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Influence of probiotic and flaxseed oil supplementation on some physiological parameters and immune response of broilers

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Abstract
The present research was aimed to study the effect of probiotic, flaxseed oil and their combination on some physiological traits and immune response of broilers. 200 one-day-old chicks (Ross 308) were randomly divided into four equal groups (50 of each) with two replicates. All the chicks were reared in farm of Veterinary Medicine College/ University of Baghdad for 42 days from 2 November 2016 up to 13 December 2016. The 1st group (control) fed on basal diet, 2nd group fed on basal diet with 0.5 gm probiotic /kg feed, 3rd group fed on basal diet with 2 gm flaxseed oil/kg feed and 4th group fed on basal diet with 0.5 gm probiotic and 2 gm flaxseed oil/kg feed. The results of the 4th groups recorded significant ($P \leq 0.05$) increase in RBC (2.03×103/ml), total protein (63.66 g/l) as compared with the 2nd, 3rd and control groups at aged 42 days. The results also showed significant reduction in cholesterol, triglycerides concentrations and H/L ratio of 3rd and 4th groups at the same time. In conclusion, dietary probiotic and flaxseed oil supplementation have improved the some hematological and biochemical parameters of broilers; also that mixture has enhanced the immune response to ND vaccine of treated chicks.

Keywords: Probiotic, Flaxseed oil, Immune response, Broilers

1. Introduction
The increase in poultry production led to the widespread use of antibiotics and chemical treatments in preventive doses in raising chickens in order to improve its performance and to obtain the desired economic profits, but there was a negative impact of this indiscriminate use of antibiotics to human health [1]. Therefore, researchers had to find alternatives to antibiotics in chicken breeding such as Probiotics. This term is derived from the Latin word pro and the other term biotic, which means life, which is the opposite of the concept of antibiotic [2]. The use of probiotics has become widely accepted as a natural means to promote health for both humans and animals. Also, probiotic cultures were used in the poultry industry for pathogen control and performance enhancement has gained attention recently due to the increasing restriction of antibiotics as growth promoting agents [3]. Probiotic organisms, like those of the genera *Lactobacillus*, *Pediococcus*, *Bifidobacterium*, and *Enterococcus*, consist of live microorganisms that exert a beneficial effect on the host by enhancing immune response, nutrient absorption, decrease lipid profile pool in blood and control of pathogen, their therapeutic use towards cholesterol-lowering activities has further increased their applications as effective probiotics [4, 5]. The researchers also have focused to reduce fat, cholesterol and triglycerides content in chicken meat by varying the composition of diet such as adding probiotic, omega 3 fatty acids or both of them in ration. Polyunsaturated fatty acids (PUFA) among the various essential nutrients that consider human requirement in daily diet particularly n-3 fatty acids plays an important role in reducing the risk of diseases like cardiovascular, hypertension, diabetes, arthritis and other autoimmune disorders [6, 7]. Also, several studies showed that incorporating n-3 FAs with poultry ration has effective means to enrich chicken meat with n-3 FAs, the chickens are normally in a positive energy balance the FAs supplied through diet are deposited mostly unaltered and as a result the composition of fat tends to reflect that of dietary fat, as well as, the dietary supplementation of flaxseed is one way to increase omega-3 long chain PUFA accumulation in chicken meat [8]. The oils that vegetable sources such as flaxseed (linseed oil) are the rich sources of alpha linolenic acid (ALA) which is the precursor of all n-3 LC-PUFA. Hence, diets enriched with vegetable n-3

PUFA sources can also be an effective method of increasing the n-3 PUFA in the meat [9, 10]. The aim of this study was to identify the effect of probiotic, flaxseed oil and their combination on some physiological parameters, biochemical parameters and immune response against Newcastle disease virus.

2. Materials and Methods

2.1 Chicks of the study

Two hundred broiler chicks (Ross 308) at one day old were used in our experiment. One-day age is not homogenized. Chicks were obtained from commercial hatchery of Baghdad province.

2.2 Study area

All chicks were reared in farm of Veterinary Medicine College/ University of Baghdad for 42 days, started from 2 November 2016 up to 13 December 2016.

2.3 Experimental deign

Two hundred chicks were randomly divided into four equal groups at 50 chicks of each group with two replicates (25 chicks of each replicate). All birds in this study were offered feed and water *ad libitum*. The first group (control) was fed with basal diet (starter and grower). The second group: chicks were fed on basal diet with adding 0.5 gm probiotic /kg feed. The third group: chicks were fed on basal diet with adding 2 gm flaxseed oil/kg feed. The fourth group: chicks were fed on basal diet with adding 0.5 gm probiotic and 2 gm flaxseed oil/kg feed.

2.4 Nutrients and Vaccines

The nutrients and the vaccines used in the experiment:

- Probiotics (Poultry Star® me) naturally ahead in poultry gut health manufactured by BIOMIN Austria. Preparation of dried probiotic bacteria (*Enterococcus sp.*, *Bifidobacterium sp.*, *Pediococcus sp*. and *Lactobacillus sp*.). Product contains a minimum of 5×10^{12} CFU/kg.
- Flaxseed Oil (linseed oil): manufactured by Hemani international Kepz Karachi-Pakistan. Flaxseed oil (100% natural herbal oil). Package size 500ml.
- Vaccines used: manufactured by Volvac® (Boehringer Ingelheim- HQ Germany). All chicks were vaccinated as in Table 1.

2.5 Diets used

The starter diet was applied for 21 days of old, then the grower diet offered up to end of the experiment, the yellow corn and wheat were the major sources of energy, while the soybean and animal protein were the major sources of protein in this diets. Nutritional requirements were adjusted according to the NRC [11].

2.6 Sampling

2.6.1 Collection of Samples

At age 42 days old of bird, the blood samples were collected randomly from the jugular vein (10 chicks from each group) for estimation of the some physiological, biochemical and immunological tests.

2.6.2 Preparation of samples

Two types test tubes were used, the first type with anticoagulant (K_3-EDTA) to measure the Erythrocytes and leukocytes counts, PCV%, Hemoglobin concentration and Heterophil/ Lymphocyte ratio. The second type (anticoagulant free) used for biochemical parameters changes and ELISA test.

2.6.3 Preparation of serum

Blood samples were immediately kept overnight at 4˚C (in the refrigerator). After then, samples centrifuged at (2300 rpm) for 5 minutes as recommended [12] to separate serum from the cellular part of blood, that serum was frozen at (-20˚C) until assayed [13]. All hematological parameters were conducted in the hematology unit/ laboratories department of Veterinary Directorate. Total serum protein concentration was measured by using kit produced by BioSystems S.A. company, Barcelona/Spain according Biuret method [14].

Table1: Vaccination program adopted in the present study.

Age/Day	disease	Type of vaccine	Administration rout
9	ND+IB	Volvac® ND-IB MLV (Boehringer Ingelheim- HQ Germany)	Via Drinking water
14	Gumboro	Volvac® IBD MLV (Boehringer Ingelheim- HQ Germany)	Via Drinking water
18	ND+IB	Volvac® ND-IB MLV (Boehringer Ingelheim- HQ Germany)	Via Drinking water

Cholesterol concentration was measured by using Kit produced by Biomaghreb Company, Paris/France. The cholesterol is determined after enzymatic hydrolysis and oxidation. The indicator quinoneimine is formed from hydrogen peroxide and 4-aminoantipyrine in the presence of phenol and peroxidase [15]. Triglycerides concentration was measured by using kit produced by Biomaghreb Company, Paris/France. The triglycerides are enzymatically hydrolyzed to glycerol according to [16]. HDL-Cholesterol concentration was measured by using Kit produced by BioSystems S.A. company, Barcelona / Spain according the principle of the method based on very low density lipoprotein (VLDL) and low density lipoprotein (LDL) in the sample precipitate with phosphating- state and magnesium ions.The supernatant contain high density lipoproteins (HDL). The HDL-cholesterol was then spectrophotometrically measured by means of the coupled reactions described below [17]. Antibody titers against Newcastle disease virus in serum samples were detected at (7, 21, 35 and 42) days of age by using ELISA test for different groups. ND virus antibody test kit ProFLOK® PLUS Synbiotics Corporation –San Diego/USA was used in this study.

2.7 Statistical analysis

The data was performed using SAS program (Statistical Analysis System - version 9.1). One-way and two-way ANOVA with Least significant differences (LSD) post hoc test were performed to assess significant differences among means. ($P \leq 0.05$) was considered statistically significant [18].

3. Results and Discussion

Results presented Table 2 revealed significant ($P \leq 0.05$) increase in chick's red blood cells count of the 4th group, higher values in PCV%, and Hb concentration of 4th and 2nd groups at age 42 day of birds. Also, white blood cells showed significant ($P \leq 0.05$) increase in 3rd and 4th group as

compared with the other treatments and the control groups. The results revealed significant ($P \leq 0.05$) reduction in H/L ratio of 3rd and 4th treatments in comparison with 2nd treatment and control group. The results demonstrated that adding probiotic with flaxseed oil to broilers diet as in the current study was beneficial to preserve RBC, WBC membranes and decrease endoplasmic degeneration might be depend on the reduction of the harmful effects of free radicals on the cells membranes by omega-3 fatty acids which present in flaxseed oil, and then preserve the stability of the cells membranes. Rymer and Givens [19] who reported that Omega-3 FA has been shown to reduce the catabolic response induced and may be effective in promoting growth. Yang *et al*., [20] concluded that the flaxseed oil supplementation may prevent lipid peroxidation and membrane dysfunction of erythrocytes in hyperglycemia, and his study indicated that flaxseed oil could reduce the lipid peroxidation level malondialdehyde, and maintain the L-glutathione contents in erythrocytes.

Table 2: Mean ± Standard Error of Red Blood Cells count (Cell x 10^6/ml), White Blood Cells count (Cell x 10^3/ml), Packed Cells Volume (%), Hemoglobin concentration (g/dl) and Heterophil /Lymphocyte ratio of Blood in different groups.

Group Parameter	Group 1 (Control) Basal diet	Group 2 Probiotic supplement	Group 3 Flaxseed oil supplement	Group 4 Probiotic + Flaxseed oil supplement
RBC	1.76±0.01 d	1.97±0.0 b	1.87±0.02 c	2.03±0.01 a
WBC	82.92±0.12 b	82.12±0.19 b	85.06±0.26 a	85.56±0.46 a
PCV%	27.36±0.33 b	29.90±0.49 a	28.11±0.14 b	30.28±0.14 a
Hb	10.86±0.26 bc	11.65±0.20 a	10.63±0.14 c	11.31±0.17 ab
H/L ratio	0.43±0.006 a	0.41±0.001 b	0.34±0.004 c	0.34±0.003 c

Different small letters horizontally denote significant ($P \leq 0.05$) differences among mean groups.

In addition, Flaxseed oil could also maintain the normal cellular shape of erythrocytes, Ross *et al*., [21] indicated that flaxseed oil contained a high content of omega-3 PUFA and many double bonds in omega-3 PUFA avoided damage on membrane form oxidative stress. Membrane incorporation of PUFAs may reduce cellular susceptibility to lipid peroxidation [22], alter membrane fluidity enhance receptor function, elevate enzyme activity and influence the production of lipid mediators [23, 24]. The observation was probably related to the reaction of free radicals with methylene groups in PUFA to avoid free radical conjugating with fatty acids of membrane [25, 26]. Roy *et al*., [27] noticed that the addition 10% of oils enriched PUFA omega-3 to ration of chickens will increase the concentration of Hb and increasing growth it at present 20%, as well as enhance the health status. On the other hand, the increase of RBC and WBC could be due to increase the ratio of omega-3: omega-6 PUFA to be more important in modulating eicosanoid synthesis. Enrichment of cell membrane with omega-3 PUFAs is associated with immune cell structure and eicosanoid formation. Omega-3 PUFAs possess anti-inflammatory or less inflammatory properties by decreasing the release of pro-inflammatory eicosanoids and cytokines [28]. The present study results are with agreement with [29, 30, 31] who showed that omega-3 led to significant increase of RBC, Hb conc. and PCV%, those researchers ascribed them results to the presence of omega-3 PUFA also the ratio of omega-3: omega-6 PUFA appears to be more important in modulating biosynthesis of eicosanoid than the absolute concentration of omega-3 PUFA in the diet. There were also some results showing that enrichment of cell membrane with omega-3 PUFAs could be decreased inflammatory response, improved of growth rate, erythropoiesis, leucopoiesis and increased specific immunity [32]. Furthermore, the results of hematological parameters of the second treatment were significant ($P \leq 0.05$) improvement in RBC count, PCV%, and Hb concentration as compared with third and control groups. That results may be due to the positive role of probiotics in the host intestinal tract, which led to improve digestion, and absorption of the most important vitamins and minerals in the ration, therefore the elevation in hematological picture was happen. The improvement in leukocytes (WBC) count occurs in flaxseed groups may be attributed to highly speed growth, which led to the direct effect on the haemopoetic tissue [33]. As the birds were vitality, this was a sign to good condition which might have been related to the immunomodulatory properties of the active components of flaxseed, namely, a high content of omega-3 fatty acids, which influence various immune mechanisms, inter alia, modulate the process of phagocytosis, the activity of natural killer cells (NK) and the production of cytokines [34].

Furthermore, the results of this study also agreed with Lands, [35] and Roy *et al*., [27] who evidenced that increase leukocyte numbers in birds which fed diet enriched with different concentration of omega -3 PUFAs specially on 6th week of age has an important role of these acids by observation the chemical transporters which control on some physiological functions in the body like growth, cells division and immunological function. The effect of stress in chickens was characterized by elevation of heterophil and reduction of lymphocyte due to the increased corticosteroid level in serum [36]. Thus, the 3rd and 4th treatments showed opposite picture to this status, these results may be attributed to PUFA omega -3 which are found in flaxseed oil that have a good role in the vitality of the body and enhancement the birds health [37]. In addition, the previous studies suggest that active substance of omega-3 fatty acids have positive role in increase in protein deposition in terms of body protein mass was due to the much faster rate of protein synthesis than that protein degradation. The difference between protein synthesis and degradation rates can be caused by the state of animal health [38].

Results presented in Table -2- indicated that H/L ratio especially in 3rd and 4th treatments were significantly lower than that of the 2nd and control group and these conditions did not affect body protein mass, this status arrives to the assumption that protein degradation was not usually affected by the increase in H/L ratio [39]. The results of total protein recorded significant ($P \leq 0.05$) increase in the 4th group (63.66±0.35**)** g/L at 42 days of age as compared with the 2nd, 3rd and the control groups (53.44±0.32, 55.97±0.40, 42.56±0.43) g/L respectively. In addition, the results of the 3rd and 4th groups showed significant ($P \leq 0.05$) decrease in cholesterol and triglycerides concentrations compared with the second treatment and control group. The 2nd treatment suggested significantly ($P \leq 0.05$) higher in HDL- cholesterol concentration than the other treatments and control as shown in Table 3. The highest significant increase of total protein may be attribute to the role of flaxseed oil enriched with omega-3 fatty acids in the improve immunity of the chicks and increasing of immunoglobulins in their serum. First,

flaxseed is one unique a food crop, its high content omega-3 fatty acid [40]. The results of the present study were agreement with that of AL-Mayah [41] who found that broilers fed 50 gm of fish oil/kg of diet were accelerated production of immunoglobulins (IgM and IgG) then increased serum total protein.

Results presented in Table 3 suggested that reduction in level of serum cholesterol and triglycerides concentrations could be attributing to the positive role of omega-3 PUFA. The antioxidant properties of omega-3 unsaturated fatty acids should be considered as a major component of its beneficial effects on tissue lipid peroxidation, and antioxidant enzyme activities, omega-3 fatty acids have important role in decreasing of plasma cholesterol, triacylglycerol, and it has a protective effect against lipid peroxidation products, by enhancing the antioxidant status [42]. The our results are in agreement with Chashnidel *et al*., [43] who mentioned that broilers dietary fish oil supplementation enrichment of diet with omega-3 PUFA at percentage (1.5, 3, 4.5)% showed significant decrease in cholesterol and triglycerides concentration in blood serum. The researcher attributes these results to reduce of hepatic synthesis by suppressive synthetic enzymes which enter cholesterol formation and increase B-oxidation process. Diets containing unsaturated fatty acids omega-3 decreased the plasma cholesterol and triglyceride levels compared to diets supplemented with saturated fatty acids [44, 45].

Table 3: Mean ± Standard Error of Total Protein (g/L), Total Cholesterol, Triglycerides and High Density Lipoprotein Cholesterol (HDL-Cholesterol) concentrations (mg/dl) in Serum for different groups.

Group Parameter	Group 1 (Control) Basal diet	Group 2 Probiotic supplement	Group 3 Flaxseed oil supplement	Group 4 Probiotic + Flaxseed oil supplement
Total Protein	42.56±0.43 d	53.44±0.32 c	55.97±0.40 b	63.66±0.35 a
Total Cholesterol	239.26±9.67 a	173.83±4.92 b	154.20±8.90 bc	152.30±2.40 c
Triglycerides	231.51±4.42 a	166.16±4.70 b	140.24±3.76 c	144.03±3.94 c
HDL-Cholesterol	45.82±0.98 d	50.82±2.19 c	83.08±1.38 a	66.61±1.06 b

Different small letters horizontally refer to significant ($P \leq 0.05$) differences among mean groups.

Analysis of data Table 4 recorded significant ($P \leq 0.05$) differences in antibody titers against Newcastle disease virus measured by ELISA among the treatments and control group at (7, 21, 35 and 42) days old of chicks. The results of the 2nd group showed significant ($P \leq 0.05$) increase antibody titers in blood serum samples in compare with the 3rd treatment and control group at (21, 35 and 42) days old (3037.0±82.41, 4992.00±25.79, 6461.67±319.21) respectively. Furthermore, the results showed significantly ($P \leq 0.05$) higher antibody titers of the 4th group (6186.83±309.24) than the 3rd and control groups (5200.83±220.15, 2321.17±111.51) respectively at 42 days of chicks age except the 2nd group there is no significant ($P > 0.05$) differences between them in the same age, there were (6461.67± 319.21) of the 2nd treatment and (6186.83±309.24) of the 4th treatment. On the other hand, the 3rd group showed a significant ($P \leq 0.05$) increased in antibody titers as compared with the control group at 42 days old. All the chicks of this study recorded significantly ($P \leq 0.05$) increment in antibody titers against Newcastle Disease virus with progress the age. This increment in Ab appear obviously in the treatment groups as compared with the control at (21, 35 and 42) days of age. These results may be obtained from the positive effect of probiotic in improving the immune response by increasing the immunoglobulins especially B-lymphocyte cells were led to increase antibody titers as indicated that defense of broiler chicks against ND virus and enhance overall animal health. These findings are in agreement with several studies. Hatab *et al.,* [46] revealed that serum antibody titers against ND virus based on HI test in chicken fed basal diet supplemented with probiotic was significantly higher ($P \leq 0.05$) than those of chickens in the control group on days (3, 7 and 9) post vaccination. The significant increase in antibody titer production against ND compared with control group he discussed may be due to immune-stimulatory and immune-modulatory effect of using biological (*B. subtilis and E. faecium*) supplementation. King and Seal [47] reported that the antibody titers against ND in broilers fed with diet-supplemented probiotics containing *B.subtilis* were significantly higher at 10 days post-immunization compared to the control birds. Many researches mentioned that probiotics have proven their ability to enhance the immune response by promoting the clearance of several economically important pathogens such as *Eimeria* spp., *Salmonella* spp., *Escherichia coli* and *Clostridium perfringens*, further asserting their potential use as an antibiotic alternative [48, 49, 50, 51]. However, the mechanism of action of probiotics in poultry production system includes establishing and maintaining healthy gut microflora, improving digestion and utilization of nutrients, competitive exclusion of harmful bacteria/pathogens, decreases pH and releases various antibacterial substances, neutralization of toxins, competition for nutrients with pathogens, reduction in ammonia production and stimulation of the immune system [52, 53, 54].

Table 4: Mean ± Standard Error of Antibody titers against Newcastle Disease Virus measured by ELISA for different groups.

Group Age/ day	Group 1 (Control) Basal diet	Group 2 Probiotic supplement	Group 3 Flaxseed oil supplement	Group 4 Probiotic + Flaxseed oil supplement
7	3070.00±98.86 a	2945.17±16.25 c	3154.00±37.77 b	3020.00±44.96 b
21	661.16±11.22 Cd	3037.00±82.41 Ac	1857.50±36.70 Bd	2017.50±28.58 Bd
35	1186.67±8.11 Cc	4992.00±25.79 Ab	2544.00±9.22 Bc	2619.33±57.80 Bc
42	2321.17±111.51Cb	6461.67±319.21 Aa	5200.83±220.15 Ba	6186.83±309.24 Aa

Different capital letters horizontally denote significant ($P \leq 0.05$) difference among mean groups.
Different small letters vertically denote significant ($P \leq 0.05$) difference among ages.

The previous study would be explained as the supplementation of dried probiotic to the basal diet resulted better absorption of iron salt from the small intestine and better produce of vitamins B that affecting positively blood

cell forming processes [55]. Moreover, increased blood WBC's count might be related to the production of more immune cells [56] that play an important role in defending the biological system against different diseases [57]. Probiotics also influenced humoral and cell-mediated immune responses by increasing antibody production and upregulating T-lymphocyte numbers and associated responses [58, 59]. Rowghani *et al*., [60] reported that broiler chickens fed a diet supplemented with probiotic had a significant increase in the ND antibody titers than the control group. Probiotics could also help in alleviating the immune response against secondary infections in birds that observed commonly during viral diseases or immunosuppressive conditions [61]. Furthermore, Mountzouris *et al*. [5] and Alkhalf *et al*. [62] reported that probiotic's immunomodulatory activity and ability to fortify beneficial members of the intestinal microflora, improving efficiency of digestion and nutrient absorption processes of the host. In the current study, the results also showed that a significant ($P \leq 0.05$) increase in antibody titers against ND virus in broilers fed with diets supplemented probiotic and flaxseed oil as compared with the control group at 42 days old. These improving in immune response may be attributed to the flaxseed oil enrich with omega-3 PUFA which have positive effect on the immune response. Many researchers have used omega-3 or its sources as feed additives alone or mixed with other compounds for enhancing the efficiency of the immune system in birds. Recently**,** Abdulwahid *et al.,* [31] concluded that in ovo injection with inactivated ND vaccine and different doses of cod liver oil richest with long chain omega-3 fatty acids could be contributed in develop the immune system and improve their function via increasing the antibody production and enhancing immune response of the broilers. Furthermore, several studies confirmed many positive effects of dietary omega-3 PUFA including immunomodulatory and antioxidative properties [63, 64]. This positive effect of omega-3 is similar to the observations of Yuming *et al.* [65] who proved that the antibody levels were higher in hens fed oils flaxseed oil (rich in n-3 PUFA) than in hens fed maize oil (rich in n-6 PUFA). Some studies also elucidated that a moderate intake of omega-3 PUFA could enhance the antioxidative properties including the activity of glutathione peroxidase (GSH-Px) in experimental animals [66]. Jameel *et al.,* [67] revealed that dietary addition of 0.5% flaxseed oil may be stimulate the development of the immune response and antibody production against ND and enhancement the blood biochemical parameters and health status of broilers. It has also demonstrated that the vitamin E and CLO have an antiviral effect in chickens when were blended with local bivalent oil based vaccine of ND and Infectious bronchitis disease may enhance the immune response of the broilers to both viruses [68]. Supplementation of dietary n-3 PUFA has shown to increase IgM level, suggesting an immuno-stimulating property of n-3 PUFA. Immunoglobulins production by B cells and interferon-g (IFN-g) is facilitated by interlukin-2 (IL-2) [69]. Al-Mayah, [41] showed that dietary fish oil as 50 gm/kg of feed will accelerates antibody production (IgG and IgM) to maintain proper immune function in chickens after vaccination against ND vaccine at 35 days old as compared with the control group. Calder [6] proved the long chain omega-3 fatty acids (EPA and DHA) have the ability to increase the immune response. In addition, Saleh *et al.,* [70] who explained that role of omega-3 fatty acids as immunity stimulators of cytokines and help B-lymphocytes to increase antibody production. The omega-3 PUFA can affect immune cell activation in both the innate and specific immune systems in different species [71, 72]. Therefore, diet enrichment with omega-3 PUFA consider as an alternative to enhance immune response and disease resistance.

4. Conclusion

From the results of this study, it can be concluded that dietary probiotic and flaxseed oil supplementation have improved the some hematological and biochemical parameters of broiler chicks; also that mixture has enhanced the immune response to Newcastle disease vaccine of treated chicks.

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