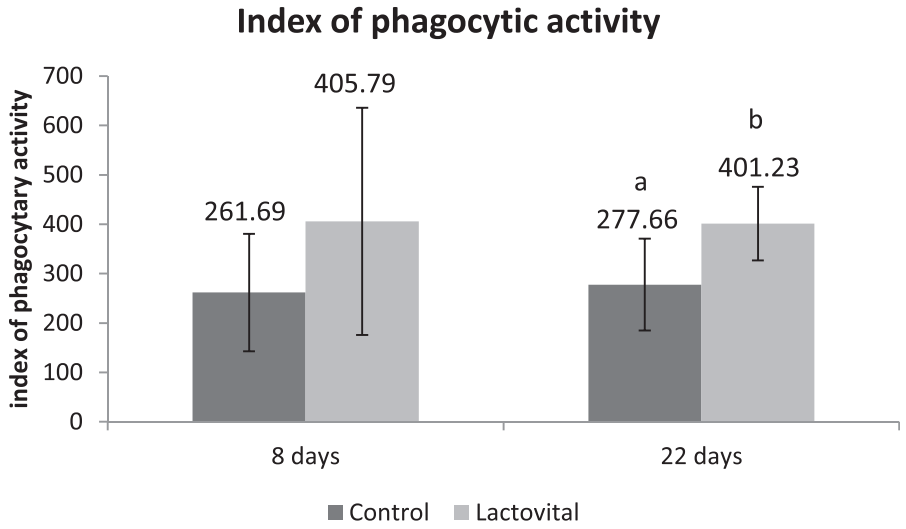

Figure 2. Index of phagocytic activity of polymorphonuclear cells (index; mean ± SD; $^{ab}P < 0.05$).

concomitant inhibition of pathogen adhesion (Jha et al., 2020). We suggest, that the decrease of relative percentage observed in our trial can be explained by formation of optimal intestinal homeostasis in gut after permanent inclusion of synbiotic in the feed.

Phagocytic activity in chickens fed diet supplemented with Lacto-Immuno-Vital was insignificantly higher on d 8 but significantly on d 22 ($P < 0.05$) of the experiment compared to control group (Figure 1). Similarly, phagocytic index improved in both samplings of the experimental group compared to control chickens with significance only on d 22 ($P < 0.05$) of the experiment (Figure 2). Synbiotics included in the diet of chickens contained β-glucans. It is known that these components are used as immunomodulators to enhance immune function in many different animals, including poultry. Exposure to yeast β-glucans has been shown to enhance proliferation and phagocytic efficiency of avian macrophages (Guo et al., 2003) as well as heterophils (Lowry et al., 2005). Inclusion of purified yeast β-glucans was shown to stimulate phagocytosis, bactericidal activity and oxidative burst in chickens (Lowry et al., 2005).

In conclusion, administration of Lacto-Immuno-Vital in drinking water of nonstressed broilers during fattening period in commercial production increased phagocytic activity and phagocytic index. Beneficial effect of

Table 6. Subpopulations of lamina propria lymphocytes (LPL) in the jejunum (relative percentage; mean ± SD).

Subpopulation	Sampling (days)	Controlgroup	Lactovitalgroup	P values
CD3	8	20.39 ± 7.39	16.83 ± 4.64	0.53
	22	68.41 ± 10.44	67.96 ± 4.65	0.95
CD4	8	2.39 ± 1.13	3 06 ± 0 92	0.42
	22	11 61 ± 5.53	9.86 ± 2.92	0,28
CD8	8	41.52 ± 8.46	30.11 ± 14.28	0.25
	22	64.43 ± 9.76^{a}	49.59 ± 7.16^{b}	0.12
IgM	8	3.12 ± 1.80	1.78 ± 0.53	0.16
	22	4.96 ± 2.63	4.88 ± 1.60	0.92
IgA	8	3.73^{a} ± 0.82	2.02^{b} ± 0.72	0.05
	22	8.08 ± 0.64	8.50 ± 2.52	0.73

$^{ab}P < 0.05$.

synbiotic preparation by process of competitive exclusion of harmful bacteria probably assisted in decrease of numbers of IgA^+ and $CD8^+$ cells in intestinal lamina propria of chickens fed diet supplemented with Lacto-Immuno-Vital in drinking water. We suggest that increased phagocytic activity and decreased number of immunocompetent cells in the mucosa of intestine was caused by improved function of systemic and local immune systems.

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DISCLOSURES

No potential conflict of interest was reported by the authors.

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