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Effects of Dietary Fish Oil and Green Tea Powder Supplementation on Broiler Chickens Immunity

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ABSTRACT

***Background*:** Poultry strains used by the industry have been submitted to intensive genetic selection for rapid growth and increased efficiency of feed utilisation along with a high metabolic rate. As major consequences a loss in the immune system competency and increased sensibility to stressors are pointed out. Several nutritional strategies have been essayed to alleviate immuno-suppression in broilers, including the administration of rich 3n- polyunsaturated fatty acids. The purpose of the current study was to investigate the effects of fish oil and green tea feeding in broilers chicks humoral immunity using different challenges and the weight of immunity-related organs.

***Materials, Methods & Results*:** In the present study, different combinations of fish oil (FO) and green tea powder (GrT) supplements were tested in a 3x3 combination of supplement proportions (0%, 1.5% and 2% FO and0%, 1% and 1.5% GrT), in a total of 270 Ross 308 broilers, to analyse the effects of supplementation on the humoral immune response to challenges against Influenza and Newcastle virus (antibody titers) and to SRBC (immunoglobulin production) and also in the weight of liver and main lymphoid organs at slaughter. At the end of the study, the carcass weight was similar between groups and to the reported in other studies for the same age at slaughter. On respect to the responses to viral challenges, the GrT supplementation failed to show advantageous effects, compared to FO supplemented groups; when combined with 2% FO the decrease in the humoral response was frequently negatively related to GrT proportions in co-supplementation treatments. However, no significant differences were found between treatment groups. As for the viral challenging, little, non-significant changes were found associated to FO or GrT supplementation. With exception for the group treated with 2% FO plus 1% GrT a slightly non-significant increase in total Igs production was recorded one and 2-weeks after challenging, reflecting the increase in IgM production. No statistical differences were found between treatments in the unpluck body weight at slaughter, suggesting that no constraints in fed intake were associated with supplementation, in contrast to the reported in some GrT studies. Moreover, little non-significant variations were found in the liver, spleen, thymus or bursa of Fabricius weights. Still, on respect to thymus weight, the supplementation with 1.5% FO significantly improved the relative organ weight, in particular if associated with 1.5% GrT. Also, the bursa of Fabricius weight was significantly increased in groups supplemented of 2% FO plus 1% GrT, compared to all the other treatment groups.

***Discussion*:** In the present study, slightly improved humoral responses against influenza were found in groups under 2% FO supplementation thought these beneficial effects could be antagonised by higher GrT co-supplements. Moreover, the humoral response to Newcastle virus challenging was positively influenced by FO supplementation and again these effects could be partially reversed by GrT co-supplementation. This is in part supported by the available literature. It has been demonstrated that improvement of humoral responses with fish oil are dose dependant, with birds fed with higher levels of FO showing higher antibody titers than those feed medium or low levels. Still, it is also acknowledged that increased FO incorporation rates (above 3 to 4%) in diets result in fish flavours in poultry meat and compromise the consumer acceptance of the product. In conclusion, no detrimental effects were found in association with the supplementation regimens tested, but the irregular responses found with some of the combinations used in the present study need clarification in further studies, with a larger number of birds.

Keywords: fish oil, green tea, broiler, immunity, virus.

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INTRODUCTION

Poultry strains used by the industry have been submitted to intensive genetic selection for specific traits. The genetic selection originated strains of rapid juvenile growth, breast-meat yield and increased efficiency of feed utilisation along with a high metabolic rate [5,12]. Accrued feed conversion is mainly associated to an increase of the intestinal mass, compared to wild chicken breeds [12]. Conversely, the genetic selection of modern poultry has also altered the immune system of birds, whilst the existing rearing systems, using high density confinement groups, put then under constant effect of different stressors and tend to intensify birds exposure to pathogenic organisms and compromise welfare [5,14]. Taken these together, it is easily previewed that poultry immune system is constantly threaten under common commercial production systems. Thus immuno-suppression is commonly found and increases the bird´ susceptibility to secondary infections and compromises the adequate response to vaccinations [12,17], as well as it presents a significant negative impact on broiler performance worldwide [2].

However, limited information exists on the effect of green tea combined with fish oil on the immunity of commercial broilers. Thereby, with this study it is intend to determine the effects of fish oil and green tea feeding in broilers chicks humoral immunity using different challenges (Influenza and Newcastle virus, and sheep red blood cells) and the weight of immunity-related organs (thymus gland, bursa of Fabricius, spleen) and liver.

MATERIALS AND METHODS

This study was developed in the poultry house of Khazar Co, Abkenar, Iran (37° 27' 38" N, 49° 20' 02" E, 18 m below sea) during the first semester of 2013. All the analyses were performed at the Rasht Branch, Islamic Azad University (37°15' N and 49°36' E, 7 m below sea level), Iran.

Facilities

Prior to birds' introduction, all non-fixed equipment such as drinkers and feeders were removed out of the hall for cleansing and disinfection. After dry cleaning, the room and non-mobile equipment was washed thoroughly with water and a flame-thrower was used for the floor. Thereafter, floor and walls were sprayed with Despatak disinfectant (solution of sodium hypochlorite) diluted at 1:200 v/v. The ventilation system, supplied using window fans and room air, was gasified with formaldehyde gas (Formalex® solution), all doors and windows closed. Twenty four hours after gasification, doors and windows were kept open, and fans were switched-on for better exhaust fumigation gas.

Mobile equipment was also washed and disinfected with the same antiseptic solution and flamed whenever the material allowed. To maintain the sanitary conditions in the hall, a disinfectant solution (20% Benzalkonium chloride[3]- Germo Killer) was used daily for cleaning the pond in the entrance hall, and the footbath was replaced daily.

During the experiments, room temperature was maintained within standard values for brooding practices and adapted to the birds rearing stages [3], using the central heat supply system. The temperature was adjusted to approximately 31ºC on day 1, and then was gradually decreased to 22ºC by day 27, thereafter maintained constant. Lighting was provided for 24 h on day 1, and thereafter for 23 h/day, with one hour of darkness, from 7 to 8 PM. The birds were housed in the main compartment, fitted with electrical heaters. Humidity was maintained at least at 55 to 65% in the early growing period, by spraying water on the floor.

Animals were housed in pens with walls in metal square mesh and wood shaving litter-bedded floor, with 1.5 × 1 m; each pen housed 10 birds aiming an adequate stocking density. All pens were located inside one hall and provided identical conditions to all the birds used in the experiments.

Animals

This experiment used 270 broilers to test the influences of fish oil and green tea powder supplementation in the immune competence of birds. Animals were grouped according to nine treatment groups, according to the combination of 3 × 3 different concentration of supplements.

One-day-old chicks (Ross 308) were purchased from a local hatchery and sexed so only male broilers were used in this study. Birds were randomly assigned into one of the nine treatment groups. For each group, three replicates were performed, each one with 10 birds. Broilers were reared until the age of 42 days. During this period, they were fed a baseline diet according to the rearing stage: starter (days 1-14), grower (days 14-28), and finisher (days 28-42), which were

supplemented with fish oil and green tea powder. The composition of the diets used in this study, formulated to meet the requirements of broilers as recommended for this strain [3], as well as their nutrient content is presented in Tables 1 to 3. Water was supplied *ad libitum* during the entire experimental period. Treatments consisted in supplementation with fish oil and/or green tea powder.

Table 1. Diet ingredients and calculated nutrient content in the starter diet (days 1-14).

Ingredient (gr/kg)	Treatments								
	1	2	3	4	5	6	7	8	9
Corn	583.1	574.2	568.2	576.5	566.5	561.6	573.8	564.1	559.3
Soybean Meal	377.7	374.2	373.7	378.9	376.3	375.0	379.6	376.8	375.4
Fish Oil	0	0	0	15	15	15	20	20	20
Green Tea Powder	0	10	15	0	10	15	0	10	15
Soybean Oil	12.6	15	16.5	3	5.6	6.8	0	2.5	3.7
DL-Methionine	1.8	1.8	1.8	1.8	1.8	1.8	1.8	1.8	1.8
Lysine-Hydro-Chloride	0.8	0.8	0.8	0.8	0.8	0.8	0.8	0.8	0.8
Mineral Mixture[1]	3	3	3	3	3	3	3	3	3
Vitamin Mixture[2]	3	3	3	3	3	3	3	3	3
$CaCO_3$	10	10	10	10	10	10	10	10	10
Phytase enzyme	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5
Multi-enzyme	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5
Ca:P (22:18) %	7	7	7	7	7	7	7	7	7
Total	1000	1000	1000	1000	1000	1000	1000	1000	1000
Energy (kcal/kg)	2969	2969	2969	2969	2969	2969	2969	2969	2969
Protein (%)	22.13	22.10	22.13	22.12	22.13	22.13	22.13	22.13	22.13
Crude fiber (%)	3.340	3.461	3.529	3.342	3.468	3.532	3.345	3.47	3.532
Calcium (%)	0.654	0.652	0.652	0.654	0.653	0.652	0.654	0.653	0.652
Tryptophan SID[3] (%)	0.420	0.415	0.413	0.419	0.415	0.412	0.419	0.414	0.412
Available Phosphorus (%)	0.246	0.244	0.244	0.245	0.244	0.243	0.245	0.244	0.243
Linoleic Acid (%)	1.768	1.858	1.915	1.324	1.422	1.467	1.185	1.279	1.323
Lysine SID (%)	1.331	1.318	1.315	1.333	1.323	1.318	1.334	1.323	1.318
Methionine SID (%)	0.567	0.563	0.561	0.567	0.563	0.560	0.567	0.562	0.560
Cysteine (%)	0.681	0.674	0.671	0.681	0.674	0.671	0.681	0.674	0.671
Sodium (%)	0.045	0.044	0.044	0.044	0.044	0.044	0.044	0.044	0.043
Price (Iranian Rial/kg)	11025	11600	11904	11058	11645	11936	11078	11660	11950
Price (Euro/kg)	0.33	0.34	0.35	0.33	0.34	0.35	0.33	0.34	0.35

[1]Calcium Pantothenate: 4 mg/g; Niacin: 15 mg/g; Vitamin B6: 13 mg/g; Cu: 3 mg/g; Zn: 15 mg/g; Mn: 20 mg/g; Fe: 10 mg/g; K: 0.3 mg/g.
[2]Vitamin A: 5000 IU/g; Vitamin D3: 500 IU/g; Vitamin E: 3 mg/g; Vitamin K3: 1.5 mg/g; Vitamin B2: 1 mg/g. [3]SID (Standardized Ileal Digestible).

Table 2. Diet ingredients and calculated nutrient content in the growing diet (days 15-28).

Ingredient (gr/kg)	Treatments								
	1	2	3	4	5	6	7	8	9
Corn	628.1	618.6	613.6	621.4	611.5	606.8	615.7	609.4	604.6
Soybean Meal	333.6	330.8	329.5	335.0	332.3	330.9	336.2	332.7	331.3
Fish Oil	0	0	0	15	15	15	20	20	20
Green Tea Powder	0	10	15	0	10	15	0	10	15
Soybean Oil	11.7	14	15.3	2	4.6	5.7	0	1.3	2.5
DL-Methionine	1.8	1.8	1.8	1.8	1.8	1.8	1.8	1.8	1.8
Lysine-Hydro-Chloride	0.8	0.8	0.8	0.8	0.8	0.8	0.8	0.8	0.8
Mineral Mixture[1]	3	3	3	3	3	3	3	3	3
Vitamin Mixture[2]	3	3	3	3	3	3	3	3	3
$CaCO_3$	10	10	10	10	10	10	10.5	10	10
Phytase enzyme	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5
Multi-enzyme	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5
Ca:P (22:18) %	7	7	7	7	7	7	8	7	7
Total	1000	1000	1000	1000	1000	1000	1000	1000	1000
Energy (kcal/kg)	3005	3005	3005	3005	3005	3005	3005	3005	3005
Protein (%)	20.54	20.54	20.54	20.54	20.54	20.54	20.55	20.54	20.54
Crude fiber (%)	3.063	3.188	3.251	3.067	3.192	3.255	3.070	3.193	3.256
Calcium (%)	0.640	0.638	0.638	0.640	0.639	0.638	0.680	0.639	0.638
Tryptophan SID[3] (%)	0.403	0.398	0.396	0.402	0.397	0.395	0.401	0.397	0.395
Available Phosphorus (%)	0.244	0.242	0.242	0.243	0.242	0.241	0.262	0.242	0.241
Linoleic Acid (%)	1.790	1.876	1.925	1.343	1.441	1.481	1.243	1.289	1.333
Lysine SID (%)	1.213	1.202	1.197	1.215	1.205	1.200	1.217	1.206	1.200
Methionine SID (%)	0.543	0.539	0.536	0.543	0.538	0.536	0.542	0.538	0.536
Cysteine (%)	0.635	0.629	0.626	0.635	0.629	0.625	0.635	0.629	0.625
Sodium (%)	0.045	0.045	0.044	0.045	0.044	0.044	0.045	0.044	0.044

[1]Calcium Pantothenate: 4 mg/g; Niacin: 15 mg/g; Vitamin B6: 13 mg/g; Cu: 3 mg/g; Zn: 15 mg/g; Mn: 20 mg/g; Fe: 10 mg/g; K: 0.3 mg/g. [2]Vitamin A: 5000 IU/g; Vitamin D3: 500 IU/g; Vitamin E: 3 mg/g; Vitamin K3: 1.5 mg/g; Vitamin B2: 1 mg/g. [3]SID (Standardized Ileal Digestible).

Study design

The experiment included nine treatments and three replicates per treatment, each one with ten animals. Commencing from day one, three replicate pens of 10 chicks each were assigned to one of the nine treatments, comprising three levels of fish oil4 (0, 1.5 and 2.0%) combined with three levels of green tea powder Camellia sinensis (0, 1 and 1.5%). According to the assayed combination of supplements, groups were created as follows:

Group 1 - Controls (no fish oil (FO) or green tea powder (GrT) was added to the diet);
Group 2 - Baseline diets incorporating only green tea powder, at 1.0%;
Group 3 - Baseline diets incorporating only green tea powder, at 1.5%;
Group 4 - Baseline diets incorporating fish oil, at 1.5%, but not green tea powder;
Group 5 - Baseline diets incorporating fish oil, at 1.5%, and green tea powder at 1.0%;
Group 6 - Baseline diets incorporating fish oil, at 1.5%, and green tea powder at 1.5%;
Group 7 - Baseline diets incorporating fish oil, at 2.0%, but not green tea powder;
Group 8 - Baseline diets incorporating fish oil, at 2.0%, and green tea powder at 1.0%;
Group 9 - Baseline diets incorporating fish oil, at 2.0%, and green tea powder at 1.5%.

Table 3. Diet ingredients and calculated nutrient content in the finisher diet (days 29-42).

Ingredient (gr/kg)	Treatments								
	1	2	3	4	5	6	7	8	9
Corn	656.2	646.7	641.8	649.5	639.8	635.0	644.1	637.6	632.7
Soybean Meal	302.7	299.7	298.4	303.9	301.1	299.7	305.0	301.5	300.2
Fish Oil	0	0	0	15	15	15	20	20	20
Green Tea Powder	0	10	15	0	10	15	0	10	15
Soybean Oil	14.5	17	18.2	5	7.5	8.7	2.8	4.3	5.5
DL-Methionine	1.8	1.8	1.8	1.8	1.8	1.8	1.8	1.8	1.8
Lysine-Hydro-Chloride	0.8	0.8	0.8	0.8	0.8	0.8	0.8	0.8	0.8
Mineral Mixture[1]	3	3	3	3	3	3	3	3	3
Vitamin Mixture[2]	3	3	3	3	3	3	3	3	3
$CaCO_3$	10	10	10	10	10	10	10.5	10	10
Phytase enzyme	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5
Multi-enzyme	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5
Ca:P (22:18) %	7	7	7	7	7	7	8	7	7
Total	1000	1000	1000	1000	1000	1000	1000	1000	1000
Energy (kcal/kg)	3050	3050	3050	3050	3050	3050	3050	3050	3050
Protein (%)	19.39	19.39	19.39	19.39	19.39	19.39	19.39	19.39	19.39
Crude fiber (%)	2.866	2.990	3.053	2.868	2.993	3.055	2.871	2.994	3.057
Calcium (%)	0.630	0.628	0.628	0.630	0.629	0.628	0.670	0.629	0.628
Tryptophan SID[3] (%)	0.390	0.385	0.383	0.389	0.384	0.382	0.388	0.384	0.381
Available Phosphorus (%)	0.242	0.241	0.240	0.241	0.240	0.239	0.260	0.240	0.239
Linoleic Acid (%)	1.954	2.049	2.094	1.516	1.610	1.654	1.407	1.462	1.506
Lysine SID (%)	1.129	1.118	1.113	1.131	1.121	1.115	1.133	1.121	1.116
Methionine SID (%)	0.525	0.521	0.518	0.524	0.520	0.518	0.524	0.520	0.518
Cysteine (%)	0.602	0.596	0.592	0.602	0.595	0.592	0.602	0.595	0.592
Sodium (%)	0.045	0.045	0.044	0.045	0.044	0.044	0.045	0.044	0.044

[1]Calcium Pantothenate: 4 mg/g; Niacin: 15 mg/g; Vitamin B6: 13 mg/g; Cu: 3 mg/g; Zn: 15 mg/g; Mn: 20 mg/g; Fe: 10 mg/g; K: 0.3 mg/g. [2]Vitamin A: 5000 IU/g; Vitamin D3: 500 IU/g; Vitamin E: 3 mg/g; Vitamin K3: 1.5 mg/g; Vitamin B2: 1 mg/g. [3]SID (Standardized Ileal Digestible).

Immunization program

To test the humoral immune competence in treated groups, the following challenging tests were performed in 3 birds/pen, unless explicit otherwise:

a) Response to the Newcastle lentogenic vaccine inoculated at days 1 and 20 was assessed in blood sampled at days 7 and 27; commercial lyophilized vaccines[6] prepared with the strains Hitchner B1 and La Sota were administered respectively at day 1 and day 7.

b) Response to the Influenza vaccine (Avian Influenza-H9N2)[7] administration at day 1 was assessed in blood sampled at days 21 and 28.

c) Response to sheep red blood cells (SRBC) inoculation – The antigenic challenge with SRBC was performed in day 14, and blood sampling was performed at days 21 and 28 for assessment of antibody production. 0.5 mL of a 10% suspension of SRBC in sterile PBS[8] (phosphate buffered saline solution; v/v) was inoculated under the broiler´s skin in the breast area. In each replicate, only two birds were inoculated. In these birds a pre-immune blood sample was collected.

The normal vaccination schedule was completed by routine vaccination against the infectious bronchitis (Infectious Bronchitis Virus (IBV) (H120)[9] at days 1 and 10, and by Gamboro vaccination (Gamboro (IBD071IR)[10] at day 1.

Blood samples (2 mL) were collected from the wing vein on the pre-scheduled days. The samples were centrifuged at 252 g for 10 min and the serum harvested and storage at -20ºC until analysis [10].

Haemagglutination inhibition assay

The haemagglutination inhibition (HI) assays were performed in a round-bottomed 96-well microplate. For each plate row (12 wells), ten wells were used for samples and 2 for controls (positive and negative controls), according to Bartlett and Smith [4]. Briefly, prior to the test the serum samples were heat-treated at 56°C for 30 min in a water bath, for complement inactivation.

Each well of the plate was filled with 50 μL PBS. The initial well in each row received 50 μL of serum sample and for the last row 50 μL of standardized antigen 4 HAU (haemagglutination antigen units) as positive control; after gently tapping, twofold serial dilutions were performed using the wells on the right, till the final column. The plate was gently tap and allowed to incubate at room temperature for 30 min. Then 25 μL of 1% SRBC was added to each well on the plate; the plate was gently mixed and allowed to incubate at room temperature for 45 min, before recording HI titers.

The described procedure was repeated to assess the production of IgG except that 50 μL of 0.01 M 2-Mercaptoethanol (2-ME) was added to the first row of wells and allowed to incubated at 37°C for 30 min, to destroy IgM.

The HI titer for the total antibodies or of IgM was considered based on the last dilution of serum that completely inhibited haemagglutination and was expressed as log2. The IgM titer was estimated form the different between total antibodies and IgG titers.

Lymphoid organs and liver weight

At the age of 42 days, after 4 h of fasting, one bird from each replicate, in a total of 3 broilers *per* group, was chosen and slaughtered to collect the main lymphoid organs (thymus, spleen and bursa of Fabricius) and liver. Care was taken to choose the most representative male birds, which would present a live body weight similar to the mean live body weight of their cohorts. Birds were fully pluck by dry pecking method and the post-slaughter weight was recorded to estimate the relative organ weight. The thymus (all the lobes), liver, spleen and bursa of Fabricius were immediately removed, stripped of adherent connective tissue, and individually weighed in an electric balance. Relative organ weights were calculated as percentage of live body weight.

Data analysis

Data were analysed by IBM SPSS Statistics Base 17.0 statistical software for Windows®, using the GLM procedure. Titer data were analysed as a two-way ANOVA procedure, using a 3 × 3 factorial design with three fish oil (0, 1.5 and 2.0%) and three green tea powder (0, 1.0 and 1.5%) dietary supplements. For mean comparisons, least significant difference (LSD) was used. The results were considered significantly different when $P < 0.05$. The results are presented as mean ± standard error of the mean.

RESULTS

Antibody Responses to Challenging

Data on humoral immune responses of broilers, including the antibody titers against Avian Influenza, Newcastle and SRBC are summarized on tables 4 to 6. For viral challenges, both the primary and secondary responses were evaluated while for the response to SRBC only the primary response was evaluated, in two distinct moments post-challenging.

Considering the primary humoral response to avian influenza virus, in general GrT supplementation showed worst response to influenza challenge than did FO groups. Still, no differences were found between groups ($P = 0.120$; Table 4). The influence of GrT powder on humoral response was mild to absent, and when combined it tend to lessen FO effects, particularly when incorporated at 1.5% (Groups 3, 6 and 9; Table 4). Antibody titers were similar between controls and groups 2, 6 and 9 and only slightly increased in groups 3 and 5. Again, the differences were independent of treatments ($P = 0.857$).

Also for the secondary immune response, no differences between groups were found, despite the improved antibody production against Influenza virus found in treated groups compared to controls, in particular in group 7 and 8 (Table 4). In all the groups, the antibody titers were increased compared to those of the primary response. In general, supplementation with FO or GrT revealed to be inefficient to elicit aconsistent immune response compared to controls ($P = 0.672$ and $P = 0.749$, respectively).

Regarding the humoral response to Newcastle challenge, GrT supplementation resulted in lowered antibody production compared to controls, whether for the primary or the secondary response was considered. An overall slight increase in antibody production was

found for the primary response to Newcastle challenge in groups supplemented with1.5% fish oil, but not with 2% FO. GrT treated groups showed a decrease in the overall Newcastle antibody titers. However, the differences were devoid of significance for both FO ($P = 0.529$) and GrT ($P = 0.276$) [Table 4]. The effect of GrT supplementation was negatively but non-significantly correlated to the amount incorporated when combined with FO. A slight non-significant increase in antibody titers was detected in groups supplemented only FO (Table 4), but no changes were recorded according to the level of FO incorporation. Little changes in the secondary response were observed in groups not supplemented with GrT in comparison to the elicited primary response. All the other treatment groups showed lower antibody titers after the second challenge than controls, even if they tend to be slightly higher than at the moment of the primary response (Table 4), with exception for group 9, which showed a notorious fall in serum antibodies against Newcastle disease compared to all groups ($P = 0.040$).

Pre-immune sera from all birds were negative against SRBC. Overall, SRBC challenge elicited a more efficient humoral response one week after the inoculation than after 2 weeks (Table 5). One week after challenging, antibody titers were significantly lower than controls in groups 2 to 5, 7 and 9. Only groups 6 and 8 showed equal or increased serum titers compared to controls. Nevertheless, these differences were found non-significant ($P = 0.851$). Two weeks after the SRBC challenge, an overall decrease of antibody titers to half was recorded for all the groups (Table 5). Supplementation did not influenced total antibody titers against SRBC in this moment.

Total antibody production titers reflect IgM production after SRBC challenge, while IgG were relatively constant and at low values after challenging whether the period in analysis (Table 5). In general, responses to immune challenges in supplementation groups did not differ from control group on respect to IgM production at 7 days post-challenge despite the non-significant increase in IgM titers found in group 6 (Table 5). Two weeks after the challenge, the IgM titers were similar between groups ($P = 0.158$; Table 5).

Lymphoid Organs and Liver Weights

No statistical differences were found in the body weight at slaughter (after plucking) among treatment groups (Table 6). Small group variation was observed in the weight of thymus amongst groups (Table 6). With exception for group 5, which showed a heavier gland ($P = 0.040$), no differences were found between groups. Considering the relative thymus weight, the findings were similar. Slightly non-significant decreases in thymus weight were found in groups supplemented with 2% FO (Groups 7 to 9) or with the combination of 1.5% FO plus 1% GrT (Group 6). Only Group 5, supplemented with 1.5% FO and 1.0% GrT showed increased relative thymus weight ($P = 0.040$).

None of the treatments influenced the absolute ($P = 0.177$) or relative spleen weight ($P = 0.222$). Still, a small decrease in absolute weight was detected in all groups excepted in group 3 (Table 6). However, the differences fail to present statistical differences. Again, considering the relative spleen weight, all treated groups, small non-statistical differences were found between controls and treated groups (Table 6). In general, FO supplemented at 2% induced a non-significant decrease in the relative spleen weight, while no important changes were observed in GrT supplementation groups.

FO treated groups showed irregular responses in the absolute or relative weight of the Fabricius bursa ($P = 0.417$ and $P = 0.427$, respectively). On the contrary, all GrT treated groups showed increased weight for bursa de Fabricius compared to controls whether absolute or relative values were considered ($P = 0.090$ and $P = 0.061$, respectively; Table 6). Group 8, supplemented with 2% FO and 1.0% GrT was the group presenting the heavier bursa, in either the absolute or relative weight (Table 6). Nevertheless, no statistical differences were found between groups for the weight of bursa de Fabricius in either case ($P = 0.782$ and $P = 0.874$, respectively for FO and GrT treatments).

Supplementation did not influence the absolute or relative liver weight ($P = 0.288$ and $P = 0.954$, respectively for FO and GrT treatments; Table 6). Still, slightly decreases in absolute liver weight in comparison to controls were found in most treated groups, with exception for group 3, supplemented only with 1.6% GrT, and group 7, supplemented with only 2% FO (Table 6), which showed heavier livers than controls. When considering the relative liver weight, all the groups supplemented with 2% FO, with or without GrT (Groups 7 to 9), as well as group 4, supplemented with only 1.5% of FO (group 4) showed non-significant decreases compared to controls, while groups devoid of FO supplementation and the one supplemented with 1.5% FO plus 1.0% GrT (Groups 2, 3 and 5) presented the heaviest livers.

Table 4. Immune response mean (± standard error of mean) after vaccination (Influenza and Newcastle) of Ross 308 broilers fed diets containing the different levels of fish oil and green tea.

Treatment		Antibody titers (Iog2)			
		Against Influenza		Against Newcastle	
		At day 21	At day 28	At day 7	At day 27
Fish oil (% in diet)	0	2.44 ± 0.50	3.33 ± 0.41	1.33 ± 0.33	2.33 ± 0.69
	1.5	2.78 ± 0.22	3.11 ± 0.11	1.78 ± 0.52	2.22 ± 0.52
	2.0	3.00 ± 0.47	3.78 ± 0.50	1.33 ± 0.65	1.56 ± 0.56
P- value		0.120	0.672	0.529	0.276
Green tea powder (% in diet)	0	3.11 ± 0.42	3.56 ± 0.48	2.00 ± 0.37	2.89 ± 0.63
	1.0	2.67 ± 0.33	3.44 ± 0.29	1.56 ± 0.58	1.89 ± 0.48
	1.5	2.44 ± 0.48	3.22 ± 0.36	0.89 ± 0.51	1.33 ± 0.55
P- value		0.521	0.749	0.257	0.069
Group 1		2.33 ± 0.33	3.33 ± 0.33	1.33 ± 0.33	$3.67^{a,b}$ ± 1.76
Group 2		2.33 ± 0.88	3.00 ± 0.58	1.33 ± 0.67	1.67^{a} ± 0.88
Group 3		2.67 ± 1.45	3.67 ± 1.20	1.33 ± 0.88	1.67^{a} ± 0.67
Group 4		3.33 ± 0.33	3.33 ± 0.33	2.33 ± 0.67	$3.00^{a,b}$ ± 0.00
Group 5		2.67 ± 0.33	3.00 ± 0.00	1.67 ± 1.33	1.67^{a} ± 0.67
Group 6		2.33 ± 0.58	3.00 ± 0.01	1.33 ± 0.88	2.00^{a} ± 1.53
Group 7		3.67 ± 1.20	4.00 ± 1.53	2.33 ± 0.94	2.00^{a} ± 1.00
Group 8		3.00 ± 0.58	4.33 ± 0.33	1.67 ± 0.67	2.33^{a} ± 1.20
Group 9		2.33 ± 0.67	3.00 ± 0.00	0.00 ± 0.00	0.33^{b} ± 0.33
P- value		0.854	0.643	0.640	0.230

*Means (± standard error of mean) within each column of dietary treatments with no common superscript differ significantly at $P < 0.05$.

DISCUSSION

Broilers immune system is easily unbalanced under modern rearing conditions leading to high productive losses, increased morbidity and mortality. Thus, intense efforts have been developed in the formulation of broilers diets in order to achieve a balance between the adequate nutrient profile for the necessary feed conversion and the support or event the strengthening of the immune system competence [11].

Immune responses are generally classified as innate and acquired or adaptive. In chickens, while a species with relatively short life span, innate immunity is quite effective at defending against most potential pathogens. Innate immunity can be affected by stressful physiologic events, such as suboptimal environmental conditions, poor management practices, mycotoxins, competition for feed, stress and suboptimal nutrition [14]. Moreover, gut physiology and nutrient availability in birds depends on the existing intestinal bacteria, which in turn can be manipulated by nutrition. Disruption of intestinal biota aggravates of immuno-suppression and predispose to increased morbidity or mortality.

Today, nutrition, genetics, vaccination and hygiene, are the core issues of the poultry health and productivity. Several nutritional strategies have been tested to alleviate immuno-suppression in broiler chickens since the antibiotics additives have been banned due to public health concerns with microbial antibioresistence [16]. Alternative substances have been studied for nutritional modulation of poultry immune competency, such as probiotics, yeast cultures, enzymes and diverse phytogenic additives. There is evidence suggesting that inclusion of fish oil in the diet has beneficial effects on the chicken immune system, because it increases the proportion of omega-3 (n-3) relative to omega-6 (n-6) PUFA (polyunsaturated

Table 5. Immune response mean (± standard error of mean) after injection of sheep red blood cell at Ross 308 broilers fed diets containing the different levels of fish oil and green tea

		Antibody titers (Iog2) against Sheep Red Blood Cell (SRBC)					
		Total antibodies		IgG		IgM	
Treatment		At day 21	At day 28	At day 21	At day 28	At day 21	At day 28
Fish oil (% in diet)	0	4.44 ± 0.67	1.89 ± 0.35	0.22 ± 0.15	0.22 ± 0.15	4.22 ± 0.64	1.67 ± 0.29
	1.5	5.00 ± 0.67	2.00 ± 0.41	0.22 ± 0.15	0.00 ± 0.00	4.78 ± 0.62	2.00 ± 0.34
	2.0	4.56 ± 0.65	1.89 ± 0.20	0.33 ± 0.24	0.00 ± 0.00	4.22 ± 0.62	1.89 ± 0.20
P- value		0.822	0.963	0.883	0.123	0.771	0.745
Green tea powder (% in diet)	0	4.44 ± 0.80	1.89 ± 0.36	0.22 ± 0.15	0.00 ± 0.00	4.22 ± 0.80	1.89 ± 0.26
	1.0	4.67 ± 0.69	2.00 ± 0.39	0.56 ± 0.24	0.11 ± 0.11	4.11 ± 0.56	1.89 ± 0.30
	1.5	4.89 ± 0.46	1.89 ± 0.42	0.00 ± 0.00	0.11 ± 0.11	4.89 ± 0.45	1.78 ± 0.43
P- value		0.894	0.963	0.073	0.613	0.638	0.959
Group 1		5.00 ± 1.53	2.00 ± 0.58	0.33 ± 0.33	0.00 ± 0.00	4.67[a] ± 1.67	2.00 ± 0.58
Group 2		4.33 ± 1.00	1.33 ± 0.33	0.00 ± 0.00	0.33 ± 0.33	4.00[a] ± 1.00	1.00 ± 0.00
Group 3		4.00 ± 1.00	1.33 ± 0.33	0.00 ± 0.00	0.33 ± 0.33	4.00[a] ± 1.00	1.00 ± 0.00
Group 4		4.33 ± 0.88	1.67 ± 0.33	0.33 ± 0.33	0.00 ± 0.00	4.00[a] ± 0.58	1.67 ± 0.33
Group 5		4.67 ± 1.86	1.67 ± 0.33	0.33 ± 0.33	0.00 ± 0.00	4.33[a] ± 1.67	1.67 ± 0.33
Group 6		6.00 ± 0.58	2.67 ± 1.20	0.00 ± 0.00	0.00 ± 0.00	6.00[a,b] ± 0.58	1.78 ± 1.20
Group 7		4.00 ± 2.08	2.00 ± 0.58	0.00 ± 0.00	0.00 ± 0.00	4.00[a] ± 2.08	2.00 ± 0.58
Group 8		5.00 ± 0.58	2.00 ± 0.00	1.00 ± 0.58	0.00 ± 0.00	4.00[a,b] ± 0.00	2.00 ± 0.00
Group 9		4.67 ± 0.33	1.67 ± 0.33	0.00 ± 0.00	0.00 ± 0.00	4.67[a] ± 0.33	1.67 ± 0.33
P- value		0.851	0.349	0.374	1.000	1.000	0.158

*Means (± standard error of mean) within each column of dietary treatments with no common superscript differ significantly at $P < 0.05$.

fatty acids) in tissues. Omega-3 PUFAs have anti-inflammatory and immunosuppressive properties and might potentially affect the poultry immune responses by altering the phospholipid composition of immune cells membranes [15]. The omega-3 PUFA enrichment of cell membrane has been associated with decreases in the inflammatory response, improvements in mediated immunity and growth rate, while its influence on specific immunity is less clear [13,15]. However, fish oil concentrations in diets present some restrictions due to the residual fish flavour in poultry meat thereby originating consumers' rejection.

Thus, one approach available is the nutritional modulation of the avian immune system. It seem possible to modulate of the bird´ immune response by manipulating the type of fat incorporated into the diet. However, fat origin (fish vs. seed) [6] and the bird genetic strain [18] may interfere with the response to supplementation. In the present study it was intend to examine the effect of the incorporation of fish oil and green tea in 2 different concentrations into baseline diets on the humoral immune responses of Ross 308 broilers challenged with SRBC and Newcastle and Influenza virus as antigens, along with the effects on the weight of lymphoid organs and liver. Fish oil contains relatively large amounts omega-3polyunsaturated fatty acids (n-3 PUFAs), which has been envisaged as an improver of broilers immune system [15]; lower levels of fish oil in diets may be advantageous for broiler recovery from immunological challenges. Moreover,

Table 6. Mean absolute (gr) and relative (%) weight (± standard error of mean) of organs related with immune system at 42nd days of age in Ross 308 broilers fed diets containing the different levels of fish oil and green tea.*

Treatment		Body weight (gr)	Weight of lymphoid organs (gr or %)							
			Thymus		Liver		Spleen		Bursa of Fabricius	
			Absolute	Relative	Absolute	Relative	Absolute	Relative	Absolute	Relative
Fish oil (% in diet)	0	2136.67 ± 75.52	8.73 ± 0.57	0.41 ± 0.02	54.76 ± 1.71	2.59 ± 0.13	3.32 ± 0.37	0.16 ± 0.02	2.07 ± 0.20	0.10 ± 0.01
	1.5	2080.56 ± 73.65	9.15 ± 0.97	0.45 ± 0.05	52.04 ± 2.30	2.52 ± 0.13	3.38 ± 0.30	0.17 ± 0.02	2.36 ± 0.12	0.12 ± 0.01
	2.0	2295.56 ± 71.53	7.15 ± 0.85	0.32 ± 0.04	53.68 ± 2.13	2.34 ± 0.09	2.83 ± 0.24	0.12 ± 0.01	2.48 ± 0.29	0.11 ± 0.01
P- value		0.092	0.208	0.089	0.649	0.326	0.400	0.180	0.417	0.427
Green tea powder (% in diet)	0	2247.22 ± 65.55	8.18 ± 0.71	0.37 ± 0.03	53.92 ± 1.49	2.41 ± 0.06	3.27 ± 0.34	0.15 ± 0.02	1.93 ± 1.67	0.09^{a} ± 0.01
	1.0	2082.56 ± 76.73	9.34 ± 1.02	0.46 ± 0.06	52.32 ± 2.31	2.55 ± 0.18	2.87 ± 0.35	0.14 ± 0.02	2.57 ± 0.24	0.12^{6} ± 0.01
	1.5	2182.78 ± 72.96	7.52 ± 0.72	0.35 ± 0.03	54.24 ± 2.33	2.49 ± 0.10	3.39 ± 0.23	0.16 ± 0.01	2.41 ± 0.20	0.11^{a,b} ± 0.01
P- value		0.284	0.308	0.152	0.786	0.697	0.465	0.773	0.090	0.061
Group 1		2171.70 ± 123.3	8.62^{a} ± 1.09	0.40 ± 0.05	54.90 ± 2.44	2.53 ± 0.08	4.03 ± 0.81	0.19 ± 0.05	1.72^{a} ± 0.31	0.08^{a} ± 0.01
Group 2		2018.03 ± 149.9	8.05^{a} ± 0.54	0.40 ± 0.14	51.87 ± 4.07	2.63 ± 0.42	2.47 ± 0.43	0.12 ± 0.42	2.17^{a} ± 0.54	0.11^{a} ± 0.02
Group 3		2222.00 ± 158.8	9.53^{a,b} ± 1.37	0.43 ± 0.43	57.52 ± 2.16	2.60 ± 0.11	3.45 ± 0.42	0.16 ± 0.02	2.33^{a} ± 0.06	0.11^{a} ± 0.01
Group 4		2211.67 ± 110.0	9.40^{a,b} ± 1.23	0.43 ± 0.05	51.58 ± 4.00	2.33 ± 0.09	3.41 ± 0.14	0.16 ± 0.03	2.13^{a} ± 0.35	0.10^{a} ± 0.03
Group 5		1961.67 ± 121.2	11.90^{b} ± 1.35	0.61 ± 0.07	52.37 ± 5.84	2.71 ± 0.39	3.36 ± 0.97	0.18 ± 0.06	2.33^{a} ± 0.24	0.12^{a} ± 0.01
Group 6		2068.33 ± 150.2	6.16^{a} ± 0.02	0.30 ± 0.02	52.18 ± 3.62	2.53 ± 0.15	3.38 ± 0.45	0.17 ± 0.03	2.62^{a} ± 0.25	0.13^{a,b} ± 0.02
Group 7		2358.33 ± 120.8	6.52^{a} ± 1.16	0.27 ± 0.38	55.29 ± 3.67	2.36 ± 0.10	2.38 ± 0.20	0.10 ± 0.01	1.94^{a} ± 0.44	0.08^{a} ± 0.02
Group 8		2068.33 ± 82.9	8.07^{a} ± 1.33	0.36 ± 0.12	52.73 ± 3.64	2.32 ± 0.11	2.76 ± 0.38	0.12 ± 0.02	3.21^{b} ± 0.10	0.14^{a,b} ± 0.01
Group 9		2260.00 ± 76.5	6.81^{a} ± 1.06	0.32 ± 0.05	53.01 ± 6.24	2.34 ± 0.27	3.35 ± 0.54	0.15 ± 0.02	2.28^{a} ± 0.63	0.10^{a} ± 0.02
P- value		0.408	0.371	0,547	0.288	0.954	0.177	0.222	0.782	0.874

*Means (± standard error of mean) within each column of dietary treatments with no common superscript differ significantly at $P < 0.05$.

it has also been demonstrated that fish oil at low levels also improved broiler body weight, by interacting with Insulin production and the energetic and lipid metabolism [9].

Green tea, made from the leaves from Camellia sinensis that have undergone minimal oxidation during processing, is a functional medicinal plant used as anti-aging herb in oriental societies. Green tea contains catechins, commonly called polyphenols, which show strong antioxidant potential. Functional features referred to green tea include antioxidant, anti-diabetogenic and cholesterol-lowering properties, which results in lean poultry meat and increased performance [21,23]. On the other hand, green tea is rich in polyphenols, mainly catechins, and studies in other species points the existence of antimicrobial activity and of anti-oxidant protective effects, which are particularly valued in immunomediated diseases, cancer or cardiovascular diseases [7,24].

In the present study, slightly improved humoral responses against influenza were found in groups under 2% FO supplementation thought these beneficial effects could be antagonised by higher GrT co-supplements. Moreover, the humoral response to Newcastle virus challenging was positively influenced by FO supplementation and again these effects could be partially reversed by GrT co-supplementation. This is in part supported by the available literature [6,15]. It has been demonstrated that improvement of humoral responses with fish oil are dose dependant, with birds

fed with higher levels of FO showing higher antibody titers than those feed medium or low levels [6,15]. Still, it is also acknowledged that increased FO incorporation rates (above 3 to 4%) in diets result in fish flavours in poultry meat and compromise the consumer acceptance of the product.

Thought devoid of significance for most cases, an antagonist effect was found for FO and GrT supplementation on respect to the humoral responses against virus and in SRCB challenges, which ought to be clarified in future works. When analysing in more detail the humoral responses to non-viral (SRBC) challenges, an overall decrease in IgM production was observed associated with FO and GrT co-supplementation. On this regard, effects of GrT incorporation were inconstant. Anti-SRBC antibody titers obtained in the present study were similar to those reported in other studies [19], despite that only one challenge was performed and a different rout for administration was used (subcutaneous vs. intravenous). Still, they were lower than those reported by Saleh *et al*. [20]. Considering that total Igs denote the chicken´ potential humoral immune response [25], the present study showed that both FO and GrT supplementation induced slight non-significant changes in total Igs content one week post-challenge.

The thymus, spleen, and bursa of Fabricius are major lymphoid organs in poultry. During the immune challenge, mature lymphocytes and other immune cells interact with the injected antigens in these tissues. It is commonly accepted that immune tissue mass may reflect the immune system functionality [8,22]. Regarding the weight of lymphoid organs, little changes in its relative weight were observed in the present study, which is in accordance with previous reports [1]. In general, inclusion of 1.5% of FO in poultry diets was accompanied by a slight decrease in the relative weight of thymus but not on the the bursa of Fabricius weight, if associated with 1.0% of GrT, but not in different combinations of FO and GrT co-supplementation. With higher FO proportions, a non-significant reduction in the weight of the lymphoid organs analysed was recorded, whether GrT co-supplementation was used or not. The slightly reduction of the spleen relative weight in all the treatments might be associated with the anti-inflammatory properties of either FO and GrT supplementation, which might limit the proliferation of the mature lymphocytes during the immune challenges. Nevertheless one cannot forget that, in birds, the spleen mass is highly variable within individuals, with season, gender, parasitic load, stress [22]. The decrease in thymus and spleen weight, on another hand, could also be a reflex of the suppressive action of n-3 fatty acids on the lymphocytes proliferation in response to viral challenge [1]. Overall, neither FO nor GrT supplementation significantly affect the relative liver weight, though changes in the organ weight followed the tendencies reported earlier for the other organs evaluated in the study.

It has been reported that FO supplementation up to 4 weeks of age will increase the weight of thymus, spleen, and bursa of Fabricius. Thereafter, with the natural decrease in weight of these immune organs and spontaneous atrophy of the bursa, supplementation effects are more difficult to ascertain [25]. Similarly, the delay between the immune challenge (between days 7 and 21) and the weighing analysis of the chicks´ lymphoid organs (day 42) in the present work might explain the inability in detect significant changes in the weight. Nevertheless, the existence of beneficial effects of FO supplementation on chicken immunity is still controversial [6,15]. In fact, Korver and Klasing [15] reported that fish oil supplementation decreased indices of the inflammatory response and either improved or did not change indices of the specific immune response of growing chicks, in a concentration-related fashion.

CONCLUSION

In the present study, no clear beneficial effects of the diets with n-3 fat sources was evidenced, but none of the tested combinations of FO and GrT supplementations compromised the productive parameter of broilers or increased largely the feeding costs. In fact, the final live weight of broilers was similar to the observed in other studies under similar conditions. Furthermore, this also suggest that inclusion of GrT did not constraint fed intake, as reported in early studies. None of the essayed treatments showed a clear favourable influence over the broilers´ humoral immune response. Nevertheless, as large individual differences among birds should be expectable, as it was found in the present study, these preliminary results ought to be confirmed in studies with a larger number of individuals.

SOURCES AND MANUFACTURERS

[1]Multifenelic- Sigma-Aldrich. St. Louis, MO, USA.

[2]Formalex®- American Master Tech. Lodi, CA, USA.

[3]Benzalkonium chloride- Sigma-Aldrich. St. Louis, MO, USA.

[4]Black Sea-Caspian Kilka. Mehregan Khazar Co. Gilan, Iran.

[5]Teaman Company, Refah Tea. Rasht, Iran.

[6]Newcastle lentogenic vaccine, strains Hitchner B1 and La Sota. Razi Co, Karaj, Iran.

[7]Avian Influenza- H9N2, Razi Co. Karaj, Iran.

[8]Phosphate Buffered saline Solution- Sigma-Aldrich. St. Louis, MO, USA.

[9]Infectious Bronchitis Virus (IBV) (H120), Razi Co. Karaj, Iran.

[10]Gamboro (IBD071IR), Razi Co. Karaj, Iran.

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***Ethical approval*.** Use of birds in this study was approved by the research committee of the Islamic Azad University, Rasht Branch (17/16/4/7829-920424).

***Declaration of interest*.** The authors report no conflicts of interest. The authors alone are responsible for the content and writing of the paper.

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