

ACCEPTED AUTHOR VERSION OF THE MANUSCRIPT:

Benefits of supplementing the diet of laying hens with levan as an additive

DOI: 10.2478/aoas-2026-0014

Damian Konkol^{1*}, Marta Kuźmińska-Bajor², Ewa Popiela³, Cassandra Ceccopieri⁴, Jan P. Madej⁴, Zbigniew Lazar², Magdalena Lis⁵, Maciej Kuczkowski⁶, Marianna Szczypka⁵, Sepideh Fallahi³, Mariusz Korczyński¹

¹Department of Animal Nutrition and Feed Sciences, Wrocław University of Environmental and Life Sciences, Chelmońskiego 38C, 51-630 Wrocław, Poland

²Department of Biotechnology and Food Microbiology, Wrocław University of Environmental and Life Sciences, Chelmońskiego 37, 51-630 Wrocław, Poland

³Department of Environment Hygiene and Animal Welfare, Wrocław University of Environmental and Life Sciences, Chelmońskiego 38C, 51-630 Wrocław, Poland

⁴Department of Immunology, Pathophysiology and Veterinary Preventive Medicine, Wrocław University of Environmental and Life Sciences, Norwida 31, 50-375 Wrocław, Poland

⁵Department of Pharmacology and Toxicology, Wrocław University of Environmental and Life Sciences, Norwida 31, 50-375 Wrocław, Poland

⁶Department of Epizootiology with Exotic Animal and Bird Clinic, Wrocław University of Environmental and Life Sciences, pl. Grunwaldzki 45, 50-366 Wrocław, Poland

♦Corresponding author: damian.konkol@upwr.edu.pl

Received date: 27 May 2025

Accepted date: 30 January 2026

To cite this article: (2026). Konkol D., Kuźmińska-Bajor M., Popiela E., Ceccopieri C., Madej J.P., Lazar Z., Lis M., Kuczkowski M., Szczypka M., Fallahi S., Korczyński M. (2026). Benefits of supplementing the diet of laying hens with levan as an additive, *Annals of Animal Science*, DOI: 10.2478/aoas-2026-0014

This is unedited PDF of peer-reviewed and accepted manuscript. Copyediting, typesetting, and review of the manuscript may affect the content, so this provisional version can differ from the final version.

BENEFITS OF SUPPLEMENTING THE DIET OF LAYING HENS WITH LEVAN AS AN ADDITIVE

Damian Konkol^{1*}, Marta Kuźmińska-Bajor², Ewa Popiela³, Cassandra Ceccopieri⁴,
Jan P. Madej⁴, Zbigniew Lazar², Magdalena Lis⁵, Maciej Kuczkowski⁶, Marianna Szczypka⁵,
Sepideh Fallahi³, Mariusz Korczyński¹

¹Department of Animal Nutrition and Feed Sciences, Wrocław University of Environmental and Life Sciences, Chelmońskiego 38C, 51-630 Wrocław, Poland

²Department of Biotechnology and Food Microbiology, Wrocław University of Environmental and Life Sciences, Chelmońskiego 37, 51-630 Wrocław, Poland

³Department of Environment Hygiene and Animal Welfare, Wrocław University of Environmental and Life Sciences, Chelmońskiego 38C, 51-630 Wrocław, Poland

⁴Department of Immunology, Pathophysiology and Veterinary Preventive Medicine, Wrocław University of Environmental and Life Sciences, Norwida 31, 50-375 Wrocław, Poland

⁵Department of Pharmacology and Toxicology, Wrocław University of Environmental and Life Sciences, Norwida 31, 50-375 Wrocław, Poland

⁶Department of Epizootiology with Exotic Animal and Bird Clinic, Wrocław University of Environmental and Life Sciences, pl. Grunwaldzki 45, 50-366 Wrocław, Poland

*Corresponding author: damian.konkol@upwr.edu.pl

ORCID:

D. Konkol – <https://orcid.org/0000-0002-3993-2847>

Abstract

This study investigated the effects of levan on various parameters in laying hens, such as: performance, egg quality, intestinal microflora formation, intestinal histomorphometry, morphometry and the composition of specific immune cells in the lymphoid organs, and the formation of blood lymphocyte and monocyte populations. A total of 120 laying hens of the Lohmann Brown line were randomly divided into five experimental groups, each group consisted of eight replications, and each replication consisted of three birds. Group C was the control group and received the basal diet with no levan supplement. The experimental groups received a basal diet supplemented with 0.1% (L0.1), 0.25% (L0.25), 0.5% (L0.5) or 1% (L1) levan. Levan, especially in a concentration of 0.5%, had a positive effect on egg production (EP) ($P < 0.05$). At low doses (0.1%), levan significantly ($P < 0.05$) improved egg breaking strength. Additionally, a significant effect ($P < 0.05$) was observed in the increase in *Lactobacillus* spp. and the decrease in *Enterobacteriaceae* in the jejunum, as well as the increase in *Bifidobacterium* spp. in the cecum. Exploratory 16S rRNA-based profiling of pooled samples suggested shifts in bacterial community composition. Levan significantly ($P < 0.05$) reduced the surface area of the villi in the ileum, and the length of the intestinal villi in the jejunum and ileum. Furthermore, an increase ($P < 0.05$) in crypt depth and a decrease ($P > 0.05$) in the ratio of the villus length to the depth of the crypts in the duodenum were observed. It can also be concluded that levan has a mild immunosuppressive effect. Levan had a positive effect on the performance of the birds. At low doses, levan had a positive effect on several egg quality parameters. The use of levan promoted the development of the gastrointestinal microflora. Levan, its fermentation products, as well as changes in the composition of the microflora of the digestive tract of laying hens may have some immunosuppressive effects.

Key words: cecal tonsil, spleen, thymus, bacteria, lymphocytes

Fructans are nonstructural carbohydrates that differ in molecular structure and molecular weight and are naturally produced by plants and some microorganisms (Zhao et al., 2013). In plants, fructans are carbohydrate-storing polymers that can increase resistance to cold and drought and are found in large amounts in roots and bulbs (Vijn and Smeekens, 1999; Versluys et al., 2018). In the case of microorganisms, fructans protect their cells from drying, help form biofilms and can be used as a carbon source (Versluys et al., 2018). These compounds can be divided into 3 main groups: inulin-type fructans, levan-type fructans and branched-chain fructans. Inulin-type fructans mainly contain a β -2,1 fructosyl-fructose bond, levan-type fructans mainly contain a β -2,6 fructosyl-fructose bond, and branched-chain fructans contain significant amounts of both β -2,1 and β -2,6 fructosyl-fructose bonds. Despite the many interesting properties of fructans, the most important from the nutritional point of view seems to be the fact that they are not hydrolyzed by digestive enzymes. The product of their partial fermentation carried out by *Lactobacillus* and *Bifidobacterium* spp. in the cecum (CEC) are short-chain fatty acids (SCFA). These acids, in turn, not only provide energy for the host, but also stimulate the growth and activity of probiotic bacteria and inhibit the growth and development of pathogens (Zhao et al., 2013; Franko-Robles and López, 2015; Madej and Bednarczyk, 2016; Ma et al., 2017; You et al., 2022). Moreover, levan-type fructans have a number of other properties, such as hypocholesterolemic effects (Yamamoto et al., 1999), the ability to bind metal ions (Ohta et al., 1993), the ability to prevent constipation (Roberfroid et al., 1993), and anticancer and immunomodulatory effects (Calazans et al., 1997).

Despite these promising characteristics, there is still a scarcity of research on the use of levan as a dietary supplement in poultry. To the best of our knowledge, unlike inulin-type fructans, levan-type fructans have been used only once in poul-

try nutrition by Zhao et al. (2013) who studied the use of levan in broiler chickens. The combined effects of levan on production traits, egg quality, intestinal histomorphometry, immune-related organ morphology, and gut microbial ecology in broiler chickens are rarely comprehensively examined, as existing studies frequently concentrate on selected physiological parameters or growth performance outcomes (Ashfaq et al., 2020; Zhao et al., 2013). Moreover, the prospective dose-dependent responses to levan supplementation have not been completely elucidated, particularly in commercial broiler chicken genotypes under practical production conditions.

In general, the effects of different feeding strategies on the performance of laying hens can be easily determined on the basis of physical characteristics such as egg production (EP), feed intake (FI), and the feed conversion ratio (FCR). However, in the case of commercial poultry farming, the impact of nutrition on the immunological competences underlying production traits is difficult to assess. Therefore, it is necessary to conduct laboratory research that will lead to a better understanding of the immunomodulatory properties of feed additives, especially in light of the need for alternatives to antibiotics.

The aim of this study was to determine the effects of levan on the performance of birds and egg quality, the microbiological composition of the digestive tract, intestinal histomorphometry, and immune responses.

Material and methods

Ethical approval

The nutritional experiment involving laying hens was approved by the Local Ethical Committee for Animal Experiments in Wrocław (No 031/2022/P3, 16 November 2022).

Animals, diets and housing

The research was carried out at the Research and Education Station in Swojczyce,

Poland (Wrocław University of Environmental and Life Sciences). A total of 120 laying hens of the Lohmann Brown line (26 wk of life) were used in the experiment. The hens were kept in a cage system under controlled microclimate conditions, which were in accordance with the line breeder recommendations (Lohmann, 2020). The birds were randomly divided into 5 experimental groups, each group consisted of 8 replications (cages), and each replication consisted of 3 hens (24 birds per group). The control group (group C) received the basal diet with no levan supplementation. The experimental groups received a basal diet supplemented with 0.1% (L0.1), 0.25% (L0.25), 0.5% (L0.5) or 1% (L1) levan (Simagchem Corporation®, Xiamen, China). The molecular weight of the levan was 504.43 Da. The hens were fed 130 g/hen/d. Access to water was unlimited. The experiment lasted 90 days. Table 1 shows the nutritional value of the levan additive, and Table 2 presents the composition and nutritional value of the basal diet. The chemical composition of the levan and the basal diet was determined according to AOAC (2005), and included dry matter (DM): AOAC, 930.15; crude ash: AOAC, 942.05; nitrogen: AOAC, 984.13; crude protein as 6.25 N; crude fat: AOAC, 920.39; crude fiber: AOAC, 978.10; and sugars: AOAC, 974.06. P and Ca contents were measured via an inductively coupled plasma emission spectrometer with an ultrasonic nebulizer (Varian VISTA-MPX ICP-OES, Victoria, Australia). All analyses were repeated 6 times.

Performance

The performance of laying hens, such as EP, egg weight (EW), daily egg mass (DEM), FI and FCR, was determined after the 30th, 60th and 90th days of the experiment. EP was defined as the number of eggs laid by birds in a given period and is presented as a percentage of laying. To determine the EW and DEM, the eggs were collected and weighed daily. To determine FI, the leftovers were also weighed daily.

The FCR was determined by dividing the FI by the DEM.

Table 1. Nutritional value of levan

Nutrient	Analyzed value
Dry matter (%)	96.81
Crude ash (% DM)	0.038
Crude protein (% DM)	bd.
Crude fiber (% DM)	bd.
Crude fat (% DM)	bd.
Nitrogen-free extract (% DM)	96.77
Sugars (% DM)	9.874

bd. – below the detection level.

Table 2. Composition and nutritional value of the basal diet

Diet composition (%)	
Wheat 10.5% CP	31.65
Maize 7% CP	30.00
Soybean meal 46% CP	15.31
Sunflower meal 34% CP	9.440
Limestone	7.720
Premix	4.000
Soybean oil	1.880
Nutritional value	
Dry matter (%) – analyzed	88.59
Crude ash (% DM) – analyzed	12.70
Crude fat (% DM) – analyzed	3.725
Crude protein (% DM) – analyzed	16.30
Crude fiber (% DM) – analyzed	4.012
Metabolizable energy (kcal/kg DM) – calculated	2640
Total Ca (% DM) – analyzed	3.802
Total P (% DM) – analyzed	0.564

*Premix composition: Ca – 213.5 g/kg; P – 45 g/kg; Na – 40 g/kg; Lys – 46 g/kg; Met – 67 g/kg; Thr – 20 g/kg; Trp – 0.700 g/kg; Arg – 3,200 g/kg; Ile – 1,600 g/kg; Val – 2,300 g/kg; vitamin A – 250,000 IU/kg; vitamin D₃ – 250,000 IU/kg; vitamin E – 750 IU/kg; vitamin K – 75 mg/kg; vitamin B₁ – 50 mg/kg; vitamin B₂ – 100 mg/kg; vitamin B₃ – 750 mg/kg; vitamin B₅ – 250 mg/kg; vitamin B₆ – 100 mg/kg; vitamin B₇ – 1250 µg/kg; vitamin B₁₂ – 625 µg/kg; choline – 10,000 mg/kg; folic acid – 25 mg/kg; Fe – 625 mg/kg; Mn – 2,500 mg/kg; Zn – 1,500 mg/kg; Cu – 375 mg/kg; I – 25 mg/kg; Se – 7.5 mg/kg; phytase – 18,725 FTU/kg.

Egg quality

Eggs for qualitative analyses were collected after the 30th, 60th, and 90th days of the experiment. Three eggs were collected randomly from each replicate (24 eggs per group). An Egg Force Reader from ORKA Food Technology (West Bountiful, UT, USA) was used to determine the breaking strength of the eggshell. Eggshell thickness was determined via a digital micrometer (SAWERON, Bydgoszcz, Poland). The shell thickness was expressed as the average of 3 measurements (sharp end, blunt end, and equator). Egg freshness based on Haugh units (HU) was determined via Egg Analyzer by ORKA Food Technology (West Bountiful, UT, USA). The color of the yolk was determined by the La Roche scale. Eggs intended for qualitative analyses were not stored but were analyzed 24 h after collection.

Microbiological analyses

On the 90th day of the experiment, 8 hens were randomly selected from each group and then euthanized. Euthanasia was carried out in accordance with Directive 2010/63/EU (European Union, 2010). Analyses of the number of bacteria of the genera *Lactobacillus* spp., *Bifidobacterium* spp., and those belonging to the *Enterobacteriaceae* family were performed. Samples of intestinal contents for tests were taken *postmortem* from the jejunum (JEJ) and the cecum (CEC). A total of 0.15 g of intestinal content was suspended in 1.35 ml of sterile PBS buffer (Merck KGaA, Darmstadt, Germany) and a series of decimal dilutions were made and inoculated into Petri dishes with MRS medium (Merck KGaA, Darmstadt, Germany) to determine the number of *Lactobacillus* spp., and surface inoculated on TOS-propionate agar (Merck KGaA, Darmstadt, Germany) with the addition of the MUP selective supplement (Merck KGaA, Darmstadt, Germany), on which bacteria from the *Bifidobacterium* spp. genus were identified. To determine

the number of bacteria from the *Enterobacteriaceae* family, the samples were surface inoculated on VRGB medium (Merck KGaA, Darmstadt, Germany). Bacterial cultures of the genus *Bifidobacterium* spp. were carried out under anaerobic conditions.

Total bacterial genomic DNA was extracted from pooled intestinal contents (0.1 g per sample) collected separately from the cecum and jejunum of euthanized hens using the QIAamp Fast DNA Stool Mini Kit (QIAGEN, Hilden, Germany). The integrity of the extracted DNA was determined via electrophoresis in 0.7% agarose stained with Midori Green DNA Stain (Nippon Genetics Europe, Germany). The concentration of DNA was determined with a Biowave II UV/Vis spectrophotometer (Biochrom WPA, UK), and the purity was determined in terms of the 260/280 nm ratio. DNA was sequenced with the Illumina MiSeq next-generation sequencing platform via the MiSeq Reagent Kit v2 500-cycles (Illumina, USA) (weSEQ.IT Poland). In accordance with the quality of a single nucleotide, the raw data were filtered primarily by Trimmomatic version 0.33 (Bolger et al., 2014). Identification and removal of the primer sequence were performed via Cutadapt version 1.9.1 (Martin, 2011). PE reads obtained from previous steps were assembled via USEARCH version 10 (Magoč and Salzberg, 2011), followed by chimera removal via UCHIME version 8.1 (Edgar et al., 2011). The high-quality reads generated from the above steps were used in the following analysis. PE reads were assembled via Usearch v10 with the following parameter settings: minimum length of overlap, 10 bp; minimum similarity within the overlapping region, 90%; and maximum mismatch, 5 bp. The operational taxonomic unit (OTU) USEARCH version 10.0 (Edgar, 2013) was applied to cluster sequences with similarity greater than 97%. The conservative threshold for OTU filtration was 0.005% (Bokulich et

al., 2013). The DADA2 method in QIIME2 version 2020.06 (Boyle et al., 2019) was applied to denoise sequences, generating ASVs. The conservative threshold for ASV filtration was 0.005%. Taxonomic annotation was performed via a classify-consensus blast in QIIME2, which identified the annotation with the highest consensus at $N = 3$ best hits, including parameters such as minimum similarity in sequence (90%), minimum coverage (90%) and minimum consensus (51%). A Venn diagram was used to illustrate shared and unique OTUs in the cecal microbiota among the dietary levan treatments. Beta-diversity was explored using NMDS based on OTU relative abundances. Due to the lack of biological replicates, the observed differences should be interpreted descriptively.

Intestinal histomorphometry

Samples of the duodenum (DUO), JEJ and ileum (IL) were collected from the birds. The surface area of the intestinal villi, the depth of the intestinal crypts, and the ratio of the length of the villi to the depth of the crypts (V/C) were determined in the collected samples. The area of the intestinal villi was calculated via the following formula: $2 \pi \times (W/2) \times L$, where W is the villus width and L is the villus length. All the samples were fixed in 4% buffered formalin and then embedded in paraffin blocks. Five-micron-thick paraffin sections were cut from the blocks prepared on an HM325 rotary microtome (Eppendorf, Kalamazoo, MI, USA) and then stained with hematoxylin according to Mayer (Roth GmbH, Karlsruhe, Germany) and eosin (Poch S.A., Gliwice, Poland) via H&E staining. Cross-sectional analysis of the intestinal fragments was performed via a Nikon Eclipse Ni light microscope (Nikon, Melville, NY, USA) equipped with a video camera. The means were calculated from 3 measurements from each sample via NIS-Elements BR 5.41 imaging software (Nikon, Melville, NY, USA).

Immune organ sample processing

The thymus (THM) samples ($n=8$) were collected, fixed, processed and stained with H&E, similar to the intestines.

Spleen (SPL) and cecal tonsil (CT) samples from eight hens from each group were collected and fixed in 4% phosphate-buffered paraformaldehyde (pH 7.4) for 1 h. The samples were subsequently washed in 0.1 M phosphate buffer for 24 h and infiltrated with phosphate-buffered 30% sucrose for 5–7 days at 4°C. The tissues were then frozen in a cryomatrix (Thermo Scientific 6769006, Shandon Cryomatrix) at -30°C and sliced with a cryostat (CryoStar NX50, Eppendorf, Kalamazoo, MI, USA). The organ samples were cut into 10 μm serial sections, mounted on glass slides (Superfrost Ultra Plus, Thermo Scientific J3800AMNZ), air-dried overnight, and frozen at approximately -30°C .

Immunohistochemical staining and morphometric analysis

The slides were retrieved from the freezer and left for 30 min to defrost and dry. The sections were then washed in distilled water and PBS, and incubated for 20 min in Antibody Diluent with Background Reducing Component (Agilent, Santa Clara, CA, USA, S3022) solution to block any nonspecific reactions. This was followed by a one-hour incubation at room temperature with monoclonal mouse anti-chicken antibodies (SouthernBiotech, Birmingham, AL, USA) directed against the Bu-1 (clone AV20, 1:500), CD4 (clone CT-4, 1:200) and CD8 α (clone CT-8, 1:200) antigens. After a few washes in PBS, the samples were incubated for 30 min with a secondary antibody via the EnVision FLEX+ system (Dako/Agilent, K8002). The signal was visualized by 3 min of incubation with diaminobenzidine chromogen (Agilent). The slides were dehydrated in a series of increasing alcohol concentrations, mounted with Euparal medium (ROTH) and covered.

The morphometric analysis consisted of measuring the areas covered by antigen-positive cells. The samples were observed with a Nikon Eclipse Ni microscope at magnifications of 200× (SPL and CT) and 100× (SPL's germinal centers). The program NIS-Elements BR 5.41 allowed the reading and calculation of the positive reaction as a percentage of the field of view in areas of 344,000 μm^2 . The field of view of the CT image was selected starting from the lamina propria of the mucosa and moving toward the lumen. In the THM, the cortex to medulla (C/M) thickness ratio was also determined.

Blood sample analyses

Blood samples were taken from the cervical vein into tubes containing ethylenediaminetetraacetic acid (EDTA) (Equimed, Kraków, Poland). The isolation of lymphocytes and monocytes from the blood was performed as described previously (Szczyńska et al., 2023). The blood was layered at a 1:1 ratio on the isolation mixture Histopaque®-1077 (Sigma-Aldrich, St. Louis, MO, USA). After centrifugation (3000 × g, 15 min, 4°C), the cells were collected from the interphase, washed with ice-cold phosphate-buffered saline (PBS) (Institute of Immunology and Experimental Therapy, Wrocław, Poland) with 1% bovine serum albumin (BSA) (Sigma-Aldrich, St. Louis, MO, USA) and centrifuged (380 × g, 7 min, 4°C). After two washes, the cells were suspended in PBS with 1% BSA, and the cell suspensions were incubated (30 min, 4°C) with monoclonal antibodies at the concentrations recommended by the manufacturer (Southern Biotech, Birmingham, AL, USA). The following monoclonal antibodies were used in this experiment: mouse anti-chicken monocyte/macrophage-FITC (KUL01), mouse anti-chicken Bu-1a-PE, mouse anti-chicken CD3-FITC, mouse anti-chicken TCR $\gamma\delta$ -PE, mouse anti-chicken CD4-PE, and mouse anti-chicken CD8a-APC. The experiment also included stain-

ing with the appropriate isotype controls: mouse IgG1-APC, mouse IgG1-PE, and mouse IgG1-FITC. After incubation with monoclonal antibodies, the cells were washed and centrifuged (380 × g, 7 min, 4°C) two times with ice-cold PBS. The cells were subsequently fixed in PBS with 2% formaldehyde (Chempur, Piekary Śląskie, Poland). Fluorescence was measured via a flow cytometer (BD FACSCalibur, BD Biosciences, San Jose, CA, USA). The distribution of the cell surface markers was analyzed via CellQuest 3.1f. Pro software (Becton Dickinson, San Jose, CA, USA). We determined the percentages and total counts of monocytes and lymphocytes and the ratio of CD4⁺/CD8⁺ lymphocytes.

Statistical analysis

Statistical analyses were performed using Statistica ver. 13.1 software (TIBCO Software Inc., 2017), with the cage with 3 hens considered a replicate experimental unit for performance data (N = 8 per group). For the egg quality data, in order to exclude the risk of pseudoreplication, the values obtained from 3 eggs were averaged at the replicate cage level before statistical analysis (N = 8 per group) and entered into the statistical model as replicate cage means. For other traits (the gut microbiota in the JEJ and CEC content; histomorphometric parameters of the intestine – DUO, JEJ and IL; immunohistochemistry and morphometry of lymphoid organs – SPL, CT, and THM; and blood analyses) the bird was used as the observation experimental unit (N = 8 per group). To determine the normality of the distribution, the Shapiro-Wilk test was performed. If the data were normally distributed, one-way ANOVA was performed, where differences between groups were assessed using the Tukey test. ANOVA was conducted according to the following model:

$$Y_{ij} = \mu + \alpha_i + \delta_{ij},$$

where Y_{ij} is the observed dependent variable, μ is the overall mean, α_i is the effect of the diet, and δ_{ij} is the random error.

If the data were not normally distributed, the Kruskal–Wallis test was performed. Differences were considered statistically significant when $P < 0.05$. The results are presented as the mean, standard error of the mean (SEM), and standard deviation (SD).

Results

Performance

Table 3 presents the performance results of laying hens after the 30th, 60th and 90th days of the experiment.

No statistically significant differences were found in the performance of laying hens after 30 days of the experiment. After 60 days of the experiment, birds from

the L0.5 group were characterized by significantly higher ($P < 0.05$) EP compared to the C group. The situation was similar after 90 days of the experiment, when birds from the L0.5 group were also characterized by significantly higher ($P < 0.05$) EP compared to the C group.

Egg quality

Table 4 shows the egg quality parameters after 30, 60 and 90 days of the experiment.

The use of different levels of levan had no effect on egg quality parameters after 30 days of the experiment. After 60 days, eggs from the L0.1 group were characterized by significantly greater breaking strength ($P < 0.05$) than eggs from the C group. After 90 days, no statistically significant differences were found in the assessed parameters.

Table 3. Performance of laying hens

Parameter	C	L0.1	L0.25	L0.5	L1	SEM	P-value
30th day							
EP (%)	90.47	92.19	92.76	93.52	92.38	0.493	0.387
DEM (g per hen)	56.00	55.35	57.05	56.05	56.60	0.252	0.268
EW (g)	57.07	56.77	58.13	57.10	58.05	0.257	0.329
FI (g)	129.1	129.6	129.6	129.6	129.9	0.118	0.587
FCR	2.306	2.346	2.273	2.314	2.298	0.011	0.351
60th day							
EP (%)	91.04 a	94.12 ab	94.42 ab	95.00 b	93.68 ab	0.392	0.015
DEM (g per hen)	58.16	57.59	59.33	57.71	58.14	0.373	0.630
EW (g)	59.71	58.94	60.81	59.01	59.73	0.397	0.544
FI (g)	129.1	129.1	128.3	129.1	129.1	0.213	0.757
FCR	2.222	2.247	2.165	2.241	2.225	0.014	0.445
90th day							
EP (%)	92.07 a	94.35 ab	95.16 ab	95.96 b	94.08 ab	0.361	0.010
DEM (g per hen)	58.85	59.17	59.83	59.14	58.82	0.257	0.759
EW (g)	60.54	60.39	61.12	60.34	60.18	0.271	0.854
FI (g)	129.5	128.9	129.7	129.3	127.2	0.552	0.773
FCR	2.205	2.180	2.170	2.188	2.165	0.012	0.886

a, b – values within a row with different letters differ significantly at the level of $P < 0.05$; EP – egg production; DEM – daily mass of eggs; EW – egg weight; FI – feed intake; FCR – feed conversion ratio; C – control group; L0.1, L0.25, L0.5, L1 – groups with the addition of 0.1%, 0.25%, 0.5% and 1% levan, respectively; SEM – standard error of the mean.

Table 4. Egg quality

Parameter	C	L0.1	L0.25	L0.5	L1	SEM	P-value
30th day							
Breaking strength (kg/cm ²)	5.518	5.942	5.759	5.801	5.863	0.049	0.168
Thickness (mm)	0.366	0.380	0.378	0.385	0.385	0.003	0.247
Yolk color	10.87	11.16	11.08	10.95	11.08	0.078	0.629
HU	96.35	96.19	93.72	93.89	94.94	0.463	0.256
60th day							
Breaking strength (kg/cm ²)	5.301 a	5.763 b	5.633 ab	5.665 ab	5.607 ab	0.048	0.035
Thickness (mm)	0.347	0.360	0.346	0.357	0.346	0.003	0.470
Yolk color	10.62	10.70	11.16	11.08	10.70	0.071	0.351
HU	83.32	85.42	85.29	82.82	83.57	0.551	0.270
90th day							
Breaking strength (kg/cm ²)	5.504	5.739	5.865	5.766	5.867	0.050	0.527
Thickness (mm)	0.368	0.363	0.361	0.360	0.361	0.002	0.921
Yolk color	8.545	8.750	8.779	8.579	8.829	0.099	0.869
HU	91.85	94.26	93.20	92.03	93.33	0.548	0.782

a, b – values within a row with different letters differ significantly at the level of $P < 0.05$; HU – Haugh units; C – control group; L0.1, L0.25, L0.5, L1 – groups with the addition of 0.1%, 0.25%, 0.5% and 1% levan respectively; SEM – standard error of the mean.

The gut microbiota

The L0.25 and L0.5 groups were characterized by a significantly greater ($P < 0.05$) number of *Lactobacillus* spp. bacteria in the JEJ than the C and L1 groups (Figure 1 a). Moreover, the L0.25 group was characterized by a significantly greater ($P < 0.05$) number of *Lactobacillus* spp. bacteria than the L0.1 group (Figure 1 a). No statistically significant differences were found for *Bifidobacterium* spp. in JEJ among the assessed groups (Figure 1 b). However, hens in the L0.1, L0.25 and L0.5 groups were characterized by a significantly lower ($P < 0.05$) number of bacteria from the *Enterobacteriaceae* family in the JEJ than those in the C group (Figure 1 c). The CEC did not significantly differ in terms of the number of *Lactobacillus* spp. or *Enterobacteriaceae* (Figure 1 d and 1 f). However, hens in the L0.1, L0.25 and

L1 groups were characterized by a significantly greater ($P < 0.05$) number of *Bifidobacterium* spp. bacteria than those in the C group (Figure 1 e).

16S rRNA sequencing was performed to characterize the microbiota composition of the contents of JEJ and CEC. In the cecal contents, the total number of OTUs/ASVs was comparable across dietary treatments. The control group contained 233 OTUs/ASVs, while hens fed diets supplemented with levan showed slightly higher richness, with 253 OTUs/ASVs at 0.1%, 265 at 0.25%, 260 at 0.5%, and 263 at 1.0% supplementation. Venn diagram analysis indicated that 110 OTUs/ASVs were shared among all groups, representing a common core microbiota. In addition, each treatment group exhibited unique OTUs/ASVs. The control group contained 28 unique OTUs/ASVs, where-

as 25 unique OTUs/ASVs were observed in the 0.1% levan group. The 0.25%, 0.5%, and 1.0% levan-supplemented groups each harbored 24 unique OTUs/ASVs. Due to the absence of biological replicates per treatment, these observations should be interpreted descriptively (Supplementary Figure 1). The Shannon diversity index showed variation among dietary treatments, with values of 6.35 in the C group, 6.80 at 0.1% levan, 6.96 at 0.25%, 6.69 at 0.5%, and 6.37 at 1.0% supplementation. Alpha diversity appeared to be higher at low to moderate levan concentrations, with the highest value observed at 0.25%, while the highest supplementation level resulted in a Shannon index comparable to the control (Supplementary Table 1). Non-metric multidimensional scaling (NMDS) analysis showed an excellent two-dimensional representation of the data, with a stress value of 0.0004, indicating minimal distortion of the original distance matrix. However, due to the limited number of samples and the lack of biological replicates, the ordination should be interpreted descriptively (Supplementary Figure 2). The 8 bacterial phyla were subjected to phylogenetic analysis. Phylogenetic classification revealed that *Actinobacteriota*, *Firmicutes* and *Bacteroidota* were the predominant phyla in laying hens from all the groups. However, in the C group, the relative abundance of *Firmicutes* (32.5%) was lower than that in the hens in the groups receiving a diet supplemented with levan (44.6–57.1%), and the relative abundance of *Actinobacteriota* was estimated to be 49.3%, whereas in the treated hens, the level of *Actinobacteriota* varied between 6.8 and 14.2% (Figure 2 a). These differences were reflected in the massive reduction in *Coriobacteriia* in samples taken from hens fed a diet supplemented with levan (4.9–12.3%) and the increase in *Clostridia* (24.6–35.1%) compared with those in the C group (42.3% and 13.6%, respectively) (Figure 2 b).

A significant dose-dependent increase in *Bacilli* was also observed (Figure 2 b). The relative abundance of bacteria in each order was also compared, confirming the analysis of phyla and classes. In hens from the C group, the relative abundance of *Coriobacteriales* (23.5%) was dramatically greater than that in hens with levan-supplemented diets (6.6–14.5%) and the abundances of *Clostridia_UCG_014* and *Lactobacillales* increased when the levan was in the diet (6.9–12.7% and 9.8–18.3%, respectively), in comparison to those in the C group (1.8 and 5.3%, respectively) (Figure 2 c). Analysis of the relative abundance of bacteria in the families revealed striking differences in the effect of supplementation with levan in increasing unclassified *Clostridia_UCG_014* (5.7–11.8%), *Bacteroidaceae* (7.9–12.2%) and *Lactobacillaceae* (9.1–17.8%) compared with those in the C group (1.6, 4.7, and 3.1%), respectively, and decreasing *Eggerthellaceae* in the C (23.5%) and treated groups (2.5–7.9%) (Figure 2 d). These results were reflected in the relative abundance of bacteria at the genus level. The relative abundances of unclassified *Muribaculaceae* and *Enterorhabdus* were greater in hens with no levan in the diet (7.8 and 14.2%, respectively) than in the levan-treated groups, with values estimated to be 3.3–5.6% and 1.2–5.1%, respectively. An increasing effect was observed for the relative abundances of *Limosilactobacillus* (4.1–8.7%) and *Bacteroides* (6.1–9.2%) in hens supplemented with levan in comparison with those in the C group (2.7 and 3.9%, respectively) (Figure 2 e). Phylogenetic analysis of the relative abundance of bacterial species clearly revealed a dose-dependent effect of the supplementation of the diet, with 0.1 and 0.25% of levan in the diet in one cluster related to the C group and 0.5 and 1.0% levan in another cluster related to the C group. The taxonomic assignments are presented in Figure 3.

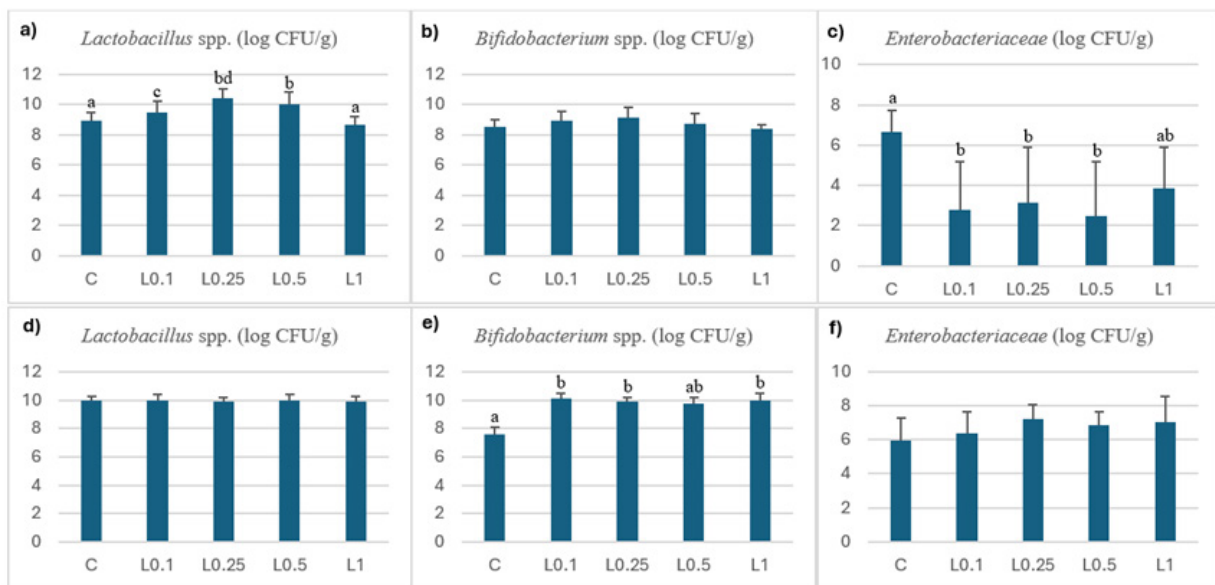


Figure 1. The number of *Lactobacillus* spp., *Bifidobacterium* spp., and *Enterobacteriaceae* family bacteria in the JEJ and CEC (mean $\pm$ SD, N=8); a, b – values above bars with different letters differ significantly at the level of $P < 0.05$; C – control group; L0.1, L0.25, L0.5, L1 – groups with the addition of 0.1%, 0.25%, 0.5% and 1% levan, respectively.

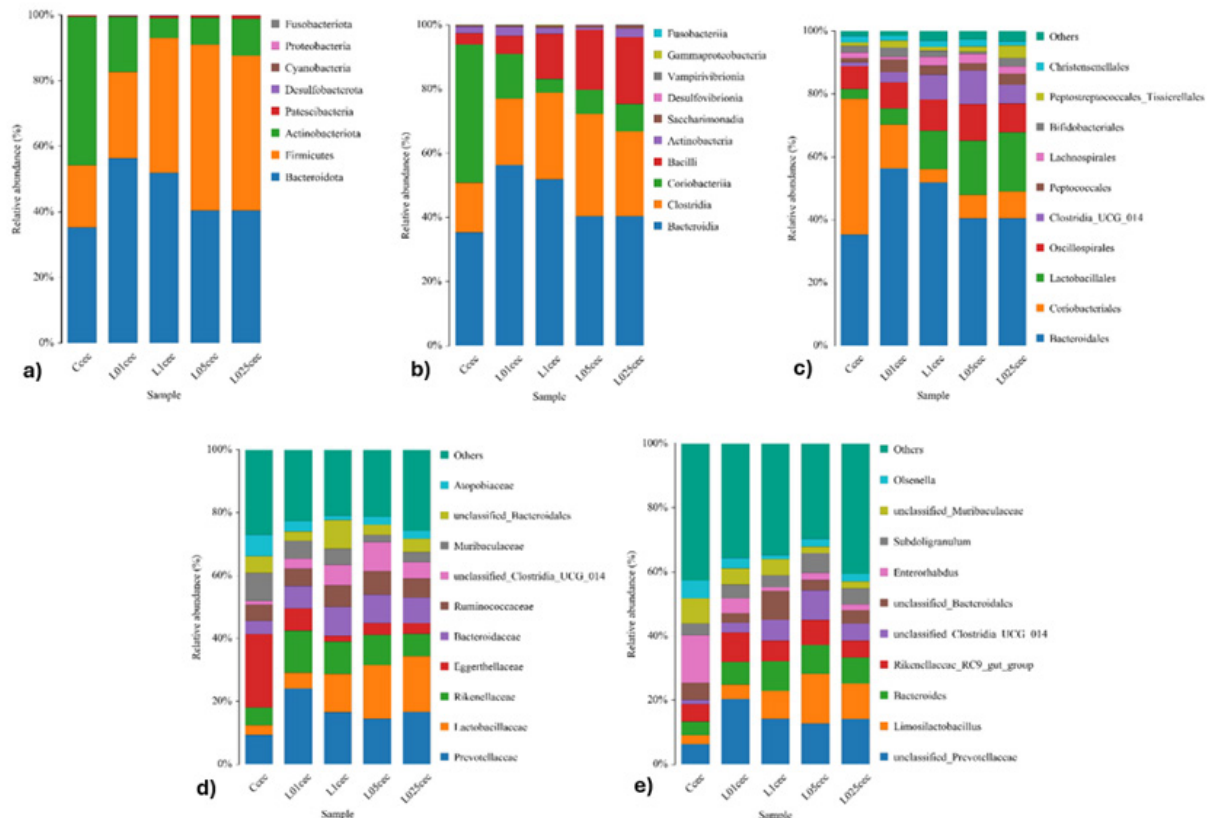


Figure 2. Relative abundance of individual phyla (2 a), classes (2 b), orders (2 c), families (2 d), and genera (2 e) of bacteria in the CEC of laying hens.

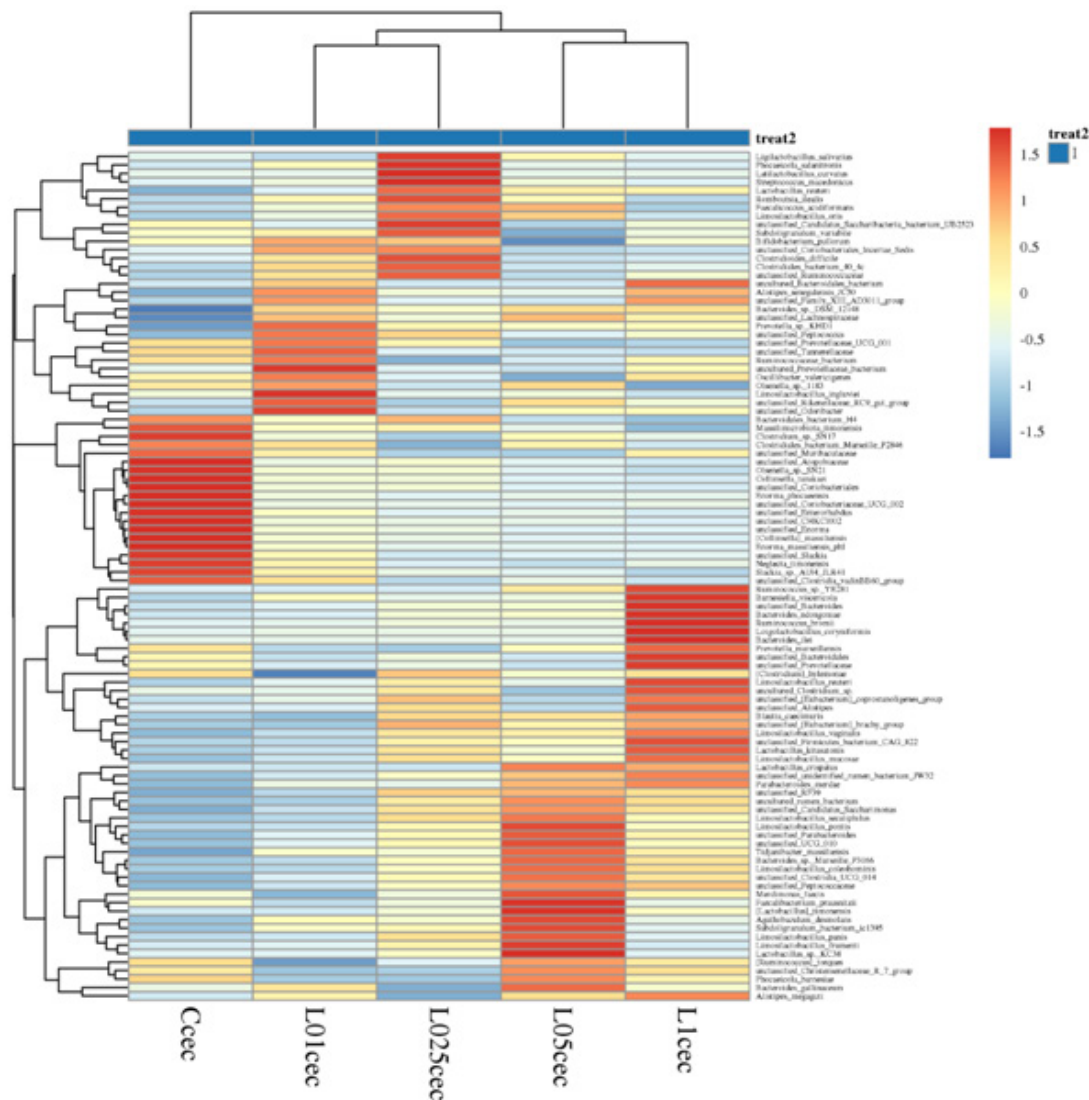


Figure 3. The taxonomic assignments in CEC.

In the samples of intestinal contents taken from the JEJ, showed lower complexity and no differences were observed.

Histomorphometry of individual parts of the small intestine

Compared with those in groups L0.25 and L1, the intestinal villi in groups C, L0.1 and L0.5 were significantly longer ($P < 0.05$) in the DUO (Figure 4). In the case of JEJ, significantly longer ($P < 0.05$) intestinal villi were observed in the C and L0.1 groups than in the L0.25 and L1 groups (Figure 4). There were no differences in the surface area of the intestinal villi in the DUO and IL, but in the JEJ, the intestinal villi from the C and L0.1 groups were characterized by

a significantly larger ($P < 0.05$) surface area than those from the other groups (Figure 5). In the L0.25, L0.5 and L1 groups, significantly deeper ($P < 0.05$) intestinal crypts were found in the DUO than in the other groups (Figure 6). Compared with the L0.25 group the C and L0.1 groups were characterized by significantly deeper ($P < 0.05$) crypts in the JEJ (Figure 6). In the IL, no statistically significant differences were found in terms of intestinal crypt depth (Figure 6). The V/C ratio was significantly lower ($P < 0.05$) in the DUO from the L1 group than in that from the C group (Figure 7). There were no statistically significant differences in the discussed ratios in the JEJ or the IL (Figure 7).

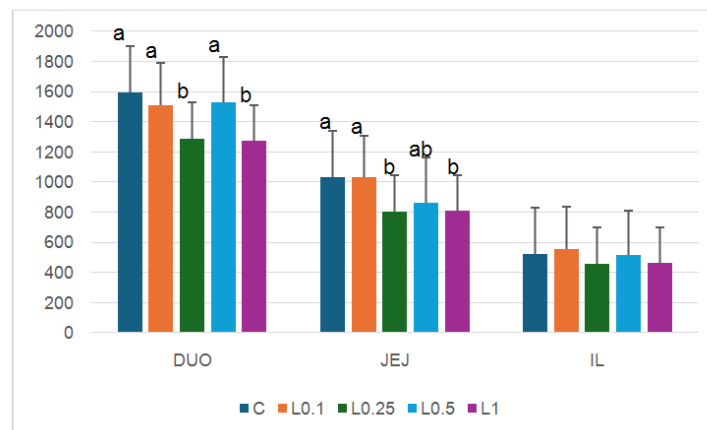


Figure 4. Length of intestinal villi (μm) in DUO, JEJ and IL (mean ± SD, N=8); a, b – values above bars with different letters differ significantly at the level of $P < 0.05$; DUO – duodenum; JEJ – jejunum; IL – ileum; C – control group; L0.1, L0.25, L0.5, L1 – groups with the addition of 0.1%, 0.25%, 0.5% and 1% levan, respectively.

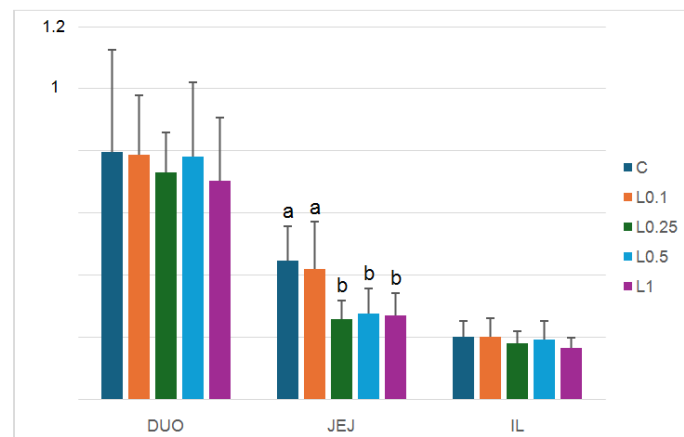


Figure 5. Intestinal villi surface area (mm²) in DUO, JEJ and IL (mean ± SD, N=8); a, b – values above bars with different letters differ significantly at the level of $P < 0.05$; DUO – duodenum; JEJ – jejunum; IL – ileum; C – control group; L0.1, L0.25, L0.5, L1 – groups with the addition of 0.1%, 0.25%, 0.5% and 1% levan, respectively.

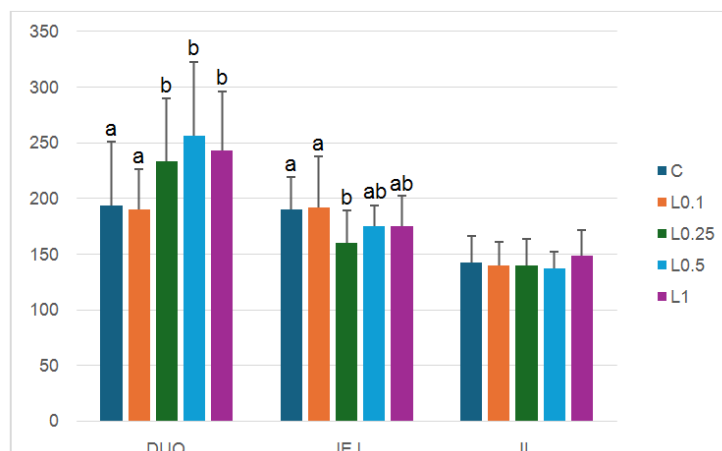


Figure 6. Depth of intestinal crypts (μm) in DUO, JEJ and IL (mean ± SD, N=8); a, b – values above bars with different letters differ significantly at the level of $P < 0.05$; DUO – duodenum; JEJ – jejunum; IL – ileum; C – control group; L0.1, L0.25, L0.5, L1 – groups with the addition of 0.1%, 0.25%, 0.5% and 1% levan, respectively.

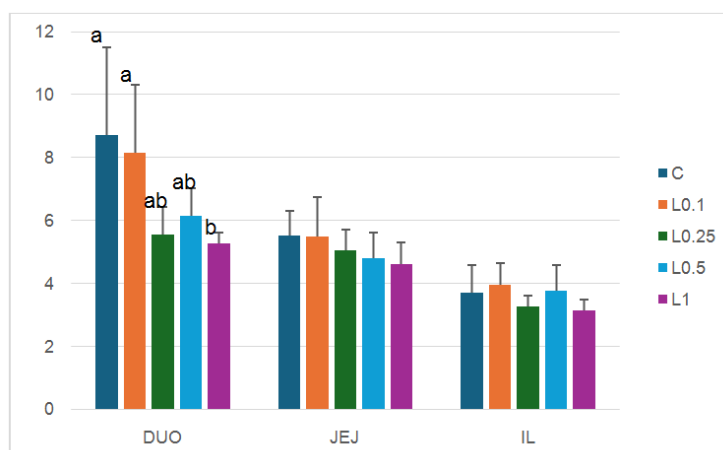


Figure 7. The V/C ratio in DUO, JEJ and IL (mean $\pm$ SD, N=8); a, b – values above bars with different letters differ significantly at the level of $P < 0.05$; DUO – duodenum; JEJ – jejunum; IL – ileum; C – control group; L0.1, L0.25, L0.5, L1 – groups with the addition of 0.1%, 0.25%, 0.5% and 1% levan, respectively.

Morphometry of the THM and specific immune cell composition in the SPL and CT

In the present study the C/M ratio was significantly lower ($P < 0.05$) in the L0.25, L0.5 and L1 groups than in the C group (Figure 8). The lowest value was noted in the L0.25 group.

In the present study, the numbers of B lymphocytes (Bu-1⁺), helper T lymphocytes (CD4⁺), and cytotoxic cells (T lymphocytes and NK cells) (CD8 α ⁺) were estimated in the CT and SPL of laying hens receiving different levels of levan in the feed (Figures 9 and 10). There were no statistically significant differences in the number of Bu-

1⁺ cells in the SPL between the assessed groups. A significantly lower ($P < 0.05$) number of CD8 α ⁺ cells was recorded in the SPL of hens in the L0.25 group than in those in the C group. In the CT, the number of Bu-1⁺, CD4⁺ and CD8⁺ cells did not differ significantly between the experimental groups and the C group (Figure 10). Only differences in the number of Bu-1⁺ and CD4⁺ cells were observed between groups receiving different concentrations of levan.

In the SPL, the CD4⁺/CD8⁺ cell ratio was also calculated (Figure 11). Compared with the C group, the L0.25 group presented a significantly greater ($P < 0.05$) CD4⁺/CD8⁺ cell ratio.

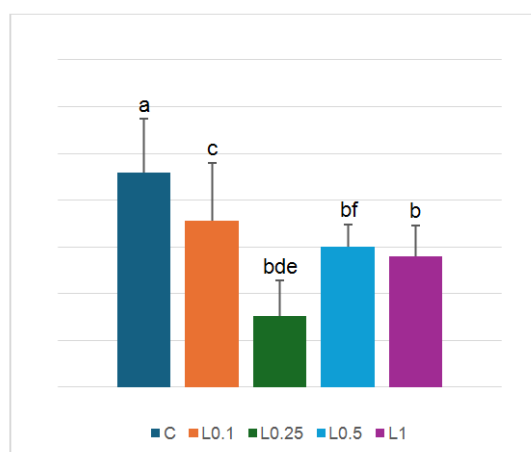


Figure 8. The cortex to medulla thickness ratio in THM (mean $\pm$ SD, N=8); a, b – values above bars with different letters differ significantly at the level of $P < 0.05$; C – control group; L0.1, L0.25, L0.5, L1 – groups with the addition of 0.1%, 0.25%, 0.5% and 1% levan, respectively.

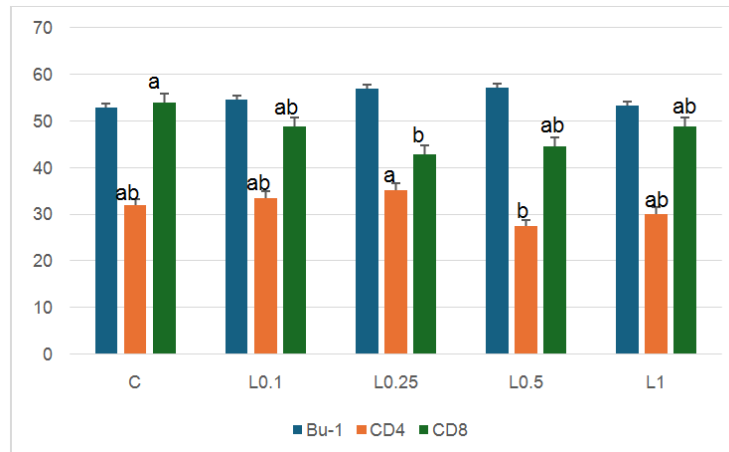


Figure 9. The number of Bu-1⁺, CD4⁺ and CD8⁺ cells in the SPL (mean ± SD, N=8); a, b – values above bars with different letters differ significantly at the level of P<0.05; C – control group; L0.1, L0.25, L0.5, L1 – groups with the addition of 0.1%, 0.25%, 0.5% and 1% levan, respectively.

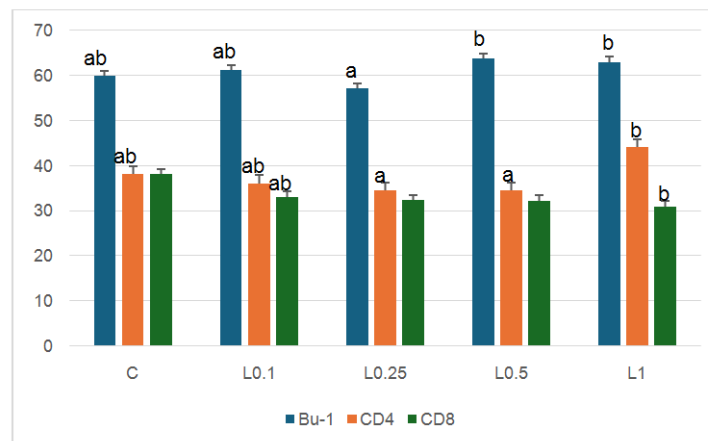


Figure 10. The number of Bu-1⁺, CD4⁺ and CD8⁺ cells in the CT (mean ± SD, N=8); a, b – values above bars with different letters differ significantly at the level of P<0.05; C – control group; L0.1, L0.25, L0.5, L1 – groups with the addition of 0.1%, 0.25%, 0.5% and 1% levan, respectively.

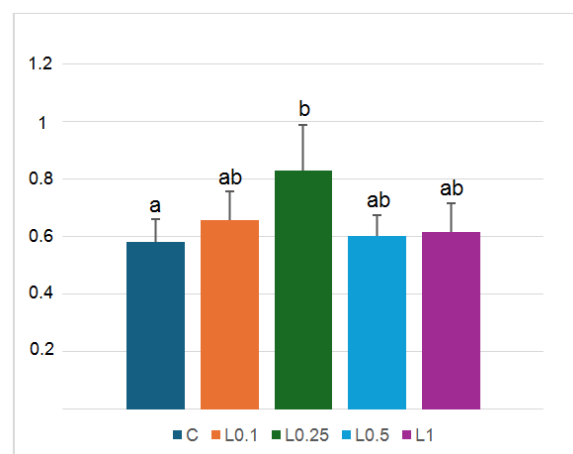


Figure 11. CD4⁺/CD8⁺ cells ratio in the SPL (mean ± SD, N=8); a, b – values above bars with different letters differ significantly at the level of P<0.05; C – control group; L0.1, L0.25, L0.5, L1 – groups with the addition of 0.1%, 0.25%, 0.5% and 1% levan, respectively.

Table 5. Populations of blood lymphocytes and monocytes

Type of cells		C	L0.1	L0.25	L0.5	L1	SEM	P-value
KUL01 ⁺ cells	%	15.46 ab	22.01 a	19.42 ab	20.13 ab	14.55 b	1.422	0.042
	Total count (×10 ⁶ /ml)	5.990	10.11	9.090	8.540	5.540	0.892	0.087
Bu-1a ⁺ cells	%	25.39	29.01	27.20	24.68	27.74	0.787	0.235
	Total count (×10 ⁶ /ml)	9.720	12.44	12.69	10.65	9.600	0.657	0.108
CD3 ⁺ cells	%	60.48 a	47.38 b	46.14 b	47.99 b	54.01 ab	2.688	0.001
	Total count (×10 ⁶ /ml)	23.52	20.63	21.11	20.19	19.58	0.677	0.693
CD4 ⁺ cells	%	40.80 ab	37.23 b	37.48 b	36.27 b	44.48 a	1.514	0.001
	Total count (×10 ⁶ /ml)	15.80	16.48	17.38	15.30	16.08	0.350	0.931
CD8 ⁺ cells	%	16.47 a	11.44 b	10.39 b	12.80 ab	11.95 b	1.040	0.002
	Total count (×10 ⁶ /ml)	6.360	5.020	4.860	5.390	4.260	0.347	0.245
CD3 ⁺ /TCRγδ ⁺ cells	%	14.97	14.28	13.22	12.67	14.60	0.432	0.578
	Total count (×10 ⁶ /ml)	5.830	6.150	6.140	5.460	5.340	0.168	0.900
CD4 ⁺ /CD8 ⁺ ratio		2.630 b	3.320 ab	3.650 ab	2.990 ab	3.930 a	0.230	0.014

a, b – values within a row with different letters differ significantly at the level of $P < 0.05$; C – control group; L0.1, L0.25, L0.5, L1 – groups with the addition of 0.1%, 0.25%, 0.5% and 1% levan, respectively; SEM – standard error of the mean.

Populations of blood lymphocytes and monocytes

Table 5 presents the results related to the populations of lymphocytes and monocytes in the blood of the tested birds.

In the L0.1 group, there was a significantly greater ($P < 0.05$) percentage of monocytes (KUL01⁺ cells) than in the L1 group. None of the concentrations of levan affected the population of Bu-1a⁺ cells, either the percentage or total count. Levan supplementation decreased ($P < 0.05$) the percentage of T cells (CD3⁺ cells) compared with that in the C group. This effect was observed

after the administration of levan at concentrations of 0.1%, 0.25% and 0.5%. A similar downward trend was observed after the addition of 1% levan; however, this change was not statistically significant. Levan, regardless of the concentration used, did not affect the percentage of CD4⁺ cells compared with that in the C group. However, statistically significant ($P < 0.05$) differences were detected between the levan groups. The percentage of CD4⁺ cells was lower in the L0.1, L0.25 and L0.5 groups than in the L1 group. Levan at concentrations of 0.1%, 0.25% and 1% decreased ($P < 0.05$)

the percentage of CD8⁺ cells compared with that in the C group. After 0.5% levan administration, a similar downward trend was observed; however, this difference was not statistically significant. Levan at a concentration of 1% increased ($P<0.05$) the CD4⁺/CD8⁺ ratio compared with that in the C group. The use of levan at other concentrations showed a similar upward trend, but it was not statistically significant. There were no changes in the total count of T cells (CD3⁺, CD4⁺ and CD8⁺ cells) after levan supplementation. Levan, regardless of the concentration used, did not affect either the percentage or total count of the TCR $\gamma\delta$ ⁺ cell subpopulation.

Discussion

The improvement in EP is probably due to the general prebiotic properties of levan causing changes in the gastrointestinal microflora, nutrient absorption and the surface of the intestinal epithelium. Notably, these are the first studies in which levan was used as a feed additive in the diet of laying hens. To date, the fructan that has been used primarily in poultry nutrition is inulin. Chen et al. (2005), who used inulin in the feed of laying hens, reported that inulin significantly increased ($P<0.05$) EP. Similar to our research, the use of this additive had no effect on EW or FI. Other results were obtained by Shang et al. (2010), who reported no significant effect of different inulin concentrations on EP or other production parameters. Świątkiewicz et al. (2010) also reported that inulin has no effect on egg performance parameters. Park and Park (2012) reported that the use of inulin in the nutrition of laying hens significantly increased ($P<0.05$) EP. Additionally, Shang et al. (2020) reported that inulin supplementation, especially at 15 g/kg, can significantly increase ($P<0.05$) EP and EW, and significantly reduce ($P<0.05$) the FCR.

The literature indicates that the use of low doses of fructans increases the absorp-

tion of nutrients, including minerals such as calcium, which should translate into improved eggshell quality (Azorin-Ortuno et al., 2009). The present study seems to confirm this tendency, especially considering that a difference in breaking strength was observed in the L0.1 group. Similar results were obtained by Shang et al. (2010), who reported that inulin had no effect on egg quality parameters. Different results were presented by Park and Park (2012), who reported that the addition of fructan in the form of inulin significantly increased HU, eggshell thickness and breaking strength. They did not observe an effect of the addition of inulin on the color of the yolk, which is natural because fructans are not dyes. Other results were also obtained by Shang et al. (2020), who reported that the addition of inulin can have a positive effect on eggshell thickness. These findings, similar to those of the present study, revealed no effect of the use of fructans on HU.

The results confirmed that levan has prebiotic properties and stimulates the growth of probiotic bacteria, while inhibiting the development of potentially pathogenic microflora. This is because bacteria from the genera *Lactobacillus* spp. and *Bifidobacterium* spp. use fructans much more efficiently in the fermentation process than other groups of bacteria do. The byproducts of this process are short-chain fatty acids (SCFAs) (Chen, 2003) which acidify the gastrointestinal environment and, in turn, limit the opportunities for the development of other microorganisms. Similar results were obtained by Shang et al. (2010), who reported that the addition of 2% inulin significantly increased ($P<0.05$) the number of *Bifidobacterium* spp. in CEC. Park and Park (2012) reported that the addition of inulin significantly increased ($P<0.05$) the number of *Lactobacillus* spp. and *Bifidobacterium* spp. and significantly decreased ($P<0.05$) the number of *Escherichia coli* and *Salmonella* in CEC. Abdelqader et al. (2013) noted that

the addition of inulin significantly increased ($P < 0.05$) the number of *Lactobacillus* spp. and *Bifidobacterium* spp. in CEC. The obtained results are, to some extent, consistent with the results of other authors who also indicated *Firmicutes*, *Actinobacteria* and *Bacteroidetes* as the dominant phyla (Li et al., 2018; Song et al., 2020). Li et al. (2018), however, did not demonstrate such a strong effect of inulin and wheat bran use on the development of the microbiological profile of birds as the present study did. However, it should be taken into account that they euthanized the birds on the 11th day of life; the lack of a visible effect on the composition of the gastrointestinal microflora may therefore be due to the young age of the birds. Xia et al. (2021) also did not find that the inclusion of inulin in the basal diet significantly affects the composition and diversity of the gastrointestinal microbiota. The differences in the results are most likely due to the effects of different durations of prebiotic use, which, in the case of this study, were longer and lasted 90 days.

The changes described in the histomorphometric parameters of the small intestine of laying hens under the influence of levan provide new information on the potential use of fructans as prebiotics. It is generally believed that a greater length of the intestinal villi increases the surface area capable of better absorbing available nutrients (Caspar, 1992). However, the increased length of the intestinal villi means that more energy and nutrients are needed to replace the intestinal mucosa more quickly. The use of additional nutrients to regenerate the mucosa may therefore come at the expense of the energy needed to maintain the animal's performance (Rehman et al., 2007). On the other hand, shortening of the intestinal villi and deepening of the crypts may lead to poor absorption of nutrients and, consequently, poorer performance of the animal (Xu et al., 2003). Deepened crypts indicate rapid tissue turnover, allowing intestinal villi

renewal in response to normal sloughing or inflammation induced by pathogens or their toxins (Yason et al., 1987). An increase in the V/C ratio is related to increased epithelial cell turnover (Chichlowski et al., 2007), and longer intestinal villi are associated with activated cell mitosis (Samanya and Yamauchi, 2002). On the basis of the results obtained in this study, the changes observed in the epithelium of the small intestine are a negative effect of the use of levan because they lead to a reduction in the absorption surface. However, it should be noted that on the surface of the intestinal villi, in addition to enterocytes (absorptive cells), there is also a variable number of goblet cells and enteroendocrine cells, which do not have absorbent properties. Therefore, the absorptive surface of the intestinal villi is largely dependent on the mutual proportions of absorptive, goblet and enteroendocrine cells (Awad et al., 2011). Therefore, this should be the aim of future research on the possibility of using fructans in poultry nutrition. The effects of fructans on the histomorphometric parameters of the small intestine of laying hens have not been widely studied. Research has focused on broiler chickens, whose production cycle is much shorter; therefore, the obtained results cannot be translated to laying hens. Abdelqader et al. (2013) reported that the addition of inulin to the diet of laying hens significantly increases ($P < 0.05$) the length of intestinal villi and the depth of crypts in DUO, JEJ and IL.

In the thymus, intensive proliferation, maturation and selection of thymocytes take place in the cortex, whereas mature $CD4^+$ or $CD8^+$ cells predominate in the medulla (Nagy et al., 2021). A decrease in the C/M ratio usually indicates thinning of the cortex or widening of the medulla, which may not be observed for similar organ weights and lobule diameters (Elmore, 2006). In chickens, the physiological involution of the thymus begins at approximately 2–4 months of age and is dependent on

the strain (Ciriaco et al., 2003; Ayman et al., 2020). In the present study, the thymuses came from layers aged 39 weeks, i.e. with an advanced involution process. In several cases, blurring of the boundary between the cortex and the medulla was observed. The decrease in the C/M ratio resulting from the relative thinning of the cortex in relation to the medulla indicates that the addition of levan may accelerate the process of thymic involution in laying hens.

It can be concluded that levan may cause some immunosuppression, especially in CD8⁺ cells in the SPL and in the CT (Figures 9 and 10). This is contrary to the results obtained by Janardhana et al. (2009), who reported that the use of mannan-oligosaccharides (MOS) and fructo-oligosaccharides (FOS) causes immunosuppression in the CT of broiler chickens. There was a significant decrease ($P<0.05$) in the number of Bu-1⁺ lymphocytes in the CT. These results can be explained by the ability of levan to promote the growth of beneficial microorganisms, such as *Lactobacillus* spp. and *Bifidobacterium* spp., and to inhibit the growth of *Enterobacteriaceae* and *Clostridium* spp. Another possible mediator of immunosuppression may be byproducts of levan fermentation, such as SCFAs, which in turn may bind to receptors expressed on leukocytes and affect their function (Janardhana et al., 2009). The effect of MOS on human lymphocytes has been well documented (Nelson et al., 1984; Muchmore et al., 1990; Nelson et al., 1991). These studies have shown that *in vivo*, mannan can cause suppression directly by blocking T or B-cell receptors or indirectly by inducing the production of prostaglandin E2 by monocytes or antigen-presenting cells, which in turn can attenuate the proliferative response of T cells to mitogens and inhibit the formation of B-cell colonies in the SPL and lymph nodes (Jelinek and Lipsky, 1985; Jelinek et al., 1985). The results of the present study re-

vealed that levan appears to have a similar effect on laying hens. Compared with the C group, the L0.25 group presented a significantly greater CD4⁺/CD8⁺ cell ratio in the SPL. This was due to a decrease in the number of CD8⁺ cells in the L0.25 group. These results are consistent with those obtained by Huang et al. (2015), who noted that the use of inulin in the diet of broiler chickens increases the ratio of CD4⁺ to CD8⁺ lymphocytes.

On the basis of the results related to the population of blood lymphocytes and monocytes, it can also be concluded that the use of levan in the nutrition of laying hens causes some immunosuppression, including T CD8⁺ cells. Kim et al. (2011), reported that the heterophil:lymphocyte ratio and basophil leukocyte count were significantly lower ($P<0.05$) in birds receiving MOS supplementation in the feed. These results are inconsistent with those obtained by Mohebbifar et al. (2013), who noted no effect of the use of prebiotics in bird nutrition on white blood cell counts in laying hens. Other results were obtained by Helal et al. (2015), who reported that the use of prebiotics in the diet of broiler chickens significantly increased ($P<0.05$) the total number of leukocytes and the percentage of lymphocytes. Additionally, Biswas et al. (2019) reported that the inclusion of a prebiotic in the form of MOS in the diet of broiler chickens significantly increased the immune response rates in the blood of tested animals.

Conclusion

The addition of levan to the diet of laying hens had a positive effect on the performance of the birds, especially in the EP context. At low doses, levan had a positive effect on several egg quality parameters, such as breaking strength. The use of levan as a feed additive promoted the development of the gastrointestinal microflora by promoting the growth of *Lactobacillus* spp. and *Bifidobacterium* spp. bacteria and in-

hibiting the growth of *Enterobacteriaceae* bacteria. Exploratory 16S rRNA-based profiling of pooled samples suggested shifts in bacterial community composition. Without additional tests, such as determining the mutual ratio of enterocytes, goblet cells and enteroendocrine cells, it is difficult to clearly determine the effect of levan on the intestinal epithelium. Levan, its fermentation products, as well as changes in the composition of the microflora of the digestive tract of laying hens may have some immunosuppressive effects, as confirmed by examination of lymphatic organs and the population of lymphocytes and monocytes in the blood. Taking into account all the indicators examined, the best solution seems to be the addition of 0.5% levan to the diet of laying hens. However, despite many confirmed positive effects, it is crucial to find solutions that allow for the effective acquisition of levan, which significantly reduces its price. Until this point, the use of levan in a large-scale production has been rather unlikely.

Competing interests

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Authors' contributions

DK: conceptualization and design, methodology, investigation, data interpretation, writing – original draft preparation, funding acquisition. MKo: conceptualization and design, methodology, supervision. JPM: methodology, data interpretation, writing – review and editing. MK-B: methodology, data interpretation, writing – review and editing. MKu: methodology, data interpretation, writing – review and editing. ML: methodology, data interpretation, writing – review and editing. MS: methodology, data interpretation, writing – review and editing. EP: formal analysis, investigation, writing –

review and editing. CC: formal analysis, investigation, data interpretation. SF: formal analysis, investigation. ZL: methodology.

Funding

The research was supported under the Preludium 20 program by the National Science Centre [2021/41/N/NZ9/00696]. The APC is financed by Wrocław University of Environmental and Life Sciences.

Availability of data and materials

All data generated or analyzed during this study are available from the corresponding author upon reasonable request.

References

- Abdelqader A., Al-Fataftah A.R., Das G. (2013). Effects of dietary *Bacillus subtilis* and inulin supplementation on performance, eggshell quality, intestinal morphology and microflora composition of laying hens in the late phase of production. *Anim. Feed Sci. Technol.*, 179: 103–111.
- Ashfaq I., Amjad H., Ahmad W., Nasir A., Ali A., Ali W.R., Khaliq S., Hayat A., Ali H., Sattar F., Ghauri M.A., Anwar M.A. (2020). Growth inhibition of common enteric pathogens in the intestine of broilers by microbially produced dextran and levan exopolysaccharides. *Curr. Microbiol.*, 77: 2128–2136.
- Association of Official Analytical Chemists (2005) Official Methods of Analysis of AOAC International, AOAC International, Gaithersburg, MD.
- Awad W.A., Ghareeb K., Bohm J. (2011). Evaluation of the chicory inulin efficacy on ameliorating the intestinal morphology and modulating the intestinal electrophysiological properties in broiler chickens. *J. Anim. Phys. Anim. Nutr.*, 95: 65–72.
- Ayman U., Alam M.R., Das S.K. (2020). Age-related development and histomorphological observations of bursa of Fabricius in Sonali chicken. *J. Adv. Biotechnol. Exp. Ther.*, 3: 20–28.
- Azarin-Ortuno M., Urban C., Ceron J.J., Tecles F., Allende A., Barberan F.A. (2009). Effect of low inulin doses with different polymer-

- ization degree on lipid metabolism, mineral absorption, and intestinal microbiota in rats with fat-supplemented diets. *Food Chem.*, 113: 1058–1065.
- Biswas A., Mohan N., Raza M., Mir N.A., Mandal A. (2019). Production performance, immune response and blood biochemical parameters in broiler chickens fed diet incorporated with prebiotics. *J. Anim. Physiol. Anim. Nutr.*, 103: 493–500.
- Bokulich N.A., Subramanian S., Faith J.J., Gavers D., Gordon J.I., Knight R., Mills D.A., Caporaso J.G. (2013). Quality-filtering vastly improves diversity estimates from Illumina amplicon sequencing. *Nat. Methods.*, 10: 57–59.
- Bolger A.M., Lohse M., Usadel B. (2014). Trimmomatic: a flexible trimmer for Illumina sequence data. *Bioinformatics.*, 30: 2114–2120.
- Boylen E., Rideout J.R., Dillon M.R., Bokulich N.A., Abnet C.C., Al-Ghalith G.A., Alexander H., Alm E.J., Arumugam M., Asnicar F., Bai Y., Bisanz J.E., Bittinger K., Brejnrod A., Brislawn C.J., Brown C.T., Callahan B.J., Carballo-Rodriguez A.M., Chase J., Cope E.K., Da Silva R., Diener C., Dorrestein P.C., Douglas G.M., Durall D.M., Duvallet C., Edwardson C.F., Ernst M., Estaki M., Fouquier J., Gauglitz J.M., Gibbons S.M., Gibson D.L., Gonzalez A., Gorlick K., Guo J., Hillmann B., Holmes S., Holste H., Huttenhower C., Huttley G.A., Janssen S., Jarmusch A.K., Jiang L., Kaehler B.D., Kang K.B., Keefe C.R., Keim P., Kelley S.T., Knights D., Koester I., Kosciolk T., Kreps J., Langille M.G.I., Lee J., Ley R., Liu Y.X., Lofffield E., Lozupone C., Maher M., Marotz M., Martin B.D., McDonald D., McIver L.J., Melnik A.V., Metcalf J.L., Morgan S.C., Morton J.T., Naimey A.T., Navas-Molina J.A., Nothias L.F., Orchanian S.B., Pearson T., Peoples S.L., Petras D., Preuss M.L., Priesse E., Rasmussen L.B., Rivers A., Robeson M.S., Rosenthal P., Segata N., Shaffer M., Shiffer A., Sinha R., Song S.J., Spear J.R., Swafford A.D., Thompson L.R., Torres P.J., Trinh P., Tripathi A., Turnbaugh P.J., Ul-Hasan S., van der Hooft J.J.J., Vargas F., Vázquez-Baeza Y., Vogtmann E., von Hippel M., Walters W., Wan Y., Wang M., Warren J., Weber K.C., Williamson C.H.D., Willis A.D., Xu Z.Z., Zaneveld J.R., Zhang Y., Zhu Q., Knight R., Caporaso J.G. (2019). Reproducible, interactive, scalable and extensible microbiome data science using QIIME 2. *Nat. Biotechnol.*, 37: 852–857.
- Calazans G.M.T., Lopez C.E., Lima R.M.O.C., de França F.P. (1997). Antitumour activities of levan produced by *Zymomonas mobilis* strains. *Biotechnol. Lett.*, 19: 19–21.
- Caspary W.F. (1992). Physiology and pathophysiology of intestinal absorption. *Am. J. Clin. Nutr.*, 55: 299–308.
- Chen T.C. (2003). Effect of adding chicory fructans in feed on fecal and intestinal microflora and excreta volatile ammonia. *Int. J. Poult. Sci.*, 2(3): 188–194.
- Chen Y.C., Nakthong C., Chen T.C. (2005). Improvement of laying hens performance by dietary prebiotic chicory oligofructose and inulin. *Int. J. Poult. Sci.*, 4: 103–108.
- Chichlowski M., Croom W.J., Edens F.W., Macbride B.W., Qiu R., Chiang C.C., Daniel L.R., Havenstein G.B., Koci M.D. (2007). Microarchitecture and spatial relationship between bacteria and ileal, cecal and colonic epithelium in chicks fed a direct-fed microbial, PrimaLac, and salinomycin. *Poult. Sci.*, 86: 1121–1132.
- Ciriaco E., Pinera P.P., Diaz-Esnal B., Laura R. (2003). Age-related changes in the avian primary lymphoid organs (thymus and bursa of Fabricius). *Microsc. Res. Tech.*, 62: 482–487.
- Edgar R.C. (2013). UPARSE: highly accurate OTU sequences from microbial amplicon reads. *Nat. Methods*, 10: 996–998.
- Edgar R.C., Haas B.J., Clemente J.C., Quince C., Knight R. (2011). UCHIME improves sensitivity and speed of chimera detection. *Bioinformatics.*, 27: 2194–2200.
- Elmore S.A. (2006). Enhanced histopathology of the thymus. *Toxicol. Pathol.*, 34: 656–665.
- European Union (2010). Directive 2010/63/EU of the European Parliament and of the Council of 22 September 2010 on the Protection of Animals Used for Scientific Purposes. Accessed June 2024. <https://eur-lex.europa.eu/legal-content/EN/TXT/HTML/?uri=CELEX:32010L0063>.
- Franco-Robles E., López M.G. (2015). Implication of fructans in health: immunomodulatory and antioxidant mechanisms. *Sci. World J.*, 1: 289267.
- Helal M.S., Youssef F.M., Moursi M.K., Khalil

- W.F., Abdel-Daim M.M. (2015). Effectiveness of prebiotic as an alternative to the antimicrobial growth promoter on growth performance, blood constituents, intestinal healthiness and immunity of broilers. *Alex. J. Vet. Sci.*, 45: 13–25.
- Huang Q., Wei Y., Lv Y., Wang Y., Hu T. (2015). Effect of dietary inulin supplements on growth performance and intestinal immunological parameters of broiler chickens. *Livest. Sci.*, 180: 172–176.
- Janardhana V., Broadway M.M., Bruce M.P., Lowenthal J.W., Geier M.S., Hughes R.J., Bean A.D.G. (2009). Prebiotics modulate immune responses in the gut-associated lymphoid tissue of chickens. *J. Nutr.*, 139: 1404–1409.
- Jelinek D.F., Lipsky P.E. (1985). The roles of T cell factors in activation, cell cycle progression, and differentiation of human B cells. *J. Immunol.*, 134: 1690–1701.
- Jelinek D.F., Thompson P.A., Lipsky P.E. (1985). Regulation of human B cell activation by prostaglandin E2. Suppression of the generation of immunoglobulin-secreting cells. *J. Clin. Invest.*, 75: 1339–1349.
- Kim G-B., Seo Y.M., Kim C.H., Paik I.K. (2011). Effect of dietary prebiotic supplementation on the performance, intestinal microflora, and immune response of broilers. *Poult. Sci.*, 90: 75–82.
- Li B., Lebloy J., Taminiau B., Schroyen M., Beckers Y., Bindelle J., Everaet N. (2018). The effect of inulin and wheat bran on intestinal health and microbiota in the early life of broiler chickens. *Poult. Sci.*, 97: 3156–3165.
- Lohmann (2020). Lohmann Brown Classic Layers Management Guide Cage Housing. Accessed June 2024. <https://lohmann-breeders.com/media/strains/cage/management/LOHMANN-Brown-Classic-Cage.pdf>
- Ma Y., Wu X., Giovanni V., Meng X. (2017). Effects of soybean oligosaccharides on intestinal microbial communities and immune modulation in mice. *Saudi J. Biol. Sci.*, 24: 114–121.
- Madej J.P., Bednarczyk M. (2016). Effect of *in ovo*-delivered probiotics and synbiotics on the morphology and specific immune cell composition in the gut-associated lymphoid tissue. *Poult. Sci.*, 95: 19–29.
- Magoč T., Salzberg S.L. (2011). FLASH: fast length adjustment of short reads to improve genome assemblies. *Bioinformatics*, 27: 2957–2963.
- Martin M. (2011). Cutadapt removes adapter sequences from high-throughput sequencing reads. *EMBnet J.*, 17: 10–12.
- Mohebbifar A., Kashani S., Afsari M., Torki M. (2013). Effects of commercial prebiotic and probiotics of diet on performance of laying hens, egg traits and some blood parameters. *Annu. Res. Rev. Biol.*, 3: 921–934.
- Muchmore A.V., Sathyamoorthy N., Decker J., Sherblom A.P. (1990). Evidence that specific high-mannose oligosaccharides can directly inhibit antigen-driven T-cell responses. *J. Leukoc. Biol.*, 48: 457–464.
- Nagy N., Olah I., Vervelde L. (2021). Structure of the avian lymphoid system. In: *Avian Immunology*, Kaspers B., Schat K.A., Gobel T.W., Vervelde L. (eds). London, UK, pp. 11–44.
- Nelson R.D., Herron M.J., McCormack R.T., Gehrz R.C. (1984). Two mechanisms of inhibition of human lymphocyte proliferation by soluble yeast mannan polysaccharide. *Infect. Immun.*, 43: 1041–1046.
- Nelson R.D., Shibata N., Podzorski R.P., Heron M.J. (1991). *Candida* mannan: chemistry, suppression of cell-mediated immunity, and possible mechanisms of action. *Clin. Microbiol. Rev.*, 4: 1–19.
- Ohta A., Osakabe N., Yamada K., Saito Y., Hidaka H.J. (1993). Effect of fructooligosaccharides and other saccharides on Ca, Mg, and P absorption in rats. *J. Jpn. Soc. Nutr. Food Sci.*, 46: 123–129.
- Park S.O., Park B.S. (2012). Effect of feeding inulin oligosaccharides on cecum bacteria, egg quality and egg production in laying hens. *Afr. J. Biotechnol.*, 11: 9516–9521.
- Rehman H., Rosenkranz C., Böhm J., Zentek J. (2007). Dietary inulin affects the morphology but not the sodium-dependent glucose and glutamine transport in the jejunum of broilers. *Poult. Sci.*, 86: 118–122.
- Roberfroid M.B., Gibson G.R., Delzenne N. (1993). The biochemistry of oligofructose, a nondigestible fiber: An approach to calculate its caloric value. *Nutr. Rev.*, 51: 137–146.
- Samanya M., Yamauchi K. (2002). Histological alterations of intestinal villi in chickens fed dried *Bacillus subtilis* var. *natto*. *Comp. Biochem. Physiol.*, 133: 95–104.

- Shang H.M., Hu T.M., Lu Y.J., Wu H.X. (2010). Effects of inulin on performance, egg quality, gut microflora and serum and yolk cholesterol in laying hens. *Br. Poult. Sci.*, 51: 791–796.
- Shang H.M., Zhao J., Dong X., Guo Y., Zhang H., Cheng J., Zhou H. (2020). Inulin improves the egg production performance and affects the cecum microbiota of laying hens. *Int. J. Biol. Macromol.*, 155: 1599–1609.
- Song J., Li Q., Everaert N., Liu R., Zheng M., Zhao G., Wen J. (2020). Dietary inulin supplementation modulates short-chain fatty acid levels and cecum microbiota composition and function in chickens infected with *Salmonella*. *Front. Microbiol.*, 11: 584380.
- Szczyпка M., Lis M., Kuczkowski M., Bobrek K., Pawlak A., Zambrowicz A., Gawel A., Obmińska-Mrukowicz B. (2023). Yolkin, a polypeptide complex from egg yolk, affects cytokine levels and leukocyte populations in broiler chicken blood and lymphoid organs after *in ovo* administration. *Int. J. Mol. Sci.*, 24: 17494.
- Świątkiewicz S., Koreleski J., Arczewska A. (2010). Laying performance and eggshell quality in laying hens fed diets supplemented with prebiotics and organic acids. *Czech J. Anim. Sci.*, 55: 294–306.
- Versluys M., Kirtel O., Toksoy Öner E., Van den Ende W. (2018). The fructan syndrome: evolutionary aspects and common themes among plants and microbes. *Plant Cell Environ.*, 41: 16–38.
- Vijn I., Smeekens S. (1999). Fructan: more than a reserve carbohydrate? *Plant Physiol.*, 120: 351–360.
- Xia Y., Miao J., Zhang Y., Zhang H., Kong L., Seviour R., Kong Y. (2021). Dietary inulin supplementation modulates the composition and activities of carbohydrate-metabolizing organisms in the cecal microbiota of broiler chickens. *PLoS One.*, 16(10):e0258663.
- Xu Z.R., Hu C.H., Xia M.S., Zhan X.A., Wang M.Q. (2003). Effects of dietary fructooligosaccharide on digestive enzyme activities, intestinal microflora and morphology of male broilers. *Poult. Sci.*, 82: 1083–1036.
- Yamamoto S., Bouvet P.J., Harayama S. (1999). Phylogenetic structures of the genus *Acinetobacter* based on *gyrB* sequences: Comparison with the grouping by DNA-DNA hybridization. *Int. J. Syst. Bacteriol.*, 49: 87–95.
- Yason C.V., Summers B.A., Schat K.A. (1987). Pathogenesis of rotavirus infection in various age groups of chickens and turkeys. *Am. J. Vet. Res.*, 6: 927–938.
- You S., Ma Y., Yan B., Pei W., Wu Q., Ding C., Huang C. (2022). The promotion mechanism of prebiotics for probiotics: A review. *Front. Nutr.*, 9: 1000517.
- Zhao P.Y., Wang J.P., Kim I.H. (2013). Effect of dietary levan fructan supplementation on growth performance, meat quality, relative organ weight, cecal microflora, and excreta noxious gas emission in broilers. *J. Anim. Sci.*, 91: 5287–5293.

Received: 27 V 2025

Accepted: 30 I 2026