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Original Article

Effect of fish oil on growth performance and immune response in experimentally infected broiler chicken with avian influenza virus H9N2

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Authors' Contribution

AA, BZ: Contributed in purchasing of broiler and kits. IA: Involved in conduction of experiment and samples collection for serological and histological studies, **BZ, IA:** Contributed in histopathology. **HR:** Analyzed the data..

Key words

Histopathology
Lymphoid organs
Broiler
Avian Influenza virus

Abstract

Avian influenza (AI) is an important infectious disease of poultry which cause immune suppression and immune organs damage in broilers. Supplementations of fish oil (FO) enhance the immune status and subside inflammation. This study was design to observe the effects of Fish oil on lymphoid organs through histopathology, growth performance and antibody response in H9N2 challenged broilers. A total of 80 chicks were divided in to 4 groups A, B, C & D. Group A and B were challenged with AI virus (H9N2) on 21 day of age, group B and C were treated with 3% FO while group A and D were treated with cooking oil. For isolation of virus, 21 samples were collected, out of which 8 (40%) were positive for H9. The virus titer was ranged from 1:64 to 1:512. The body weight gain of FO supplemented broilers was higher than broilers receiving cooking oil (CO) while the FCR of FO treated broilers was significantly lower than broilers receiving diet with CO before virus inoculation, furthermore a significant difference was observed in FCR after challenged, with highest value of group A, followed by group D, B and C. The Geometric mean titer (GMT) for H9N2 and Newcastle Disease Virus (NDV) were recorded weekly up to 42 day of age by haemagglutination inhibition test. The mean GMT for H9N2 of group A, B, C, and D after challenge were 76.0, 273.7, 0.5 and 0.35, respectively. However, after challenged the highest GMT for NDV was observed in group C with 158.8, followed by group B, D and A with GMT of 72.6, 49.3, and 11.32, respectively. Congestion, depletion of lymphocytes, dysplasia of thymic lobules, necrosis, disappearance of lymph follicles and interfollicular edema were the prominent histopathological changes observed in challenged group. It is concluded that fish oil improve the growth performance and immune response in broilers chicken against avian influenza virus infection..

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INTRODUCTION

Pakistan poultry industry is an important sub-sector of livestock which play a major role in the economy of country and made considerable contribution to food production (Alam and Khan, 2000; Muhammad *et al*., 2005). In intensive culture systems, bird intensity is higher and infectious diseases can be easily transmitted from one bird to the other (Irfan *et al*., 2017). Avian influenza (AI) is one of the most prevalent and devastating viral infection of poultry (Capua and Alexander, 2004). The AI viruses are classified into low pathogenic avian influenza viruses (LPAIV) and high pathogenic avian influenza viruses (HPAIV) (Alexander, 2000; Swayne, 2007). The LPAI subtype H9N2 virus is currently circulating in birds and cause economical losses to poultry industry of Pakistan (Subtain *et al.,* 2011). Beside the respiratory and gastrointestinal system the H9N2 virus also affect the immune system of birds and cause immune suppression. The immune organs damage in broilers through H9N2, with an

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associated loss of response to Newcastle disease vaccine (Qiang and Youxiang, 2011).

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 improve the antioxidative status, reduce lipid peroxidation, enhance the antibody response and bone morphological characteristics in Japanese quail (Ebeid *et al*., 2011). Feeding laying chickens with diets rich in n-3 PUFA promote the growth of thymus, spleen, and bursa up to 4th week of age (Al-Khalifa *et al*., 2012). The current project was designed to evaluate the effects of fish oil on histopathology of lymphoid organs and also to evaluate their response on antibody titer and growth performance in broilers during H9N2 infection.

MATERIALS AND METHODS

Isolation and identification of virus

For isolation of H9N2 viruses, 21 samples of trachea were collected from 7 broiler flocks (3 samples per flock) exhibiting mild signs of respiratory distress. Trachea samples were taken in phosphate buffered saline (PBS) centrifuged for 30 minutes at 1500 rpm and the collected supernatant mixed with antibiotics (Penicillin10, 000 IU/ml, Gentamycin 1 mg/ml, Streptomycin 10,000 µg/ml) and antifungal agents (Amphotericin B) (Nili and Asasi, 2002). Inoculum was prepared by passing through a 0.45 µm filter and incubating at 37 °C for 1 hour. Virus isolation was performed by inoculating 10 days old embryonated chicken eggs with 0.2 ml of inoculum according to the protocol adopted by Office International des Epizooties (OIE 2005). Harvesting of eggs was done 48 hours post inoculation (PI) and chorioallantoic fluid (CAF) was collected with a sterile syringe and centrifuged at 3000 rpm for 5 minutes to remove mixed blood and tissues. Presence of virus was confirmed by haemagglutination (HA) and haemagglutination inhibition (HI) tests as described by WHO (2005). H9N2 virus confirmed through Enzyme linked Immunosorbent Assay commercial ELISA kit, IDEXX Flock Chek standard (IDEXX Corporation, Westbrook, ME, USA).

Experimental design

A total of 80 commercial day old Ross broiler chicks were purchased from Hi-Tech poultry breeder (Pvt) Ltd. All chicks were kept under standard conditions of management. The birds (n = 80) were divided into 4 groups (A, B, C & D) and each group contained 20 chicks. Group A and B were challenged with AIV (H9N2), group B and C were treated with fish oil while group A and D were treated with cooking oil (vegetable oil) in day 1, blood was collected from 5 randomly selected chicks for screening of maternal antibodies against H9N2 and Newcastle disease (ND) viruses and for vertically transmitted *Mycoplasma gallisepticum* and *Salmonella pullorum* at University Diagnostic Laboratory, UVAS Lahore, Pakistan. The birds of group A and B were challenged with 0.1 ml of H9N2 of known EID_{50} ($10^{-6.72}$/ ml) intranasally on 21st day of age (Reed and Muench, 1938).

Composition of diet

All birds were fed with diet to which either cooking oil or fish oil was added at 3 g/100 g diet. The diets were formulated according to National Research Council (NRC) guidelines 1994.

Feed consumption and body weight gain

Feed intake (FI) per group was recorded every week till 42 days. The weight of all chicks were determined individually on very first day of experiment and then on weekly basis up to 42 days of age. Data recorded regarding FI and body weight gain (BWG) were used to calculate feed conversion ratio (FCR) (Gharaibeh, 2008).

Clinical signs

During experimental period all the birds were examined twice daily for the development of clinical signs and mortality and all the observations were recorded (Subtain *et al.,* 2011).

Histopathology of lymphoid organs

On day 20, 22, 29, 36 and 42, 5 birds from each group were slaughtered and all gross lesions swollen kidneys, cloudy air sacs, slight hyperemia and congested trachea and gross hemorrhages on lungs were observed. Samples like (thymus, bursa of Fabricious, spleen and cecal tonsils) were collected and fixed in 10% neutral buffered formalin and processed for fixation, dehydration, clearing, embedding, sectioning and staining as described by Drury and Wallington (1980).

Measurement of serum antibody titer

Aseptically blood was collected from the wing vain of 5 randomly selected birds from each group on day 20, 22, 29, 36 and 42 for monitoring the antibody titers for ND virus and AIV subtype H9N2 by haemagglutination inhibition (HI) technique as described by (Ghaniei et al., 2013).

Statistical analysis

Data were statistically analyzed using Statistical Package for Social Science (SPSS) version 16.00 (Chicago,IL,USA). The BWG and feed conversion ratio were analyzed through repeated measure analysis, while the data were analyzed by one-way analysis of variance. The data were presented as mean ± S.D. The difference between groups was compared by using Duncan's Multiple Range Test. The difference was considered significant at $P < 0.05$.

RESULTS

Isolation and identification of virus

Avian influenza virus was isolated from 21 tracheal samples that were collected from suspected H9 positive broilers, and inoculated in 10 days old embryonated chicken eggs for cultivation. Out of 21 samples, 8 samples (38%) were positive for H9 through HA and HI tests. The virus titers were calculated through HA test and ranged from 1:164 to 1:512. Out of 8 positive samples, 2 samples showed the titer of 1:64, 3 represented 1:128, 2 have 1:256 and 1 sample showed 1:512.

Body weight gain (BWG)

BWG of group A and D and that of group B and C remained same before H9N2 challenged, however, the weight gain differ significantly ($P < 0.05$) with advancement in age. Comparatively the BWG of 3% FO supplemented broilers (group B and C) was significantly ($p < 0.05$) differ higher than broilers receiving feed with cooking oil (group A and B) before challenged. However, after challenged the BWG of group C was significantly higher than group B among broiler supplemented with fish oil. Similarly, the BWG of group D was significantly ($p < 0.05$) higher than group A among broilers receiving feed with cooking oil (Fig. 1).

Feed conversion ratio (FCR)

The results revealed that feed conversion ratio (FCR) of group A and B and that of group C and D remained same before challenged, however, the FCR differ significantly ($p < 0.05$) with advancement in age. The comparative results of fish oil supplemented broilers and broilers receiving feed with cooking oil indicated significant difference in feed conversion ratio during 1st and 3rd week of age. The results regarding FCR after challenge the broilers with H9N2 showed significant difference ($p < 0.05$) with highest value of FCR by group A followed by group D, B and C (Fig. 2).

Table I: Geometric Mean Titer for Newcastle Diseases Virus and Avian Influenza virus H9N2 before and after challenge with H9N2

Group	Before Challenge		After Challenge									
	Day 20		Day 22		Day 29		Day 36		Day 42		Average	
	ND	H9	ND	H9	ND	H9	ND	H9	ND	H9	ND	H9
A	3.2	1.6	4.0	1.2	5.7	4.6	13.0	90.5	22.6	207.9	11.32	76.0
B	5.7	2.3	8.0	1.4	22.6	5.7	52.0	256	207.9	831.7	72.6	273.7
C	8.0	3.2	21.1	2.0	64.0	0.0	256	0.0	294.1	0.0	158.8	0.5
D	2.8	1.6	5.7	1.4	18.4	0.0	45.3	0.0	128.0	0.0	49.3	0.35

Geometric mean titer

Antibody titer from each group on 20, 22, 29, 36 and 42 days of age was measured for ND virus and AIV subtype H9N2 by haemagglutination inhibition (HI) technique. Before challenged, group C showed highest GMT value (8.0) for NDV followed by group B (5.7), A (3.2) and D (2.8), However, after challenged the highest average GMT value for NDV was observed in group C with 158.8, followed by group B, D and A with average GMT of 72.6, 49.3, and 11.32 respectively. The GMTs for H9N2 of group A, B, C and D before challenged were 1.6, 2.3, 3.2 and 1.6

respectively, however after challenge the highest average GMT value for H9 was observed in group B followed by group A, C and D with average GMT of 76.0, 0.5 and 0.35 respectively (Table I).

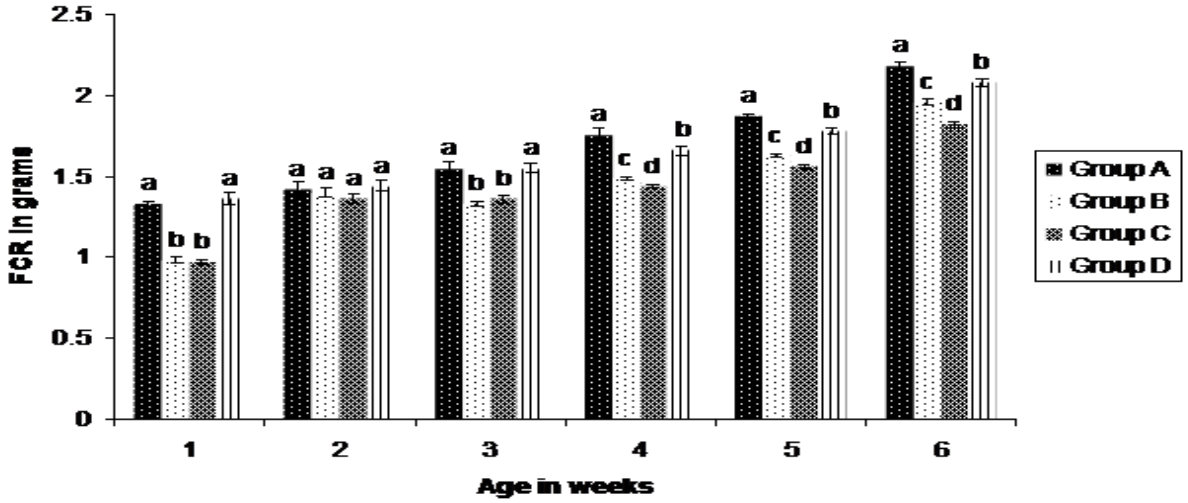

Figure 1: Mean body weight gain (Mean ± St.D) of broilers at 1st, 2nd, 3rd, 4th, 5th and 6th week of age on different diets

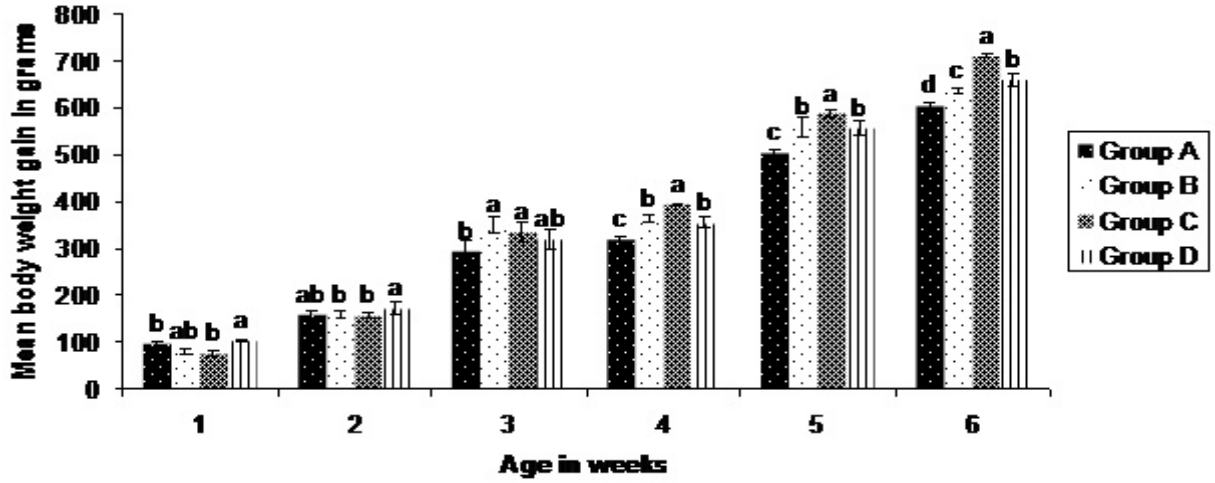

Figure 2: FCR (Mean ± St.dev) of broilers at 1st, 2nd, 3rd, 4th, 5th and 6th week of age on different diet

Clinical signs and gross pathology

Before challenge, birds of all groups were healthy and did not show any signs of disease and mortality. However, after virus inoculation the birds of group A showed anorexia, conjunctivitis, depression, nasal and ocular discharges, coughing, gasping, sneezing and huddling from 4 to 10 days post inoculation while that of group B exhibited anorexia, depression, conjunctivitis, slight sneezing and coughing from 4 to 8 days of virus inoculation. All the visceral organs of control birds were found normal with no prominent abnormal gross lesions while in infected birds swollen kidneys, cloudy air sacs, slight hyperemia and congested trachea and lungs were observed in birds slaughtered at 7 days post challenge. Two mortalities, one at day 5 and another at day 7 were observed in group A while no mortality occurred in any other group.

Histopathological changes

Histopathological changes like congestion, depletion of lymphocytes, dysplasia of thymic lobules, necrosis, lymphocytolysis, cellular atrophy, rupture of blood capillaries, disappearance of lymph follicles and interfollicular edema were observed in lymphoid organs of challenged birds as shown in Fig. 5-6.

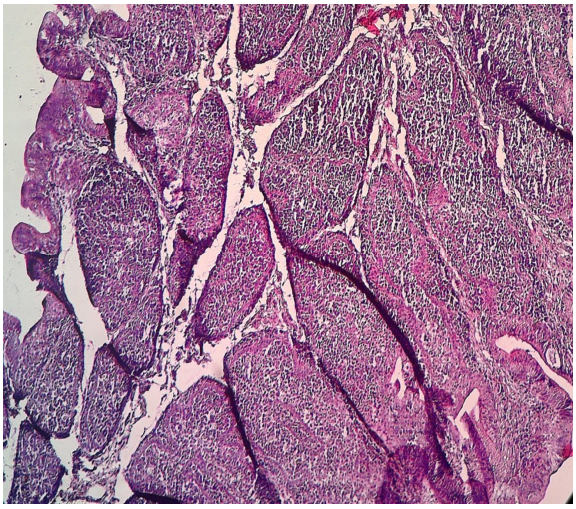

Figure 3: Bursa of Fabricious of 20-day-old chicken, supplemented with 3% fish oil in diet. Showed hypertrophy of lymphoid follicles (black arrow) and increased lymphocytes population (H&E ×400).

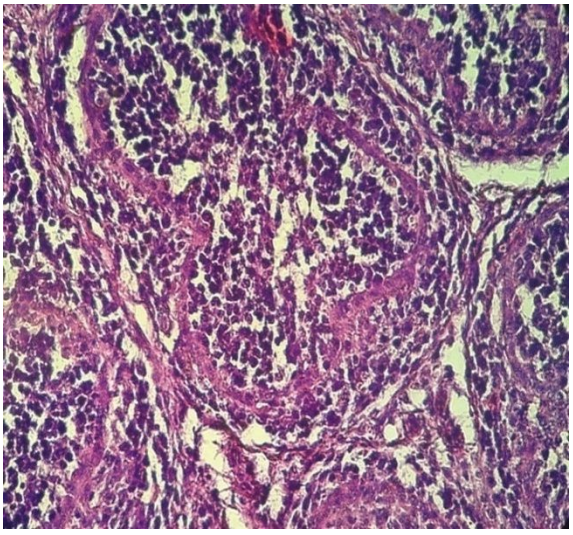

Figure 4: Bursa of Fabricious of 22-day-old chicken, supplemented with 3% fish oil in diet. Indicated increase lymphocytes population (H&E ×400).

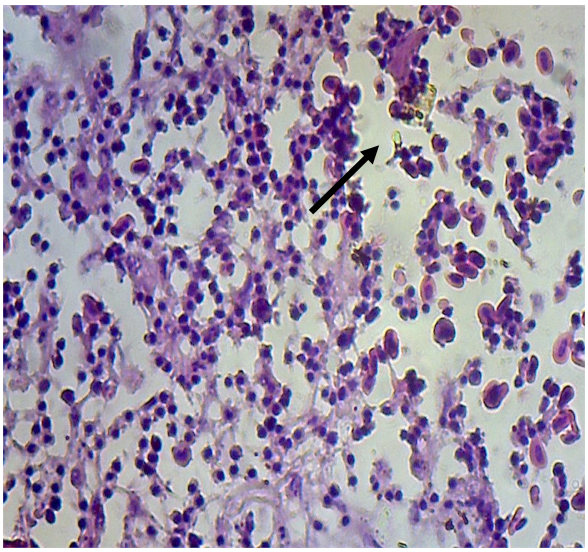

Figure 5: Thymus of 29-day-old chicken of group A, challenged with H9N2 subtype. Demonstrated depletion of lymphocytes (black arrow) and degeneration (H&E ×400).

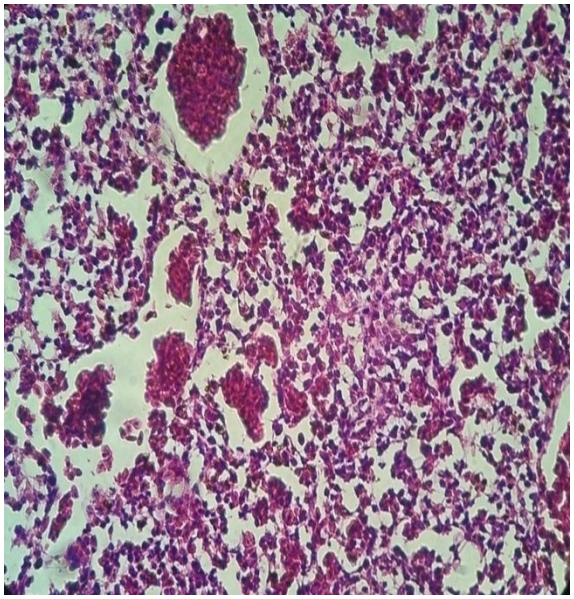

Figure 6: Spleen of 29-day-old chicken (group A) inoculated with H9N2 subtype. Represented atrophy (black arrow) and degeneration of lymph follicles (H&E ×400).

Cellular and glandular hypertrophy, increased mature lymphocytes population and presence of lymphoblasts were the important histological lesions observed in lymphoid organs of fish oil treated birds as indicated in Fig. 3-4

DISCUSSION

Avian influenza (AI) is one of the most prevalent and devastating viral infection of poultry. The AI viruses are classified into low pathogenic avian influenza viruses (LPAIV) and high pathogenic avian influenza viruses (HPAIV). Avian influenza (AI) is an important infectious disease of poultry which cause immune suppression and immune organs damage in broilers. Therefore, present experiment was conducted to evaluate the immunomodulatory effect of fish oil (FO) against AI infection in broiler chicken. The avian influenza virus H9 was isolated from tracheal samples, out of 21 samples 8 were positive through HI test and the virus titers were calculated with the help of HA (Subtain *et al.,* 2011). These observations are in agreement with (Nagarajan *et al*. 2008) who reported the HI test positive with 1:16 to 1:512 titers for H9N2 virus isolates from India. The consumption of feed and weight gain of all birds increased in regular ascending order with advancement in age. However, fish oil supplemented broilers consumed less feed as compare to the broilers receiving diet with cooking oil. The low level of FI is likely due to increasing fishy smell in the feed reported by Hulan *et al*. (1988) and Saleh *et al*. (2009). The weight gain of 3% fish oil treated birds was significantly higher than the birds receiving feed with cooking oil. This is probably due 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). The significant difference ($p < 0.05$) in mean BWG and feed conversion ratio observed in challenge birds and control group from day 1^{st} to day 10^{th} post infection (Gharaibeh , 2008, Vasfi Marandi ,2002).

Fish oil improves the antibody titers against NDV and H9 in broilers. This is probably due to the immunomodulatory activities of fish oil, as fish oil greatly increase the activation and number of T lymphocytes in the body and hence the ability of body to fight disease. Furthermore, it is concluded that the immune system improved because of the effect of fish oil on eicosanoid (leukotriene) and interleukin levels (Kidd, 2004; Klasing, 1998). The average geometric mean titer (GMT) for NDV of infected birds was lower than unchallenged birds. It is concluded that, the low values of GMT of group A and B for NDV were due to avian influenza subtype H9N2 virus as the protective antibody titer against NDV cannot be formed in the presence of avian influenza subtype H9N2. These observations regarding immunosuppressive effects of avian influenza were supported by the previous study of Qiang and Youxiang D, (2011).

The histopathological effects of LPAIV H9N2 on lymphoid organs of broilers were evaluated that cause dysplasia of thymic lobule, thinning of the cortex, depletion lymphocytes in the medulla and hypertrophy of epithelial reticular cells of thymus. Similar, histopathological changes were seen in broilers challenged with H9 reported by several other researchers (Pazani *et al*. 2008, Qiang and Youxiang, 2011, Hadipour *et al*., 2011). Increased lymphocytes population and cellular hypertrophy in lymphoid organs of FO supplemented broilers may be due to the hyper activation of lymphoid organs because fish oil increase the activation and number of T lymphocytes in the body. This immune enhancing ability of fish oil may exert hypertrophic effect on lymphoid organs (Wang *et al*., 2000). Al-Khalifa *et al*., (2012) reported that feeding laying chickens with diet containing 50 gm/kg of fish oil increase the weight of thymus, spleen and bursa significantly. The results showed that, in the presence of low pathogenic avian influenza (H9N2), the protective antibody titer cannot be achieved. When the birds were feed with diet containing cooking oil birds achieved protective titer even in the presence of low pathogenic avian influenza (H9N2). Furthermore supplementations of fish oil also improve the meat quality by increasing the n-3-PUFA content in meat and promote the morphological characteristics of bones. Fish oil has also beneficial and economical if they provided in the feed of commercial poultry. Fish oil contains unsaturated fatty acids which incorporate in the meat of poultry bird. The extra cost due to fish oil is 7.8 rupees per bird but beside growth performance, the fish oil has also good effects on antibody titer against NDV and H9N2. It is concluded from the current research that provision of fish oil improve the growth performance and immune response in broilers. Furthermore the low pathogenic avian influenza H9N2 exert in immune suppressive effect due to which the protective antibody titer against NDV cannot be formed instead of vaccination.

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