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Effect of different levels of dietary sweet orange (*Citrus sinensis*) peel extract on humoral immune system responses in broiler chickens

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Running title: Dietary sweet orange peel extract and broiler immunity

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Abstract

The experiment was conducted to evaluate the effects of different levels of sweet orange (*Citrus sinensis*) peel extract (SOPE) on humoral immune system responses in broiler chickens. Three-hundred one-day broilers (Ross-308) were randomly allocated to treatments varying in supplemental SOPE added in the drinking water. The experimental groups consisted of three treatments fed for 42 days as follow: a control treatment without feed

extract, a treatment containing 1000 ppm of SOPE and a treatment containing 1250 ppm of SOPE. All treatments were isocaloric and isonitrogenous. Broilers were vaccinated with Newcastle disease virus (NDV), avian influenza (AI), infectious bursal disease (IBD) and infectious bronchitis virus (IBV) vaccines. Antibody titer response to sheep red blood cells (SRBC) was higher in group fed 1250 ppm of SOPE ($P< 0.05$) as well as for IgG and IgM. Similarly, antibody titer responses to all vaccines were constantly elevated ($P< 0.05$) by SOPE enrichment in a dose-dependent manner. Relative weights of spleen and bursa Fabricius were unaffected by treatments. Dietary SOPE supplementation may improve the immune response and diseases resistance, indicating that it can constitute a useful additive in of broiler feeding. Thus, supplying SOPE in ration may help to improve relative immune response in broiler chickens.

Keywords: broiler, *Citrus sinensis*, immune system, peel extract

Introduction

The success in poultry production is mainly due to the nutrition, breeding, management as well as the optimal developed of birds' immune status. Modern nutritional and farming strategies have been designed to produce broilers with high potential for growth, yield and feed efficiency that have resulted in compromised health status (Khan *et al.* 2012a). Under these conditions, broilers are exposed to pathogens and other environmental stressors at an age when they are not fully immunocompetent. One of the many approaches proposed to enhance immune response is to supplement the diet with natural products which can modulate immune responses due to their extensive involvement in structural components and molecular mechanisms (Khan *et al.* 2012b). Compared with synthetic antibiotics or inorganic chemicals, these plant-derived products have proven to be natural, less toxic, residue free and are thought

to be ideal feed additives in food animal production (Wang *et al.* 1998). Advances in chemistry and identification of plant compounds which are effective in the treatment of certain diseases have renewed interest in herbal medicines (Khan *et al.* 2012b).

Sweet orange (*Citrus sinensis*) is one of the most important and oldest horticulture products in many tropical and sub-tropical areas. The orange peels are a primary by-product produced by the fruit processing industry, so attempts were made to use sweet orange peel extract as natural feed additive, and even as medicinal supplement for animals (Callaway *et al.* 2008). Sweet orange peels contain high concentrations of phenols, specially flavonoids (Manthey 2004). Citrus fruit extracts are also found to have several inhibitory activities, such as anti-inflammatory, antitumor, antifungal, and blood clot inhibition activities (Middleton and Kandaswami 1994). The health benefits of Citrus fruit have mainly been attributed to the presence of bioactive compounds, such as phenolics (Ross et al. 2000), vitamin C (Nagy 1980) and vitamin E (Craig 1997). Studies have found that the peel had the highest levels of vitamins C and E followed by the pulp then the juice. Thus, the fruit extracts could increase the production of antibody improving indirectly the immune system by their antivirus and antibacterial effects. Therefore, this study was performed to examine the effect of different levels of SOPE as feed additive on immune system response and blood parameters in broiler chickens.

Material and Methods

Birds, Housing and Treatments

The trial was conducted in Sowme'eh Sara, Gilan province (Iran) observing the animal welfare procedures of the Islamic Azad University, Rasht Branch, Rasht, Iran. The trial lasted 42 days during 2012 and used scaffoldings, pens with dimensions of 2×1 m and 1 m height installed, and each pen was assigned to a repetition. In preparation to trial, the poultry facility

was carefully cleaned and rinsed using pressurized water in order to sanitize the facilities (Ebrahimi *et al.* 2013). After drinkers and feeders installation and 24 h before broilers allocation, the hall was gasified. These procedures were repeated before each of the four replicates.

A total of 300 day-old male chicks (Ross-308) from a commercial hatchery were raised in a conventional environment. The study was conducted in a completely randomized design with three treatments. Each treatment was replicated five times, with each replicate comprising one pen of 20 birds. The average weight of broilers was 43.5 g and the respective breeders were 38 weeks of age. Vaccination program applied to birds was reported in Table 1, and vaccines were used following the drinking practices in order to ensure the optimal utilize of the vaccine on all chickens. Further, in order to reduce the stress caused by vaccination, 24 h before and after vaccination, multi-electrolyte solutions were added in the drinking water. Poultry facility had thermostatically controlled curtains and cross-ventilation as well as lighting program (Laudadio *et al.* 2012). Pens were equipped with a pan feeder, a manual drinker and wood shavings. Drinkers were regularly washed to prevent with faecal and microbial contaminations.

For the preparation of sweet orange (*Citrus sinensis*) peel extract (SOPE), 40g of peels were mixed in 320 mL of 72% ethanol and then in water bath at 50°C for 3 h. The suspension was centrifuged at 3000 × *g* for 10 min. The upper liquid was filtered (Wathman No. 42 filter paper) and the concentrate with router evaporator set, and the concentrate were dried under lab room temperature. The obtained extract was added to the drinking water. The chemical composition (% on dry matter, DM) of sweet orange peel extract used for the trial was: 97.5% DM, 5.1% crude protein, 28.5% ether extract, 23.2% carbohydrates, 5.7% ash. A two phase feeding regime consisting of starter (1 - 21 days) and grower (22 - 42 days) was used in the study. Experimental treatments included: SOPE-0, control treatment without feed extract;

SOPE-1000, treatment supplemented with 1000 ppm of feed extract; and SOPE-1250, treatment supplemented with 1250 ppm of feed extract. Diets were formulated to meet or exceed broiler nutrients' requirements (NRC 1994). Broilers had *ad libitum* access to feed and water, and birds and feeders were weighed at one week intervals to monitor the average daily gain, feed intake and feed conversion ratio. The drinking water supplied to broilers was equal for each experimental group in order to assure the same daily water intake.

Samples of the diet were ground in a hammer mill with a 1 mm screen and analysed in triplicate according to the methods of AOAC (2000). The metabolisable energy (ME) of the basal diet was estimated using the Carpenter and Clegg equation (Leeson & Summers 2001): ME (MJ/kg) = 53 + 38 × [crude protein (%) + 2.25 × ether extract (%) + 1.1 × starch (%) + sugar (%)].The amount of available phosphorus of diet was calculated from the difference between total and undigestible phosphorus. Ingredient and chemical composition of the basal diets is shown in Table 2.

Serology and Lymphoid Organ Weight

At 21 and 35 days of age SRBC suspension (5% PBS) was injected in breast muscle of 12 birds per treatment. Total antibody titers to SRBC were determined by hemagglutination assay in serum from birds. Therefore, 7 days after each sensitization (28 and 42 days, respectively) antibody titers against SRBC were measured. In U-bottom microtiter plates, two fold serial dilutions of heat-inactivated (at 56 °C) serum were made with PBS (0.01 M; pH 7.4) for total antibody, or PBS with 1.4% 2-mercaptoethanol for IgG antibody. All antibody titers were recorded as $\log_2$ of the highest dilution of serum that agglutinated an equal volume of a 0.5% SRBC suspension in PBS. The IgM titer was determined by the difference between total and IgG titer.

126 Ten birds from each treatment were chosen at random on days 14, 21 and 42, and blood
127 samples were collected from the brachial vein. Serum was separated by centrifugation (3000
128 × *g* for 15 min) and antibody titers against IBD and IBV were measured using commercially
129 available ELISA kits (Bio-check BV, Gouda, Holland) according to manufacturer's
130 instructions. The absorbance of controls and samples were read at 405 nm using an ELISA
131 reader (Bio-Tek Instruments Inc. ELX 800; Winooski, VT). On days 7, 14, 28, 35 and 42 the
132 blood sample blood samples were collected from the brachial vein and antibody titers against
133 ND and AI were measured in birds through haemagglutination inhibition test according to
134 Cunningham (1971).
135 A total of ten blood samples per treatment were collected from wing vein. Serum was
136 separated by centrifugation at 1300 × *g* for 15 min following a 1 h incubation at room
137 temperature and stored at -20°C until the analysis. White blood cells (WBC) were counted
138 using a bright line hemocytometer at 400× magnification. Blood samples were diluted 20
139 times with a diluter fluid (3 mL acetic acid glacial + 97 mL distilled water + some Lushman
140 stain) for WBC determinant (Hepler 1966). Differential leukocyte counts were examined
141 using four samples per treatment on Giemsa-stained blood smears using a light microscope.
142 One hundred cells were counted and differentiated into heterophils (H), lymphocytes (L) and
143 monocytes. The mean H/L ratio was calculated from individual H/L ratios. At the end of
144 feeding period (42 d), eight birds from each group were chosen randomly and killed by
145 cervical dislocation and the spleen and bursas of Fabricius were removed and their weight
146 recorded .
147
148 ***Statistical analysis***
149 A completely randomized design (CRD) with five replicates was employed for this study.
150 Statistical analyses were conducted using one-way ANOVA general linear models of SAS v8

(SAS Institute Inc., Cary, NC, USA). Means were separated by Tukey-Kramer's multiple range tests. Antibody titers were logarithmically transformed prior to analysis to achieve homogeneity of variance and were expressed as $\log_{10}$. The results were expressed as mean ± SEM. Statistical significance was considered at $P < 0.05$.

Results

Serology

Dietary treatment did not affect (P > 0.10) body weight, daily gain, average daily feed intake and feed conversion ratio of broilers (data not shown).

Chicks given supplemental SOPE tended to express higher immune response for SRBC than those receiving the control treatment. The IgG and IgM titers also increase significantly with SOPE supplementation (P < 0.05). The effect of 1000 and 1250 ppm SOPE was marked (Table 3). The effect of dietary SOPE supplementation on serum antibody titer in broilers vaccinated against ND and AI virus is reported in Tables 4 and 5, respectively. Serum ND antibody titers were positively influenced by treatments on day 28 to 42 days after vaccination (P < 0.05), whereas there was a significant improvement of serum AI antibody titers from 7 to 42 days. The lowest and highest ND and AI antibody titers were in SOPE-0 and SOPE-1250 ppm broilers' groups, respectively. The influence of SOPE enrichment on serum antibody titer in chickens vaccinated against IBD and challenged with IBV virus is shown in Table 6. Broilers in both groups including SOPE showed (on days 14, 21 and 42, respectively) the highest immunological response compared to unsupplemented group (P < 0.05).

Blood traits and lymphoid organ weight

Dietary SOPE had significant effects on serum components, increasing the WBC and lymphocytes concentrations, and decreasing heterophils percentage ($P < 0.05$). The heterophil/lymphocyte ratio (H/L) was significantly decreased by SOPE ($P > 0.05$). Bursa of Fabricius weight was not influenced by SOPE supplementation ($P > 0.05$) (Table 7). There were no significant differences in spleen weigh after 42 days of feeding period ($P > 0.05$).

Discussion

Although a considerable body of evidence have demonstrated the beneficial effect of natural extracts supplementation on the immune system of avian species, several studies have failed to show improvement in disease resistance or enhancement of the immuno-competence due to dietary extract supplementation. However, the herbal extracts have been reported to protect the cells involved in the immune response against oxidative damage and to enhance the function and proliferation of these cells (Khan *et al.* 2012c).

Sweet orange peel extract is a valuable source of antioxidants (Hasin *et al.* 2006; Yang *et al.* 2011) and it was reported that its content of polyphenols increased the antioxidant enzymes in red blood cells (Dragsted *et al.* 2001). Mona and Hanan (2007) showed in laying hens that *Citrus sinensis* peels can significantly improve the immune system activities due to their antioxidant properties. The vitamin C concentration found in *Citrus sinensis* increases humoral response and cellular response as well as bird's resistance to infections such as *Escherichia coli*, *Mycobacterium avium*, ND and IBD diseases (Karthiyaini & Philiomina 2009). Similarly to the findings of this last study, adding the *Citrus sinensis* peel extract in the drinking water improved the antibody titer against ND and AI viruses in broilers. These results can be attributed to the increased activity of T lymphocyte and B lymphocyte of birds. Furthermore, it was also found that dietary vitamin E content have significant influence on the antibody produced by the ND and AI (Leshchinsky & Klasing 2001). In experimental models,

many parameters of the immune system, including the resistance to infections, specific antibody production, numbers of antibody-producing cells, *in vitro* mitogenic responses to lymphocytes and the phagocytic index, are modified by diets supplemented or not with antioxidant substances (Leshchinsky & Klasing, 2001). Boa-Amponsem *et al.* (2000) investigated the cell-mediated and humoral immune response in three pure lines of broiler chicks by feeding either 10 or 300 mg/kg of vitamin E. In this experiment, it was found that antibody titer against SRBC was dose-dependent. Similarly, the production of IgM and IgG were affected by dose, genetic stock as well as the measurement criteria. Analogous results were also observed in terms of H/L ratio.

In a previous study, Hejazi *et al.* (1993) showed an increase of T and B lymphocyte cells as well as an higher antibody titers (up to 40 days of age) in broilers fed diets supplemented with propolis, reporting the positive effect of propolis on the growth of the bursa Fabricius.

It seems that the flavonoids contained in *Citrus sinensis* peel are substances stimulating the immune system through the increase of IgG and IgM antibodies production that reinforce the broiler's humoral immune system. On the other hand, by stimulating macrophages, increased cytokines production, especially the amounts of interferon-γ. Primarily due to the close relationship between the immune system and cancer, treatment with some herbs has been relied to stimulate the immune system by them, while lymphocyte count increasing influence in the intestinal mucosa, as a measure to stimulate the immune system is of interest to researchers (Khan *et al.* 2012d). These findings directly reflect the increased proliferation of immune cells that can either confirm the findings of our study.

Several reports are available advocating the strong immune potentiating effects of natural feed additives (Khan *et al.* 2012b). Ahsan-ul-Haq *et al.* (1999) reported that feeding broilers with garlic powder improved significantly antibody titers against ND virus and IBD, whereas Jafari *et al.* (2008) found that including the garlic powder in chicks diet does not have any

beneficial effects on antibody production. Moreover, feeding garlic powder enhanced titer against ND virus and SRBC in broilers (Hanieh *et al.* 2010) and laying hens (Dorhoi *et al.* 2006). In line with our findings, Rahimi *et al.* (2011) found that 0.1% of coneflower (*Echinacea purpurea*) extract in broiler diet improved antibody response against SRBC, however no was found against ND virus. It was observed that the immuno-modulating effects of natural feed extracts are associated with their ability to enhanced phagocytosis of peritoneal macrophages, increased production of interleukins, interferon-γ and tumor necrosis factor secretary metabolism of macrophages, antigen presenting cells and antioxidant function (Khan *et al.* 2012d).

In conclusion, the sweet orange peel extract supplementation improved the immune response and resistance to diseases, and it seems that dietary extract does not modulate the broiler chicken immune response to diseases in a dose-dependent manner. Thus, a moderate level (1000 ppm) of dietary sweet orange peel extract may help to enhance the relative immune response in broiler chickens.

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References

Ahsan-Ul-Haq, Mera KA, Rasool S. 1999. Effect of supplementing *Allium sativum* (Garlic) and *Azadirachta indica* (Neem) leaves in broiler feeds on their blood cholesterol, triglycerides and antibody titre. *International Journal of Agriculture and Biology* **1**, 125-127.

AOAC. 2000. *Official methods of analysis*. 17th edn. Association of Official Analytical Chemists, Washington DC, USA.

Boa-Amponsem K, Price SEH, Picard M, Geraert PA, Siegel PB. 2000. Vitamin E and immune responses of broiler pureline chickens. *Poultry Science* **79**, 466-470.

Callaway TR, Carroll JA, Arthington JD, Pratt C, Edrington TS. 2008. Citrus products decrease growth of *E. coli* O157: H7 and *Salmonella Typhimurium* in pure culture and in fermentation with mixed ruminal microorganisms in vitro. *Foodborne Pathogens and Disease* **5**, 621-627.

Craig WJ. 1997. Phytochemicals: guardians of our health. *Journal of the American Dietetic Association*. **97**, S199–S204.

Cunningham CH. 1971. *Virologia practica*. 6th edn. p. 260, Acribia, Zaragoza.

Dorhoi A, Dobrean V, Zăhan M, Virag P. 2006. Modulatory effects of several herbal extracts on avian peripheral blood cell immune responses. *Phytotherapy Research* **20**, 352–358.

Dragsted LO, Young JF, Loft S, Sandstrom B, Nesaretnam K, Packer L. 2001. *Biomarkers of oxidative stress and of antioxidative defense: relationship to intervention with antioxidant-rich foods*. In: Nesratnem K, Packer L. (eds) Micronutrients and health: molecular biological mechanisms, pp. 272-278, Danish Food Administration, Søborg, Denmark.

Ebrahimi A, Qotbi AAA, Seidavi A, Laudadio V, Tufarelli V. 2013. Effect of different levels of dried sweet orange (*Citrus sinensis*) peel on broiler chickens growth performance. *Archiv Tierzucht* **2**, 11-17.

Hanieh H, Narabara K, Piao M, Gerile C, Abe A, Kondo Y. 2010. Modulatory effects of two levels of dietary *Allium*s on immune response and certain immunological variables, following immunization, in White Leghorn chickens. *Animal Science Journal* **81**, 673–680.

Hasin BM, Ferdaus AJM, Islam MA, Uddin MJ, Islam MS. 2006. Marigold and orange skin as egg yolk color promoting agents. *International Journal of Poultry Science* **5**, 979-987.

Hejazi AG, Berding EF, Assily S, Khashabah E, Hassan N, Popv S. 1993. Study on some aspect of antiviral activity. 1-Influence of propolis on NDV. *Veterinary Medicine Journal* **41**, 53-56.

Hepler OE. 1966. *Manual of Clinical Laboratory Methods*. Thomas Spring Field, Illinois.

Jafari RA, Jalali MR, Ghorbanpoor M, Saraei SM. 2008. Effect of dietary garlic on immune response of broiler chicks to live Newcastle Disease vaccine. *Pakistan Journal of Biological Sciences* **11**, 1848- 1851.

Karthiyaini K, Philiomina PT. 2009. *Effect of vitamin c supplementation on some biochemical profile stressed broiler*. Proceedings of National Seminar of Recent Trends in Animal Welfare and Sustainable Livestock Production, (PTAWSLPO9), Thrissur, India, p. 109.

Khan RU, Naz S, Javdani M, Nikousefat Z, Selvaggi M, Tufarelli V, Laudadio V. 2012a. The use of Turmeric (*Curcuma longa*) in poultry feed. *World's Poultry Science Journal* **68**, 97-103.

Khan RU, Nikousefat Z., Tufarelli V, Naz S, Javdani M, Laudadio V. 2012d. Garlic (*Allium sativum*) supplementation in poultry diets: effect on production and physiology. *World's Poultry Science Journal* **68**, 417-424.

Khan RU, Naz S, Nikousefat Z, Tufarelli V, Laudadio V. 2012b. *Thymus vulgaris*: alternative to antibiotics in poultry feed. *World's Poultry Science Journal* **68**, 401-408.

Khan RU, Rahman ZU, Nikousefat Z, Javdani M, Tufarelli V, Dario C, Selvaggi M, Laudadio V. 2012c. Immunomodulating effects of vitamin E in broilers. *World's Poultry Science Journal* **68**, 31-40.

Laudadio V, Dambrosio A, Normanno G, Khan RU, Naz S, Rowghani E, Tufarelli V. 2012. Effect of reducing dietary protein level on performance responses and some microbiological aspects of broiler chickens under summer environmental conditions. *Avian Biology Research* **5**, 88-92.

300 Leeson S, Summers JD. 2001. *Scott's Nutrition of the Chicken*. 4th edn University Books,
301 Guelph, Ontario, Canada.
302 Leshchinsky TV, Klasing KC. 2001. Relationship between the level of dietary vitamin E
303 and the immune response of broiler chickens. *Poultry Science* **80**, 1590-1599.
304 Manthey JA. 2004. Fractionation of orange peel phenols in ultrafiltered molasses and mass
305 balance studies of their antioxidant levels. *Journal of Agriculture and Food Chemistry* **15**,
306 7586-7592.
307 Middleton EJ, Kandaswami C. 1994. Potential health promoting properties of citrus
308 flavonoids. *Food Technology* **48**, 115–120,
309 Mona SR, Hanan A. 2007. Effects of using dried Egyptian clover and orange peels as natural
310 feed additives on egg production egg quality and immune response of laying hens. *Fayoum*
311 *Journal of Agricultural Research & Development* **21**, 188-205.
312 Nagy S. 1980. Vitamin C contents of citrus fruit and their products: a review. *Journal of*
313 *Agriculture and Food Chemistry* **28**, 8–18.
314 NRC. 1994. *Nutrient Requirements of Poultry*, 9th rev. ed., National Research Council,
315 Washington, USA.
316 Rahimi S, Zadeh ZT, Torshizi MK, Omidbaigi R, Rokni H. 2011. Effect of the three herbal
317 extracts on growth performance, immune system, blood factors and intestinal selected
318 bacterial population in broiler chickens. *Journal of Agricultural Science and Technology*
319 **13**, 527-539.
320 Ross SA, Ziska DS, Zhao K, Elsohly MA. 2000. Variance of common flavonoids by brand of
321 grapefruit juice. *Fitoterapia* **71**, 154–161.
322 Wang R, Li D, Bourne S. 1998. *Can 2000 years of herbal medicine history help us solve*
323 *problems in the year 2000? Biotechnology in the feed industry*. Proceedings of Alltech's
324 14th Annual Symposium, Kentucky, USA, pp. 273-291.

325 Yang XY, Xie JX, Wang FF, Zhong J, Liu YZ, Li GH, Peng SA. 2011. Comparison of
326 ascorbate metabolism in fruits of two citrus species with obvious difference in ascorbate
327 content in pulp. *Journal of Plant Physiology* **168**, 2196-2205.
328

329 **Table 1.** Vaccination program

Type of vaccine	Age at vaccination (day)	Method of vaccination
Inactive IBV[1], AI[2], ND[3]	0	Spray
ND, IBV	7	Oral
IBD[4]	16	Oral
ND Clon 30	20	Oral
ND Clon 30	30	Oral

330 [1] Infectious Bronchitis Virus (IBV)

331 [2] Avian Influenza (AI)

332 [3] Newcastle Disease (ND)

333 [4] Infectious Bursal Disease (IBD)

334

335 **Table 2.** Ingredient and chemical composition of diets fed to broilers during the experimental

336 periods

Ingredient, % as fed-basis	**Starter diet (1-21 d)**	**Grower diet (21-42 d)**
Corn	54.32	58.69
Soybean meal (44% CP)	39.43	31.87
Corn oil	2.16	5.83
Dicalcium phosphate	2.05	1.68
Oyster shells	0.90	0.79
Vitamin-mineral premix[1]	0.50	0.50
Sodium chloride	0.37	0.37
DL-Methionine	0.20	0.22
L-Lysine	0.07	0.05
Chemical composition		
Metabolizable Energy (kcal/kg)[2]	2,900	3,200
Crude Protein (%)	22.16	19.20
Lysine (%)	1.15	0.96
Methionine (%)	0.50	0.48
Methionine + Cystine (%)	0.83	0.78
Threonine (%)	0.79	0.71
Calcium (%)	1.00	0.85
Available Phosphorus (%)[2]	0.50	0.42
Dietary cation-anion balance (mEq/kg)	236	202

337

338 [1] Supplied per kg of diet: Vitamin A 12000 IU, Vitamin E 10 mg, Vitamin D 2200 IU, Niacin
339 35 mg, D-pantothenic acid 12 mg, riboflavin 3.63 mg, Pyridoxine 3.5 mg, Thiamine 2.4 mg,
340 Folic acid 1.4 mg, Biotin 0.15 mg, Vitamin B 0.03 mg, Manganese 60 mg, Zinc 40 mg, Iron
341 1280 mg, Copper 8 mg, Iodine 0.3 mg, Selenium 0.2 mg. [2] Calculated analyses.

342

343 **Table 3.** Immune response for total SRBC, IgG and IgM (log_{10}) values of broilers fed different sweet orange peel extract levels

Treatment	SRBC[1]		IgG		IgM	
	(28 d)	(42 d)	(28 d)	(42 d)	(28 d)	(42 d)
SOPE-0	3.00b	4.50c	1.50c	1.87b	1.50b	2.63b
SOPE-1000 ppm	4.87a	7.25b	2.87b	4.62a	2.25a	3.12a
SOPE-1250 ppm	5.25a	8.37a	3.00a	4.87a	2.25a	3.62a
SEM	0.41	1.01	0.66	0.95	0.21	0.16
P-value	0.027	0.019	0.039	0.033	0.041	0.045

344

345 SOPE, sweet orange peel extract; $n = 5$ birds/replicate (25 birds/treatment)

346 [1] Sheep Red Blood cell (SRBC); Immunoglobulin G (IgG); Immunoglobulin M (IgM);

347 a-c Means within a column with the same letter are not significantly different ($P < 0.05$).

348

349

Table 4. Anti-Newcastle disease haemagglutination-inhibition (log_{10}) titers of broilers fed different sweet orange peel extract levels

Treatment	Newcastle Disease titers				
	7 d	14 d	28 d	35 d	42 d
SOPE-0	5.00	5.75	4.75b	5.00b	5.50c
SOPE-1000 ppm	5.25	6.50	5.75a	6.00a	6.25b
SOPE-1250 ppm	5.75	6.50	6.00a	6.25a	7.00a
SEM	0.16	0.19	0.22	0.26	0.29
P-value	0.071	0.066	0.039	0.041	0.037

SOPE, sweet orange peel extract; $n = 5$ birds/replicate (25 birds/treatment)

a-c Means within a column with the same letter are not significantly different ($P < 0.05$).

355 **Table 5.** Anti-avian influenza haemagglutination-inhibition (log_{10}) titers of broilers fed different sweet orange peel extract levels.

Treatment	Avian Influenza titers				
	7 d	14 d	28 d	35 d	42 d
SOPE-0	3.50b	4.25c	3.50b	4.75b	4.50c
SOPE-1000 ppm	5.25a	5.50b	5.50a	5.50a	5.50b
SOPE-1250 ppm	5.50a	6.50a	5.25a	5.75a	6.50a
SEM	0.22	0.21	0.15	0.19	0.27
P-value	0.031	0.042	0.044	0.035	0.029

356

357 SOPE, sweet orange peel extract; $n = 5$ birds/replicate (25 birds/treatment)

358 a-c Means within a column with the same letter are not significantly different ($P < 0.05$).

359

360 **Table 6.** The Infectious Bursal Disease (IBD) and Infectious Bronchitis Virus (IBV) (log_{10}) titers of broilers fed with different sweet orange peel

361 extract levels.

Treatment	Treatment					
	IBD (14 d)	IBD (21 d)	IBD (42 d)	IBV (14 d)	IBV (21 d)	IBV (42 d)
SOPE-0	6.82b	3.68b	2.10b	4.17c	2.73c	0.34b
SOPE-1000 ppm	13.10a	7.38a	7.71a	8.23b	5.01b	1.54a
SOPE-1250 ppm	14.37a	8.96a	8.15a	9.28a	6.75a	2.19a
SEM	0.95	0.77	0.58	0.42	0.29	0.12
P-value	0.019	0.021	0.012	0.041	0.031	0.035

362

363 SOPE, sweet orange peel extract; $n = 5$ birds/replicate (25 birds/treatment)

364 a-c Means within a column with the same letter are not significantly different ($P < 0.05$).

365

366 **Table 7.** Blood parameters and lymphoid organs weight of broilers fed different sweet orange peel extract levels.

Treatment	WBC ($n \times 10^3$/ml)	Heterophils (%)	Lymphocytes (%)	Monocytes (%)	H/L	Spleen (g/kg BW)	Bursa Fabricius (g/kg BW)
SOPE-0	34.16b	45.25a	51.50b	3.25	0.88a	2.89	1.15
SOPE-1000 ppm	44.54b	33.75b	64.10a	2.25	0.52b	2.73	1.27
SOPE-1250 ppm	59.38a	33.70b	63.50a	2.75	0.53b	2.90	1.02
SEM	5.11	2.38	3.22	0.45	0.02	0.21	0.09
P-value	0.041	0.045	0.039	0.059	0.028	0.133	0.084

367

368 SOPE, sweet orange peel extract; n = 5 birds/replicate (25 birds/treatment).

369 WBC, white blood cells; H/L, heterophils to lymphocytes ratio; BW, body weight

370 a,b Means within a column with the same letter are not significantly different ($P < 0.05$).