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Impact of multistrain probiotics on growth performance, immune response, and gut morphometry in broiler chicken *Gallus gallus domesticus*

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ABSTRACT

The objective of this investigation was to examine the impact of four lab-isolated probiotics Enterococcus faecium (OR563785.1), Weissella confusa (OR563786.1), Weissella cibaria (OQ543569.1), Lactiplantibacillus plantarum (OQ689085.1) in 1:1:1:1 of CFU dilution as multistrain probiotics (MSP) regarding growth performance, haemato-biochemical indices and immune function in broilers. Ninety uniformly weighed broilers were divided into five groups at random with (n = 18/group). NC: negative control (basal diet); PC: commercial probiotic, G1: MSP supplemented, G2: MSP + vaccinated, G3: (vaccinated). Blood samples were collected at 42 days of age to assess immunological, haemato-biochemical parameters, and intestinal morphometry. Compared to the group of negative control, the broiler chicks' body weight was considerably ($p < 0.05$) higher in MSP-treated groups (G1, G2). This study found that, as compared to the NC, there was a substantial rise ($p < 0.05$) in RBC and hemoglobin in the probiotic-supplemented bird group. The results indicated that cholesterol and triglyceride remarkably decreased compared to control in probiotic-treated groups. There was no discernible change in the enzyme activity of ALT, AST, and ALP across the groups ($p > 0.05$). The findings indicated higher levels of immunoglobulin and interleukins in the MSP group than in the control (NC). The villus's height to crypt depth ratio was higher in the MSP groups (G1, G2) in contrast with the PC group ($p < 0.05$). The haemagglutination inhibition test (HI) revealed that the probiotic-treated groups had greater New Castle disease virus (NDV) antibodies than the other groups. The humoral response to live NDV vaccinations may be enhanced by multistrain probiotics. These results revealed MSP significantly affected growth performance, haemato-biochemical parameters, and immunity through alteration in intestinal morphology which helps in nutrient uptake.

Introduction

The consumption of chicken meat is anticipated to reach 145 million tons worldwide by 2029. OECD/FAO, (2020) Antibiotic usage has increased as a result of the expansion of meat chicken production to enhance growth performance and stop the spread of illness. Long-term subtherapeutic antibiotic usage has led to bacterial resistance and residue buildup in meat products, which harms both human and animal health Soumeh et al., (2021). Probiotics have been proposed as an antibiotic substitute in light of the strong limitations or outright prohibition on the use of antibiotics to boost chicken development Wang et al., (2020).

Probiotics are now recognized as potential dietary supplements that can strengthen the microbiota, immune system, and gut health of chickens through several different action mechanisms, including competitive exclusion of pathogens, enhancement of gut barrier function, modulation of the immune response, and production of beneficial metabolites such as short-chain fatty acids Yousaf et al., (2022).

A probiotic having one strain of a certain species is known as a monostrain. Strains of various probiotic species that are preferred members of one or more genera are referred to as multispecies probiotics. *Lactobacillus, Streptococcus, Enterococcus, Lactococcus,* and *Bifidobacteria, Bacillus,* as well as *Candida pintolopesii, Aspergillus oryzae*, and *Saccharomyces* continue to be the most often employed probiotic agents in livestock Lambo et al., (2021). Multi-strain probiotics involve the use of multiple bacterial strains in a single formulation, typically combining strains from different species or genera to achieve a synergistic effect. These probiotics are designed to enhance the overall efficacy by

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leveraging the unique properties and benefits of each strain. The rationale behind multi-strain formulations is a broader spectrum of health benefits compared to single-strain probiotics Kilvington et al., (2011).

The overall effectiveness of the given type of feed additive depends on the birds' age and health, the diet that is used, as well as environmental factors (Sandvang et al., 2021; Zou et al., 2022). The multistrain probiotics increase the effectiveness of nutrient consumption and absorption and improve body weight gain (Korada et al., 2018; Kwoji et al., 2021; McFarland, 2021; Dias et al., 2022). There were significant differences in broiler's body weights when they fed on multistrain probiotics as compared to single-strain probiotics Fathima et al., (2022).

Both the adaptive and innate immune system of chickens is significantly impacted by probiotics which is achieved by modulating the GALT (gut-associated lymphoid tissue), which is essential for immune monitoring and pathogen response. Probiotics assist in preserving the intestinal barrier's integrity by halting the spread of dangerous bacteria and toxins and promoting the generation of immunoglobulins and other immune components (Kasarello and Sajdel-Sulkowska, 2019; Biswas et al., 2022).

Probiotics also modulate interleukin cytokinesis, influencing the production and activity of cytokines, which are key signaling molecules in the immune system. Cytokines regulate the intensity and duration of immune responses, playing a crucial role in inflammation and immune regulation. Probiotics can shift the cytokine balance towards an anti-inflammatory profile, which is beneficial for maintaining gut health and preventing chronic inflammation Caro et al., (2005).

The broiler chickens' intestinal morphology is one of the vital measures of the birds' health and nutrient absorption and digesting efficiency Cassani et al., (2020). According to recent studies, probiotics visualize a favorable impact concerning intestinal morphology by raising the height of the small intestine's villi and simultaneously deepening crypts, which are beneficial for better absorption of nutrients, intestinal tract health, and overall outlook of intestinal tissues (Forte et al., 2018; Rodrigues et al., 2019). The improved growth performance was a result of improved gut health due to relatively higher gut bacteria diversity and improved healthy villi in the small intestine portion Markowiak and Śliżewska, (2018). The research by Hossain, (2019) demonstrated that broilers given multistrain probiotics impacted higher villus height and depth of the crypt ratio in comparison to birds given single-strain probiotics.

Furthermore, it has been noted that the immune response to vaccination is significantly influenced by the GIT microbiota (Jamieson, 2015; Zimmermann and Curtis, 2018). Protexin® is a multi-strain probiotic preparation that has been shown to increase development and the immune system's response to the Newcastle disease virus and also reduce the proinflammatory cytokine levels in birds (Han et al., 2018; Elsayed et al., 2021). Some commercially available multistrain probiotics are available today e.g., PoultryStar® (a combination of *B. animalis, L. reuteri, P. acidilactici, E. faecium, L. salivarius*) and Floramax® B11 (*P. parvulus* and *L. salivarius*). The present study's objective was to evaluate the growth performance of broiler chickens and their general state of health as the impact of multistrain probiotics demonstrated by immunological, biochemical, intestinal morphology, and haematological variables.

Material and methods

In the current investigation, four strains of laboratory-isolated probiotics were used. These probiotics were screened using the catalase test, NaCl tolerance test, bile tolerance test, phenol tolerance test, and antimicrobial activity assessment. Finally, the isolates were subjected to 16S rRNA gene sequencing for species-level confirmation.

These strains included *Enterococcus faecium* (Pro 1-GCU-DAB-S-41) (OR563785.1), isolated from gizzard, *Weisella confusa* (Pro2-GCU-DAB-S-6) (OR563786.1), isolated from garlic, *Weisella cibaria* (Pro 3-GCU-DAB-S-24) (OQ543569.1), isolated from cucumber and *Lactiplantibacillus plantarum* (Pro 4-GCU-DAB-S-43) (OQ689085.1), isolated from honey. Commercial probiotic *Lactobacillus acidophilus* was bought (Fazal Din Pharmacy Lahore). A 1:1 ratio of CFU dilutions was used to unite all strains. Each strain was maintained at−80°C in De Man, Regosa, and Sharpe (MRS) media in stock cultures with 30% (v/v) glycerol added (Prabhurajeshwar and Chandrakanth, 2019). All the strains were refreshed in MRS agar plates.

Experimental birds and housing

A total of ninety one-day-old chicks were bought from a local hatchery near Riawind Lahore. The experiment was conducted at Government College University, Lahore, Pakistan. Before the arrival of the birds, the facilities were meticulously cleaned and prepped. The five groups of chicks were randomly assigned, with three replicates with six chicks in each for 6 weeks. The birds in the various groups initially weighed between 46.0 and 46.5 g on average. The experiment was conducted maintaining the room's temperature at 33±1°C during the first three days and then dropped to 27±1°C until the experiment was over. The chicken house was cleaned every morning and evening and the ventilation system was kept in place. There were two phases to the feeding period: the feeding phase (0–3 weeks) and the finisher phase (4-6 weeks) (Wang et al., 2018).

Five dietary groups were G1 (basal diet + Pro1 + Pro2 + Pro3 + Pro4), G2 (basal diet + Pro1 + Pro2 +Pro3 + Pro4 + vaccinated), G3 (basal diet + vaccinated), G4 (positive control-*Lactobacillus acidophilus*), G5 (negative control-basal diet). Throughout the testing periods, the broilers have unrestricted food and water availability. Throughout the experiment, mortality was noted. As per the standard immunization schedule, G2 and G3 received vaccinations with ND+IB, Gumboro, and ND Lasota (Fig. 1).

Performance

Throughout the trial, the body weight of chicks was noted to calculate the weekly and total BWG Biswas et al., (2020).

Slaughtering and organ weighting

After eight hours of fasting, one bird was randomly chosen and weighed for each replicate on the final day of the trial and was slaughtered via the jugular vein. Internal organs were removed after dissection and weighed separately (bursa of Fabricius, liver, thymus, spleen, and intestine). The following equation was applied to determine the relative weights of each organ to the total body weight. (Organ weight/ live body weight) × 100 = Relative weight (Hidayat et al., 2020).

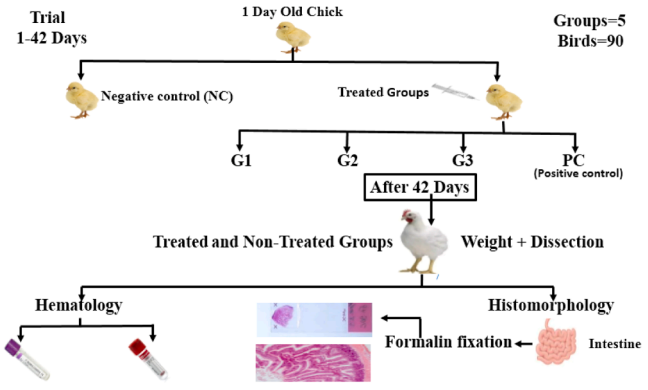

Fig. 1. Experimental layout: The trial consists of five groups (G1, G2, G3, PC, NC), each with 18 birds (3 replicates/ group). The histomorphology and hematology of the samples were done after 42 days.

Haemato-biochemical parameters

Before slaughtering, blood samples from each bird ($n = 3$/group) were drawn aseptically from wing veins were collected on day 42 of the study. The samples were placed into anticoagulant tubes for hematological analysis and plane tubes for biochemical tests. The blood samples were used to measure the physiological response specifically the number of red blood cells (RBCs), total leucocyte count (TLC), differential leucocyte (monocytes and eosinophils), Mean corpuscular haemoglobin (MCH), Mean corpuscular haemoglobin concentration (MCHC), packed cell volume (PCV), Mean corpuscular haemoglobin (MCH), Mean Corpuscular Volume (MCV) and platelets. After centrifugation, the serum of blood samples was stored at −20°C until biochemical analysis. Globulin, total protein, albumin, triglycerides, glucose, cholesterol, aspartate aminotransferase (AST), alkaline phosphatase (ALP), alanine aminotransferase (ALT), amounts in serum were measured by using commercially available kits (Liquick, Germany).

Serum immunoglobulins and interleukins

The chicken blood was collected from a wing, and centrifuged, and serum was kept at −20°C. Using commercial ELISA kits (Cusabio, China), the levels of immunoglobulins (IgM, IgA, IgG,) Han et al., (2018) and interleukins (IL-2, IL-4, and IL-10) Wu et al., (2019), were measured.

Intestinal morphometry

The duodenum's medial region was taken from each replicate (3 birds/group). The samples were rinsed in distilled water and preserved in a neutral buffered formalin (10%) solution. The samples underwent a series of dehydration steps with increasing alcohol concentrations, followed by xylene cleaning, paraffin embedding, hematoxylin, and eosin staining for histological examination. At random, five villi were chosen for every slide which were inspected using an Olympus CX40 light microscope with a magnification of 10X attached to a digital camera. The crypt depth, villus width, and villus height were measured using the software Image J, and the villus height to crypt depth ratio was computed.

Hummoral immune response

Although hens' level of immunity and NDV-specific antibody levels are positively correlated, protective response is often evaluated using serological testing. To find NDV-specific antibody titers, haemagglutination inhibition (HI) was employed Dall'Ara et al., (2021), using 1% of chicken red blood cells and four HA units of NDV antigen in a U-shaped HA plate OIE, (2022). About 6–10 days after vaccination antibodies against NDV can be found. Since the average death period following NDV infection is 2 to 6 days. After vaccination, in the first, second, and third weeks, the blood samples were taken in heparinized tubes.

Statistical analysis

Utilizing the statistical program IBM SPSS Statistics 20, the experimental findings were put through a one-way ANOVA. Utilizing a significance level of $p < 0.05$, the Tukey post-hoc analysis was performed to examine any noteworthy mean variations across the groups. The findings are displayed as means ± SEM. By the use of the GraphPad Prism 5, the figures were created.

Ethical statement

This study's procedures were all authorized by Government College University's Ethics Committee in Lahore, Pakistan, and adhered to the standards set out by the Animal Care and Use Committee (Permission No: GCU-IIB-116).

Results

Growth Performance

The average weekly body weight is displayed in Table 1 for each group. There was no difference between the groups up to three weeks but at the end of the trial significant increase ($p < 0.05$) in the weight of multistrain probiotic-treated groups (G1) followed by PC was seen (Fig. 2). Body weight gain (BWG) of G1 supplemented by multistrain probiotics was substantially ($p < 0.05$) higher than PC and NC (Fig. 3).

Organ relative weight

A considerable ($p < 0.05$) change in the immune system's linked organs' weight (bursa of Fabricius, thymus, and spleen) was shown in the MSP group. Groups G1 and G2 showed higher values of spleen relative weight, whereas in the case of bursa, G3 showed greater weight compared to negative control. Higher thymus relative weight was shown by groups G2 and G3. The G1 group was noted for the high value of the liver in contrast to NC (Fig. 4).

Haematobiochemical indices

Table 2 shows the impact of multistrain probiotics (MSP) on the haematological indices of broiler chickens. This study found a notable rise ($p < 0.05$) in red blood cells (RBC) and haemoglobin in the probiotic-supplemented bird group, as well as a slight increase in PCV, but the difference was not statistically significant ($p > 0.05$).

The G2 and PC groups had significantly higher hemoglobin levels than the NC. Concerning the impact of multistrain probiotic supplementation on chicken haematological parameters, it was observed that there was not a noticeable change ($p > 0.05$) in mean corpuscular haemoglobin (MCH), mean corpuscular volume (MCV), mean corpuscular haemoglobin concentration (MCHC), and monocytes and heterophils. Only the PC group demonstrated a noteworthy ($p < 0.05$) increase in total platelet count than NC (Table 2).

Fig. 5 displays the serum biochemical parameters. Even though, as compared to NC, the probiotic-supplemented groups demonstrated a decrease in total cholesterol that was statistically significant. The HDL levels of all study probiotic-treated groups increased numerically, with the G1 group's birds displaying the highest value (194.33 mg/dl) (Fig. 5b). No discernible change in the enzyme activity of ALP, AST, and ALT across the groups ($p > 0.05$) was seen (Fig. 5a). This study also found that birds fed commercial probiotics had decreased levels of triglycerides ($p < 0.05$) when compared to the NC (Fig. 5c). A substantial change ($p < 0.05$) was seen in the birds' blood glucose levels from the G1 and PC groups.

Serum cytokines and immunoglobulins

The G2 group's birds had greater IgG, IgM, and IgA levels at day 42 ($p < 0.05$) than the NC. Statistically high levels of IL-4 and IL-10 were found in the G2 and G1 groups of birds, respectively. On the other hand, the G1 group of birds was noted for a high level ($p < 0.05$) of interleukin IL-2 (Table 3).

Intestinal morphometry

The impact of multistrain probiotics (MSP) on broiler intestine morphology is displayed in Table 4. In the duodenum, the G1 group's villus heights were greater than PC's ($p < 0.05$). In contrast to PC, the G1 group had a smaller crypt depth in their duodenum. The groups that received multistrain probiotic treatment (G1, G2) had a high value ($p < 0.05$) of villus height to crypt depth ratio in contrast to the PC (positive

Table 1
Impact of multiple strain probiotics on broiler chicken weight.

Days	G 1	G 2	G 3	PC	NC	P value
Initial	$47.30^{a}\pm0.35$	$47.83^{a}\pm0.44$	$47.00^{a}\pm0.57$	$46.33^{a}\pm1.85$	$46.66^{a}\pm0.66$	0.826
7th day	$176.66^{a}\pm4.40$	$173.33^{a}\pm6.00$	$167.33^{a}\pm5.04$	$178.33^{a}\pm1.66$	$168.33^{a}\pm6.00$	0.453
14 th day	$404.00^{a}\pm3.05$	$381.66^{a}\pm17.40$	$373.66^{a}\pm12.01$	$400.00^{a}\pm5.77$	$387.33^{a}\pm8.19$	0.287
21st day	$810.00^{b}\pm20.81$	$691.66^{a}\pm24.88$	$702.66^{a}\pm5.36$	$858.59^{b}\pm7.02$	$736.00^{a}\pm7.02$	<0.001
28 th day	$1133.33^{b}\pm16.66$	$940.00^{a}\pm10.00$	$910.33^{a}\pm6.06$	$1143.33^{b}\pm6.66$	$951.66^{a}\pm13.01$	<0.001
35th day	$1500.00^{c}\pm10.00$	$1345.00^{b}\pm24.66$	$1341.66^{b}\pm9.02$	$1488.33^{c}\pm6.00$	$1230.00^{a}\pm15.00$	<0.002
42 day	$1926.66^{b}\pm14.52$	$1726.66^{a}\pm14.52$	$1701.00^{a}\pm4.93$	$1836.66^{b}\pm31.79$	$1650^{a}\pm28.86$	<0.001

Data are shown as mean ± SEM. The values in a row with no common superscript are substantially different from one another ($p < 0.05$).

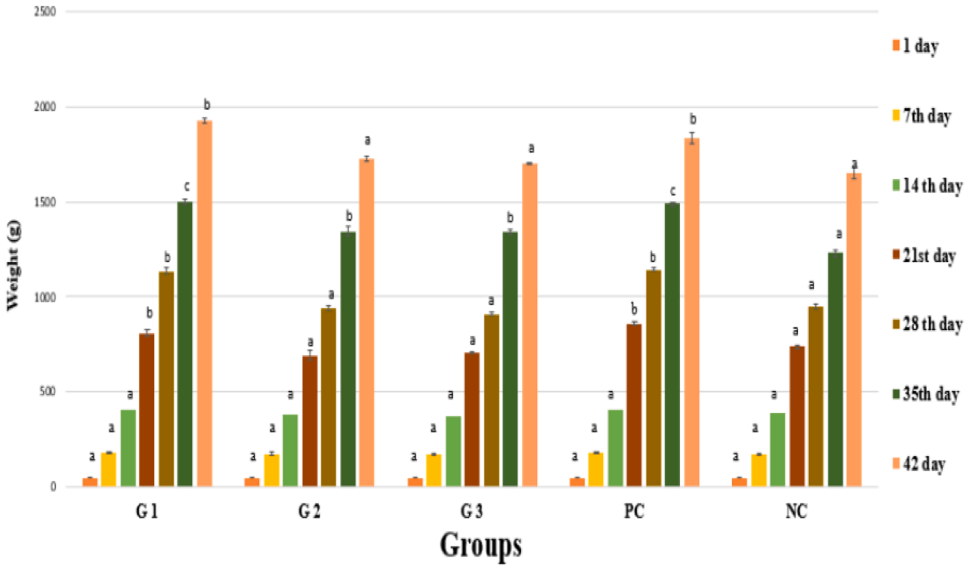

Fig. 2. Graph showing the mean weekly body weight of birds across experimental groups. Error bars represent ($n = 3$, means±SEM). Different letters indicate significant differences ($p < 0.05$). Groups: G1 (Pro 1-4), G2 (Pro 1-4, Vaccinated), G3 (Vaccinated), PC (Positive Control), and NC (Negative Control).

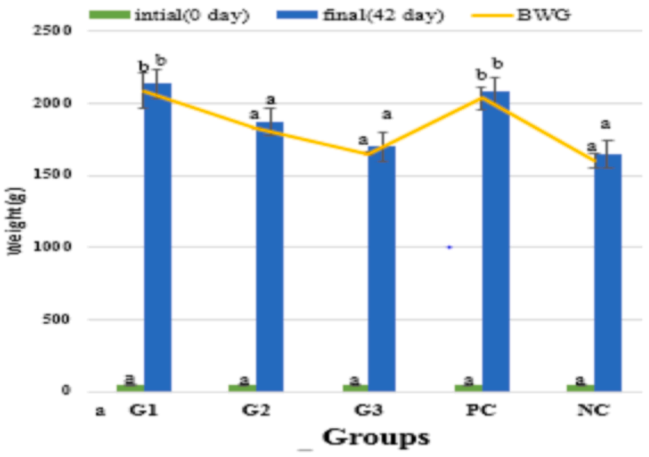

Fig. 3. Graph showing birds' mean initial and final body weight across experimental groups. Error bars represent ($n = 3$, means±SEM). Different letters indicate significant differences ($p < 0.05$). Groups: G1 (Pro 1-4), G2 (Pro 1-4, Vaccinated), G3 (Vaccinated), PC (Positive Control), and NC (Negative Control).

control). Moreover, there was a noteworthy variation ($p < 0.05$) in the thickness of duodenal mucosa among the groups. In contrast to NC, probiotic-supplemented groups G2 and PC showed a considerable increase in VW (Fig. 6; Table 4).

Humoral immune response

Supplementing birds' diets with MSP has a considerable impact on their immunological response. Log2 HI titers were used to express the results of antibodies that are specific to NDV. Following immunization, G2 and G3 had the highest HI titer values from the first week to the third week and this difference from the other groups was statistically significant (Fig. 7). Fourteen days after vaccination, high values were seen in the HI titers of the G1, G2, and G3 groups, while the PC and NC groups showed decrease value. In the third week, a decline was observed in maternal antibodies that reached baseline in the NC group. HI-NDV Ab levels in our investigation were greater in probiotic-treated groups compared to the control group (NC). The humoral response to live NDV vaccinations may be enhanced by multistrain probiotics.

Discussion

The study aimed to find the impact of multistrain probiotics on the immune response and gut morphology of broiler chickens. The current research results showed improved growth performance in chickens administered multistrain probiotics compared to the negative control group.

This investigation showed that growth performance has increased in multistrain probiotics-fed groups in comparison to NC. Comparable results were documented by (Fathima et al., 2022; Dias et al., 2022; Zeng et al., 2021; McFarland, 2021 and Korada et al., 2018), that multistrain probiotics might aid in nutrient assimilation and utilization, thereby improving the feed conversion ratios and weight gain. These results contradict the studies of (Ray et al., 2022; Aalaei et al., 2019).

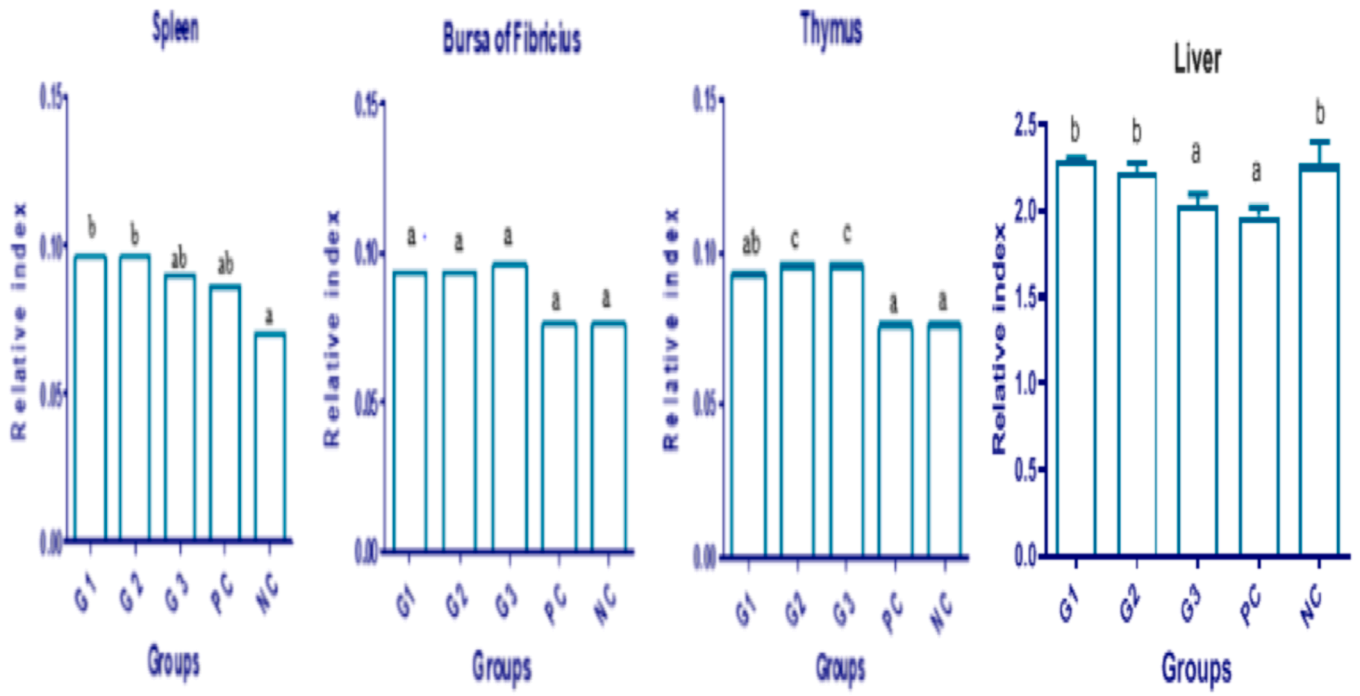

Fig. 4. Graph showing the mean relative weight of spleen, bursa of Fabricius, thymus, and liver across experimental groups. Error bars represent ($n = 3$, means ±SEM). Different letters indicate significant differences ($p < 0.05$). Groups: G1 (Pro 1-4), G2 (Pro 1-4, Vaccinated), G3 (Vaccinated), PC (Positive Control), and NC (Negative Control).

Table 2
Hematological parameters in blood of broiler chickens.

Parameters	G 1	G 2	G 3	PC	NC	p value
Hb (g/dl)	13.03^{b}±0.26	14.36^{bc}±0.47	13.16^{bc}±0.16	14.46^{c}±0.29	10.80^{a}±0.20	<0.001
RBCx106 /ml	3.09^{bc}±0.08	3.67^{c}±0.17	2.41^{ab}±0.22	3.10^{bc}±0.10	2.16^{a}±0.16	<0.001
PCV (%)	36.46^{ab}±0.85	40.50^{b}±1.04	37.60^{ab}± 1.62	36.34^{ab}±0.97	35.05^{a}± 1.10	0.063
MCV (fl)	137.59^{a}±0.55	139.36^{a}±2.95	139.07^{a}±1.57	143.44^{a}±2.32	151^{a}±8.62	0.245
MCH (pg)	44.26^{a}±0.88	40.76^{a}±0.76	42.33^{a}±1.45	49.47^{a}±3.67	47.36^{a}±1.77	0.063
MCHC (%)	31.03^{a}±0.57	31.50^{a}±0.28	33.56^{a}±0.72	41.23^{a}±1.75	32.83^{a}±0.27	0.072
PLT 10^{3}/ul	21.66^{ab}±1.20	26.66^{bc}±0.88	20.00^{a}±1.15	32.32^{c}±1.45	24.00^{ab}±2.08	<0.001
TLC10^{3}/ul	13.16^{ab}±0.88	19.66^{c}±1.45	16.00^{bc} ±0.57	17.00^{bc}±1.00	10.50^{a}±0.76	<0.001
Hetero(%)	41.66^{a}±4.40	46.66^{a}±10.13	35.00^{a}±2.88	44.00^{a}±1.00	41.66^{a}±1.66	0.612
Lympho(%)	51.33^{abc}±5.81	38.33^{a}±4.40	67.00^{c}±1.52	57.00^{bc}±3.00	45.00^{ab}±2.88	<0.003
Mono(%)	3.33^{a}±0.88	3.33^{a}±0.66	3.33^{a}±0.66	3.66^{a}±0.88	3.00^{a}±0.57	0.980
Eosino(%)	5.53^{a}±0.33	3.66^{a} ±0.33	4.66^{a}±0.66	4.33^{a}±1.20	4.33^{a} ±0.66	0.599

Data is displayed as, mean± SEM. When values in a row dont share a superscript, they differ from one another significantly (p<0.05). Eosino-Eosinophil, Hb-Haemoglobin, TLC- Total leukocyte count, MCHC (Mean corpuscular haemoglobin concenteration), MCV (Mean Corspuscular Volume), Mono-Monophils, PCV-Packed cell volume, Hetero-Heterophiles, MCH- Mean corpuscular haemoglobin, PLT-Platelets, RBC-Red blood cell, Lympho- lymphocyte.

A significant rise in haemoglobin concentration and total red blood counts was observed which is in line with the results of (Deraz, 2018; Reuben et al., 2022) and contradicts Alkhalf et al., (2010). This study's findings about the decline in important biochemical markers, such as LDL and cholesterol, are consistent with those of Arczewska-Włosek et al., (2022) who also noted a noteworthy decline in triglycerides and cholesterol by adding Protexin (multistrain probiotic) in feed. On the other hand, Aalaei et al., (2019) observed that probiotic combinations comprising *L. casei, B. thermophilum, L. acidophilus,* and *E. faecium* had no impact on cholesterol. Additionally, as this study's results show, probiotic addition had a substantial impact on total protein in comparison to NC. Similar outcomes were reported by Wang et al., (2021). This contradicts investigations carried out by Abdel-Hafeez et al., (2017), who found no difference in the total protein concentration of hens given probiotic supplements.

The present results revealed that multistrain probiotics added to broiler feeds led to a substantial rise in the height of the villus and the ratio of villus height to crypt depth in the duodenum compared to the control groups ($p < 0.05$). Similar outcomes were noted by (Bogucka et al., 2019; Wieërs et al., 2020), those broilers fed with pellets containing multistrain probiotics that contained five bacterial strains yielded enhancement in the intestinal morphology than the ones fed with single strain probiotics. Additionally, Kazemi et al., (2019), gave broiler chicks two commercial multistrain probiotic supplements. These two products enhanced intestinal structure and overall performance in chickens. Conversely, however, Han et al., (2018), demonstrated that morphometric parameters were not notably impacted by the addition of microencapsulated *C. butyricum* and *L. plantarum*. Hence evidence from positivist literature supports the positive outcomes of multistrain probiotics on intestinal morphology, yet the findings need to be well standardized.

Typically, serum immunoglobulins are utilized as metrics to assess animal immunological health Wang et al., (2018). Probiotics in feed can lead to increased systemic antibody response by the generation of IgA levels at mucosal surfaces, a variety of anti and pro-inflammatory cytokines, immunoglobulins (IgG, IgM), interferon, and macrophage

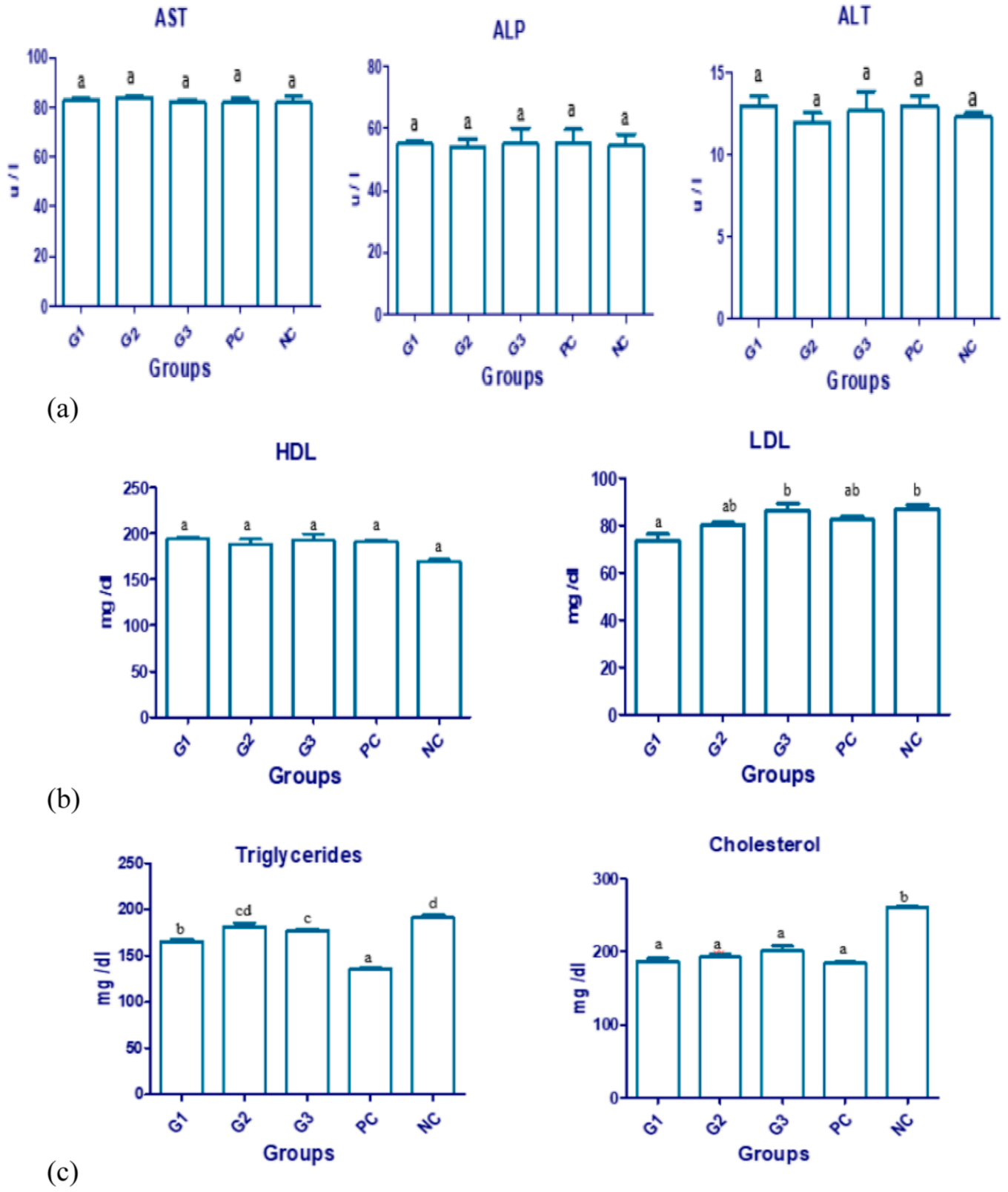

Fig. 5. Impact of Multistrain probiotics (a) on liver enzymes, ALP-alkalinephosphatase, AST-aspartateaminotransferase, ALT-alanine aminotransferase, (b) high-density lipoprotein (HDL), low-density lipoprotein (LDL) (c) Triglycerides, Cholesterol in broilers. No significant differences ($p > 0.05$) were observed in ALP, AST, and ALT enzyme activity. HDL levels showed the highest value in the G1 group. Probiotic-supplemented groups showed a significant decrease in total cholesterol compared to the negative control (NC) group.

Table 3
Impact of Multistrain probiotics on Interleukins and inmunoglobulins in broilers.

Parameters	G 1	G 2	G 3	PC	NC	p- Value
IL2(pg/ml)	$2.91^{a}\pm0.22$	$5.94^{d}\pm0.09$	$7.30^{e}\pm0.19$	$5.06^{c}\pm0.07$	$3.89^{b}\pm0.08$	<0.001
IL4(pg/ml)	$93.14^{c}\pm4.74$	$183.52^{d}\pm0.51$	$33.66^{a}\pm0.69$	$80.49^{b}\pm0.96$	$33.50^{a}\pm0.58$	<0.001
IL10(pg/ml)	$85.22^{e}\pm0.56$	$83.46^{d}\pm0.30$	$16.83^{a}\pm0.22$	$31.92^{c}\pm0.43$	$24.30^{b}\pm0.22$	<0.001
IgG(ng/ml)	$1654.85^{a}\pm7.17$	$4099.33^{c}\pm45.85$	$1059.37^{a}\pm16.92$	$3362.26^{b}\pm319.00$	$2990.32^{b}\pm56.41$	<0.001
Ig A(ng/ml)	$1835.99^{c}\pm22.80$	$3057^{e}\pm2.37$	$945.52^{a}\pm3.47$	$1970.30^{d}\pm3.08$	$1167.10^{b}\pm5.06$	<0.001
IgM(ng/ml)	$343.04^{d}\pm3.71$	$520.76^{e}\pm2.44$	$43.34^{b}\pm0.51$	$25.69^{a}\pm0.34$	$224.61^{c}\pm3.08$	<0.001

Data is shown as mean± SEM. When there is no common superscript, values in a row differ considerably from one another ($p < 0.05$).

Table 4
Impacts of multi-strain probiotics on duodenum morphology.

Parameters	G 1	G 2	G 3	PC	NC	p- Value
VH (μm)	$991.86^{d}\pm16.03$	$712.79^{c}\pm17.72$	$231.56^{a}\pm17.63$	$401.63^{b}\pm15.61$	$267.85^{ab}\pm5.59$	<0.001
CD(μm)	$94.52^{ab}\pm7.17$	$128.2^{bc}\pm3.86$	$132.49^{c}\pm8.37$	$111.35^{bc}\pm6.70$	$80.34^{a}\pm6.24$	<0.001
VW(μm)	$60.32^{b}\pm2.01$	$110.04^{c}\pm10.46$	$146.84^{d}\pm28.23$	$85.23^{bc}\pm1.81$	$23.74^{a}\pm1.97$	<0.001
VH:CD(μm)	$7.74^{bc}\pm0.26$	$7.80^{c}\pm1.81$	$1.77^{a}\pm0.24$	$3.64^{b}\pm0.31$	$3.38^{ab}\pm0.34$	<0.001
Mucosa(μm)	$155.15^{b}\pm5.92$	$113.66^{c}\pm2.47$	$98.33^{ab}\pm7.26$	$119.28^{ab}\pm15.94$	$80.66^{a}\pm2.54$	<0.001

Data is presented as mean± SEM. Within a row, values without a common superscript exhibit significant differences from one another ($p < 0.05$). VH: villus height, VD: villus width, CD: crypt depth.

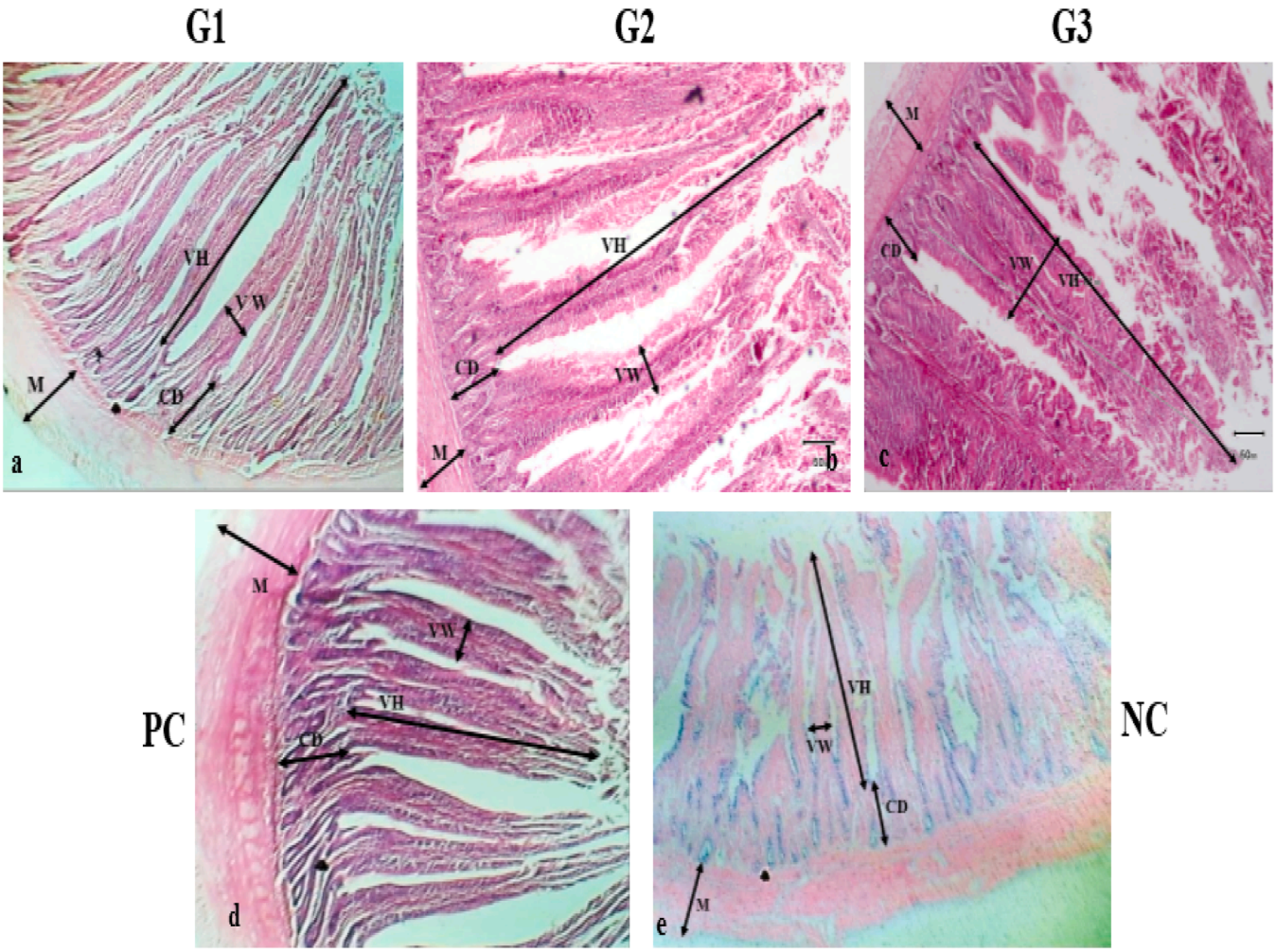

Fig. 6. H&E stained histological sections of duodenum. (a): G1, (b): G2, (c): G3, (d): PC, (e): NC group, C.D- crypt depth, V.W-, villus width M- mucosa, Scale bar =50 mm.

activity (Biswas et al., 2022; Atela et al., 2019). Present findings of increased levels of immunoglobulins align with the research by Elsayed et al., (2021), which showed that Protexin, a commercial multistrain probiotic, had immunomodulatory effects. Similarly, serum levels of IgA and IgM are raised by multi-strain probiotics that contain *B. subtilis, L. acidophilus, and C. butyricum* reported by Zhang and Kim, (2014). Our

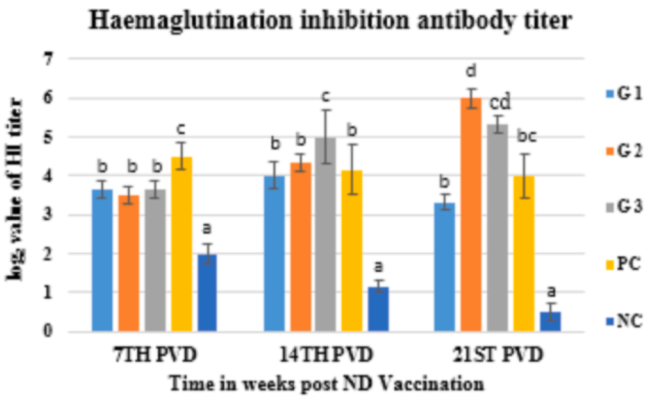

Fig. 7. Antibody titers, expressed as the mean HI titer log2± SEM.

results are also supported by Gong et al., (2018), who reported that serum IgA concentration increased significantly when broiler chicks were given probiotics, such as *Bacillus* species (*B. licheniformis, B. subtilis*). Other researchers' findings, in contrast, show that probiotic microorganisms do not affect the amount of serum immunoglobulins (Mountzouris et al., 2010; Żbikowski et al., 2020).

The results of the ND titer are in line with Houshmand et al., (2012), who discovered that the probiotics (*B. subtilis* and *C. butyricum*) groups had higher antibody titers to the ND virus than the control group. However, according to research by Rehman et al., (2020), probiotics did not affect the titer against ND. Increased ND antibody titers may be the consequence of cytokines regulating immunity, which immune cells produce in response to probiotic microbial stimulation.

Conclusion

Multistrain probiotics are an effective substitute for antibiotics in chickens and also enhance growth in animal husbandry. The results of this investigation demonstrated that multistrain probiotics consisting of *Enterococcus faecium* (OR563785.1), *Weissella confusa* (OR563786.1), *Weissella cibarian* (OQ543569.1), and *Lactiplantibacillus plantarum* (OQ689085.1) had a favorable impact on broiler performance, gut morphometry, and immunology. Chicks-fed probiotics had higher specific antibody titers after being immunized against the New-Castle disease (ND) virus, demonstrating the useful adjuvant action of probiotics. For **future research**, further investigations are needed to study how different probiotic combinations affect various chicken types and the correct dosage amounts. Observation of probiotic molecular processes will lead to the complete understanding of these microorganisms. The evaluation of financial potential alongside research confirmation requires testing that must be done in commercial agricultural conditions.

Declaration of competing interest

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.

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Supplementary materials

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