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Effect of omega-3 rich diet on the response of Japanese quails (*Coturnix coturnix japonica*) infected with Newcastle disease virus or avian influenza virus H9N2

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ABSTRACT

This study was performed to evaluate the effects of omega-3 supplementation on growth performance, clinical signs, post-mortem lesions, haemagglutination inhibition (HI) antibody titres, gene expression and histopathology in quails (*Coturnix coturnix japonica*) infected with Newcastle disease virus (NDV) and avian influenza virus (AIV) H9N2. One hundred, 40-day-old male quails were divided into 5 groups: G1, fed a control basal diet; G2A, infected with NDV; G2B, infected with H9N2; G3A, infected with NDV and given omega-3, and G3B, infected with H9N2 and given omega-3. The dietary omega-3 supplementation was continued for 4 weeks: two weeks before infection and two weeks after intranasal infection with virulent NDV and AIV H9N2. Our results revealed significant differences ($P < 0.05$) in growth performance, HI antibody titres, clinical signs, post-mortem lesions, mortality, viral shedding rates, immunological parameters, and histopathological lesions between the treated (G3A and G3B) and untreated (G2A and G2B) groups. In conclusion, dietary omega-3 supplementation for 4 weeks can improve growth performance and alleviate the deleterious immunological and pathological effects of NDV and AIV H9N2 infection in quails.

1. Introduction

Japanese quail (*Coturnix coturnix japonica*) is one of the smallest avian species reared for meat and egg production with high nutritive value. Quail rearing is characterized by rapid growth, can be sold at 5 weeks, and low feed intake (Ebeid et al., 2011; Ali et al., 2017). The feathers of adult Japanese quail are sexually dimorphic and adult male quails have a cloacal gland which secretes a white foamy material. Body weight of adult male quail varies from 160 to 250 g (Baer et al., 2015).

Poultry meat has high levels of polyunsaturated fatty acids but low levels of antioxidants (Grashorn, 2007). Hence, poultry meat undergoes lipid oxidation after storage, and lipid oxidation leads to the loss of nutritional value and the production of toxic compounds that reduce the meat quality and shelf life (Cortinas et al., 2005). Therefore, alternative methods to enrich poultry meat or eggs with omega-3 fatty acids and simultaneously reduce lipid oxidation are extremely important. Recently, the awareness of the effects of omega-3 fatty acids, including α-linolenic (LNA, 18:3 n-3), eicosapentaenoic (EPA, 20:5 n-3), docosapentaenoic acid (DPA, 22:5 n-3) and docosahexaenoic acid (DHA, 22:6 n-3), and their metabolites in poultry health has increased (Cortinas et al., 2005).

Quails were also found to be susceptible to natural infection with a

Abbreviations: LPAIV, low pathogenic avian influenza virus; NDV, Newcastle disease virus; vNDV, virulent Newcastle disease virus; IFN-γ, interferon-γ; IL, interleukin; EID50, 50% embryo infective dose; SPF, specific pathogen-free; HI, haemagglutination inhibition; ME VAC, Middle East for Veterinary Vaccines; NLQP, national laboratory for veterinary quality control of poultry production; DPI, days post-infection; BW, body weight; BWG, body weight gain; g, gram; min, minutes; s, seconds; SFA, saturated fatty acids; MUFA, monounsaturated fatty acids; PUFA, polyunsaturated fatty acids

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velogenic strain of NDV (Lima et al., 2004). Low pathogenic AIV (LPAIV) can infect different poultry species, including Japanese quails. Quails carry sialic acid receptors that are suitable for avian and human influenza virus binding; therefore, they act as a potential intermediate host (Wan and Perez, 2006). The first isolation of AIV H9N2 from commercial bobwhite quail (*Colinus virginianus*) in Egypt was performed by El-Zoghby et al. (2012).

Fish oil is a rich source of long chain polyunsaturated omega-3 fatty acid and responsible to reduce inflammation throughout the body. Inclusion of fish oil in diets at moderate levels can improve the antioxidative status and antibody response in Japanese quails (Ebeid et al., 2011). Moreover, Ali et al. (2017) found that the weight gain of broiler chickens challenged with AIV H9N2 and supplemented with 3% fish oil was significantly higher than the broiler chickens receiving feed with cooking oil and challenged with AIV H9N2.

The dietary supplementation of 0.5% flaxseed oil in chickens stimulates the immune response to NDV (Jameel et al., 2015). NDV produces a significant overexpression of different pro-inflammatory cytokines, such as TNF-α, IL-1β and IL-6, within 5 days of infection (Rajasekaran et al., 2019). Furthermore, Taseer et al. (2017) indicated that dietary supplementation of vitamin E plus omega-3 in chickens infected with LPAIV significantly reduces the histopathological lesions in the trachea and lungs.

In this study, we investigated the effect of omega-3 dietary supplementation on the growth performance, haemagglutination inhibition (HI), clinical signs, mortality rate, viral shedding rate, gene expression levels of immune genes, and histopathology in quails experimentally infected with NDV or AIV H9N2.

2. Materials and methods

2.1. Quails

One hundred male Japanese quails (*C. c. japonica*) (originating from the same hatching batch) were purchased from a commercial quail farm (Dakahlia, Egypt) that had no history of infection with NDV or AIV H9N2. The birds were kept in strictly isolated cages in a well-ventilated room and were provided with a balanced basal diet (NRC, 1994; Table 1). All hygiene and biosecurity features were considered. The birds did not receive any medications. Food and water were available to the birds *ad libitum*.

Table 1
Ingredients (%) and proximate composition of experimental diets.

Ingredients%	Control basal diet	Diet with omega-3 source
Yellow corn (8.5%)	55	55
Soybean meal (44%)	35.3	35.3
Corn gluten (62%)	6	6
Vegetable or Fish oil	0.7	0.7
Limestone	1.3	1.3
Dicalcium phosphate	1.00	1.00
Vitamin and mineral premix*	0.25	0.25
Salt	0.3	0.3
DL-methionine	0.1	0.1
Chemical composition		
CP%	23.89	23.89
ME (Kcal/Kg)	2914	2914
Ca%	0.84	0.84
Available P%	0.32	0.32
Analyzed fatty acids		
Myristic (14:0)	0.09	0.12
Palmitic (16:0)	12.50	11.49
Palmitoleic (16:1)	0.122	0.162
Stearic (18:0)	2.67	2.5
Oleic (18:1)	20.42	20.57
Linoleic (18:2 n-6)	56.17	55.5
α-Linolenic (18:3 n-3)	4.43	4.54
Archidonic acid (20:4n6)	0.00	0.5
Eicosapentaenoic acid (EPA, 20:n3)	0.00	2.25
Docosahexanoic (DHA, 22:n3)	0.00	1.34

In the present study, all birds were managed in accordance with the "Guide for the Care and Use of Laboratory Animals" that was approved by the Ethics Committee of the Faculty of Veterinary Medicine, Mansoura University. Embryonated egg isolation and HI testing were used to confirm the absence of ND, H9 or H5 viruses or antibodies in the birds (Iqbal et al., 2013).

2.2. Fatty acid analysis

Fatty acids profile of used oils and diet was determined. Samples of 250 µl from well mixed fresh samples of each of corn oil, and fish oil ($n = 6$) were resolubilized into 2 ml of boron trifluoride-methanol-hexane solution (35% boron trifluoride, 45% methanol, 20% hexane). The tubes containing the resolubilized oil samples were heated in a water bath (90–100 °C) for 60 min. After cooling, 2 ml of hexane and 2 ml distilled water were added. The samples were mixed and allowed to separate. The hexane (upper) layer was withdrawn. Two µl of hexane layer was taken for separation of fatty acids by gas chromatography. Fatty acid analysis was performed with focus gas chromatograph (Thermo-nicolet, USA) equipped with TR-5 fused silica column (30 mm × 0.25 mm i.d.). The initial oven temperature was set at 110 °C, held for 0.50 min and then increased by 20 °C/min to 190 °C, and held for 7 min, then increased by 5 °C/min to 210 °C and held for 8 min and increased by 20 °C/min to 230 °C and held for 0.2 min (Cherian and Sim, 1992). Inlet and detector temperatures are 250 °C. Helium was used as the carrier gas at a flow rate of 1.0 ml/min. Fatty acid methyl esters were identified by DSQ11 mass spectrometer.

2.3. Antigens and challenge strains

- **The AI H9N2 standard diagnostic antigen is derived from the strain** A/Chicken/Egypt/11490v/NLQP/2011 belonging to group B of G1-like lineage viruses. It is an LPAIV antigen. This antigen was obtained from Middle East for Veterinary Vaccines (MEVAC) and used for the HI test.
- **The LPAIV strain AIV H9N2** containing A/chicken/Egypt/Mansora-36/2015 (accession number KX663332), which was kindly provided by Eladl et al. (2019a), is an AIV H9N2 challenge strain related to group B of the G1-like lineage viruses. The titration of AIV H9N2 was performed in 10-day-old specific pathogen-free (SPF) embryonated chicken eggs and was calculated as previously described by Reed and Muench (1938). At 54 days of age, the quails were infected intranasally with a dose of 0.5 ml of 10^6 EID_{50}/bird (Nili et al., 2007; Arafat et al., 2018).
- **The velogenic strain of NDV (vNDV)** was kindly provided by Saif et al. (2017) and used as a virulent VIId genotype challenge virus. The nucleotide and amino acid sequences of this strain were deposited in the GenBank database with accession number MG257769. At 54 days of age, the birds were infected intranasally with a dose of 0.5 ml of 10^6 EID_{50}/bird (Lima et al., 2004; Sharawi et al., 2015; Igwe et al., 2018).

2.4. Experimental design

At 40 days old (start of the experiment), 100 male quails were divided into 5 groups (10 birds × 2 replicates per group) and reared for the 4-week duration of the experiment. The G1 group was kept as a negative control and fed a basal diet, the G2A group was infected with NDV, the G2B group was infected with H9N2, the G3A group was infected with NDV and given omega-3, and the G3B group was infected with H9N2 and given omega-3. Dietary omega-3 supplementation was continued for 4 weeks; two weeks before infection and two weeks after infection with the virulent NDV and AIV H9N2 strains. The code liver oil was the source of omega-3

Table 2
Fatty acid composition percentages of experimental oils used in quail diets.

Fatty acids	% of FA in supplemented oils	
	Corn oil	Cod liver oil
Myristic (14:0)	0.19	4.06
Palmitic (16:0)	12.13	10.88
Palmitoleic (16:1)	0.04	5.85
Stearic (18:0)	3.41	3.79
Oleic (18:1)	21.54	43.61
Linoleic (18:2 n-6)	61.8	2.2
α-Linolenic (18:3 n-3)	0.88	1.33
Eicosonoic acid (20:1)	ND	5.45
Archidonic acid (20:4n6)	ND	1.05
Eicosapentaenoic acid (EPA, 20:n3)	ND	10.65
Docosahexanoic (DHA, 22:n3)	ND	8.64
Σ SFA[a]	15.73	18.73
ΣMUFA[b]	21.58	54.91
Σ Omega 6	61.8	3.25
Σ Omega 3	0.88	20.62

Note: ND = not detected.
[a] SFA: saturated fatty acids.
[b] MUFA: monounsaturated fatty acids.

and the corn oil was used as a vegetable oil in the basal control diet. Analysis of oils that used in the experiment was shown in Table 2. Omega-3 level in the diets after supplementation was checked.

2.5. BW, BWG, clinical signs, post-mortem lesions and mortality

Random birds ($n = 6$) from each group were weighed initially at 40 days of age (initial BW) and at 14 DPI (final BW). Then the body weight gain (BWG) was calculated at 14 DPI (68 days of age). The clinical signs, post-mortem lesions and mortality rates were also recorded at 14 DPI with NDV and H9N2.

2.6. Haemagglutination inhibition (HI) test

The AI H9N2 and ND antibody titres were detected using the HI (beta procedure) at 0, 7 and 14 DPI, as previously described by Beard (1989). The antigens used for the HI test were the AIV H9N2 standard antigen and the NDV LaSota antigen.

2.7. H9N2 virus shedding rates in tracheal swabs

Tracheal swabs were collected from birds (n = 6 per group) infected with H9N2 at 3, 5 and 9 DPI to assess the virus shedding rates. Sterile phosphate-buffered saline (PBS, 2 ml) containing 1% gentamicin was used to elute the swabs. A QIAamp Viral RNA Mini kit (Qiagen, Germany, GmbH) was used for viral RNA extraction, and PCR amplifications were performed in a 25 μl final volume containing 7 μl of RNA template, 12.5 μl of 2× QuantiTect Probe RT-PCR Master Mix, 4.125 μl of PCR-grade water, 0.5 μl of each primer (50 pmol conc.), 0.125 μl of probe (30 pmol conc.) and 0.25 μl of QuantiTect RT Mix. The following real-time RT-PCR primers and probe sequences were used as previously described (Ben Shabat et al., 2010): H9F: 5′-GGAAGAATTAATTATTA TTGGTCGGTAC-3′; H9R: 5′-CACCTTTTTCAGTCTGACATT-3′; and H9 probe: [FAM] 5′-AACCAGGCCAGACATTGCGAGTAAGATCC-3′ [TAMRA]. Reverse transcription was performed at 52 °C for 30 min, followed by 40 cycles of denaturation at 94 °C for 15 s, annealing, and extension at 60 °C for 45 s. The Ct values were detected after the calculation of the standard curve.

2.8. NDV shedding rates in tracheal swabs

Tracheal swabs were gathered from birds ($n = 6$ per group) infected with vNDV at 3, 5 and 9 DPI to evaluate virus shedding rates. A QIAamp viral RNA Mini kit (Qiagen, Germany, GmbH) was used for the extraction of RNA for NDV from tracheal swabs. A PCR was performed in a final volume of 25 μl containing RNA template (7 μl), 2× QuantiTect Probe RT-PCR Master Mix (12.5 μl), PCR-grade water (3.625 μl), each primer (50 pmol conc., 0.25 μl), each probe (30 pmol conc., 0.125 μl), and QuantiTect RT Mix (0.25 μl). The following real-time RT-PCR primers and probe sequences were used as previously indicated (Wise et al., 2004): NDVF: 5′- CCGGAGGATACAAGGGTCT-3′; NDVR: 5′-AGCTGTTGCAACCCCAAG-3′; and NDV probe: [FAM] 5′-AAGCGTT TCTGTCTCCTTCCTCCA-3′ [TAMRA]. Primary denaturation was performed at 50 °C for 30 min; reverse transcription was performed at 95 °C for 15 min, followed by 40 cycles of denaturation at 94 °C for 10 s, annealing at 52 °C for 30 s and extension at 72 °C for 10 s. The Ct values were detected after the calculation of the standard curve.

2.9. Tissue sample collection

At 5 DPI, four quails were randomly selected and slaughtered. Tissue specimens from the duodenum and spleen were collected from each bird in all groups and then held at −20 °C until the time of RNA isolation and complementary deoxynucleic acid (cDNA) synthesis for the detection of the gene expression of IFN-γ, interleukin (IL)-1β and IL-6. Moreover, specimens of liver, spleen and duodenum were collected from the same quails in all groups for histopathological examination after fixation in 10% neutral buffered formalin.

2.10. Immune gene expression analysis

For RNA isolation, 100 mg of frozen duodenal and splenic tissue was homogenized in TRIzol™ reagent (Invitrogen, UK) as described in the manufacturer's instructions (Simms et al., 1993). A nanospectrophotometer (Q5000 UV–Vis spectrophotometer, San Jose, USA) was used to check the RNA purity and concentration. RNA integrity was also checked using gel electrophoresis. The equivalent of 1 μg of RNA was transcribed to cDNA with a High Capacity cDNA.

Reverse Transcription kit (Applied Biosystems) using random hexamers in a 20 ml reaction volume that was further diluted 1:20 for downstream analyses. Real-time PCRs for the amplification and relative quantification of immune genes in the current study were conducted on a Pikoreal real-time PCR system (Thermo Fisher Scientific). Real-time PCR was conducted on the immune genes indicated in Table 3 with their associated primers. The primers (Metabion, Germany) were based on those described by Uno et al. (2012). Real-time PCR was performed using TOPreal qPCR 2× premix (Enzynomics, South Korea) with the following cycling conditions: initial denaturation at 95 °C for 8 min, followed by 40 cycles of 95 °C for 40 s, 55 °C for 30 s and 72 °C for 40 s, and a final elongation step at 72 °C for 7 min. The expression analysis was performed using the 2-ΔΔCT method described by Livak and Schmittgen (2001), where the fold expression was normalized to that of β-actin as a housekeeping gene.

Table 3
Primers sequences of cytokine genes.

Primer	Sequence	Reference
β-actin	F: CTGGCACCTAGCACAATGAA R: CTGCTTGCTGATCCACATCT	Uno et al. (2012)
IFN-γ	F: CAACCTTAATGATGGCACGA R: CTTTGCGGTGGATTCTCA	
IL-1β	F: CTTCCTCCAGCCAGAAAGT R: CAGCTTGTAGCCCTTGAT	
IL-6	F: CAACCTCAACCTGCCCAA R: GGAGAGCTTCCTCAGGCATT	

2.11. Histopathology

Formalin-fixed tissue specimens were dehydrated in graded concentrations of alcohol, cleared in xylene, and melted paraffin was used for embedding. Three sets of 5 μm thick paraffin sections were cut with a microtome. The first set was again cleared in graded concentrations of xylene, dehydrated in graded concentrations of alcohol and then stained with haematoxylin and eosin according to Bancroft and Gamble (2007). Stained sections were examined using a light microscope, and pictures were taken with a microscope camera.

2.12. Statistical analysis

Statistical analysis was performed using SPSS 19 software (SPSS Inc., Chicago, Illinois). BW, HI titers and gene expression levels were tested using two-way analysis of variance (ANOVA) with Duncan's multiple comparisons of the means to compare the difference between means. Data were expressed as the mean ± standard error. Differences between means were considered significant at values of $P < 0.05$.

3. Results

3.1. The mean BW, BWG, clinical signs, gross lesions, HI antibody titers, and virus shedding and mortality rates

Non-significant changes ($P > 0.05$) were detected in the initial BW among the experimental groups. The final BW was significantly higher ($P < 0.05$) in the G3A and G3B groups than in the G2A and G2B groups. In addition, the BWG followed a similar trend (Table 4). Two way Anova showed a significant change in BW, when both dietary regimen and viral infections effects as well as the interaction between both dietary regimen and viral infections were compared ($P < 0.05$).

No clinical signs were detected in birds in the G1 and G3B groups, whereas mild respiratory signs with diarrhoea were observed in birds in the G3A group. The birds infected with NDV (G2A) showed mild respiratory signs and nervous system manifestations, including uncoordinated gait, opisthotonos, torticollis, and paralysis of the legs and wings. The birds infected with H9N2 (G2B) showed depression with loss of appetite, nasal discharge, and diarrhoea. Signs were observed between 2 and 7 DPI. The lesion analysis revealed congestion of the internal organs, petechial haemorrhage of the proventriculus and trachea, pneumonia, enlarged liver and spleen, and pale kidneys in dead quails from the G2A and G3A groups. Moreover, the lesion analysis revealed mild air sacculitis, tracheal congestion and nephritis in dead quails from the G2B group. No observed gross lesions were detected in quails from the G1 and G3B groups.

Maternally derived NDV and H9N2 HI antibody titres were not detected in quails before infection. The HI antibody titres were significantly higher ($P < 0.05$) in the G3A and G3B groups than in the G2A and G2B groups at 7 and 14 DPI (Table 4). Viral shedding was positively detected in the tracheal swabs from G2A and G2B group birds at 3, 5 and 9 DPI, and shedding was detected in the swabs from G3A and G3B group birds at 3 and 5 DPI, revealing that the quails in the G3A and G3B groups had a short period of viral shedding. No viral shedding was detected in the G1 control group at any time. The number of shedders and shedding rates in tracheal swabs at 3, 5 and 9 DPI were higher in the G2A and G2B groups than in the G3A and G3B groups. The two way Anova analysis revealed that diet and viral infections produced a significant change regarding HI-titers ($P < 0.05$).

The mortality rates were approximately 45% in the G2A group, 20% in the G2B group, 15% in the G3A group and 0% in the G3B and G1 groups. The NDV and H9N2 viruses in the dead quails were re-isolated from swabs (tracheal and cloacal) and tissues (trachea and lung) in embryonated eggs, and the haemagglutination test gave positive results. Real-time RT-PCR was used to confirm the NDV and H9N2 virus detection.

3.2. Immune gene expression analysis

The expression of duodenal IFN-γ was significantly higher in the G2A and G2B groups than in the other experimental groups ($P < 0.05$). A significant difference was observed between the G3A group and G3B group supplemented with omega-3 fatty acids ($P < 0.05$). Splenic IFN-γ was significantly overexpressed in the G2B group when compared with that in the G3A and G3B groups ($P < 0.05$). The magnitude of splenic IFN-γ expression in the G3B group was not significantly different from that in the control group (G1; Fig. 1A). In Table 5, two way Anova showed a significant change in IFN-γ when both dietary regimen and viral infection effects as well as the interaction between both dietary regimen and viral infections in duodenum and spleen ($P < 0.05$).

Duodenal IL-1β expression was highest in the G2A group, followed by the G2B group ($P < 0.05$). Duodenal IL-1β expression was significantly lower in the G3B group than in the other experimental groups

Table 4
Body weight (BW), body weight gain (BWG), HI antibody titers (log2), virus shedding rates and mortality % of infected quails with NDV or AIV H9N2 received omega-3 (Mean ± S.E).

Groups	Growth performance ($n = 6$)			HI (Log2) titers (DPI) ($n = 6$)			Virus shedding rates (Shedders %)* (DPI) ($n = 6$)			Mortality %
	Initial BW(g) (40 days old)	Final BW(g) (68 days old) (14 DPI)	BWG (g) (68 days old) (14 DPI)	0	7	14	3	5	9	
G1 Control	192.1 ± 5.4^{B}	250.1 ± 3.7^{Ab}	58.2 ± 1.3^{c}	0.0 ± 0.0	0.0 ± 0.0^{c}	0.0 ± 0.0^{c}	0/6	0/6	0/6	0/20 (0)^{c}
G2A NDV	202.5 ± 4.9^{B}	231.2 ± 7.5^{Ac}	29.1 ± 2.1^{d}	0.0 ± 0.0^{C}	3.1 ± 0.7^{Ab}	4.2 ± 1.3^{Ab}	5/6	4/6	2/6	9/20 (45)^{a}
G2B H9N2	197.3 ± 6.2^{B}	235.5 ± 4.1^{Ac}	38.6 ± 3.5^{d}	0.0 ± 0.0^{C}	4.1 ± 1.2^{Ab}	5.2 ± 0.9^{Ab}	6/6	5/6	4/6	4/20 (20)^{b}
G3A Omega-3 + NDV	200.7 ± 1.5^{B}	265.3 ± 1.2^{Aa}	65.2 ± 2.2^{b}	0.0 ± 0.0^{C}	6.4 ± 1.2^{Aa}	7.2 ± 0.2^{Aa}	4/6	3/6	0/6	3/20 (15)^{b}
G3B Omega-3 + H9N2	195.2 ± 3.1^{B}	274.5 ± 3.2^{Aa}	79.3 ± 2.4^{a}	0.0 ± 0.0	6.2 ± 0.2^{Ba}	8.3 ± 0.2^{Aa}	3/6	2/6	0/6	0/20 (0)^{c}

The different small letters within the same column were significantly different at $P < 0.05$.
The different capital letters within the same row were significantly different at $P < 0.05$.
* Positive shedders % = number of positive quail shedding virus/number of tested quails X 100; DPI, days post-infection.

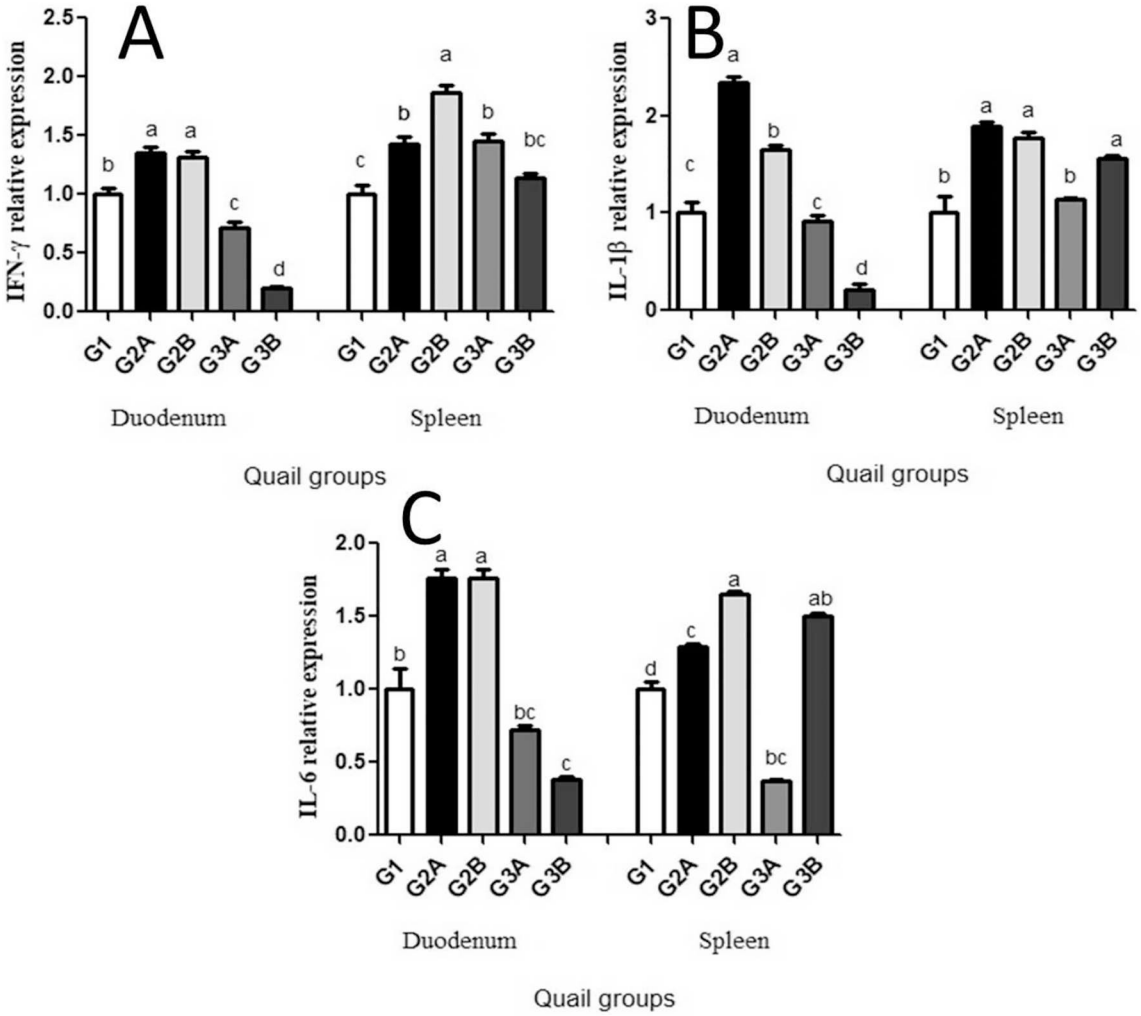

Fig. 1. Statistical analysis of duodenal and splenic IFN-γ (A), IL-1β (B) and IL-6 (C) showing significant variation in experimental groups. Different small alphabetical letters mean significant when $P < 0.05$.

Table 5
Two way Anova analysis studying the effect of different dietary regimen of infected quails with NDV or AIV H9N2 received omega-3.

	IFN-γ		IL-1β		IL-6	
Source	Duodenum	Spleen	Duodenum	Spleen	Duodenum	Spleen
Diet	0.000	0.000	0.000	0.000	0.000	0.281
Viruses	0.000	0.000	0.000	0.000	0.000	0.000
Diet * viruses	0.000	0.000	0.93	0.011	0.046	0.008

($P < 0.05$). Splenic IL-1β expression was significantly higher in the G2A and G2B groups ($P < 0.05$) than in the G1 group and significantly lower in the G3A group than in the G2A, G2B and G3B groups (Fig. 1B). Two way Anova analysis revealed that diet and viral effects produced a significant change regarding IL-1β expression ($P < 0.05$). However, the interaction between both dietary regimen and viral infection didn't show a significant change in IL-1β expression in duodenum ($P > 0.05$) with a significant change in spleen ($P < 0.05$).

Duodenal IL-6, which was significantly upregulated in the G2A and G2B groups compared with the other studied groups but was significantly downregulated in the G3A and G3B groups compared with the G2A and G2B groups ($P < 0.05$). Splenic IL-6 expression in the G2B group was the highest and was significantly higher than that in the G1 group. A significant reduction in IL-6 was observed in the G3A group compared with the G1 and G2B groups ($P < 0.05$; Fig. 1C). The dietary regimen effects on two way Anova analysis indicated that duodenum IL-6 showed a significant change ($P < 0.05$) with a non-significant change in splenic IL-6 ($P > 0.05$). Moreover, the effects of viral infections and the interaction between both dietary regimen and viral infections produced a significant change in both duodenal and splenic IL-6 expression ($P < 0.05$).

3.3. Histopathology

Microscopic examination of the liver revealed normal histological picture in the G1 group, marked portal congestion and fibrosis, and a focal area of necrosis with heterophil infiltration in the G2A group, marked congestion, sinusoidal dilation and hepatocyte vacuolar degeneration in the G2B group, mild congestion and hepatocyte vacuolar degeneration in the G3A group, and very mild hepatocyte vacuolar degeneration in the G3B group (Fig. 2). Microscopic examination of the spleen revealed normal histological picture in the G1 group, marked congestion in the red pulp, severe necrosis and depletion of lymphocytes from the white pulp that was replaced with fibrin deposits in the G2A group, moderate necrosis and depletion of lymphocytes from the white pulp that was replaced with fibrin deposits, and moderate congestion in the red pulp in the G3A group, and mild depletion of lymphocytes from the white pulp in the G3B group (Fig. 3). Microscopic examination of the duodenum showed a normal histological picture in the G1 group, necrotic enteritis with heterophil and macrophage

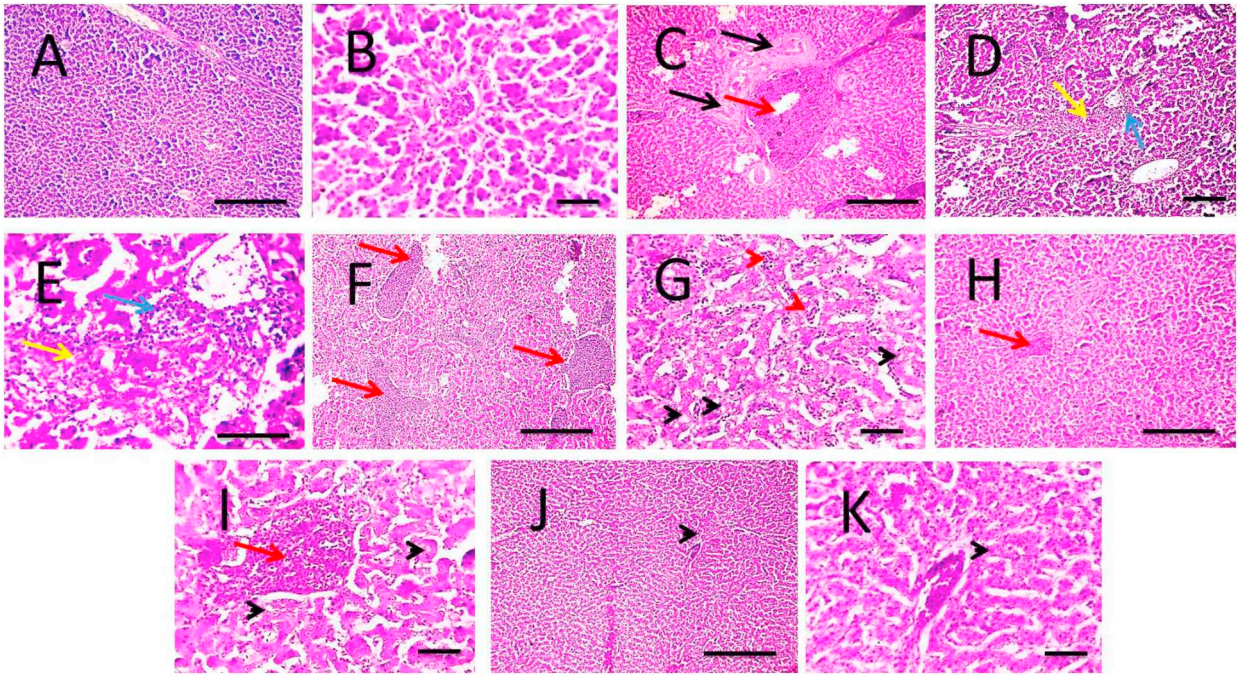

Fig. 2. Microscopic pictures of H&E stained liver sections showing normal histological picture in G1 (A & B), marked portal congestion (red arrow) and fibrosis (black arrow) (C), focal area of necrosis (yellow arrow) with infiltration of heterophils (blue arrow) (D & E) in G2A, marked congestion (red arrows) (F & G), sinusoidal dilation (red arrowheads) and hepatocytes vacuolar degeneration (black arrowheads) (G) in G2B, mild congestion (red arrow) (H) and mild hepatocytes vacuolar degeneration (black arrowheads) (I) in G3A, very mild hepatocytes vacuolar degeneration (black arrowheads) (J & K) in G3B. X:100 bar 100 (A, C, D, F, H, J), X:400 bar 50 (B, E, G, I, K).

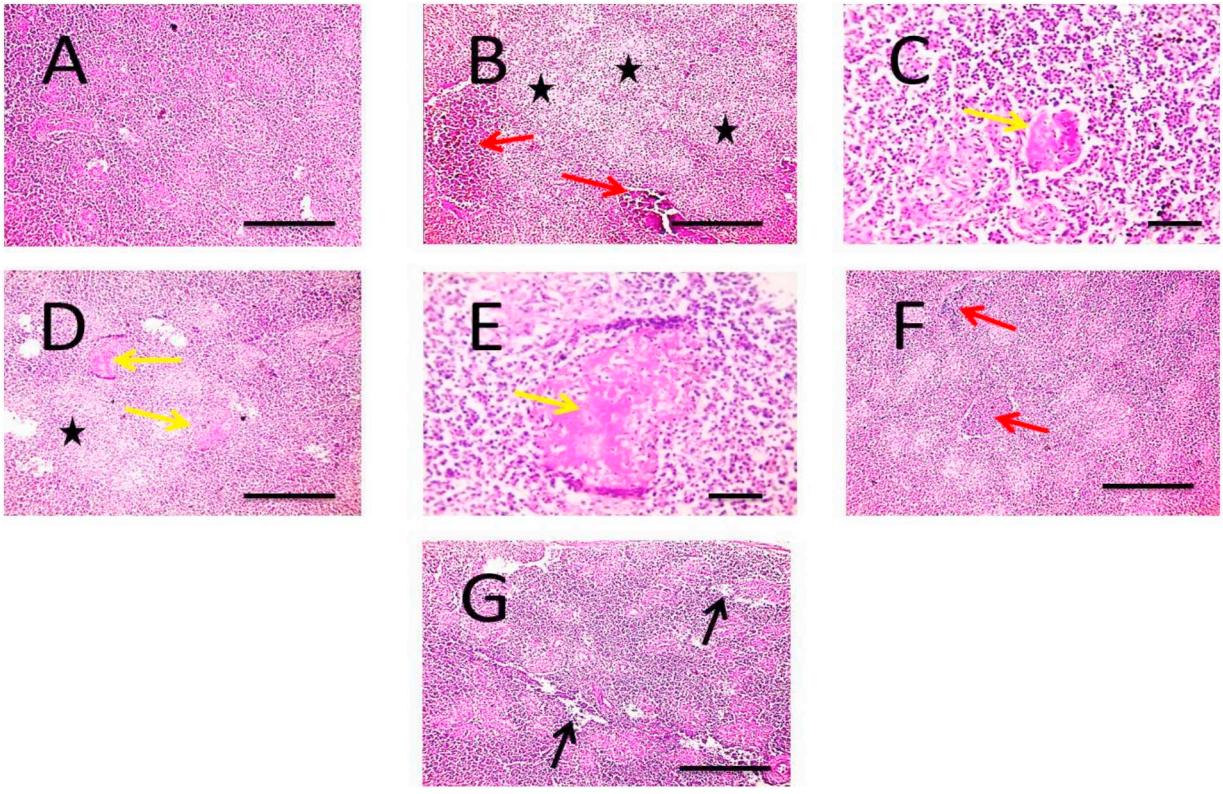

Fig. 3. Microscopic pictures of H&E stained splenic sections showing normal histological picture in G1 (A), marked congestion in red pulp (red arrows), severe necrosis and depletion of lymphocytes from white pulp (asterisks) (B) that replaced with fibrin mat (yellow arrow) (C) in G2A, moderate necrosis and depletion of lymphocytes from white pulp (asterisks) that replaced with fibrin mat (yellow arrow) (D & E), moderate congestion in red pulp (red arrows) in G3A (F), mild depletion of lymphocytes from white pulp (black arrows) in G3B (G). X:100 bar 100 (A, B, D, F, G), X:400 bar 50 (C & E).

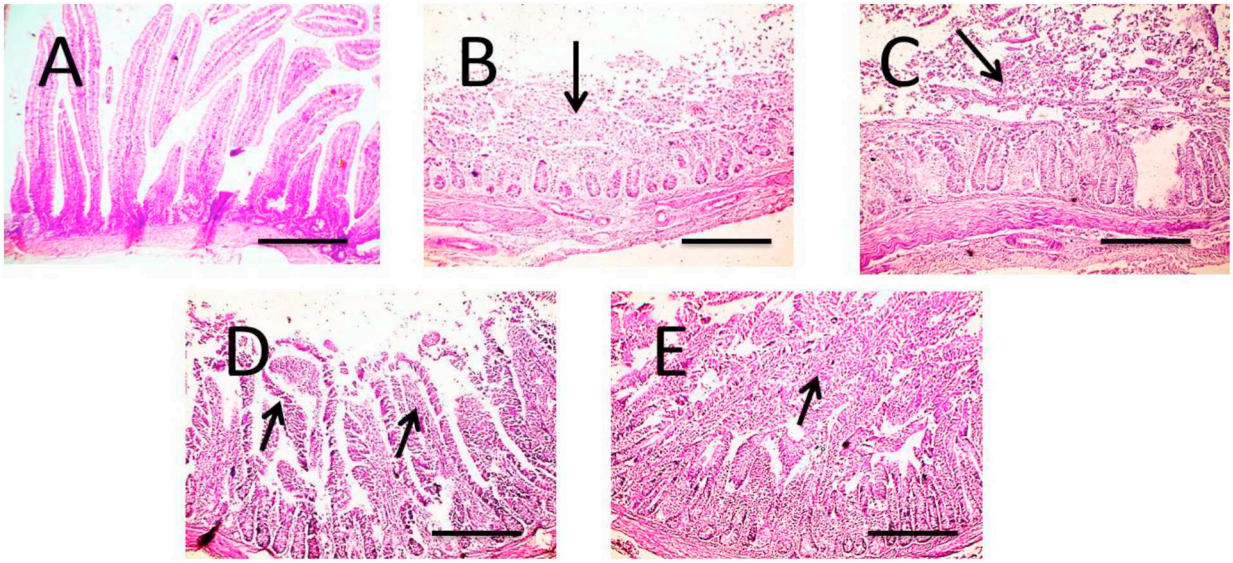

Fig. 4. Microscopic pictures of H&E stained duodenal sections showing normal histological picture in G1 (A), necrotic enteritis with heterophils and macrophages infiltration in lamina propria (black arrow) in G2A (B), desquamated villi (black arrow) in G2B (C), widened villous lamina propria (black arrows) in G3A (D), desquamated epithelial covering villi (black arrow) (E) in G3B X:100 bar 100.

infiltration in the lamina propria in the G2A group, desquamated villi in the G2B group, widened villous lamina propria in the G3A group, and desquamated villi covering the epithelium in the G3B group (Fig. 4).

4. Discussion

In the current study, we investigated the effects of omega-3 on the immune response of Japanese quails (*C. c. japonica*) infected with NDV or AIV H9N2. The virulent NDV challenge strain was related to a VIId genotype strain and isolated from a broiler farm in Dakahlia Governorate, Egypt (Saif et al., 2017).

ND virus can infect quails naturally or experimentally causing changes in HI titers and injury to different organs (El-Tarabili et al., 2009). NDV was recovered from tracheal and intestinal samples and identified in quails by HI test using NDV-specific antiserum (Sharawi et al., 2015). The pathogenicity of NDV in quail depends chiefly on the strain of the virus, its dose and route of administration (Oladele et al., 2008). The intramuscular or intravenous routes of NDV infection appear to enhance neurological signs, while natural routes of infection (nasal, oral and ocular) appear to confirm the respiratory nature of the disease in quails (Oladele et al., 2008). Therefore, in the current study, we used the intranasal route as a natural route of infection instead of intramuscular route.

Meanwhile, the H9N2 challenge strain was an LPAIV belonging to the G1 lineage that was isolated from an infected commercial layer farm in Dakahlia Governorate, Egypt (Eladl et al., 2019a). The mortality of quails infected with either NDV or H9N2 may be due to the virulence of the challenge strain or the pathogenicity of infection in stressed birds (Eladl et al., 2011; Ali et al., 2013; Eladl et al., 2019b).

In the present study, omega-3 supplementation improved the BW, BWG and HI antibody titres for NDV and H9N2 and protection rates, and it reduced shedding rates, clinical signs and lesions post-infection with NDV and H9N2. The previous research conducted by Maroufyan et al. (2012) showed that dietary n-3 PUFA supplementation to infectious bursal disease challenged broiler chickens increased body weight and improved FCR at 42 days of age. The authors returned this result to the superior digestibility of unsaturated fatty acid compared to saturated type. Newman et al. (2007) showed the effects of dietary n-3 and n-6 PUFA on FCR in avian metabolism was through the modulation of lipid deposition and oxidation.

Ali et al. (2017) found that the weight gain of broiler chickens challenged with AI virus (H9N2) and supplemented with 3% fish oil was significantly higher than the broiler chickens receiving feed with cooking oil and challenged with AI virus (H9N2). It could be attributed to the fact that fish oil improves the absorption of poly unsaturated fatty acids from intestine, which enhance the metabolic energy and because of the dietary fat composition that makes it possible to increase diet digestibility and to stimulate growth (Farhoomand and Chekani-Azar, 2009).

Omega-3 polyunsaturated fatty acids play immunomodulatory and anti-inflammatory roles (Feng et al., 1999); particularly during viral infection (Kolawole and Evavold, 2019). Oladele et al. (2008) showed that HI antibody titres are elevated in quails administered 0.3 ml of the NDV Kudu 113 strain either orally or intramuscularly. Jameel et al. (2015) showed that dietary supplementation of broilers with 0.5% flaxseed oil can stimulate the immune response to NDV.

Oral administration of 1% Immulant® containing *Echinacea* (coneflower) extract and *Nigella sativa* (black seed) extract for six weeks can enhance the immune response after AI-H9N2 vaccination and reduce the pathogenicity of infection in stressed chickens (Eladl et al., 2019b). Vitamin E with Fetomune Plus® supplementation for four weeks also improved the immunological and pathological effects of H9N2 infection on chickens (Awadin et al., 2019).

In this study, H9N2 infection produced a significant overexpression of IFN-γ. In mice experimentally infected with H5N1 and H9N2, a significant increase in serum IFN-γ was observed 3 days post-infection (Arai et al., 2019). Generally, pro-inflammatory cytokine expression is increased during equine influenza virus infection (Nang et al., 2011). In the current study, IFN-γ overexpression was observed in quail groups exposed to NDV. The infection of chickens with a highly virulent strain of NDV elicited the expression of IFN-γ before death, which highlights the intrinsic role of IFN-γ as an antiviral cytokine with important immunomodulatory effects (Susta et al., 2013). Similarly, NDV elicited an innate immune response and significantly increased pro-inflammatory cytokine expression in chicken splenocytes and lymphocytes at 48 h post-inoculation (Kapczynski et al., 2013). Two way Anova analysis showed a significant interaction between the effect of dietary regimen and different viral infection in both duodenal and splenic IFN-γ expression.

In the present study, omega-3 fatty acid dietary supplementation reduced the expression of IFN-γ during NDV and H9N2 infection in quails. Omega-3 polyunsaturated fatty acids can reduce the number of

IFN-γ receptors, which highlights the role of omega-3 fatty acids as an immunomodulatory and an anti-inflammatory agent in mice (Feng et al., 1999). In general, high-concentration omega-3 fatty acid supplementation results in the decreased production of arachidonic acid-derived chemical mediators, which can efficiently decrease the expression of pro-inflammatory cytokines in macrophages (Calder, 2001).

IL-1β was significantly overexpressed in quails exposed to H9N2, which is consistent with the finding of Wang et al. (2014). The highly pathogenic avian flu virus H5N1 induces the overexpression of pro-inflammatory cytokines (IL-1β, Il-18 and TNF-α) in chickens in the mid and late stages of infection (Kalaiyarasu et al., 2016). The significant overexpression of IL-1β and IL-6 has been observed after 24 h of inoculation with two Egyptian strains of H9N2, V3 and RCF/1 in chickens (Ahmed et al., 2016). IL-1β overexpression has been observed in quail exposed to NDV. IL-1β is strongly overexpressed in macrophages in chickens with certain viral infections, such as poult enteritis (Heggen et al., 2000). In chicken macrophages, the class II strain of LaSota virus was found to induce the expression of M1-associated genes (Zhang et al., 2018). M1 genes are derived from macrophages that is polarized into specialized functional proinflammatory cells and named as M1 (Chawla, 2010). In this study, two way Anova analysis revealed that the interaction between different dietary regimen and viral infections in quails showed a non-significant change in IL-1β expression, while the interaction in splenic IL-1β showed a significant change.

The duodenal and splenic expression of IL-6 in quails exposed to AIV was also significantly increased when compared with that in the control group quails. Intranasal inoculation of H9N2 in chickens induces the expression of IL-6 in lung specimens as a result of viral replication in lung tissues (Guan et al., 2015). The expression of IL-6 is also increased in quails exposed to NDV, as reported by Rue et al. (2011). NDV-CA-02 increases the expression of IL-6 by 35-fold in chicken splenocytes, whereas LaSota increases the IL-6 expression by nearly 2-fold. A viscerotropic strain of NDV (D165) caused a significant overexpression of several pro-inflammatory cytokines, such as TNF-α, IL-1β and IL-6, within five days of infection (Rajasekaran et al., 2019). Omega-3 fatty acids have been proven to play an effective role in the immune system through the manipulation of structural lipid content in the cell membrane, which reduces the recognition of antigens by T cells, leading to decreased, but still effective, immune response during viral infection (Kolawole and Evavold, 2019). In broilers, the dietary supplementation of 0.5% flaxseed oil results in the stimulation of the immune response against NDV (Jameel et al., 2015) through decreasing proinflammatory cytokines. Two way Anova analysis revealed that the effect of dietary regimen showed a non-significant result regarding IL-6 expression.

In this study, compared with the no supplementation control, omega-3 fatty acid supplementation improved the immune response, reduced the expression of inflammatory markers and reduced the severity of the histopathological lesions in the liver, spleen and duodenum due to NDV and AIV infection. Taseer et al. (2017) revealed that dietary vitamin E supplementation alone followed by vitamin E plus omega-3 supplementation in broilers infected with LPAIV significantly reduced the histopathological lesions in the trachea and lungs. Moreover, the immune-enhancing ability of fish oil supplementation in broilers may be due to the increase in lymphocyte populations and hypertrophy in lymphoid organs (Wang et al., 2000). Ali et al. (2017) found that fish oil supplementation in birds infected with H9N2 induced cellular and glandular hypertrophy and increased the size of the mature lymphocyte population and the presence of lymphoblasts in lymphoid organs. Finally, dietary omega-3 supplementation in quails ameliorated the deleterious effects of NDV and AIV H9N2.

5. Conclusion

Our results showed significant changes in growth performance, HI antibody titres, clinical signs, post-mortem lesions, mortality, virus shedding rates, immunological parameters, and histopathological lesions between omega 3-treated and untreated groups. The expression of pro-inflammatory cytokines was significantly increased in quails infected with both NDV and AIV H9N2 and this increased expression was significantly downregulated in infected quails receiving supplementary omega-3 fatty acids due to the anti-inflammatory role of omega-3 fatty acids in the duodenum and spleen. In conclusion, dietary omega-3 supplementation for 4 weeks can improve growth performance and the deleterious immunological and pathological effects of NDV and AIV H9N2 infection in quails.

Declaration of competing interest

The authors declare that they have no conflict of interest.

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