



The Potential Diversity of Intestinal Enterobacteriaceae in Broiler Chickens is Associated with Infectious Bursal Disease Virus Infection

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THE aim of this work is to study the Enterobacteriaceae-related bacteria of the gut microbiota with infectious bursal disease virus infection (IBDV) in naturally and experimentally infected broiler chickens. From the field, samples were collected from 20 suspected Gumboro-infected and 5 apparently healthy farms. For the experimental study, 36-day-old chicks were allocated to 2 groups of 18 birds each, then at 14 days old, G1 was challenged via eye drop with IBDV strain MK088026, and G2 was kept as control negative. RT-PCR revealed that 75% of farms were IBD-positive. The aerobic bacterial counts in positive farms were significantly higher than in apparently healthy individuals. The bacterial counts on MacConkey, IBD-positive farms were significantly higher than apparently healthy. Also, positive farms showed significantly higher lactose and non-lactose fermenter counts than apparently healthy ones. Serologically, the bacterial isolates from positive farms were 5 serotypes of *E. coli* identified as *E. coli* O78, O127H6, O91H21, O159, and O1H7. On apparently healthy, 3 *E. coli* serotypes were recorded as O128 H2, O146H21, and O2H6. Also, in positive farms, the non-lactose fermenter was *Proteus mirabilis*, *Providencia rettgeri*, *Salmonella kentucky*, and *Salmonella typhimurium* while, in apparently healthy, 3 isolates were *Salmonella enteritidis*, *Salmonella larochella*, and *Salmonella typhimurium*. On the experimental level, the bacterial counts on different media in challenged birds were higher than control. The serologically identified bacteria in G1 were 3 *E. coli* serotypes (O26H11, O78, and O128H2) while in G2, 3 *E. coli* (O26H11, O78, and O55H7). Our results indicated that IBDV infection was associated with an increased number of Enterobacteriaceae-related bacteria in the chicken gut.

Keywords: Chickens; Enterobacteriaceae, *E. coli*; Gut; IBD; Microbiome; PCR; *Salmonella*

Introduction

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Infectious bursal disease (IBD) is a viral disease of young chickens which is also called Gumboro disease [1]. It is an acute and highly contagious immunosuppressive disease that is caused by IBD virus (IBDV), a non-enveloped virus belonging to the genus *Avibirna* virus and the family *Birnaviridae*

[2,3]. IBD is characterized mainly by severe lesions in the bursa of Fabricius (BF) causing fatal conditions with immunosuppression in chickens [4]. Since the first record of IBD in Egypt by El-Sergany in 1974 [5], Egypt's broiler farms have been very concerned about IBD. According to [4], the economic losses caused by this disease are either attributable to mortality or indirect losses linked to induced immunodeficiency, which may lead to problems with secondary infections and gut-associated diseases in the future and further economic losses in the poultry industry [6]. During the pathogenesis of IBD virus, the virus replicates in gut-associated lymphoid tissue causing microscopical lesions, immune cells alterations, and changes to intestinal microbial population [7,8].

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Chicken intestinal microbiota (CIM) is a complex ecosystem that has a vital role in the development of intestinal immunity, nutrition, physiology, and health [9,10]. At the phylum level, the CIM includes hundreds of bacterial species dominated [11]. Firmicutes, Bacteroidetes, Tenericutes, Proteobacteria, and Actinobacteria are the most predominant phyla of bacteria detected in the intestinal tract of chickens [9]. The members of Proteobacteria are mainly *Escherichia* and *Enterococcus* which have been found in the ileum [12,13]. Enterobacteriaceae normally constitutes a small proportion of the healthy human gut microbiota at 0.1–1% relative abundance [14], and due to their relatively higher tolerance of oxygen, they localized near the intestinal mucosa [15].

Intestinal inflammation leads to a reduction in butyrate-producing microbiota, which results in an increase in nitrate production and then the blooming of Enterobacteriaceae [16]. The alterations in the gut microbiota trigger dysbiosis so, the disruption of the intestinal eubiotic status can be considered a cause rather than simply a consequence of the chronic gut inflammation [17]. Also, an increase in the proportion of potentially harmful Proteobacteria, especially of the Enterobacteriaceae family has been reported in gastrointestinal (GI) inflammations [18,19]. Currently, the interaction between viruses and microbiota is an area of intensive research in human and other animal models [20]. However, the interaction between IBDV and the intestinal microbiota was investigated in a few research conducted under the experimental conditions as the microbiota composition was determined by molecular-based techniques [8,21]. Therefore, our objective was to investigate the effect of IBD virus on the intestinal aerobic Enterobacteriaceae as the model for microbiological study in commercial broiler chickens from farms naturally infected by IBDV and confirm our results by conducting experimental infection by very virulent IBD (vvIBD) virus in broiler chickens.

Material and Methods

Ethical approval:

The institutional animal care and use committee of the Faculty of Veterinary Medicine, University of Cairo, Egypt, ensured that the handling of chickens complied with all applicable laws (Vet CU 2009 2022526).

1. Field investigation:

On the base of clinical signs and PM lesions, a total of 25 chicken farms with capacity ranging from

The virus titration and propagated according to OIE [23] on Specific pathogen-free (SPF)

10000 to 25000 birds have been investigated (20 suspected broiler chicken flocks from different breeds with natural IBDV infection and five apparently normal chicken farms). The tested flocks with signs were confirmed to be positive by RT-PCR for IBDV infection. The investigated flocks were aged from 22-32 days, and located in 6 Egyptian governorates, including (Giza, Menofia, Mansura, Fayum, Behera and Menia) during 2022-2023.

Sample collection:

From each investigated flock, a total of 5 birds (freshly dead or living) were collected and hygienically transferred to the laboratory for further examination. Live birds ethically euthanized for bursal and intestinal sample collection.

2. Experimental investigation:

Chicks and managemental procedures:

Thirty-six one-day old commercial broiler chicks (Ross, mixed sex) were purchased from a certified local hatchery. The chicks were housed in sterilized units and fed *ad libitum* on commercial rations according to the NRC [22]. and given pelleted starter (Crude Protein “CP” not less than 23%) and growing (CP not less than 21%) rations. All birds were vaccinated by eye drop with ND clone 124 + IB H120 (POLIMUN[®], lot no. 1663), and NDV- Lasota (MEVAC[®], lot no.2206150401) at 6 and 20 days of life; respectively.

Experimental design:

At the 13th days old the 36 chicks were separated equally and randomly into two groups (18 birds/group). At 14 days old one group was infected via eye drop with 0.2 ml of 10⁴ EID₅₀ in 0.2 ml per bird [23] IBDV strain accession number (MK088026) “IBDV/Egypt/Qalubia/17” [24] and other group remain as control negative group administered the same dose of sterile saline with the same route and dose at the same age. At age 22 days three birds from each group were euthanized and samples from the small intestine specifically from the last third of the intestine were collected and directly transferred to the lab to count the aerobic and facultative anaerobic bacteria mainly *Enterobacteriaceae*. In addition, serum samples were collected and kept at -20°C from three birds in each group on days 1, 14, and 24 to determine antibodies titer against IBD virus.

Virus titration for experimental challenge and inoculum preparation

embryonated chicken eggs (ECEs) aged 11 days old by using the chorioallantoic-membrane (CAM)

method. The experimental strain titration was calculated using the endpoint titration method [25]. A titer of 10^4 (EID)₅₀ was used to infect the birds. The virus had been stored at -80°C [23].

Sample collection for lab work:

Samples from both field and experimental birds were collected and used as follows:

1. Intestinal content:

About 1 gm of the intestinal content from the middle part of ileum of 5 birds/flock were harvested individually from each bird (25 farms X 5 birds = 125 intestinal samples). The samples were kept under cooling till used For Counting isolation, and identification of Enterobacteriaceae.

2. The bursa of Fabricius:

The bursa of Fabricius was collected from each bird in phosphate-buffered saline (PBS) then transported to the laboratory in an icebox for the detection of IBDV using RT-PCR. All the collected bursal samples were kept at -20°C until usage for molecular detection of IBDV.

TABLE 1. Nucleotide sequence of PCR primers (specific for Segment A, VP2 gene)L

Primer	Sequence	Length	Reference
Forward	5'-TGT-AAA-ACG-ACG-GCC-AGT-GCA-TGC-GGT-ATG-TGA-GGC-TTG-GTG-AC-3'	604 bp	[23]
Reverse	5'-CAG-GAA-ACA-GCT-ATG-ACC-GAA-TTC-GAT-CCT-GTT-GCC-ACT-CTT-TC-3'		

d. RT-PCR amplification:

The PCR reaction was performed in a total volume of 50 μL per sample, containing 5 μL of extracted RNA, 25 μL of 2x RT-PCR buffer, 1 μL forward primer, 1 μL reverse primer and nuclease-free water to a final volume of 50 μL . PCR thermocycler was programmed as follows: RT reaction for 20 minutes at 50°C ; initial denaturation at 95°C for 15 minutes; followed by 40 cycles of denaturation at 95°C for 30 sec, annealing at 59°C for 30 sec and extension at 72°C for 1 minute; then one cycle of final extension at 72°C for 5 minutes.

Detection of IBDV-antibodies in the experimentally inoculated chicks.

Ten serum samples were collected from one-day-old chicks to determine maternally derived antibodies (MDA) for choosing the proper time of IBD inoculation and Three serum samples per group were collected from two-week-old chicks and ten days post-infection. The detection of IBDV-antibodies was performed using ELISA test. The ELISAs were performed using a commercially available kit (iD.Vet kit) according to the guidelines

Molecular detection of IBD by RT-PCR:

a. Preparation of tissue samples:

By using a sterile mortar and pistol, collected bursa of Fabricius samples (five from each farm) have been pooled and ground [23]. With sterile saline solution, a 20% (w/v) suspension was made. The suspensions were centrifuged for 20 minutes at a rate of 2000 rounds per minute (rpm) then supernatants were collected and stored at -20°C until use.

b. Extraction of nucleic acid:

Following the manufacturer's instructions, 300 μL of the supernatant was utilized to extract the total viral nucleic acid using the viral RNA/DNA extraction kit (Applied biotechnology).

c. Primer's oligonucleotides:

VP2 is the target gene of a set of primers used in the RT-PCR for the detection of the IBDV (Table 1). The IBD strain IBDV/Egypt/Qalubia/17" with an accession number of (MK088026) recovered and identified by Elsamadony [24] was used as positive control.

of the manufacturer. Sample to positive (S/P) and titer values were derived using optical density measurements of the samples and the positive and negative control sera at wavelength 450 nm.

Detection of intestinal Enterobacteriaceae:

a. Colony count:

To create a representative sample, the collected intestinal contents of IBD positive samples were processed and pooled. One gram of that pooled homogenate into sterile test tubes containing 9 mL of 0.1 percent sterile buffered saline (10^{-1} dilution) and subsequent dilutions up to 10^{-8} were generated. To count the aerobic and facultative anaerobic bacteria mainly Enterobacteriaceae, 200 μL of the 10^{-2} to 10^{-8} were transferred into two petri dishes (100 μL per each); one contains nutrient agar and the other has MacConkey agar and incubated at 37°C for 24 hours. A plate that has less than 250 colonies is considered countable. The averages of the colony forming unit (CFU) were transformed into log CFU/gram [26].

b. Purification of bacterial isolates:

Each bacterial colony from MacConkey agar was morphologically described and then picked up to be cultured on a new MacConkey plate by streaking method to have a single pure colony of lactose fermenter or non-lactose fermenter bacteria.

c. Selective media:

Suspected isolates of *Escherichia coli* (*E. coli*) bacteria were further cultured on eosin methylene blue (EMB) media, and suspected isolates of salmonella were cultured on Xylose Lysine Deoxycholate (XLD) agar and salmonella shigella (SS) agar media.

d. Identification of bacterial isolates:

The obtained isolates were morphologically a Gram stain identified by using microscopical examination [27]

as well as motility test. For Biochemical identification, Indole, Methyl Red Test, Voges – Praskauer test, Citrate utilization test, Urease test, Hydrogen sulfide production test, Gelatin hydrolysis test Oxidation–Fermentation test, Nitrate reduction test, detection of Ornithine decarboxylase (ODC), Detection of L- lysine decarboxylase (LDC), Detection of Arginine decarboxylase (ADH), Detection of β - galactosidase (ONPG) and Fermentation of sugars by following previous studies [28].

c. Serological identification of Enterobacteriaceae isolates:

The *E. coli* isolates were serologically identified according to the author [29] by using rapid diagnostic *E. coli* antisera sets (DENKA SEIKEN Co., Japan) for diagnosis of the Enteropathogenic types. Serological identification of salmonellae was carried out according to Kauffman – White scheme [30] for the determination of Somatic (O) and flagellar (H) antigens using *Salmonella* antiserum (DENKA SEIKEN Co., Japan).

Statistical analysis:

Bacterial count data are represented as mean \pm standard deviation. Statistical analysis started by validating the assumptions of normal distribution and homogeneity of variance. Then, differences in bacterial count between IBD-positive and IBD-negative farms were tested using an independent sample t-test. All statistical analysis and graphs were produced using RStudio-2023.06.1-524 [31] and R programming language v4.3.1 [32]. *p*-values < 0.05 were considered statistically significant.

Results**Clinical signs, mortalities, and PM lesions:**

Clinical signs and PM in 20 investigated suspected field cases, the observed clinical signs were depression, ruffled feathers, whitish diarrhoea, dehydration and a high mortality rate (15-20%) among the chicken flocks analysed in the present study. However, the other apparent healthy 5 farms showed neither clinical signs of illness nor mortalities.

The PM findings in all clinically diseased birds had lesions of petechial hemorrhages on thigh and chest muscles, swollen kidney with accumulation of ureates in ureters. Hemorrhagic enlargement of BF, some had yellowish gelatinous exudates, and some showed bursal atrophy. While the apparently healthy five farms had no PM changes.

IBD experimentally infected group of birds, the IBDV-infected group showed the same symptoms of field investigation but with 11 % mortality. In addition, the PM findings revealed that the IBD-infected group had lesion of muscular hemorrhages and bursal lesions as hemorrhage, yellowish exudates at 3-day post-infection (dpi), and bursal atrophy was observed at 8 days post the experimental infection.

RT-PCR testing revealed that 15 out of 20 (75%) examined samples were positive by RT-PCR which produced a band of 604 bp corresponding to the partial amplification of VP2 gene of IBDV (Figure 1).



Fig. 1. Visualization of 604-bp PCR product of VP2 gene of IBDV by agarose gel electrophoresis (1.5%) after staining with ethidium bromide. Lane M: 100bp DNA ladder, Lanes 2, 3, 4, 5, 7, 8, 9, 11: positive samples, Lane 12: Negative control, Lane 1: Positive control.

The ELISA test of antibody titer against IBD virus revealed that the one-day-old chick had MDA ranging from 2107 to 10305 which declined after two weeks to range from 126 to 1671 after 10 days post

infection of the positive group with IBD virus the IBD antibody titer increased and ranged from 2508 to 5256 while IBD antibody titer of negative group ranged from 1 to 171 (Table 5).

TABLE 5. Antibody titer against IBD virus by ELISA in experimental infection

	IBD antibody titer			
	One day old (n=10)	14 days old (n=3)	24 day old (10 days PI)	
			Negative group (n=3)	Positive group (n=3)
Minimum	2107	126	1	2508
Maximum	10305	1671	171	5256
Geometric mean	5263.7	705.2	5.5	3638.1

MDA: maternally derived antibody.

PI: post infection

Mean aerobic bacterial count on nutrient agar \log_{10} (Table 2). The aerobic bacterial count in IBD-infected 20 farms ranged from 8.1 ± 0.96 to 10.69 ± 0.69 with a mean of 9.31 ± 0.79 . However, in the case of apparently healthy farms, the bacterial counts ranged from 6.95 ± 0.29 to 7.84 ± 0.21 with a mean of 7.38 ± 0.36 . In addition, by using statistical analysis on bacterial count there is a significant difference between bacterial count on nutrient agar in the case of IBD-infected farms and IBD non-infected farms at p -values < 0.001 (Figure 2).

Moreover, the mean of aerobic bacterial count on MacConkey agar, lactose fermenter bacterial and non-lactose fermenter bacteria in form of \log_{10} are shown in Table 2. The total bacterial count on MacConkey in the case of IBD infected farms is from 7.42 ± 0.51 to 10.21 ± 0.46 with mean of 8.85 ± 0.79 while the bacterial count in case the apparently healthy farms is from 7.08 ± 0.14 to 7.75 ± 0.21 with mean of 7.35 ± 0.25 . There is a significant difference

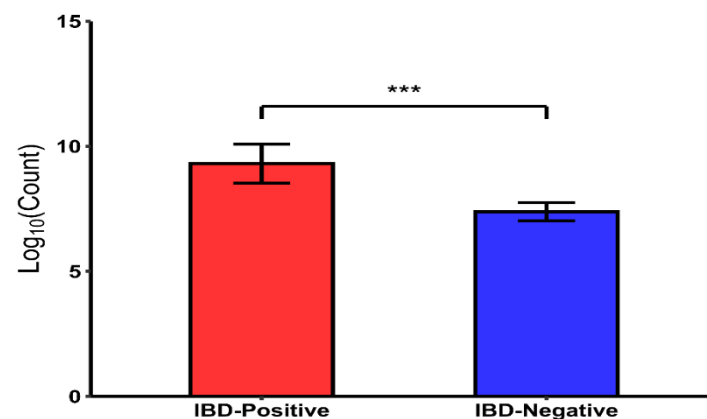
between bacterial count on MacConkey agar in case of IBD infected farms and IBD non infected farms (p -values < 0.001) (Figure 3).

In IBD infected farms the count of lactose fermenter bacteria is ranged from \log_{10} 7.42 ± 0.51 to 9.74 ± 0.65 with a total mean of 8.78 ± 0.73 (Table 2), while in apparently healthy farms is ranged from \log_{10} 7.08 ± 0.14 to 7.53 ± 0.08 with mean of 7.23 ± 0.18 which is significantly lower than in case of IBD infection (p -values < 0.001) (Figure 4). In addition, the non-lactose fermenter bacterial count in infected farms with IBDV is ranged from 0 ± 0 to 9.94 ± 0.34 with mean of 8.56 ± 0.84 (Table 2) but in case of the apparently healthy farms which were ranged from 6.23 ± 0.33 to 7.69 ± 0 with mean of 6.80 ± 0.59 (Table 2). However, the bacterial count of infected farms is statistically significantly higher than the apparently healthy farms (p -values < 0.001) (Figure 5).

TABLE 2. Bacterial count on Nutrient agar, MacConkey agar, lactose fermenter bacteria and non-lactose fermenter bacteria in IBD naturally infected and non-infected birds, (\log_{10} mean \pm SD).

Farm No.	Status	Nutrient agar Mean \pm SD	MacConkey agar Mean \pm SD	lactose fermenter bacteria Mean \pm SD	Non-lactose fermenter bacteria) Mean \pm SD
1.	IBD clinical signs positive / PCR positive	10.65 \pm 0.73	10.03 \pm 0.45	9.66 \pm 0.29	9.47 \pm 0.58
2.		9.96 \pm 0.98	9.55 \pm 0.96	9.12 \pm 0.99	9.34 \pm 0.95
3.		10.47 \pm 0.37	9.66 \pm 0.52	9.64 \pm 0.55	8.3 \pm 0
4.		8.57 \pm 0.77	8.16 \pm 0.73	8.01 \pm 0.83	7.53 \pm 0.43
5.		9.36 \pm 0.65	9.02 \pm 0.5	8.96 \pm 0.43	9 \pm 0
6.		8.31 \pm 0.35	8.22 \pm 0.46	8.22 \pm 0.46	0 \pm 0
7.		9.56 \pm 0.84	9 \pm 0.82	8.91 \pm 0.86	8 \pm 0
8.		8.64 \pm 0.6	7.42 \pm 0.51	7.42 \pm 0.51	0 \pm 0
9.		8.59 \pm 0.86	8.07 \pm 0.96	8.1 \pm 0.44	0 \pm 0
10.		8.92 \pm 0.15	8.22 \pm 0.1	8.22 \pm 0.1	0 \pm 0
11.		8.1 \pm 0.96	7.59 \pm 0.77	7.59 \pm 0.77	0 \pm 0
12.		9.81 \pm 0.59	9.75 \pm 0.66	9.74 \pm 0.65	8.3 \pm 0
13.		9.68 \pm 0.28	9.38 \pm 0.22	9.53 \pm 0.59	7.47 \pm 0
14.		8.75 \pm 0.21	8.51 \pm 0.12	8.51 \pm 0.12	0 \pm 0
15.		9.27 \pm 0.65	8.65 \pm 0.06	8.65 \pm 0.06	0 \pm 0
16.	IBD clinical signs positive / PCR negative	10.69 \pm 0.69	10.21 \pm 0.46	9.84 \pm 0.61	9.94 \pm 0.34
17.		9.65 \pm 0.73	9.16 \pm 0.94	9.13 \pm 0.92	7.8 \pm 1.2
18.		9.61 \pm 0.39	9.13 \pm 0.9	9.03 \pm 0.76	8.97 \pm 0.98
19.		9.07 \pm 0.45	9.05 \pm 0.28	9.05 \pm 0.28	0 \pm 0
20.		8.38 \pm 0.12	8.18 \pm 0.15	8.18 \pm 0.15	0 \pm 0
21.	Apparently healthy (PCR negative)	6.95 \pm 0.29	7.24 \pm 0.23	7.2 \pm 0.22	6.23 \pm 0.33
22.		7.59 \pm 0.11	7.39 \pm 0.3	7.16 \pm 0.38	7.08 \pm 0.29
23.		7.84 \pm 0.21	7.75 \pm 0.21	7.53 \pm 0.08	7.69 \pm 0
24.		7.42 \pm 0.23	7.27 \pm 0.35	7.14 \pm 0.37	6.39 \pm 0.61
25.		7.09 \pm 0.63	7.08 \pm 0.14	7.08 \pm 0.14	6.57 \pm 0.69

SD: standard deviation

**Fig. 2. Bacterial count in \log_{10} on nutrient agar in IBD naturally infected and non-infected chickens. Data are expressed as mean \pm standard deviation.**

***Asterisks indicate significant differences between them (p-values < 0.001)

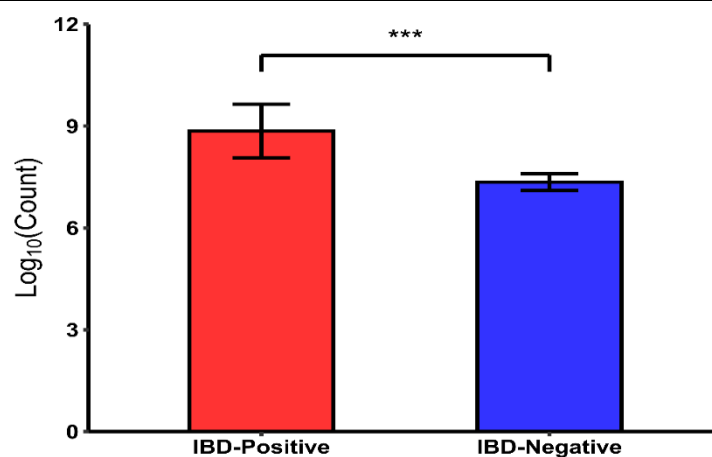


Fig. 3. bacterial count in log₁₀ on MacConkey agar in IBD naturally infected and non-infected chickens, Data are expressed as mean \pm standard deviation.

***Asterisks indicate significant differences between them (p-values < 0.001).

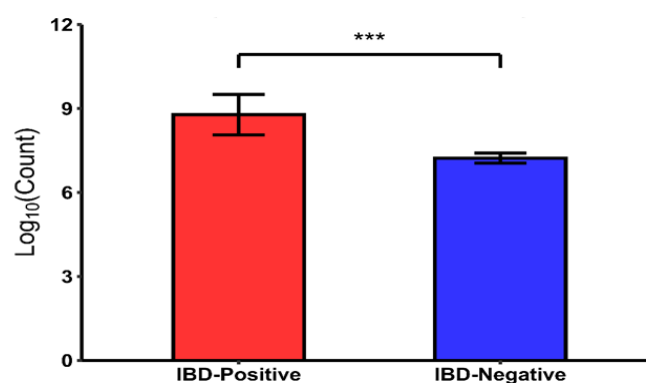


Fig. 2. Bacterial count in log₁₀ of lactose fermenter bacteria on MacConkey agar in IBD naturally infected and non-infected birds, Data are expressed as mean \pm standard deviation.

*** Asterisks indicate significant differences between them (p-values < 0.001).

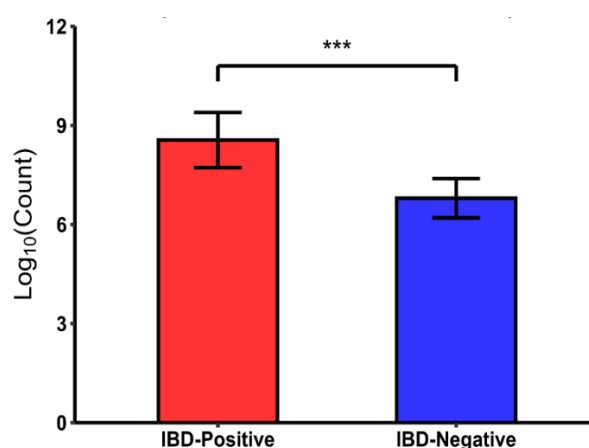


Fig. 3. Bacterial count in log₁₀ of non-lactose fermenter bacteria on MacConkey agar in IBD naturally infected and IBD non infected birds, Data are expressed as mean \pm SD.

***Asterisks indicate significant differences between them (p-values < 0.001).

Serologically, the bacterial isolates were identified. The prevalence of lactose fermenter bacterial isolates and non-lactose fermenter bacteria (Figures 6 and 7). Five serotypes of *E. coli* were identified as *E. coli* O₇₈, *E. coli* O₁₂₇H₆, *E. coli* O₉₁H₂₁, *E. coli* O₁₅₉ and *E. coli* O₁H₇ with a prevalence of 95%, 25%, 15%, 5% and 5% of the 20 investigated IBD infected farms, also *Enterobacter* bacteria was recorded with prevalence of 10%. However, three serotypes of *E. coli* were identified as *E. coli* O₁₂₈ H₂, *E. coli* O₁₄₆H₂₁ and *E. coli* O₂H₆

with prevalence of 40%, 40% and 20% of the 5 investigated apparently healthy farms, as seen in (Figure 6). In addition, the non-lactose fermenter bacteria were serologically identified as *Proteus mirabilis*, *Providencia rettgeri*, *Salmonella kentucky* and *Salmonella typhimurium* with a prevalence of 40%, 5%, 5% and 5% of the 20 investigated IBD infected farms while in apparently healthy farms three isolates were identified as *Salmonella enteritidis*, *Salmonella larochella* and *Salmonella typhimurium* with prevalence of 40%, 40% and 20% (Figure 7).

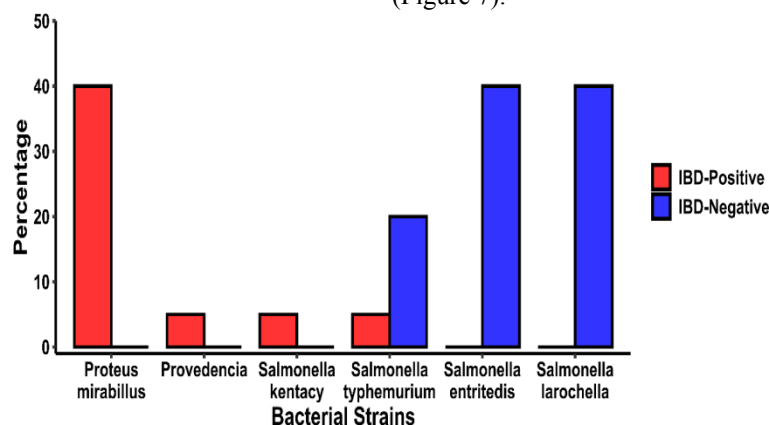


Fig. 4. Prevalence of lactose fermenter bacteria on MacConkey agar in IBD infected and non-infected birds.

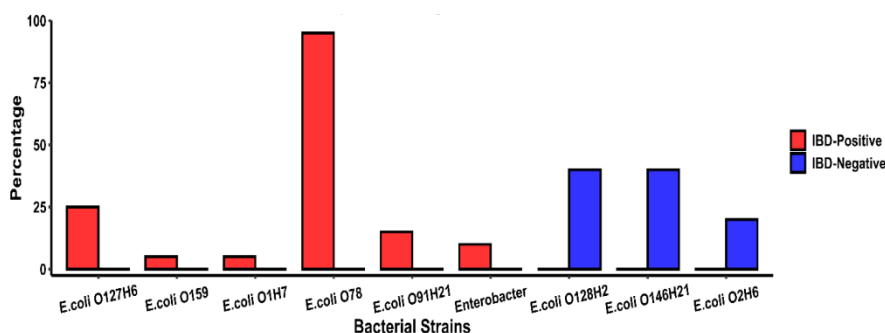


Fig. 5. Prevalence of non-lactose fermenter bacteria on MacConkey agar in IBD infected and non-infected birds.

The bacterial counts on Nutrient agar of IBD experimentally infected group in log₁₀ mean (7.45±0.76) was higher than in the case of control negative group (5.81±0.25), receptively see (Table 3). In addition, the facultative anaerobes count on MacConkey agar in IBD infected group was 6.35±0.25, higher than 5.5±0.13 in control negative group, respectively. lactose fermenter bacteria were 6.22±0.34 in the infected as compared with 5.5±0.13 in control group and non-lactose fermenter were detected in IBD infected group with 5.77±0.24. Moreover, by using serological identification, the bacterial isolates were identified as (Table 3).

Serological identification and bacterial count of lactose fermenter bacteria in IBD experimentally infected group were 3 *E. coli* types (O26H11, O78 and O128H2) with bacterial count in log₁₀ mean (6±0.26, 5.61±0.37, 5.36±0.58) receptively and one *Providencia rettgeri* (5.77±0.24), while control negative group showed only 3 *E. coli* (O26H11, O78 and O55H7) with bacterial count in log₁₀ mean (5.14±0.21, 4.3±0.49, 4.93±0.49) receptively see (Table 4).

TABLE 3. Bacterial count on Nutrient agar, MacConkey agar, lactose fermenter bacteria, and non-lactose fermenter bacteria in IBD experimentally infected and control negative groups, (\log_{10} mean \pm SD).

Treatment	Nutrient agar mean \pm SD	MacConkey agar mean \pm SD	lactose fermenter bacteria mean \pm SD	Non-lactose fermenter bacteria mean \pm SD
Negative group	5.81 \pm 0.25	5.5 \pm 0.13	5.13 \pm 0.13	0 \pm 0
Positive group	7.45 \pm 0.76	6.35 \pm 0.25	6.22 \pm 0.34	5.77 \pm 0.24

SD: standard deviation

TABLE 4. Serological identification and bacterial count of lactose fermenter bacteria in IBD experimentally infected group and control negative group, Data are expressed as \log_{10} mean \pm SD.

Identified bacteria	Strain characterization	Bacterial count (\log_{10} mean \pm SD)	
		Negative group	Positive group
<i>E. coli</i> O26H11	EHEC	5.14 \pm 0.21	6 \pm 0.26
<i>E. coli</i> O78	EPEC	4.3 \pm 0.49	5.61 \pm 0.37
<i>E. coli</i> O55H7	EPEC	4.93 \pm 0.49	0 \pm 0
<i>E. coli</i> O128H2	EPEC	0 \pm 0	5.36 \pm 0.58
<i>Providencia rettgeri</i>		0 \pm 0	5.77 \pm 0.24

Discussion

Infectious Bursal disease (IBD) causes immunosuppression and economic losses in the poultry industry [1,2]. In Egypt, IBDV has become endemic a half-century ago [1]. However, its economic problems have not been solved yet [33]. In addition, both vaccinated and not vaccinated chickens are vulnerable to IBDV infection opening the door to other opportunistic and destructive invaders [4].

Gut health is a crucial component of poultry production and has a big impact on a flock's overall health and performance [34]. When the mucosal intestinal barrier and gut immunity are compromised, the risk of gut infections and systemic infections is increased, which might have a detrimental effect on the bird's development [8,34]. Immunosuppressive disorders may impact the intestinal barrier by affecting the makeup of the microbiota and how the gut develops its responses [35].

A few data have recorded the effect of IBDV infection on the composition of gut microbiota therefore, this study intended to investigate the gut status in healthy and naturally IBDV-infected commercial broiler flocks. The clinically suspected IBDV-infected farms IBDV-infection was confirmed by RT-PCR. The 20 of the investigated flocks showed clinical manifestations of depression, dehydration, and whitish diarrhoea, and mortality ranged from 15-20%. These findings are in concur with [4,36]. In addition, the predominant necropsy findings included lesions of the bursa of Fabricius:

atrophy, enlargement, oedema, hemorrhages, and congestion with petechial and ecchymotic hemorrhages in the pectoral muscles. These lesions match with previously reported [2,4]. The clinically suspected IBDV-infected farms, the IBDV-infection was confirmed by RT-PCR which is a rapid, and specific molecular test for amplification of IBDV-VP2 in bursal tissue extract of IBDV infection in chicken flocks [2,4,5]. All tested flocks showed clinical pictures suspecting IBDV. However, 15 flocks out of 20 tested (75%) farms were PCR positive. These findings are like those of [37,38]. The examined bursa of different stages of the disease course may justify the rapid escape of the virus or virus clearance [39,40] or being at an undetectable level by molecular technique. As time of sampling (phase of infection) is an important factor that influences the level of success in IBDV detection during an infection [41].

ELISA test is one of most important serological tests to determine MDA of one-day-old chicks to choose the proper time of experimental infection [23] when MDA reaches the break-through levels of the virus [4]. which was 14 days old. In addition, IBD antibodies increased due to infection as an immunological response so by using ELISA we determined antibodies against IBD virus 10 dpi as we found an increase in titer compared to the negative group that is similar to [8].

GIT is recorded as the primary site of exposure to pathogens causing intestinal inflammation and subsequently causing microbial imbalance [42].

There is limited literature on dysbiosis caused by viruses, especially IBD in chickens. Interestingly, the authors in this study found that the mean of total colony counts on either Nutrient or MacConkey agar was significantly higher in the diseased flocks compared with the healthy ones. These outcomes may explain the suppressive effect of IBDV in the gut-associated lymphoid tissues leading to exacerbating the susceptibility to opportunistic and pathogenic bacterial agents, especially members of Enterobacteriaceae. Daines [21] found that at 3 days DPI by IBDV either a very virulent strain (UK661) of IBDV or a classical strain (F52-70) the infection results in an increase in the percentage of Enterobacteriaceae in the caecum also these findings were observed in chickens experimentally infected with H9N2 AIV [43]. In addition, these results are like the recorded findings in chickens experimentally infected with *Eimeria tenella* (*E. tenella*) infection in the cecum [44]. However, our finding did not match with [8] who found a decrease in the abundance of Enterobacteriaceae in cecum of the vvIBDV experimentally infected birds compared to virus-free controls [8]. The abundance of the phylum Proteobacteria has been recognized as an indicator of dysbiosis and disorder in humans [45]. The phylum Proteobacteria contains many opportunistic bacteria, including *Escherichia*, *Salmonella*, and *Proteus* [34]. Therefore, an increase in the abundance of Proteobacteria could be a helpful predictor of dysbiosis. More specifically family Enterobacteriaceae was found to increase in intestinal epithelial dysfunction [46,47]. This can explain our results as the counting of lactose fermenter and non-lactose fermenter bacteria was significantly higher in the IBD diseased farms compared with the healthy ones.

Using serological identification of lactose fermenter bacterial isolates from MacConkey agar we found that *E. coli* O78 was observed in 95% in investigated IBD infected farms which not observed in healthy farms. In addition, other serotypes was observed in IBD infected farms in lower prevalence as *E. coli* O127H6 25%, *E. coli* O91H21 15%, *E. coli* O159 5%, *E. coli* O1H7 5% and Enterobacter 10%. In contrast, other serotypes of *E. coli* was observed in healthy farms as *E. coli* O128H2, *E. coli* O146H21 and *E. coli* O2H6. That may explain as IBD infection may lead to increase certain serotypes of *E. coli* because of intestinal inflammation and dysbiosis. In addition, the non-lactose fermenter bacteria were serologically identified as *Proteus mirabilis* 40%, *Providencia rettgeri* 5%, *Salmonella kentucky* 5% and *Salmonella typhimurium* 5% of the 20 investigated IBD infected farms while in apparently healthy farms three isolates were identified as *Salmonella enteritidis*, *Salmonella larochella* and *Salmonella typhimurium* with prevalence of 40%, 40% and 20%.

To confirm our results and minimize the managemental variation between IBD-infected and non-infected chicken, an experimental infection with IBD virus clarify that the experimental condition has resulted in similar bacteriological finding to the field investigation, where the mean of total colony count on both Nutrient and MacConkey agar was higher in the IBD infected group compared with the control negative one (Table 2). Moreover, by using serological identification of bacterial isolates we found four isolates in IBD infected group *E. coli* O26H11, *E. coli* O78, *E. coli* O128H2 and *Providencia rettgeri*: 7 ± 0.26 , 6.57 ± 0.38 , 6.36 ± 0.58 , 6.68 ± 0.12 receptively. We also found *E. coli* O26H11, and *E. coli* O78 in control negative group but lower than in IBD infected group: 6.14 ± 0.21 , 5.63 ± 0.49 receptively. In addition, in control negative group only *E. coli* O55H7: 5.94 ± 0.49 was found, which was absent in IBD infected group therefore we suggest that the IBD virus makes intestinal inflammation as well as disturbance of microbiota equilibrium [8] which leading to expansion of facultative anaerobic bacteria of the family Enterobacteriaceae [48]. The fact that the IBD virus infection increased the relative abundance of the Enterobacteriaceae, particularly *E. coli*, is similar to the change of intestinal microbiota observed in H9N2 AIV [43]. infection in chickens as well as in Crohn's disease [49,50].

IBD virus cause increase in facilitative anaerobes mainly Enterobacteriaceae in both field condition and experimental condition. By comparing field results with experimental results, we found ten bacterial isolates, four of them were *E. coli* while in IBD experimentally infected group we detected four bacterial isolates, three of them were *E. coli* as well as serological findings revealed that *E. coli* O78 was found in IBD infected chickens in both field and experimental conditions. The variation between bacterial serotypes in field and experimental conditions may be due to the variable stressors in field conditions in comparison with experimental conditions.

Conclusion and Recommendation

Our results indicated that IBD virus infection was associated with increased numbers of Enterobacteriaceae-related bacteria in the gut of broiler chickens. Five genera were detected including *E. coli*, Enterobacter, *Proteus*, *Providencia*, and *Salmonella*. Most of these bacterial species and isolates are pathogenic. Therefore, prevention of IBD in broiler chicken is important to minimize losses due to both virus infection and increase population of gut with bacterial pathogens as well as their spread. further studies are suggested to evaluate the role of prebiotics and probiotics in the correction of gut dysbiosis related to IBD virus infection, as well as

alleviating the virus negative impacts on birds' health.

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Authors' contributions

M. I. M. and H.M.S. collected samples, experimental and laboratory investigations. M.M.A. and MAB supervised the work. All team members wrote, revised the original draft, and approved the final manuscript.

Data availability

The authors confirm that the data supporting the findings of this study are original, resulted from experimental work and available within the article [and/or] its supplementary materials.

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Competing interests:

The authors declare that they have no competing interests.

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التنوع المحتمل للبكتيريا المعوية في دجاج التسمين والمرتبطة بالعدوى بفيروس غده فبريشى المعدى

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لدراسة المكونات البكتيرية ذات الصلة بالبكتيريا المعوية في ميكروبات الأمعاء (GM) لدجاج التسمين المصاب بشكل طبيعي وتجريبي. في 20 مزرعة ظهرت عليها العلامات السريرية لمرض التهاب الأمعاء، تمت مقارنة معدل الوفيات والآفات بنسبة 20-15% مع 5 مزارع سليمة ظاهرياً. وفي الحالة التجريبية، ظهرت نفس الأعراض والآفات لدى المصابين بفيروس IBDV بنسبة وفيات 11%. أظهر اختبار RT-PCR أن 75% (20/15) من القطعان كانت إيجابية لمرض التهاب الأمعاء. كان عدد البكتيريا الهوائية في المزارع المصابة بـ 10.69 ± 0.69 (log10 8.1 ± 0.96) IBD بمتوسط إجمالي قدره 9.31 ± 0.79 (أعلى بكثير) القيم ($p < 0.001$) مقارنة بالمزارع السليمة ظاهرياً 7.84 ± 0.21 (log10 6.95 ± 0.29) بمتوسط 7.38 ± 0.36 (كان إجمالي عدد البكتيريا في المزارع المصابة بـ IBD في مأكوني 7.42 ± 0.51 : 10.21 ± 0.46 بمتوسط 8.85 ± 0.79) أقل بكثير من المزارع السليمة ظاهرياً (7.08 ± 0.14 : 7.75 ± 0.21 بمتوسط 7.35 ± 0.25). أظهرت المزارع المصابة بـ IBD متوسطاً أعلى بكثير لعدد مواد تخمير اللاكتوز وغير اللاكتوز قدره 8.78 ± 0.73 و 8.56 ± 0.84 مقارنة بالمزارع السليمة ظاهرياً 7.23 ± 0.18 و 6.80 ± 0.59 ؛ على التوالي.

مصلياً، تم تشخيص العزلات البكتيرية للمزارع المصابة بـ IBD بخمسة أنماط مصلية من *E. coli* O78 وهي *E. coli* O127H6 و *O127H6* و *O91H21* و *O159* و *O1H7* مع انتشار 95 و 25% و 15% و 5% و 5% من الحالات. 20، وكذلك كانت بكتيريا *Enterobacter* منتشرة بنسبة 10%. تم الكشف عن ثلاثة أنماط مصلية للإشريكية القولونية في الكائنات الصحية ظاهرياً *O128 H2* و *O146H21* و *O2H6* بمعدل انتشار 40% و 40% و 20% على التوالي. وكانت الكائنات المخمرة غير اللاكتوزية هي *Proteus mirabilis* و *Providencia rettgeri* و *Salmonella kentucky* و *Salmonella typhimurium* بنسبة انتشار 40%، 5%، 5% و 5% في المزارع المصابة بالـ IBD بينما في المزارع السليمة ظاهرياً تم تحديد ثلاث عزلات هي *Salmonella enteritidis*، *Salmonella larosilla* و *Salmonella typhimurium* بنسب انتشار 40، 40 و 20%. على التوالي.

كان عدد البكتيريا في أجار المغذيات وأجار مأكوني وبكتيريا تخمير اللاكتوز في الطيور المصابة بالـ IBD (متوسط log10 يعني 7.45 ± 0.76 و 6.35 ± 0.25 و 6.22 ± 0.34) أعلى من السيطرة السلبية (5.81 ± 0.24 و 5.5 ± 0.13 و 5.5 ± 0.13).، متقبلاً. مصلياً كانت البكتيريا في المجموعة المصابة بـ 3 IBD أنواع من الإشريشيا القولونية (*O26H11*، *O78* و *O128H2*) وواحدة من *Providencia rettgeri*، بينما أظهرت المجموعة السلبية 3 أنواع من الإشريشيا القولونية فقط (*O26H11*، *O78* و *O55H7*). أشارت نتائجنا إلى أن عدوى IBDV ارتبطت بزيادة عدد البكتيريا المرتبطة بالبكتيريا المعوية في أمعاء الدجاج. ولذلك، فإن الوقاية من مرض التهاب الأمعاء في الدجاج اللاحم أمر مهم لتقليل الخسائر الناجمة عن الإصابة بالفيروسات وزيادة عدد الأمعاء بمسببات الأمراض البكتيرية وكذلك انتشارها.

الكلمات الدالة: دجاج؛ البكتيريا المعوية، مرض التهاب غده فبريشى. القناة الهضمية. ميكروبيوم. PCR. الميكروب القولوني. *Salmonella*.