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Supplementation of clove essential oils and probiotic on blood components, lymphoid organs and immune response in broiler chickens

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Abstract

This study was carried out to determine the effect of different levels of clove essential oil, as a natural growth promoter and probiotic on blood components, immune response and lymphoid organs in broiler chickens. A total of 240 straight run one day old commercial broiler chicks (Cobb 500) were distributed randomly into five groups. Each group was subdivided into four replicates with 12 chicks each. The control diet, diet with probiotic protexin® and diets supplemented with 150, 300 and 450 ppm clove essential oil were fed to the chickens. The results showed that different levels of clove essential oils and probiotic had no significant effect on aspartate aminotransferase, alanine aminotransferase, glucose, and albumin at 21 and 42 days of age, but at 42 days of age uric acid was significantly decreased ($P<0.05$) in group treated with probiotic. Administration of probiotic and clove essential oils had no effect on cutaneous basophils hypersensitivity (CBH) and antibody levels against Newcastle disease virus and avian influenza ($P>0.05$) but antibody response to sheep red blood cells was higher in the control group which received a diet supplemented with 450 ppm clove essential oils. It can be concluded that probiotic Protexin and 450 ppm clove essential oil can enhance antibody titre but had no significant effect on cellular immunity and antibody response to Newcastle and influenza disease and relative weight of Bursa of Fabricius, thymus and spleen.

Keywords: phytogenics; probiotic; serum constituents; immunity; broilers

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Introduction

Intensive poultry production causes significant stress on the birds due to the limited amount of space allowed per bird. Overcrowding, vaccination, transportation, heat stress and chilling are some of the major stressors of chicken (Panda et al., 1999). Antibiotics have been used in poultry nutrition to improve growth performance by targeting some specific pathogenic microorganism and promoting the proliferation of useful microorganism in intestinal microflora. However, antibiotics have been banned due to their contribution to the development of resistance which negatively impact human health. Research is currently focusing on natural alternatives additives in place of antibiotics to supplement poultry feeds (Weber et al., 2012).

Probiotics as alternatives to antibiotics in poultry diets are live microbial feed supplements which beneficially affect the host by improving its intestinal microbial balance (Fuller, 2001; Midilli and Tuncer, 2001). Other alternatives are plant extracts that contain many active phytochemical components, including essential oils, which have a wide range of pharmacological activities (Lewis et al., 2003). The major constituents of the clove essential oils are eugenol, caryophyllene, humulene and humulene epoxide. The essential oils extracted from aromatic plants have been shown to have antibacterial (Mitsch et al., 2004), anticoccidial (Gianneanas et al., 2003), antifungal (Janatan et al., 2003), antioxidant (Bostoglou et al., 2004) properties. The health promoting effect of probiotics in the gastrointestinal tract has been mainly

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associated with their capacity to stimulate the immune response and to inhibit the growth of pathogenic bacteria (Rahimi et al., 2011).

Protexin® is a highly concentrated pre-mix containing seven strains of bacteria and two yeasts *(Lactobacillus plantarum* 1.89×10^{10} cfu/kg, *Lactobacillus delbrueckii* sub sp. *Bulgaricus* 3.09×10^{10} cfu/kg, *Lactobacillus acidophilus* 3.09×10^{10} cfu/kg, *Lactobacillus rhamnosus* 3.09×10^{10} cfu/kg, *Bifidobacterium bifidum* 3.00×10^{10} cfu/kg, *Streptococcus salivarius* subsp. *Thermophilus* 6.15×10^{10} cfu/kg, *Enterococcus faecium* 8.85×10^{10} cfu/kg, *Aspergillus oryza* 7.98×10^{9} cfu/kg, *Candida pintolopesii* 7.98×10^{9} cfu/kg). These combinations promote the population of helpful microorganisms (Kabir et al., 2004).

Clove extract is commonly used in the food industry because of its special aroma and natural safety. In addition, the essential oil from clove also exhibits strong antibacterial properties. Antiseptic, appetite and digestion stimulant (Kamel, 2001), anti-inflammatory and antioxidant activities of clove and its ingredients have been reported (Dragland et al., 2003).

The objective of this study was to investigate the effects of supplementing commercial broiler feeds with Protexin and clove essential oils on blood components, lymphoid organs and immune response in broiler chickens.

Materials and Methods

Experimental design and diets

A total of 240 mixed one day old commercial Cobb 500 broiler chicks were randomly divided into five groups. Every group was replicated four times with 12 chicks each. All diets were formulated to meet or exceed the nutrient requirements of broilers as recommended by Cobb 500 (Cobb, 2007). The compositions of basal diets are shown in Table 1. Dietary treatments are one, basal diet with no additive; two, basal diet supplemented with 120 ppm probiotic Protexin; three, four and five were supplemented with 150, 300 and 450 ppm clove essential oil, respectively. Clove oil was dissolved in vegetable oil and then gently mixed with the above mentioned diets. The chicks were raised on floor pens (1 m^2) for 6 weeks and had *ad libitum* access to feed and water. Lighting was provided 24 h per day throughout the trial. The ambient temperature was gradually decreased from 33 to 25°C on day 21 and was then kept constant.

Determination of blood factors

At 21, 35 and 42 d of age, for measurement of blood biochemical factors, one bird from each replicate with average pen weight was selected. Blood samples were taken from wing vein and kept at the room temperature for 30 minutes. Then blood was centrifuged for 15 min at 3000 rpm to obtain serum. The serum was then transferred to Eppendorf tube and kept at -20°C till pending assays. Serum AST, ALT, albumin, uric acid and glucose concentration were determined calorimetrically using an auto-analyzer system (Bio Systems S.A., Costa Brava 30, 08030 Barcelona, Spain) using commercially available kits (Bio Systems Co. Spain).

Table 1: Nutrient content of the basal diet over different periods of production

Ingredients (%)	Starter (1-10 d)	Grower (11-22 d)	Finisher (23-42 d)
Corn	55.59	61.27	63.19
Soybean meal (44% CP)	36.74	31.04	28.31
Soybean oil	3.56	4.07	5.05
Common salt	0.30	0.30	0.35
Dicalcium phosphate	1.85	1.79	1.66
Limestone	1.25	1.22	1.15
DL-methionine	0.21	0.22	0.23
L-lysine HCl	0.00	0.09	0.06
Mineral and vitamin premix [1]	0.50	0.50	0.50
Calculated composition			
ME (kcal/kg)	2990	3085	3176
CP (%)	21.00	19.00	18.00
Calcium (%)	1.00	0.96	0.90
Available P (%)	0.50	0.48	0.45
Na (%)	0.20	0.17	0.16
Lysine (%)	1.20	1.10	1.05
TSAA	0.89	0.84	0.82

1Vitamin premix per kg of diet: vitamin A (retinol), 2.7 mg; vitamin D3 (Cholecalciferol), 0.05 mg; vitamin E (tocopheryl acetate), 18 mg; vitamin k3, 2 mg; thiamine 1.8 mg; riboflavin, 6.6 mg; panthothenic acid, 10 mg; pyridoxine, 3 mg; cyanocobalamin, 0.015 mg; niacin, 30 mg;biotin, 0.1 mg; folic acid, 1 mg; choline chloride, 250 mg; and Antioxidant 100 mg. Mineral premix per kg of diet: Fe (FeSO4.7H2O, 20.09% Fe), 50 mg; Mn (MnSO4.H2O, 32.49% Mn), 100 mg; Zn (ZnO, 80.35% Zn), 100 mg; Cu (CuSO4.5H2O), 10 mg; I (KI, 58% I), 1mg; and Se (NaSeO3, 45.56% Se), 0.2 mg.

Determination of CBH

The cell mediated immune response was assessed using the cutaneous basophil hypersensitivity response to phyto hemagglutinin. At 35 days of age, a cutaneous basophil hypersensitivity test was administered to 2 chickens per replicate (n=8). Phytohaemagglutinin-P (PHA-P) was injected intradermally [100 µg of PHA-P suspended in 0.1 ml of phosphate buffered saline (PBS) per bird] into the web between the third and fourth toes of the left foot. For the control, an equal volume of PBS was injected into the same toes web of the right foot. The thickness of the two toes webs was measured before the intervention and at 12 and 24 h after the injections with a pressure sensitive caliper. The swelling response was measured by subtracting the pre-injection measurement from the post-injection measurement (12 and 24 h afterwards) of the PHA-P-injected toe web.

Antibody response

One chicken per replicate (n=4) was selected and was intramuscularly injected with 1 ml of 15% sheep red

Table 2: The effects of clove essential oils and probiotic on blood components at 21 and 42 d

Treatments	AST (IU/l)	ALT (IU/l)	Glucose (mg/dl)	Albumin (mg/dl)	Uric acid (mg/dl)
21 d					
Control	224.63	9.69	237	13.30	5.45
Probiotic	225.23	9.26	235.75	13.02	3.91
150 ppm clove essential oil	233.55	8.98	233.08	13.45	5.18
300 ppm clove essential oil	229.53	10.18	224.15	13.27	5.18
450 ppm clove essential oil	236.33	9.76	226.25	13.07	4.88
SEM	8.134	0.509	7.188	0.349	0.361
P value	0.809	0.524	0.642	0.906	0.067
42 d					
Control	249.42	10.53	246	13.82	4.152^{a}
Probiotic	231.65	9.52	250.5	13.47	2.635^{b}
150 ppm clove essential oil	244.60	8.92	237.5	14.62	3.732^{ab}
300 ppm clove essential oil	252.37	10.98	214.43	14.20	3.702^{ab}
450 ppm clove essential oil	243.80	10.18	243.48	14.80	3.725^{ab}
SEM	4.76	0.658	9.093	0.766	0.287
P value	0.066	0.241	0.092	0.727	0.002

[a,b]Means with no common superscripts in each column are significantly different (P<0.05)

Table 3: Effects of different levels of clove essential oils and probiotic on production of immunoglobolin M, immunoglobolin G and immunoglobolin T in broiler chickens

	IgG	IgM	IgT
35 d			
Control	2.00^{ab}	2.25	4.25^{c}
Probiotic	3.25^{a}	3.50	6.75^{a}
150 ppm clove essential oil	1.75^{b}	2.75	4.50^{c}
300 ppm clove essential oil	2.00^{ab}	3.50	5.50^{b}
450 ppm clove essential oil	2.75^{ab}	2.75	5.50^{b}
SEM	0.413	0.551	0.273
P value	0.006	0.454	0.0001
42 d			
Control	2.00^{b}	1.50	3.50^{c}
Probiotic	3.50^{a}	2.25	5.75^{a}
150 ppm clove essential oil	2.00^{b}	2.25	4.25^{bc}
300 ppm clove essential oil	2.50^{ab}	2.00	4.50^{bc}
450 ppm clove essential oil	3.25^{a}	2.00	5.25^{ab}
SEM	0.33	0.37	0.37
P value	0.015	0.628	0.005

[a,b]Means with no common superscripts in each column are significantly different (P<0.05)

blood cells (SRBC suspension in PBS at day 28). SRBC were used as an antigen to quantify the antibody response. Blood samples were collected at 7 and 14 days post-injection respectively. The serum from each blood sample was heat inactivated at 56°C for 30 min and then analyzed for IgG (mercaptoethanol-resistant), IgM (mercapto-ethanol-sensitive) and anti-SRBC antibodies as described by Cheema et al. (2003). The SRBCs used for inoculation and antibody titration were obtained from the same donor sheep.

Newcastle and influenza disease vaccine response

The chicks were vaccinated with Newcastle Disease Virus (NDV) and Avian Influenza (AI) on d 8 (14 and 20 days of age). One chick was selected randomly per replicate (n=4) and blood samples were taken from wing vein. The sera were separated by centrifugation (15 min at 3000 rpm) and stored at -20°C in separate sterile vials prior to analysis. The antibody titre was measured using Haemaglutinin Inhibition test and antibody titre expressed as reciprocal log2 values for the highest dilution that displayed HI.

Determination of immune organ masses

One bird per replicate (n=4) with similar average pen weight was selected on d 21 and 42. Bursa of Fabricius, spleen and thymus (all lobes on the left side of the neck) were removed, weighed and expressed as a percentage of live weight.

Statistical analysis

The data were analyzed using the GLM procedure of SAS 9.1 (SAS, 2004). Tukey's Studentized Range (HSD) test was used to compare the means. Statements of statistical significance are based on P<0.05.

Results

Blood factors

In the present study, different levels of clove essential oils and probiotic Protexin had no significant effects on blood concentration of AST, ALT, glucose, albumin and uric acid at 21 days of age (Table 2) but at 42 days, Protexin significantly reduced the level of uric acid as compared with control group (P<0.05).

Immune response

The results of different levels of clove essential oils and probiotic Protexin on IgM, IgG and IgT production are shown in Table 3. The results showed that at 35 and 42 days of age, the IgG and IgT levels improved significantly in group which was treated with Protexin. Protexin and 450 ppm clove essential oil caused a significant increase in serum IgG level compared to other treatments at 42 days of age.

Table 4: Effects of different levels of clove essential oil and probiotic on antibody response to Newcastle disease virus (NDV) and Avian influenza (AI) of broiler chickens

	ND	AI
6 days after vaccination		
Control	2.25	0.25
Probiotic	3.25	2.25
150 ppm clove essential oil	2.75	0.50
300 ppm clove essential oil	2.75	0.75
450 ppm clove essential oil	4.50	1.00
SEM	0.85	0.72
P value	0.43	0.36
12 days after vaccination		
Control	3.25	2.00
Probiotic	4.50	2.75
150 ppm clove essential oil	2.75	1.00
300 ppm clove essential oil	3.00	1.75
450 ppm clove essential oil	3.75	3.00
SEM	0.82	0.19
P-value	0.60	0.76

[a,b]Means with no common superscripts in each column are significantly different (P<0.05)

Table 5: Effects of different levels of clove essential oils and probiotic on Cutaneous Basophil Hypersensitivity response to phytohemagglutinin (CBH) in broiler chickens

	12 h after injection	24 h after Injection
Control	0.44	0.68
Probiotic	0.46	0.65
150 ppm clove essential oil	0.43	0.69
300 ppm clove essential oil	0.45	0.73
450 ppm clove essential oil	0.45	0.70
SEM	0.00	0.01
P value	0.44	0.14

[a,b]Means with no common superscripts in each column are significantly different (P<0.05)

The results showed that antibody response to Newcastle and Influenza disease (Table 4) and cutaneous basophils hypersensitivity response (Table 5) was unaffected by treatments (P>0.05). Relative to the mass of bursa of Fabricius, thymus and spleen were not affected (P>0.05) by treatments at 21 and 42 days of age (Table 6).

Discussion

Results of the current study showed that the clove essential oil and Protexin had no effects on serum AST, ALT, glucose and albumin concentrations (Table 2). The result showed that probiotic decreased the levels of uric acid at 42 days of age is in agreement with the reported decrease in uric acid in broilers (Isshiki, 1979). Shams-Shargh et al. (2008) showed that supplementing of probiotic to basal broiler diets increased productive performance and decreased serum uric acid, cholesterol triglyceride concentrations and nitrogen excretion and decreased litter pH. Suppressing urease activity and ammonia production can be beneficial for improving animal health and enhancing growth because ammonia locally produced by ureolysis in the intestinal mucosa can exert a significant damage to the surface cells (Isshiki, 1979). Urease has been known to play an essential role in pathogenesis of gastritis induced by *Helicobacter pylori* (Yeo and Kim, 1997).

The data showed that CBH and antibody response to ND and AI were unaffected by the treatments (P>0.05). The results showed that at 35 and 42 day of age, the antibody levels (IgG and IgT) were improved significantly in group which was treated with Protexin. It was also observed that at 42 day of age, 450 ppm clove essential oils and Protexin could increase the levels of IgG compared with other groups. Haghighi et al. (2005) reported that probiotic-treated birds had significantly more serum antibody than the control group. The results showed that treatment with different levels of clove essential oils had no pronounced effect on relative weight of bursa of Fabricius, thymus, spleen and CBH but at 42 days of age, supplementing 450 ppm clove essential oils and Protexin could increase the levels of IgG compared with other groups. Emerging evidence indicates that herbal plants exert their beneficial effects on animal immune system mostly by plant secondary metabolites (Hashemi et al., 2008).

Wang et al. (1998) reported that clove essential oils contain eugenol (which has antibacterial properties), improved immunity by increasing synthesis of IgG in body and IgA in saliva.

Moghaddam et al. (2009) indicated that relative weight of spleen in broilers at 28 days of age and relative weight of Bursa of Fabricius at 42 days of age were not influenced by probiotic administration. In contrast, Alkhalf et al. (2010) suggested that probiotic supplementation significantly increased the relative weight of spleen at 42 d of age compared to the control group. It has been shown that probiotics stimulate different subsets of immune system cells to produce cytokines, which in turn play a role in the induction and regulation of immune response. Some evidence showed that cytokines such as IL-4 and IL-10 are induced by Lactobacilli (Lutful Kabir, 2009). Competitive exclusion cultures are able to exclude enteric pathogens competition for receptor sites, production of volatile fatty acids that are inhibitory to certain enteric pathogens and native flora limiting nutrients (Hashemzadeh et al., 2010), competition for binding sites on the intestinal epithelium and stimulation of immune system (Anjum et al., 2005).

Differences in results are consequences of numerous factors such as type and part of plant used and their physical properties, harvest time, phytogenic additive preparation method, herbal extraction methods and compatibility with other food components (Hashemi et al., 2008). In addition, quality of chickens, health

Table 6: Effects of different levels of clove essential oils and probiotic on on Bursa Fabricius, spleen and thymus on broiler chickens (percent body weight)

	Bursa of Fabricius		Spleen		Thymus	
	21 d	42d	21d	42d	21d	42d
Control (Basal diet)	0.22	0.17	0.23	0.11	0.40	0.33
Probiotic	0.22	0.20	0.24	0.07	0.47	0.38
150 ppm clove essential oil	0.25	0.15	0.25	0.12	0.40	0.34
300 ppm clove essential oil	0.23	0.26	0.25	0.12	0.54	0.38
450 ppm clove essential oil	0.23	0.17	0.23	0.11	0.46	0.35
SEM	0.02	0.02	0.01	0.35	0.08	0.04
P value	0.90	0.12	0.52	0.57	0.79	0.87

[a,b]Values in the same columns and variable with no common superscript differ significantly (P<0.05)

condition, environmental management and production facility can also affect the performance (Hashemi, 2010).

It can be concluded that 450 ppm clove essential oil and probiotic Protexin can increase the levels of antibody (IgG and IgT) with no effect on response to cutaneous basophils hypersensitivity, Newcastle and influenza virus. Also different levels of clove essential oils and probiotic had no significant effect on relative weight of bursa of Fabricius, thymus and spleen.

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