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ORIGINAL RESEARCH

Feeding of dried sweet orange (*Citrus sinensis*) peel on humoral immune response of broiler chickens

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Abstract

Purpose An experiment was conducted to evaluate the effect of dried sweet orange (*Citrus sinensis*) peel (DCSP) on humoral immune response of broiler chickens.

Methods Four hundred 1-day-old Ross 308 broilers were distributed according to a completely randomized design into five treatments with four replicates of 20 chicks each. The following five dietary treatments were applied: control group with 0% DCSP; diet containing 1.5% DCSP from 1 to 21 days of age (starter phase) and from 1 to 42 days of age, respectively; and diet containing 3% DCSP from 1 to 21 days of age (starter phase) and from 1 to 42 days of age, respectively.

Results The DCSP treatments influenced positively total anti-SRBC and IgG titers on days 28 and 42, whereas no differences were found for IgM titer. The anti-NDV titers were not affected by diets; conversely, the anti-AIV titers were different among treatments. The anti-IBD titers were different on days 14 and 42 among diets, whereas anti-IBV titers were not influenced by treatments. Average white blood cell, heterophil, lymphocyte, and monocyte counts, as well as heterophil/lymphocyte (*H*/*L*) ratio, were different among treatments.

Conclusion The findings suggested that the dietary inclusion of dried *Citrus sinensis* promoted some effects on the immune humoral response of chickens; however, these effects were not completely effective to protect birds from the main diseases.

Keywords Nutrition · *Citrus sinensis* · Broiler · Immunity

Introduction

The significant level of consumer expectations and sensitivity relative high protein intake will increase the demand for poultry meat (Laudadio et al. 2012). The use of additives in poultry nutrition is considered a solution for better nutrient utilization. The use of growth promoters has resulted in a high rate of broiler production in recent years. At present, this trend has been changing due to the increase of production and concerns with environmental safety and public health (Gibson and Roberfroid 1995; Tufarelli et al. 2017).

Infectious diseases of domestic animals and poultry cause huge economic losses worldwide. One solution to reduce the risk of infectious diseases is by using immune system stimulants (Dong et al. 2007; Qorbanpour et al. 2018). Herbal plants and their products such as essential oils, flavonoids, carotenoids, saponins, plant steroids, phenolic compounds, tannins, quinones, coumarin, lectin, polypeptides, insoluble non-starch polysaccharides, and oligosaccharides have been shown to enhance immune system and improve the poultry performance (Chen et al. 2003; Azizi et al. 2018).

The immunity of birds can be divided into humoral and cellular immunity. Humoral immunity is characterized by an adaptive function of the immune system, which produces antibodies in response to an antigen. Cellular immunity involves mechanisms by which cells infected with foreign agents such as viruses are destroyed directly by an effector (e.g., activated T cell) in contact with the target cell (Weinstock et al. 1989).

Lymphoid and non-lymphoid organs are two structural categories of immune system of birds. The bursa of Fabricius and the thymus, where B and T cells develop and

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differentiate, respectively, are considered primary lymphoid organs, whereas the spleen is regarded as a secondary lymphoid organ. Additionally, the lymphoid structures distributed along the gut also play an essential role in birds' immune protection. These immune structures represent an important immune barrier against many economically significant pathogens that replicate in the intestinal epithelium. Non-lymphoid components of the immune system include cells that provide a non-specific immune defense of the host (Qureshi et al. 1998; Marech et al. 2018). Klasing (1998) reports that factors related to bird genetics, frequency of their exposure to pathogens, and effectiveness of vaccination programs influence the incidence of infectious diseases in birds. However, diet characteristics, such as nutritional levels and types of feedstuffs, may affect the susceptibility of birds to infectious diseases.

The peel from *Citrus sinensis* is a significant source of pectin (a non-digestible carbohydrate), which stimulates the growth of probiotic bacteria in the colon, thereby preventing the growth of pathogenic bacteria. Sweet *Citrus sinensis* peel contains ethyl acetate extracts, which have been shown to inhibit the growth of Gram-positive bacteria (*Staphylococcus aureus, Bacillus cereus, Listeria monocytogenes*), yeasts, and molds (Chanthaphon et al. 2008; Pourhossein et al. 2015).

Therefore, considering all of the above, the aim of this study was to evaluate the effect of the dietary inclusion of different levels of dried *Citrus sinensis* peel on the humoral immune response of broilers.

Materials and methods

The experiment was conducted on a farm located in Some'esara, Guilan province, Iran. In total, 400 1-day-old Ross 308 broilers were housed in 20 cages (2 × 1 × 1 m) and distributed according to a completely randomized design into five treatments with four replicates of 20 birds each. The farm building was thoroughly cleaned and disinfected. House temperature was maintained by three gasoline rocket heaters and was controlled by three thermostats installed in different parts of the building. In order to provide moisture, water was sprayed on the floor in order to maintain 50–60% moisture during the experimental period. Lighting was provided by windows and 26-watt fluorescent bulbs in three rows distant approximately 3 m from each other. The different treatments used to evaluate the humoral immune response of broilers were as follows (Table 1).

Control: Basal diet without any additive (Pourhossein et al. 2015).

Table 1 Ingredients and nutritional composition of diets

Ingredients (%)	Starter	Grower
Corn grain	54.32	58.69
Soybean meal	39.43	31.87
Oyster shell	0.90	0.79
Vitamin and mineral premix[a]	0.50	0.50
Salt	0.37	0.37
DL-methionine	0.20	0.22
L-Lysine	0.07	0.05
Corn oil	2.16	5.83
Dried *Citrus sinensis*	2.05	1.68
Nutritional composition		
Metabolizable energy (kcal/kg)	2.900	3.200
Crude protein (%)	22.16	19.20
Calcium (%)	1.00	0.80
Available phosphorus (%)	0.50	0.42
Lysine (%)	1.15	0.96
Methionine (%)	0.50	0.48
Methionine + cystine (%)	0.83	0.78
Threonine (%)	0.79	0.71
Dried *Citrus sinensis* (mEq/kg)	236.00	202.00

[a]Supplied per kg of diet: 12,000 IU vitamin A, 10 mg vitamin E, 2200 IU vitamin D, 35 mg niacin, 12 mg D-pantothenic acid, 3.63 mg riboflavin, 3.5 mg pyridoxine, 2.4 mg thiamine, 1.4 mg folic acid, 0.15 mg biotin, 0.03 mg vitamin B, 60 mg manganese, 40 mg zinc, 1280 mg iron, 8 mg copper, 0.3 mg iodine, and 0.2 mg selenium

Basal diet + 1.5% dried *Citrus sinensis* peel for 1–21 days of age;
Basal diet + 1.5% dried *Citrus sinensis* peel for 1–42 days of age;
Basal diet + 3.0% dried *Citrus sinensis* peel for 1–21 days of age;
Basal diet + 3.0% dried *Citrus sinensis* peel for 1–42 days of age;

The feeds supplied during the experimental periods were formulated according to NRC (1994) and the chemical composition of dried sweet orange peel was analyzed using the AOAC (1990) method (Table 2).

Birds were vaccinated according to established research program against Infectious Bronchitis Virus (IBV), Avian Influenza virus (AIV), Newcastle disease virus (NDV)*** and Bursal infectious disease (IBD) by spray or drinking water. The antibody titers of the birds were analyzed on different days according to the type of vaccine. Serum antibodies were measured in one bird per replicate by ELISA (Bio-Check) test. On days 21 and 35, a 0.5% of sheep red blood cells (SRBC) (0.1 mL per kg body weight) was injected into the wing vein of the birds. A week later (days 28 and 42) blood samples were taken, and serum was separated, followed by decomplementation at 56 °C for 30 min, using as

Table 2 Chemical composition of the orange peel

Composition	Amount (%)
Dry matter	88.00
Protein	5.46
Calcium	1.10
Phosphorous	0.05
Ash	7.00
Carbohydrate	63.54
Ether extract	2.00
Fiber	10.00

diluent buffer PBS with 1% bovine serum albumin (BSA/Fort Dodge®-22%). Serum dilutions (1:2, 1:4, 1:8, 1:16, 1:32, 1:64, 1:128, 1:256, 1:512, 1:1024 and 1:2048) were prepared in 96-well microtiter plates followed by the addition of 25 µL of 1% SRBC in each well, using the technique of simple hemagglutination, as described by Wegmann and Smithies (1965). After incubation for 45 min, HI titers were reported based on $\log_2$. Serum dilutions (1:2, 1:4, 1:8, 1:16, 1:32, 1:64, 1:128, 1:256, 1:512, 1:1024 and 1:2048) were prepared in 96-well microtiter plates followed by the addition of 25 µL of 1% SRBC in each well. After incubation for 45 min, HI titers were reported based on $\log_2$. Since IgM is sensitive to 2-Mercaptoethanol (2Me), IgM and IgG titers were obtained by fractionation of the total anti-SRBC titers. On day 42, blood samples were collected in tubes containing EDTA. Total leukocytes were counted in Neubaur hemocytometer. Blood smears were prepared and stained, and heterophils, monocytes, and lymphocytes were counted.

Data were submitted to analysis of variance using the General Linear Model (GLM) procedure of SAS statistical package (SAS Institute 2002) and in case of significant effect, means were compared by the test of Duncan at 5% probability level.

Results and discussion

The applied treatments influenced ($p < 0.05$) total anti-SRBC titers evaluated on days 28 and 42. On both days of data collection, average total anti-SRBC titer values were higher in the birds fed the diet with 1.5% of DCSP for 1–42 days and in those fed the diet with 3% of DCSP for 1–21 days when compared with the other treatments. Average IgG titer values on days 28 and 42 were significantly different ($p < 0.05$) among treatments. No differences ($p > 0.05$) in average IgM titer values were found among treatments (Tables 3, 4, respectively).

Antioxidants are abundant in fruits and vegetables and have the ability to neutralize free radicals and convert them into harmless molecules (Leonard et al. 2002). Therefore, according to Byer et al. (2001), the increase of antioxidant levels decreases free radical reactions which may have beneficial effects on cell activity. Mona and Hanan (2007) showed in laying hens that *Citrus sinensis* peel can improve immune system activities due to their antioxidant properties.

According to Kayvan and Eshtiyaghi (1992), the living environment is full of pathogens, but the animal and human bodies have the ability to protect themselves with the help of the immune system. *Citrus* peel contains antioxidants (flavonoids), which are absorbed and metabolized by the body (Oluremi et al. 2010). The strong total anti-SRBC titer response of the chickens fed the diets with DCSP indicates that *Citrus* peel added to broiler diets enhances immunity. Yamamoto and Glick (1982), comparing the antibody-mediated immunity of broilers selected according to bursa of

Table 3 Anti-SRBC (total), IgG, and IgM titers determined in 28-day-old broilers fed different dietary *Citrus sinensis* peel levels

Antibody titers at 28 days			
Treatments	Anti-SRBC[a]	IgG[b]	IgM[c]
Control	3.00b	1.50c	1.50
1.5% DCSP 1–21 days	3.12b	1.75bc	1.37
1.5% DCSP 1–42 days	4.75a	2.87a	1.88
3.0% DCSP 1–21 days	4.12a	2.25ab	1.87
3.0% DCSP 1–42 days	3.12b	1.50c	1.62
SEM	0.32	0.24	0.23

a–b Means within the same column followed by different letters are significantly different ($P < 0.05$) (Duncan's test)

DCSP dried *Citrus sinensis* peel

[a]Total antibody titers against sheep red blood cell (TSRBC)

[b]Immunoglobulin G (IgG)

[c]Immunoglobulin M (IgM)

Table 4 Anti-SRBC (total), IgG, and IgM titers determined in 42-day-old broilers fed different dietary *Citrus sinensis* peel levels

Antibody titers at 42 days			
Treatments	Anti-SRBC[a]	IgG[b]	IgM[c]
Control	4.50b	1.87b	2.63a
1.5% DCSP 1–21 days	5.50b	2.62ab	2.88
1.5% DCSP 1–42 days	7.00a	3.62a	3.38
3.0% DCSP 1–21 days	5.87ab	2.62ab	3.25
3.0% DCSP 1–42 days	4.75b	2.00b	2.75
SEM	0.46	0.36	0.27

a–b Means within the same column followed by different letters are significantly different ($P < 0.05$)

DCSP dried *Citrus sinensis* peel

[a]Total antibody against sheep red blood cell (TSRBC)

[b]Immunoglobulin G (IgG)

[c]Immunoglobulin M (IgM)

Fabricius size, reported, as shown in the present study, that increase percentage of SRBC injection concentration could achieve higher titers. Scott (1993), studying the diffusion of antibodies ac a sheep red blood cell membrane, observed that after birth, the sheep blood antibodies had parental origin and their values gradually decreased from 6 to 21 days, after which the body started to produce antibodies, thereby increasing their titers.

According to Leshchinsky and Klasing (2001) in experimental models, many parameters of the immune system, including the resistance to infections, specific antibody production, and numbers of antibody producing cells, are modified by diets supplemented or not with antioxidant substances. On both evaluation days (28 and 42), the lowest average IgG titer was obtained in the birds that received the control treatment and those fed the diet with 3% DCSP for 1–42 days; however, the highest average value was observed in the birds fed the diet with 1.5% of DCSP for 1–42 days. According to Nysather et al. (1976), IgG protects the chicken by activating the complement system and by helping the antigen to be phagocytized by macrophages. Flavonoids act through antioxidant effects, direct removal of free-radicals, and effect on nit enzymatic systems. *Citrus* fruit flavonoids have synergistic effect on the immune system (Nijveldt and Boelens 2001).

The anti-NDV titers were not affected ($p > 0.05$) by the treatments (Table 5).

On the other hand, average anti-AIV titers were significantly different ($p < 0.05$) among treatments on days 14 and 28 (Table 6), with the control treatment promoting higher or statistical equal average titers when compared with the other treatments. The results indicated that *citrus* peel does not enhance the immune response of broilers against all pathogens and its action is selective in nature.

Results reported by Chen et al. (2003), who studied the effects of two Chinese herbal polysaccharides [achyranthan (ACH), a low-molecular-weight polysaccharide, and astragalan, a high-molecular-weight polysaccaride] on the immunity and growth performance of young broilers, showed that

Table 5 Anti-newcastle disease virus (NDV) titers (log_2) of broilers fed different dietary *Citrus sinensis* peel levels as determined on 7, 14, 28, 35, and 42 days of age

Anti-NDV titers					
Treatments	7	14	28	35	42
Control	5.00	5.75	4.75	5.00	5.50
1.5% DCSP 1–21 days	4.50	5.50	4.50	5.00	6.00
1.5% DCSP 1–42 days	4.25	4.75	4.00	4.75	4.75
3.0% DCSP 1–21 days	4.50	5.50	4.50	5.00	6.25
3.0% DCSP 1–42 days	5.00	5.00	4.25	5.00	5.25
SEM	0.33	0.40	0.31	0.11	0.50

Table 6 Anti-avian influenza virus (AIV) titers (log_2) of broilers fed different dietary *Citrus sinensis* peel levels as determined on 7, 14, 28, 35, and 42 days of age

Anti-AIV titers					
Treatments	7	14	28	35	42
Control	3.50	4.25a	3.50a	4.75	4.50
1.5% DCSP 1–21 days	3.25	4.25a	3.00b	4.75	5.00
1.5% DCSP 1–42 days	4.00	3.25b	3.00b	3.75	3.75
3.0% DCSP 1–21 days	4.25	4.00ab	3.00b	4.50	4.75
3.0% DCSP 1–42 days	4.00	4.00ab	3.00b	4.50	4.50
SEM	0.33	0.27	0.13	0.37	0.40

a–b Means within the same column followed by different letters are significantly different by the test of Duncan ($P < 0.05$)

T and B lymphocyte counts increased with herbal polysaccharide consumption and antibody titers against a pathogenic ND virus. The supplementation of enzymes to wheat-based diets significantly increased the weight of the spleen, which is the main organ of the immune system (Guo et al. 2004). These enzymes increased antibody titers against ND, indicating enhanced humoral immune response. This enzyme increased lymphocyte and natural killer cell proliferation, showing that cell-mediated immunity was also affected, as also found in the present study. Average anti-IBD titers showed were significantly different ($p < 0.05$) among treatments on days 14 and 42 (Table 7). Average anti-IBV titers were not influenced ($p > 0.05$) by the treatments (Table 8). The highest average anti-IBD titer was determined in the broilers fed the diet with 3% of DCSP from 1 to 21 days, and the lowest titer in those fed the diet with 1.5% of DCSP from 1 to 42 days. The results of the present study are similar to Puthpongsiriporn and Scheideler (2005), who showed that feeding laying hens with flaxseed in the diet did not affect the concentration of IBD and IBV vaccine titers.

Table 7 Anti-infectious bursal disease (IBD) titers (log_2) of broilers fed different dietary *Citrus sinensis* peel levels as determined on 14, 21, and 42 days of age

Anti-IBD titers			
Treatments	14	21	42
Control	6815c	3678	2098b
1.5% DCSP 1–21 days	8583bc	4744	3847b
1.5% DCSP 1–42 days	774c	6177	6612a
3.0% DCSP 1–21 days	12595a	4699	3320b
3.0% DCSP 1–42 days	10943ab	6204	4499b
SEM	805	848	1115

a–b Means within the same column followed by different letters are significantly different by the test of Duncan ($P < 0.05$)

Table 8 Anti-infectious bronchitis virus (IBV) titers (log_2) of broilers fed different dietary *Citrus sinensis* peel levels as determined on 14, 21, and 42 days of age

Anti-IBV titer			
Treatments	14	21	42
Control	4166.5	2727.3	340.5
1.5% of DCSP 1–21 days	3816.3	3765.5	1032.8
1.5% of DCSP 1–42 days	3186.3	3750.5	1430.0
3.0% of DCSP 1–21 days	5299.5	3374.5	746.3
3.0% of DCSP 1–42 days	4542.0	4255.0	906.0
SEM	509	557	388

Table 10 Heterophil/lymphocyte ratio and spleen and bursa weights of broilers fed different dietary *Citrus sinensis* peel levels

Treatments	*H/L*	Spleen (g)	Bursa (g)
Control	88.25a	2.89	1.15
1.5% DCSP 1–21 days	71.24ab	3.35	1.11
1.5% DCSP 1–42 days	77.90a	3.66	1.31
3.0% DCSP 1–21 days	69.50ab	2.90	1.62
3.0% DCSP 1–42 days	54.41b	2.95	1.31
SEM	6.55	0.15	0.10

a–b Means within the same column followed by different letters are significantly different by the test of Duncan ($P < 0.05$)

H/L heterophil to lymphocyte ratio

Average white blood cell, heterophil, lymphocyte, and monocyte counts, as well as heterophil/lymphocyte ratio were different ($p < 0.05$) among treatments (Tables 9, 10, respectively).

Average spleen and bursa weights were not significantly different ($p > 0.05$) among treatments (Table 10). The results of the present study are consistent with those presented by Mona and Hanan (2007), who studied the effects of dried Egyptian clover and orange peels as natural feed additives on immune response of laying hens. Those authors found significantly different hematocrit and red blood cell and white blood cell counts among treatments (0.4 of dry clover; 0.2 of dry clover + 0.2 of *Citrus sinensis* and 0.4 of dry clover + 0.2 of *Citrus sinensis*). It may be due to the adequate amount of alpha-tocopherol in blood that simultaneously ingested the highest amounts of the vitamin C. The enhancement of the function of broilers fed orange peel in the present trial is in agreement with the results of Ding et al. (2004), who found that orange peel greatly improved the immune function. The effect of orange peel may be attributed to its antioxidant activity. Manthey (2004) showed that most of the antioxidant activity of orange peel was due to minor-occurring flavones. Orange peel extract components may counteract enzymatic lipid peroxidation processes (Malterud and Rydland 2000).

Vitamin C and polyphenols present in *Citrus sinensis* enhance the activity of antioxidant enzymes in red and white blood cells (Dragsted et al. 2001). According to Imboden et al. (1985), the polysaccharides found in *Citrus sinensis* significantly increase calcium concentration, which is an important regulator of lymphocyte activity and conduct lymphocyte signals, promoting the lymphocytes' proliferation. Vitamin C and vitamin E are important antioxidants that strengthen leukocyte membranes and, in appropriate amounts, increase heterophil phagocytosis activity (McFarland et al. 2005). Under heat stress situations, vitamin C may alleviate the stress effects by reducing the release of glucocorticoids. The addition of 1% vitamin C in the diet may reduce the immune function due to reduction of heat stress and increase of corticosterone. Differences in the beneficial effects of adding vitamin C to the diet can be attributed to vitamin C instability under storage conditions (Spinosa et al. 2002).

According to Mogenet and Youbicier-Simo (1998), acute heat stress affects the immune responses of lymphoid organs (bursa, thymus, spleen), increasing monocyte and heterophil counts, and heterophil/lymphocyte ratio (*H*:*L* ratio), and the ascorbic acid in the plasma stimulates the immune response and increases IgG and IgM titers in broiler chickens. Karthiyaini and Philiomina (2009) reported that the birds under stress-fed diets with 0.03% of vitamin C presented lower *H*:*L* ratio than those not fed vitamin C, indicating that this

Table 9 White blood cell (WBC) counts of broilers fed different dietary *Citrus sinensis* peel

Treatments	WBC ($\times 10^3$)	(%)		
		Heterophil	Lymphocyte	Monocyte
Control	34.16b	45.25a	51.50b	3.25a
1.5% DCSP 1–21 days	42.58b	40.75ab	57.75ab	1.50b
1.5% DCSP 1–42 days	43.30b	42.50a	55.00b	2.50ab
3.0% DCSP 1–21 days	30.69a	39.25ab	57.75ab	3.00a
3.0% DCSP 1–42 days	44.38b	34.25	63.75a	2.00ab
SEM	4.4	2.2	2.3	0.46

a–b Means within the same column followed by different letters are significantly different by the test of Duncan ($P < 0.05$)

vitamin C reduces the effects of stress, suggesting that *Citrus sinensis* can be used in broiler diets for this purpose.

Conclusions

The inclusion of dried *Citrus sinensis* in broiler diet promoted positively some effects on the immune response; however, these effects were not completely effective to protect the birds against diseases such as infectious bursal disease, infectious bronchitis, Newcastle disease, or avian influenza.

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