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Research article

Application of 16S rRNA gene sequencing in evaluation of prebiotics or probiotics administration to restore gut dysbiosis induced by infectious bursal disease virus in broiler chickens

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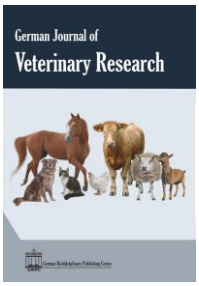

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Abstract

Infectious bursal disease (IBD) affects young chicks, inducing immune suppression, intestinal dysbiosis, poor growth performance, and mortalities. Our aim is to study the role of prebiotics and probiotics in overcoming dysbiosis related to IBD experimental infection. One-day-old chicks were divided into six groups (n=18) as four groups were given *Enterococcus faecium* (T1), *Saccharomyces cerevisiae* (T2), organic acids (T3), and symbiotic (T4) for five days at the 3rd-day post-infection. Groups 5 and 6 were considered negative control (NC) and positive control (PC). At 14 days old, all groups were challenged via eye drops with very virulent IBDV strain (MK088026), except the NC group. Feed conversion ratio (FCR), histopathological changes, and gut microbiome profile using 16srRNA gene sequencing were studied. Severe depletion of the bursa was observed in the PC group but was less severe in treated infected groups. 8 dpi, a significant decrease in cecum villous length (p<0.05) was observed in the PC group compared to the NC group, while it was corrected in treated infected groups. Cecal microbiome profile was studied on the 3rd and 8th-day post-infection (dpi). The IBD virus caused a decrease in the family *Lachnospiraceae* and family *Ruminococcaceae* compared to the NC group. In contrast, the treated infected groups showed an increase in the relative abundance of phylum Bacteroidetes, family *Ruminococcaceae*, and family *Lachnospiraceae* than the PC group. IBD infection caused poorer FCR than the NC group. In contrast, treated infected groups showed higher improvement in FCR than the PC group.

Keywords: *Enterococcus faecium*, Gumboro, *Lactobacillus*, Microbiome, Organic acids, *Saccharomyces cerevisiae*

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Introduction

Infectious bursal disease (IBD) is one of the major viral diseases in the poultry industry (Setta et al., 2024). The disease is caused by the infectious bursal disease virus (IBDV), which targets the bursa of young chickens. This results in an acute, highly contagious, and fatal immunosuppressive disease, leading to significant economic losses in the poultry industry and a dramatic decline in birds' productivity. (Eterradossi and Saif, 2019; Mosa et al., 2023). Bursal B-lymphocytes targeted with IBD leading to depletion of lymphoid tissues, suppression of humoral immunity (Vukea et al., 2014), and dysbiosis of gut microbiota (Li et al., 2018; Daines et al., 2019; Mosa et al., 2024).

The intestinal microbiota in human and other animal models and how it interacts with viruses were intensively studied (Robinson et al., 2014). However, in poultry, few studies have focused on the interaction between IBDV and intestinal microbiota (Li et al., 2018; Daines et al., 2019; Mosa et al., 2024). This intestinal dysbiosis is attributed to IBDV replication in gut-associated lymphoid tissue (GALT) and alteration of immune

cells (Li et al., 2018). Also, it has been found that the immune system has an important role in the control of microbiota composition and equilibrium (Hooper et al., 2012).

The vital role of intestinal microbiota in the health, nutrition, immunity, and physiology of birds has become evident in recent years (Bindari and Gerber, 2022). The commensal microbiota plays a vital role in maintaining homeostasis and in the protection against pathogens colonization (Diaz Carrasco et al., 2019). Also, microbiota helps in the development of intestine microstructure as the epithelial monolayer, mucus layer, and lamina propria by metabolites production such as short-chain fatty acids (SCFAs), vitamins, indoles, and anti-microbial compounds that increase the absorption capacity of the epithelial surface as well as bird productivity (Bindari and Gerber, 2022). Therefore, feed additives are an important tool used in animal food production to enhance performance and improve poultry health (Maki et al., 2019).

Prebiotics affect the host by selectively stimulating the activity of one or a limited number of bacteria in the colon, influencing intestinal bacteria, improving the immunity of chickens, and increasing SCFAs (Adhikari and Kim, 2017; Shehata et al., 2022). The predominant prebiotics in chickens include types of oligosaccharides like fructo-oligosaccharides (FOS), inulin, mannan-oligosaccharides (MOS), and xylo-oligosaccharides (XOS) (Adhikari and Kim, 2017; Maki et al., 2019).

Probiotics are live microorganisms that, when administered in adequate amounts, confer a health benefit on the host (Abd El-Hack et al., 2022). Commonly used probiotics in animals are *Lactobacillus* (*L. bulgaricus, L. plantarum, L. acidophilus, B. subtilis*), *Enterococcus* (*E. faecalis, E. faecium*), *Bifidobacterium* spp. and yeast (*S. cerevisiae*) (Adhikari and Kim, 2017). *Lactobacillus* and *Enterococcus* spp. are the most common bacterial probiotics in poultry (Maki et al., 2019). Probiotics are characterized by competitive exclusion (CE) of pathogenic microorganisms, improvement of bird immune system, and production of SCFAs and bacteriocins from metabolic reactions as antimicrobial compounds (Adhikari and Kim, 2017).

Lactobacillus spp. and *Enterococcus* spp. are lactic acid-producing bacteria that have antimicrobial activities by reducing the pH of the gut (Adhikari and Kim, 2017; Maki et al., 2019). *Lactobacillus* spp. have been associated with increased body weight, enhanced goblet cell counts, decreased colonization of pathogenic bacteria in the gut, and improved poultry flocks health (Maki et al., 2019). *Enterococcus* spp. supplementation has a role in increasing feed conversion ratio (FCR), improving bird growth performance, and enhancing immunity (Royan, 2018).

Organic acids (OA) reduce the growth and colonization of many pathogenic intestinal bacteria, decrease inflammatory processes at the gut mucosa, and stimulate specific and non-specific gut immune functions (Ganguly, 2013).

Therefore, the present study aims to study the effect of the IBD virus on the microbiota of the cecum with experimental trial to overcome the dysbiosis related to IBD infection by administration of bacterial role of bacterial probiotic (*E. faecium*), yeast probiotic (S. *cerevisiae*), organic acids and symbiotic with analysis of microbiota taxa using metagenomic analysis of 16srRNA gene sequencing.

Materials and methods

Ethics approval

All experimental procedures and handling of chickens were compliant with the laws of the institutional animal care and use committee of the Faculty of Veterinary Medicine, University of Cairo, Egypt, with approval number (Vet CU 2009 2022526).

Challenge

The challenge was done using very virulent IBDV (IBDV/Egypt/Qalubia/17) with the accession number MK088026 (El-Samadony et al., 2019). The challenge experiment was conducted in chicks at the age of 14 days through eye drop inoculation of 0.2 ml /bird, containing 104 eggs with infectious dose 50 (EID_{50}) (OIE, 2018).

Treatment

It was applied in the drinking water for five days after infection (17-21 days old) using one commercial product of the following per group:

T1= Protexin®: It is a probiotic manufactured by ADM Protoxien LTD, UK (Batch no. 124496) containing per kg *Enterococcus faecium* (NCIMB 11181) 4b 1708. <1.0% total viable count 2x10*12*

CFU/kg. Ingredients: Dextrose up to 1kg, crude protein < 1.0%, crude fiber < 1.0%, crude oil < 1.0%, crude ash < 1.0%, and trace, with a dose of 0.5 g / Liter.

T2= Bio site for feed additives®: It is composed of 100% dried yeast extract *Saccharomyces Cerevisiae* 128gm/ 1kg in a dose of 1 g / Liter.

T3= Fortibac®: It is manufactured by ADDICC GROUP s.r.o, Checa (Batch no.2208181183) each 1000 mL contains formic acid (45%) 95 g, propionic acid (50%) 95 g, butyric acid (53%) 95 g, caprylic acid (56%) 95 g, pelargonic acid (48%) 95 g, capric acid (55%) 95 g, lauric acid (46%) 95 g, the fatty acids in form of esters with glycerol and water up to 1000 ml, in a dose of 1 ml / Liter.

T4= Amino-Zyme®: It is manufactured by 2M group, Egypt (Batch no. 2389). It is composed of beta-glucan 48.6 g, fructo oligosaccharide 8.3 g, DL-methionine 0.5 g, L-carnitine 15.3 g, L-lycine HCL 4.47 g, Mono propylene glycol 45.25 g, and purified water up to 1 liter. Also, it contains spirulina, L-valine, taurine, threonine, L-arginine, leucine, isoleucine, *Lactobacillus acidophilus, Lactobacillus subtillis, Bifidobacterium*, phytase, protease, amylase, xylase. Dose 1 ml / Liter

Experimental design

A total of 121 one-day-old mixed Ross broiler chicks were purchased from a local hatchery. On arrival, ten chicks were randomly selected and individually weighted, and blood samples were collected to determine IBDV maternal-derived antibodies (MDA). One hundred eleven chicks were reared in well-prepared units. At 13 days old, 18 chicks were transferred to a separate room as a negative control group (NC). On day 14, the remaining 93 chicks were infected with vvIBDV (IBDV/Egypt/Qalubia/17 with the accession number MK088026 (El-Samadony et al., 2019) through eye drop inoculation of 0.2 mL /bird, containing 10^4 egg infectious dose 50 (EID_{50}) (OIE, 2018).

At 3 dpi, three infected chicks were euthanized for sample collection, and the 90 chicks were randomly divided into five groups (T1, T2, T3, T4, and PC) (n=18). Chicks in groups T1-T4 were received *E. faecium*, *S. cerevisiae*, organic acids, and symbiotic, respectively, for five days between 17–21 days old, while the remaining group considered as positive control PC (infected, not treated). All groups were observed daily for clinical signs, mortalities, and post-mortem lesions, as well as feed intake was recorded. Weekly FCR was calculated. Blood samples were collected from all groups (10 birds/group) at 14 and 24 days old to determine anti-IBD antibody titer. At 17 and 22 days old, three birds per group were randomly collected and euthanized for post-mortem examination, bursal index calculation (BI), and cecal contents collection (collected aseptically and kept at -20° C) for microbiome analysis. Parts of the Bursa of Fabricius and cecum were kept in 10% formalin until further examination.

Birds' management

All broiler management was done in compliance with the Ross 308 broiler rearing standards (Delezie et al., 2012). Each group of chicks was reared in an isolated unit on wood-shaving bedding. According to NRC (1994), chickens were fed on commercial pelleted rations (starter, 23%; grower, 21%; and finisher, 19%). Drinking water and rations were given to chickens *ad libitum*.

Feed conversion ratio (FCR) and bursal index (BI)

Weekly and total FCR were calculated according to Prakash et al. (2020) to evaluate growth performance. The bursal weight index was calculated according to Zaheer et al. (2022) to confirm IBD infection, as a BI of less than 0.7 is considered atrophy of the bursa (Thai et al., 2022).

Antibody detection by ELISA

The ELISAs were performed using a commercially available kit (ID Screen® IBD Indirect kit, Innovative Diagnostics company, brand name IDvet Grabels, France), according to the manufacturer's guidelines, to detect circulating anti-IBDV-specific IgG antibodies. Anti-IBDV antibody titers were calculated based on the OD values and are presented as mean titer ± standard deviation (SD) per group.

Histological investigations

Samples of bursa and cecum of 22-day-old chicks were preserved in 10 % neutral buffered formalin, followed by routine processing to obtain H&E-stained tissue sections. The severity of the lesion of collected tissue was evaluated by a scoring system previously described by (Zahedi et al., 2023).

Gut microbiota composition

Sample collection

Cecal contents (1 gm) were aseptically collected in sterile Eppendorf from all groups at 17 days and 22 days (5 separated samples /group) for a total of 24 samples. Then, samples were kept at -80°C until use.

DNA extraction

Using the commercially available kit (FavorPrepTM Stool DNA Isolation Mini Kit, Favorgene Biotech Corp. company Ping Tung, Taiwan), complete genomic DNA was extracted from the cecal sample from 200 mg per sample with a total of 600 mg per group, in accordance with the manufacturer's instructions. A nanophotometer (NanoPhotometer P360, Implen GmbH company, Munich, Germany) was used to measure the concentration and purity of DNA. Three DNA samples from each group were pooled into one sample per group by pooling equal concentrations from each sample of the same group and kept at -80°C until further processing. Thus, eight samples were created by pooling the 24 samples. Pooled samples from the same group and sample characterization are shown in (Table 1).

Table 1: Characterization of DNA samples from groups. Each sample is a pool from 3 collected samples per group at the age of 17 (3 dpi with IBD) and 22 (8 dpi with IBD) days.

Group	Sample code	Age/days	IBD infection	Treatment
NC	Healthy.D17	17	-	-
	Healthy.D22	22	-	-
PC	Control.D17	17	3 dpi with IBDV	+
	Control.D22	22	8 dpi with IBDV	+
T1	T1. D22	22	8 dpi with IBDV	+
T2	T2. D22	22	8 dpi with IBDV	+
T3	T3. D22	22	8 dpi with IBDV	+
T4	T4. D22	17	3 dpi with IBDV	+

dpi: day post-infection; IBD: infectious bursal disease; NC: negative control; PC: positive control; T1: *E. faecium*; T2: *S. cerevisiae;* T3: organic acids; T4: symbiotic.

Sequencing and microbiome analysis

Next-generation sequencing was performed. The high-throughput Illumina-MiSeq sequencing (2×300 bp paired-end protocol) (software, American biotechnology company California, United States) was used to investigate the dynamics of the gut microbiota by targeting the bacterial V3 and V4 regions of the 16S rRNA gene. Generated Illumina reads were analyzed using QIIME tool version 1.9.117 (QIIME software, Boulder, Colorado, United States). Sequences were clustered to the same OTUs (Operational Taxonomic Unit). The alpha diversity was investigated as the number of observed OTUs per sample and the number of observed species were obtained and evaluated using good's coverage, Simpson's, and Shannon's metrics. Beta diversity was investigated using the Unifrac distance metrics based on the principal coordinates analysis (PCoA). Taxa analysis of the microbiome was investigated at the levels of phylum, class, order, family, genus, and species.

Statistical analysis

Statistical significance between the 17-day-old NC and PC chicks was tested using the independent sample t-test. In other data, the groups were compared using one-way analysis of variance (ANOVA) followed by post-hoc analysis according to the Benjamini-Hochberg procedure. In both scenarios, normal distribution was examined using the Shapiro-Wilk test, and Levene's test assessed homogeneity of variance. Statistical analysis was performed in RStudio-2023.06.1-524 (Posit Company, Boston, Massachusetts, United States) (Posit team, 2023) using R programming language v4.3.1 (R Core Team, 2023). $p<0.05$ were considered statistically significant.

Regarding the microbiome analysis, PERMANOVA (permutational multivariate analysis of variance) and ANOSIM (analysis of similarities) using Python script embedded in QIIME (1.9.1) were used for the microbiome samples. These two tests were used for the analysis of the statistical significance and the strength of samples of different groups. The differential abundance analysis was done to find the differences in the relative abundance of each taxon between samples, and then we used statistical analysis to assign a significant value to each comparison. We used Kruskal–Wallis and

Bonferroni's multiple comparisons tests using Python scripts embedded in QIIME (1.9.1); for each taxon, the *p-value* and the false positive adjustment of the p-value (FDR *p-value*) corrected by the Benjamini–Hochberg FDR procedure for multiple comparisons were calculated.

Results

Clinical signs, postmortem examination, and bursal index

Signs of ruffled feathers, dullness, huddling, and whitish diarrhea were observed in the PC group at 3 dpi of IBD, while the NC group showed no clinical signs of illness. The treated infected groups showed less severe clinical signs. The total mortality due to IBDV infection was observed only in PC (2 out of 18) (11.11%) and T1 group (1 out of 18) (5.56%), while other groups showed no mortalities. IBDV induced inflammatory lesions in the bursa of Fabricius at 3 dpi, as petechial hemorrhage, and inflammatory exudates with changes of bursal size as it started to atrophy according to bursal index while at 8 dpi, the main bursal lesion was severe atrophy in all infected groups (Table 2). As at 3 dpi (age 17 days), there was no significant difference. However, at 8 dpi (22 days of age), there was significant (p<0.05) in all infected groups compared to the NC group; the bursal index was less than 0.7, which indicates bursal atrophy (Table 2).

IBDV-antibody titer

MDA at one day old was 5249 ± 2362, which declined at age 14 days to 624 ± 786. After infection, IBDV antibody titers significantly increased in infected groups compared to the negative control group at 10 dpi. The mean of IBDV antibody titers was higher in T3, T4, and T1 than in T2 receptively in comparison with the positive control group (Table 3).

FCR

At age three weeks, FCR improved in comparison with NC (1.22), as it was lowest in groups of organic acid T3 (1.09), symbiotic T4 (1.11), and *Enterococcus* T1 (1.17). In contrast, PC (1.23) and *S. cerevisiae* T2 (1.25) had poor FCR. The final FCR at 35 days of age was better in T3 (1.58), NC (1.59), T1 (1.60), T2 (1.62), and PC (1.63) than T4 (1.64), as seen in Table S1 in the supplementary file.

Histopathological findings

At age 22 days, the examination of bursal sections revealed that the NC group showed normal bursal follicles without any histological alterations. On the contrary, the PC group exhibited variable pathological lesions, including massive lymphoid depletion with necrosis in the follicular lymphoid cells associated with karyorrhectic debris mixed with proteinaceous exudate and expansion of interfollicular connective tissue, edema, and intraepithelial cysts. Meanwhile, treatment with the *E. faecium* (T1) group showed expansion of interfollicular connective tissue with heterophilic infiltration and vacuolation of inner medullary zones of bursal follicles. Similarly, the findings in groups treated with *S. cerevisiae* (T2), organic acids (T3), and symbiotic (T4) presented moderate lymphoid depletion with vacuolation of inner medullary zones adjacent to the expansion of interfollicular space (Figure S1 in the supplementary file). The bursal lesion score in the PC group was 3.60±0.50, ameliorated in all treated groups. The lesion score in T1 was (2.13±0.13), T2 (2.60±0.13), T3(2.40±0.16), and T4 (2.60±0.163) without recording any significant difference between them (Table 4).

Table 2: Bursal index of infected treated, infected non-treated, and control negative groups.

Group	Age/Days	Bursal index (BI) Mean ± SEM
NC	17	1 ± 0.577[a]
	22	1 ± 0.274[a]
T1	22	0.284 ± 0.126[b]
T2	22	0.31 ± 0.066[b]
T3	22	0.259 ± 0.054[b]
T4	22	0.354 ± 0.037[b]
PC	17	0.6528 ± 0.338[a]
	22	0.353 ± 0.132[b]

NC: negative control; PC: positive control; T1: *E. faecium;* T2: *S. cerevisiae;* T3: organic acids; T4: symbiotic. Different superscripts (a-b) reveal a significant difference between values p<0.05.

Table 3: IBDV-antibody titer (mean ±SD) as detected by ELISA at 24 days (n=10) (10-dpi).

parameter	NC	T1	T2	T3	T4	PC
Anti-IBD Titer (24 days)	77 ± 87 [b]	6,781 ± 5,686 [a]	4,260 ± 2,593 [a]	8,747 ± 2,4767 [a]	7,277 ± 1,465 [a]	3,801 ± 1,382 [a]

dpi: day post-infection; IBD: infectious bursal disease; NC: negative control; PC: positive control; T1: *E. faecium;* T2: *S. cerevisiae*; T3: organic acids; T4: symbiotic. Data represented as mean ± SEM. Different superscripts (a-b) reveal a significant difference between values $p \leq 0.05$.

Table 4: Illustrates the histopathological lesion score of the bursa, cecum, and cecal villous length(µm) among different groups at age 22 days (8 dpi) (data expressed as mean ± SEM).

Organs	NC	PC	T1	T2	T3	T4
Bursa	0.0±0.0[a]	3.60±0.507[b]	2.13±0.133[c]	2.60±0.130[c]	2.40±0.163[c]	2.60± 0.163[c]
Intestine	0.0±0.0[a]	3.53±0.13[b]	1.66±0.21[c]	2.20±0.20[c]	1.80±0.20[c]	0.80±0.177[d]
Cecal villous length	450.9±52.8[a]	277.9±58.9[b]	456.7±77[a]	417.9±50.3[a]	482.5±86 [a]	458.4±50.06[a]

IBD: infectious bursal disease; NC: negative control; PC: positive control; T1: *E. faecium*; T2: *S. cerevisiae;* T3: organic acids; T4: symbiotic. Different superscripts (a-d) reveal a significant difference between values. Statistically significant differences were considered when $p \leq 0.05$.

At age 22, the examined cecal section revealed normal mucosa and submucosa with intact epithelial lining in the NC group. In contrast, the PC group exhibited sloughing and necrosis of lining epithelium with inflammatory cell infiltration in lamina propria. The cecal section of the T1-treated group revealed mild congestion of blood vessels with mild inflammatory cell infiltrations. The intensity of inflammatory cells was increased in the T2-treated group. The T3-treated group showed minor inflammatory cells in lamina propria with sloughing in some parts of the villous epithelial lining. In the T4-treated group, minor inflammatory cells were infiltrated in the cecal mucosa (Figure S2 in the supplementary file). The positive control group recorded the highest lesion score (3.53±0.13) that inclined in treated groups. T1 group recorded (1.66±0.21), in T2, was (2.20±0.20) and in T3 was (1.80±0.20) without any statical significance difference between them. The T4-treated group (0.80±0.17) recorded the lowest score among the treated groups (Table 4). In addition, cecal villous length was studied in all groups. PC group revealed a significant decrease in villi length (277.9±58.9) compared to NC one (450.9±52.8) ($p<0.05$). At the same time, all infected treated groups showed higher villous length than the PC group. The T2 group showed a non-significant decrease in villous length (417.9±50.3) compared to the NC group, while T1, T3, and T4 recorded non-significant increases in villous length (456.7±77, 482.5±86, 458.4±50.06), receptively, compared to NC group (Table 4).

Microbiome analysis

The alpha diversity in the six tested groups was calculated and evaluated. The species diversity was investigated using Simpson's and Shannon's metrics (Figure 1, A, B). No significant difference was recorded between IBDV-infected and non-infected groups in terms of Simpson's and Shannon's metrics (Monte Carlo permutations (999), (p=0.85 and 0.43), respectively. The number of observed OTUs per sample ranged from 3082 to 1531. The Good's coverage of the sequenced samples ranged from 95 to 97.5%.

The beta diversity between samples was investigated. By studying the unweighted and weighted beta diversity, it was noted that most of the non-infected samples clustered together away from the PC sample (Figure 2, A, B). The impact of using treatment during IBD infection on the overall microbial community diversity between samples was investigated. There was a significant difference between infected treated and infected non-treated samples (PERMANOVA, p=0.058 and ANOSIM, p=0.07).

The microbiome profiles of all groups were investigated. Eight phyla were observed: *Firmicutes, Bacteroidetes, Actinobacteria, Proteobacteria, Tenericutes, Cyanobacteria, Fusobacteria,* and *Lentisphaerae,* which *Firmicutes* dominated. At 3 dpi there was an increase in relative abundance of the phylum *Actinobacteria* and a decrease in the phylum *Bacteroidetes* in PC group (2.18%, 0.48%, respectively), compared to the NC group (0.05%, 24.22%, respectively), while at 8 dpi both phyla increased as PC (1.19%, 0.46%, respectively), and NC (0.39%,0.41%, respectively). At 8 dpi, phylum

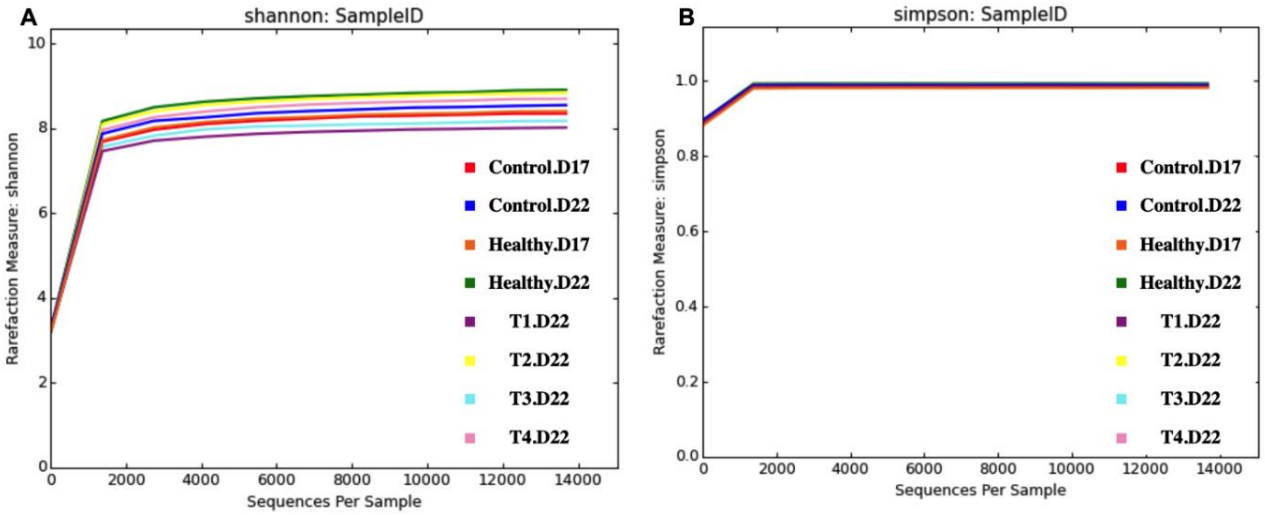

Figure 1: Rarefaction curves of alpha diversity metrics were assessed for the sequenced samples. (A) Shannon's metrics and (B) Simpson's metrics.

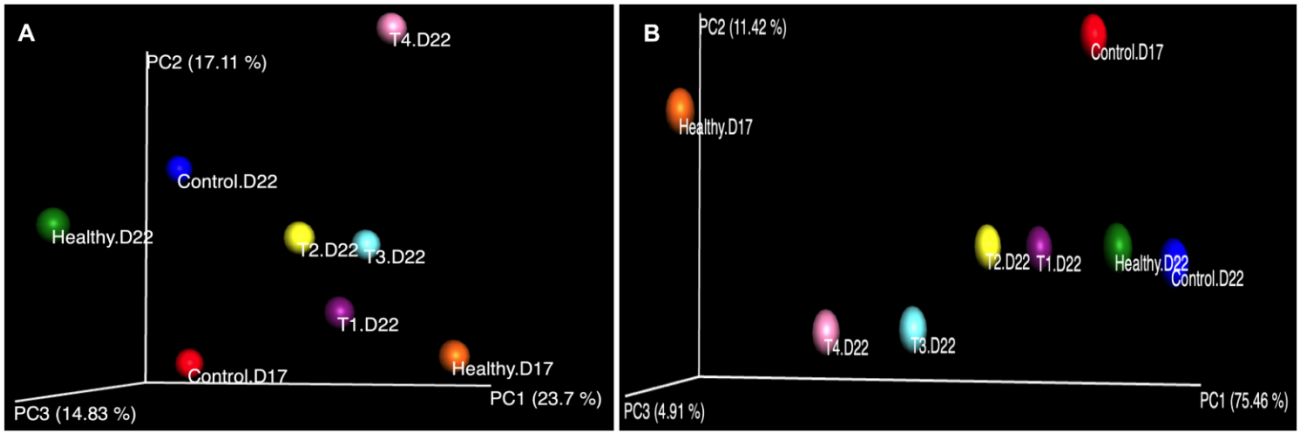

Figure 2: Principal coordinates analysis (PCoA) demonstrating the beta diversity evaluated using the unweighted UNIFRAC (A) and weighted UNIFRAC (B) methods. Red: positive control (PC) at day 17, blue: positive control (PC) at day 22, orange: negative control (NC) at day 17, green: negative control (NC) at day 22, purple: T1 (*Enterococcus faecium*), yellow: T2 (*Saccharomyces Cerevisiae*), aqua: T3 (organic acids) and pink: T4 (symbiotic).

Firmicutes increased in the PC (96.45%) and T1 group (97.03%) but decreased in all the infected treated groups compared to the NC group (96.27%) with the following values T2 (91.22%), T3 (86.75%), and T4 (71.93%) (Figure 3, A). In addition, there was a non-significant increase (p=0.064) in the relative abundance of phylum *Bacteroidetes* in the IBD infected-treated group, and the positive control group (11.12%, 0.47%, respectively) and in the T1, T2, T3, and T4 groups were (1.56%, 5.64%, 11.45%, 25.83%) compared to the PC group. There was a non-significant decrease (p=0.064) in the relative abundance of the phylum *Actinobacteria* in the IBD infected-treated groups and the PC group (0.24%,1.68%, respectively) and in the T1, T2, T3, and T4 groups were (0.21%, 0.37%, 0.18%, 0.18%) compared to the PC group.

The most predominant bacterial orders that were observed in all tested samples were orders *Clostridiales, Lactobacillales, Bacteroidales,* and *Erysipelotrichales* (Figure 4). At 8 dpi, IBD infection was accompanied by an increase in the relative abundance of order *Lactobacillales, Coriobacteriales,* and order *RF32* of class *Alphaproteobacteria* in the PC group (49.98%, 0.93%, 0.23%, respectively), compared to the NC group (40.66%, 0.37%, 0.02%, respectively). In contrast, the relative abundance of the order *Clostridiales* and order *Erysipelotrichales* decreased in the PC group (45.32% and 1.02%, respectively) compared to the NC group (54.26% and 1.23%, respectively). In infected treated groups, the relative abundance of order

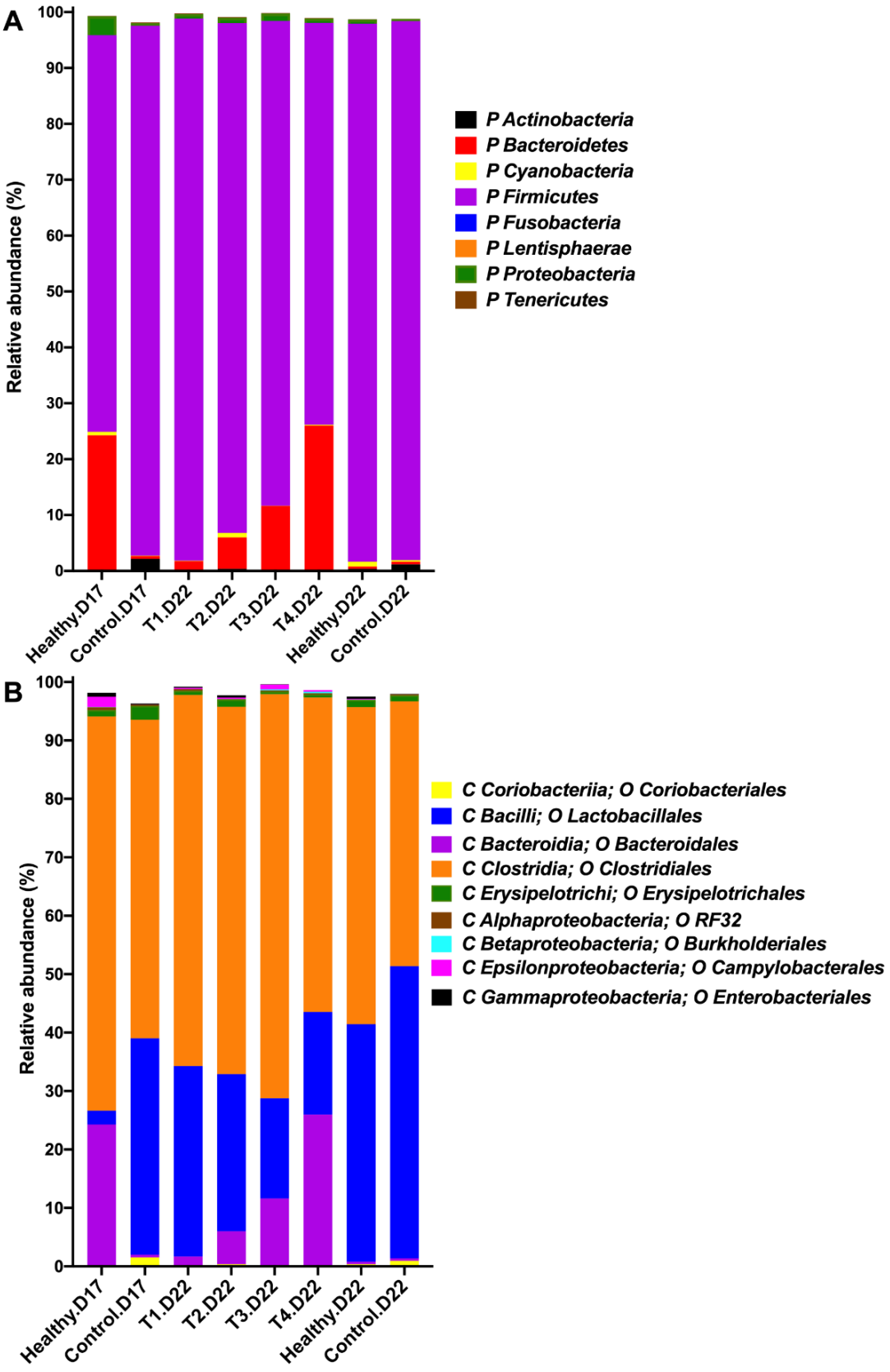

Figure 3: The relative abundance of the most dominant bacteria in sequenced cecal samples of different groups at 17 days old (3 dpi) and 22 days old (8 dpi). (A) At phylum level and (B) At class level.

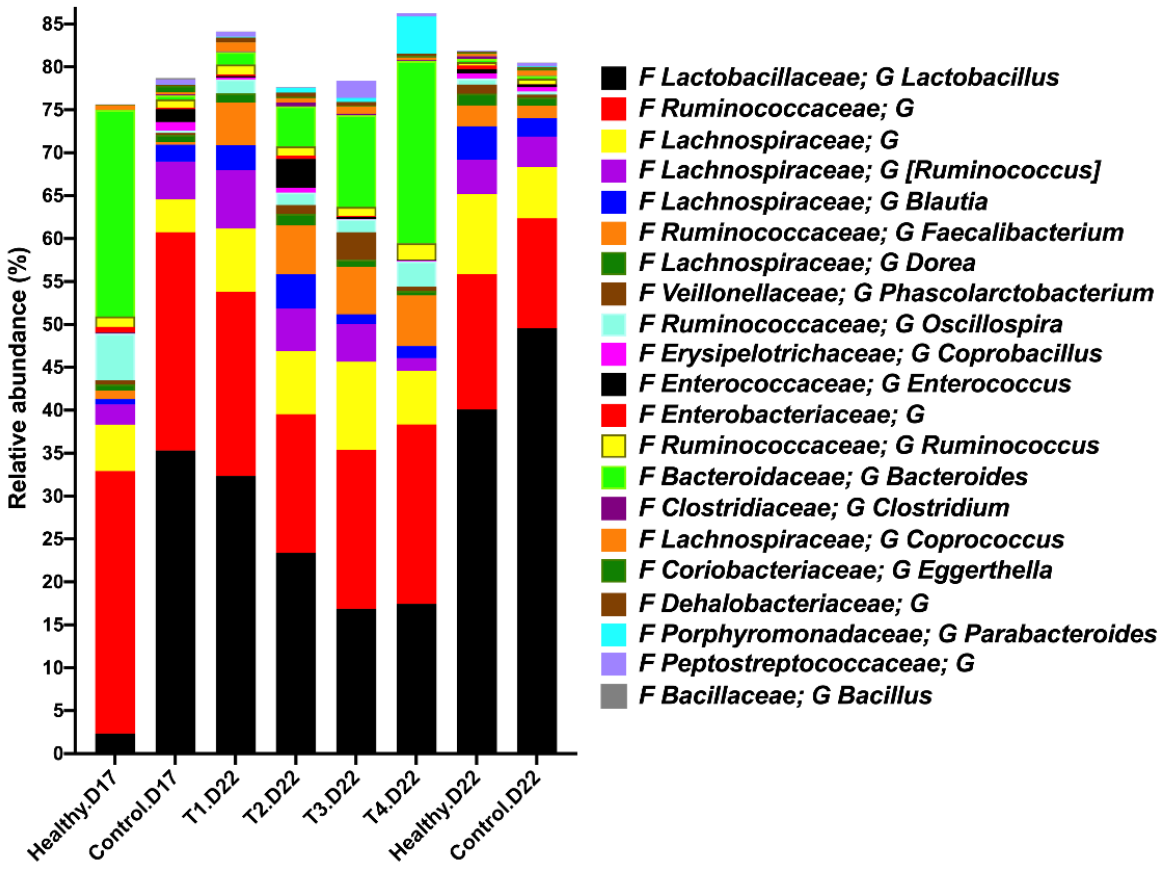

Figure 4: The relative abundance of the most dominant bacterial genera in sequenced cecal samples of different groups at 17 days old (3 dpi) and 22 days old (8 dpi).

Lactobacillales, order *RF32* of class *Alphaproteobacteria* and order *Coriobacteriales* decreased in all the treated groups compared to the NC group as T1 (32.61%, 0.3%, 0.11%), T2 (26.89%, 0.08%, 0.36%), T3 (17.12%,0.18%, 0.18%) and T4 (17.55%, 0.16%, 0.17%), respectively. In comparison with the NC group, the relative abundance of order *Erysipelotrichales* decreased in all infected treated groups except the T2 group as T1 (0.69%), T2 (1.24%), T3 (0.45%), T4 (0.55%). While the relative abundance of the order *Clostridiales* increased in all infected treated groups except the T4 group compared to the NC group as T1 (63.5%), T2 (62.87%), T3 (69.17%), T4 (53.81%) (Figure 3B).

Generally, IBDV-infected samples showed a decrease in order *Clostridiales* (58.19%), order *Bacteroidales* (7.58%) and order *Erysipelotrichales* (1.06%) compared to non-infected samples (60.87%, 12.32%, 1.15%, receptively), with a non-significant difference (p>0.05). While IBD infection caused an increase in order *Lactobacillales* (30.19%), *Coriobacteriales* (0.548%), *Bacillales* (0.254%), *Actinomycetales* (0.167%), order *Burkholderiales* (0.081%) of class *Betaproteobacteria,* order *Pseudomonadales* (0.002%) of class *Gammaproteobacteria*, two orders of phylum *Tenericutes* (order *RF39* and order *Anaeroplasmatales*) (0.077%, 0.027%, receptively), and order *Streptophyta* (0.006%) of phylum *Cyanobacteria* compared to non-infected samples as (21.5%, 0.21%, 0.229%, 0.008%, 0.046%, 0 %, 0.048 %, 0.002%, 0 %, receptively), with no significant difference (p>0.05).

At age 22 days, in both NC and PC groups the most abundant family was *Lactobacillaceae* (40.09%, 49.59%) followed by *Ruminococcaceae* (19.36%, 15.27%), *Lachnospiraceae* (19.15%, 13.33%) then an unidentified family from order *Clostridiales* (10.29%, 11.4%, receptively), see (Figure 5). As at 8 dpi, IBD infection in the PC group was accompanied by an increase in the relative abundance of the family *Lactobacillaceae* and an unidentified family from order *Clostridiales* and a decrease in the relative abundance of family *Ruminococcaceae* and family *Lachnospiraceae* compared to the NC group. In addition, the relative abundance of family

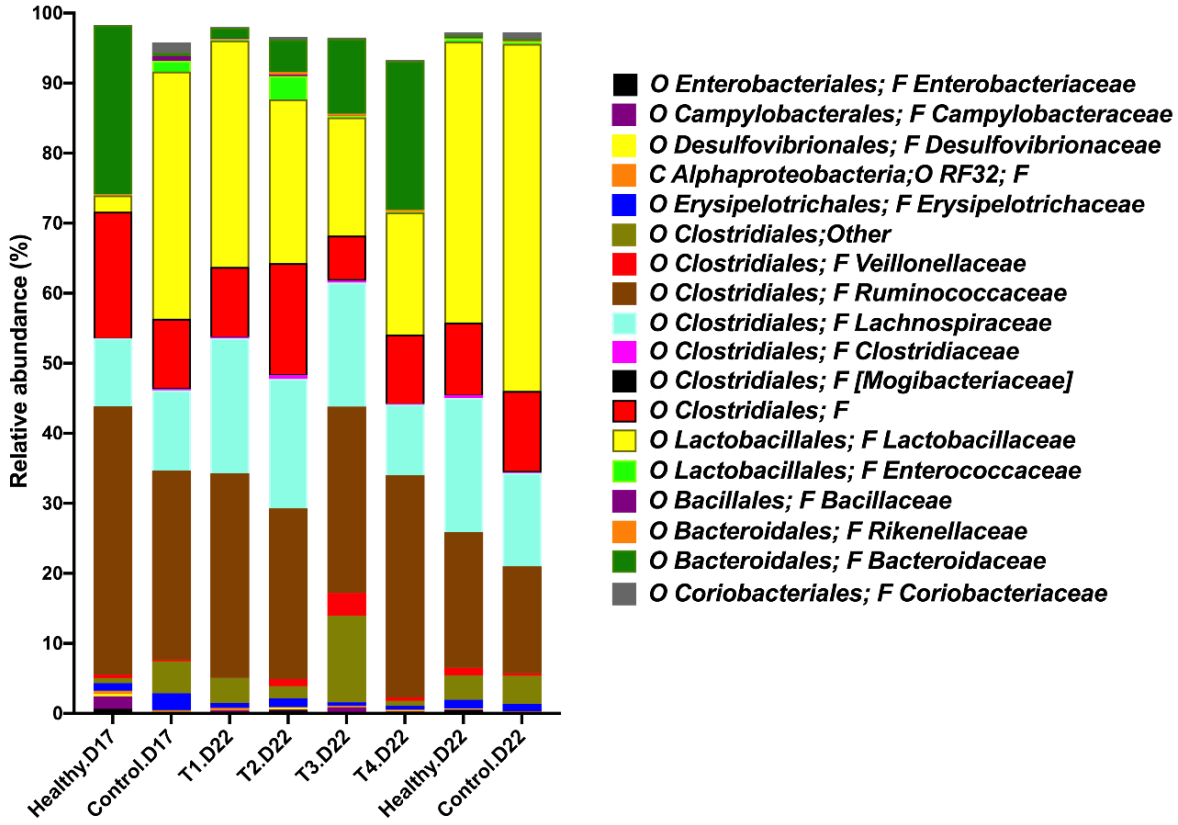

Figure 5: The relative abundance of the most dominant bacterial families in sequenced cecal samples of different groups at 17 days old (3 dpi) and 22 days old (8 dpi).

Bacteroidaceae was decreased in the PC group (0.31%) compared to the NC one (0.36%). On the other hand, the infected treated groups showed different microbiome profiles as family *Ruminococcaceae* and family *Bacteroidaceae* relative abundance increased in all IBD infected treated groups compared to NC group as T1 (29.21%, 1.45%), T2 (24.36%, 4.69%), T3 (26.6%, 10.68%) and T4 (31.71%, 21.23%) while the relative abundance of family *Lactobacillaceae* decreased as T1 (32.34%), T2 (23.38%), T3 (16.87%) and T4 (17.44%). In comparison to NC group, the relative abundance of family *Lachnospiraceae* decreased in all IBD treated infected groups except T1 (19.26%) as T2 (18.5%), T3 (17.72%) and T4 (10.07%), also unidentified family from order *Clostridiales* decreased in all IBD infected treated groups except T2 (15.82%) as T1 (9.89%), T3 (6.19%) and T4 (9.83%), see (Figure 5). The relative abundance of family *Rikenellaceae* increased in all IBD infected groups compared to the NC as NC (0.004%), T1 (0.015%), T2 (0.356%), T3 (0.290%), T4 (0.277%) and PC (0.029%). The relative abundance of family *Enterococcaceae* decreased in all infected groups except T2 (3.34%), which revealed a marked increase of this family compared to the NC group as NC (0.5%), T1 (0.19%), T3 (0.24%), T4 (0.277%) and PC (0.33%).

The studied groups were further examined at the genus level (Figure 5); at 8 dpi, IBD infection increased the relative abundance of genus *Ruminococcus* and genus *Coprococcus* in all IBD infected groups compared to the NC one as NC (0.4%, 0.27%), T1 (1.13%, 0.99%), T2 (1.07%, 0.52%), T3 (1.06%, 0.86%), T4 (1.02%, 0.31%), and PC (0.64%, 0.6%, receptively). The relative abundance of genus *Facalibacterium* and genus *Oscillospira* decreased in the PC group (1.48%, 0.36%) compared to the NC group (2.43%, 0.72) but both genera increased in infected treated groups compared to the NC and groups as T1 (4.98%, 1.6%), T2 (5.68%, 1.44%), T3 (5.52%, 1.49%) and T4 (5.91%, 2.96%). In addition, the relative abundance of genus *Blautia* and genus *Dorea* decreased in all IBD infected groups except T2 compared to the NC group as NC (3.89%, 1.37%), T1 (2.9%, 1.05%), T2 (4%, 1.37%), T3 (1.1%, 0.83%), T4 (1.38%, 0.53%), and PC (2.13%, 0.9%), receptively. At 8 dpi, the relative abundance of genus *Bacillus* and genus *Coprobacillus* decreased in all IBD infected groups compared to the NC group as NC (0.03%,

0.59%), T1 (0.01%, 0.19%), T2 (0.02%, 0.58%), T3 (0%, 0.06%) and T4 (0%, 0.06%) and PC (0.01%, 0.51%, receptively). However, the relative abundance of genus *Parabacteroides* and genus *Anaeroplasma* increased in all IBDV-infected groups compared to the NC group as NC (0.05%, 0%), T1 (0. 1%, 0.01%), T2 (0.6%, 0.01%), T3 (0.47%, 0.07%) and T4 (4.33%, 0.05%) and PC (0.09%, 0.02%, receptively). The relative abundance of genus *Eggerthella* increased in the PC group but decreased in all treated groups compared to the NC group as NC (0.18%), T1 (0.07%), T2 (0.15%), T3 (0.09%), and T4 (0.16%) and PC (0.32%).

Discussion

IBD is a viral disease that affects chickens and has a significant economic impact. It disrupts microbial equilibrium (dysbiosis) and alters the diversity of the intestinal microbiota (Li et al. 2018). This study examined the effects of probiotics and prebiotics on the cecal microbiota composition, clinicopathological picture of IBD infection, and growth parameters when used after IBD infection. In this study, all IBD-infected groups displayed clinical symptoms and IBDV PM lesions, whereas the treated groups exhibited less severe symptoms. Similar results were observed by (Ghetas et al., 2022; Mosa et al., 2024). The PC group noted a higher cumulative mortality rate than the T1 group, while there was no mortality in the other infected groups, which reflected the beneficial role of probiotics and prebiotics on birds' health and immunity (Abdel-Fattah et al., 2008). The bursal index calculations indicate mild bursal atrophy at 3 dpi and severe atrophy in all IBDV-infected groups at 8 dpi due to the destructive effect virus on bursal tissue before using the treatments (Eterradossi and Saif, 2019). The prebiotic and probiotic had a correction effect on the microscopical level of the bursa of Fabricius and its lesion score, but this effect was not observed macroscopically. Microscopically, at 8 dpi, IBD infection caused severe bursal microscopical lesions, which were due to viral replication in the bursa of Fabricius by targeting IgM+ B cells (Eterradossi and Saif, 2019) and were previously recorded by (Ahmed et al., 2023; Li et al., 2018). The infected treated groups showed less severe microscopical bursal lesions than the PC group, as the *E. faecium* (T1) group had the lowest bursal lesion score than the organic acids group (T3), *S. cerevisiae* (T2) and symbiotic (T4), receptively. These findings revealed that probiotics and prebiotics had a role in the improvement of bursal cell health and decreased the adverse effect of viral replication in bursa. It was recorded that *E. faecium* probiotic can improve the development of immune organs (Luo et al., 2013), and it was found that organic acids have a vital role in the stimulation of immune organs histologically (Mohamed et al., 2014).

Comparing the NC group with the infected groups' IBDV ELISA antibody titers increased significantly ten days after infection. This could be attributed to the beneficial effects of probiotics and prebiotics on immunity (Ghazalah et al., 2011; Abdel-Fattah et al.,2008).

Growth parameters expressed as FCR where the PC group showed poor FCR compared to NC one. These findings concur with (Getachew and Fesseha, 2020; Setta et al., 2024), who found that IBD infection has adverse effects on bird performance. Therefore, we studied the role of prebiotics, probiotics and symbiotics in improving the adverse effect of the IBD virus on bird performance. The best FCR in infected treated groups compared to PC was observed in organic acids (T3), then *E. faecium* (T1), and *S. cerevisiae* (T2) receptively. The positive effect of organic acids on bird performance was previously recorded (Yang et al., 2018). Additionally, organic acids raise pepsin activity, decrease ammonia synthesis, and decrease endogenous nitrogen losses, all of which improve the digestibility of proteins (Attia et al., 2018; Yang et al., 2018). Also, our findings concurred with those of Zheng et al. (2015), who discovered that FCR was improved by the addition of *E. faecium* to the diet.

The examination of intestinal lesions and villous height of the cecum revealed that IBD infection in the PC group caused severe significant pathological changes compared to the NC group; this finding agrees with (Li et al., 2018). The PC recorded the highest intestinal lesion score, which decreased in infected treated groups as the lowest lesion score observed symbiotic (T4), then *E. faecium* (T1), organic acids (T3), and *S. cerevisiae* (T2) receptively. Interestingly, the cecum villous length of all infected treated groups was higher than the PC group and was higher than the NC group in the case of organic acids (T3), symbiotic (T4) than *E. faecium* (T1), receptively. These findings confirm that prebiotics and probiotics have a crucial role in the maintenance of intestinal health, which

reflects on birds' performance and productivity. It has been documented that those organic acids increase the height of the intestinal villus and directly stimulate the proliferation of the intestinal cells (Adil et al., 2010) as short-chain fatty acids raise the expression of the glucose transporter (GLUT2), plasma glucagon-like peptide-2 (GLP-2) and ileal proglucagon mRNA, and proteins, all of which may be involved in the proliferation of gut epithelial cells (Adil et al., 2010). The symbiotic (T4) used in this study contains prebiotics and *L. acidophilus.* It was recorded that *L. acidophilus* can influence villus height, thus inducing small intestinal goblet cell hyperplasia to increase surface area for greater absorption of available nutrients (Chichlowski et al., 2007; Forte et al., 2018). Additionally, probiotics containing *E. faecium* have been shown to improve gut microvilli, affect the shape of the intestinal mucosa in chickens, and reduce mucosal inflammation (Luo et al., 2013). The small intestine morphology of broilers may benefit from dietary *E. faecium* probiotics (Royan 2018).

Eight phyla were observed in the cecum samples: *Firmicutes, Bacteroidetes, Actinobacteria, Proteobacteria, Tenericutes, Cyanobacteria*, *Fusobacteria,* and *Lentisphaerae*, which *Firmicutes* followed by *Bacteroidetes* dominated, these findings agreed with (Bindari and Gerber, 2022; Li et al., 2018). At 8 dpi, IBD viral infection caused a decrease in the relative abundance of phylum *Bacteroidetes* compared to the NC group; a similar finding was observed by (Daines et al., 2019). Interestingly, the using of prebiotics, probiotics, and organic acids after infection caused an increase in the relative abundance of phylum *Bacteroidetes* compared to the PC group. *Bacteroidetes* are important commensals in the chicken gut that play a significant part in the breakdown of complex carbohydrates and the production of short-chain fatty acids (SCFAs). They are particularly well-suited to the distal gut (Fan et al., 2023).

Moreover, at 8 dpi, IBD infection caused a decrease in the relative abundance of the family *Ruminococcacaea* in the PC group, while it increased in all IBDV-infected treated groups compared to the NC group; a similar result was observed previously with *Eimeria* inoculation (Wu et al., 2014). While the relative abundance of family *Lachnospiracaea* decreased in all infected groups except T1. Most bacteria in the families *Ruminococcaceaea* and *Lachnospiracaea* produce butyrate, which triggers the secretion of glucagon-like peptide 2 (GLP-2), maintaining the integrity of intestinal epithelial cells. As a result, a decrease in these families' numbers may be a sign of damage to the gut barrier (Bindari and Gerber, 2022). IBD infection caused an increase in the relative abundance of family *Rikenellaceae* in all infected groups compared to the NC group. Since mucin is essential for inhibiting the adherence of different pathogens and toxins found in the intestinal lumen (Macfarlane et al., 2005), *Rikenellaceae's* degradation of mucin would compromise the integrity of the intestinal mucosal barrier (Ruas-Madiedo et al., 2008). *Rikenellaceae* also uses mucin as a carbon and energy source (Bomar et al., 2011). In addition, the relative abundance of the family *Enterococcaceae* decreased in IBD infected PC group.

Interestingly, after 8 days of IBDV inoculation, we found that IBD infection decreased the abundance of two genera of family *Lachnospiracaea* (genus *Blautia* and genus *Dorea*), as these findings were observed in all infected groups except *S. cerevisiae* (T2). Genus *Blautia* has a role in fermentation and promotes the synthesis of lactate, butyrate, and succinate molecules that would give energy and reduce inflammation (Abaidullah et al., 2019) as well as it is important for scavenging free hydrogen released by many anaerobes during fermentation (Rychlik, 2020). While genus *Dorea* members are important acetic acid producers and have a vital role in regulating chicken growth (Zhu La et al., 2023). The relative abundance of genus *Faecalibacterium* and genus *Oscillospira* decreased in the PC group but increased in all IBDV-infected treated groups. Genus *Faecalibacterium* increases the synthesis of butyrate, succinate, and lactate, decreasing inflammation and supplying energy (Abaidullah et al., 2019), which reflects the positive role of prebiotics and probiotics in the correction of dysbiosis. Previously, the genus *Faecalibacterium* was reported to be decreased with human diseases (Heinken et al., 2014) and during exposure to heat stress in chickens (Shi et al., 2019). Genus *Coprococcus* is also a butyrate-producing member of the *Lachnospiraceae* family.

Conclusion

IBD disrupts the balance of the gut microbiota.

Also, it damages the integrity of the intestinal mucosa and depletes the bursa, all of which have an adverse effect on the immune system and FCR of chickens. Using organic acids and symbiotics after infection can reduce the negative effects of IBD by improving gut dysbiosis associated with the virus and reducing its detrimental effects on the performance of birds. We suggest using morphometric histology and metagenomic analysis of 16sr RNA gene sequencing more frequently for the study of the relation between different microbial infections and gut microbiome and the role of prebiotics and probiotics in the correction of the dysbiosis due to different infections.

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