Antioxidant and anticoccidial effects of garlic powder and sulfur amino acids on *Eimeria*-infected and uninfected broiler chickens

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Summary

To study the anticoccidial and antioxidant effects of garlic powder (GP) and total sulfur amino acid (TSAA) on growth performance, faecal oocyst output, oxidative stress indicators and antioxidant status markers in broiler chickens, a 2 × 2 × 2 split-plot-factorial arrangement of treatments was used. A total of 480-day-old male broiler chickens were equally assigned to two plots. Two hundred forty chickens were challenged with *Eimeria* oocysts species mix by oral inoculation at day 34 (infected plot) and the other half was left unchallenged. In each plot, chickens were randomly assigned to four treatments with 5 replicates of 12 birds each, and fed one of the following diets: basal diet, basal diet plus 0.5% GP, basal diet plus 50% more TSAA, basal diet plus 0.5% GP and 50% more TSAA. The results showed that inoculation with 7.5×10^2 oocysts of *Eimeria* mixed species significantly reduced growth performance (P<0.05) and increased nitric oxide (NO) and malonyldialdehyde (MDA), but did not change the hepatic activity of superoxide dismutase (SOD) and the glutathione peroxidase (GPX) activity. Growth depression and increased NO and MDA were greater in infected than uninfected birds. In the infected birds fed with the basal diet without any supplementation, NO and MDA were significantly greater (P<0.05) in comparison with other groups. Faecal oocyst output significantly decreased with the supplementation of GP and TSAA in infected birds (P<0.05). In conclusion, the supplementation of GP and 50% TSAA individually or in combination with the basal diet significantly improved ADG (P<0.05) and decreased OPG, MDA and NO. Moreover, no changes in the antioxidant enzymes were observed in birds infected with *Eimeria*.

Key words: Coccidiosis, Broiler chickens, Oxidative stress, Garlic powder, Sulfurs amino acid

Introduction

Coccidiosis, an intestinal infection of broiler chickens caused by different species of *Eimeria,* invades the mucosal epithelium of the intestines and causes enormous economic losses, particularly by reducing growth rate (Conway and McKenzie, 1991; Lillehoj and Lillehoj, 2000; Guo *et al*., 2007). Invasion of the intestinal epithelium by *Eimeria* sporozoites results in inflammation leading to the initiation of the immune response to the production of nitric oxide (NO) and other powerful pro-oxidants that belong to reactive oxygen species (ROS). Nitric oxide and other ROSs are not only toxic for sporulated oocysts but also have negative side effects on the host if not protected by an antioxidant system (Halliwell and Gutterige, 1989). In normal physiological conditions, oxidative damage is minimized by antioxidant defense systems. However, it is not surprising that an imbalanced oxidant/antioxidant status is observed in a wide range of pathologies such as cocidiosis (Allen, 1997). Numerous reports have indicated the efficacy of garlic on coccidia infection in rabbits (Toulah and Al-Rawi, 2007), mice (Dkhil *et al*., 2011) and broilers (Nidaullah *et al*., 2010). Garlic extract is believed to be capable of reducing coccidial oocysts without affecting the growth of the broilers (Nidaullah *et al*., 2010). In one study, when garlic was added to a high-fat diet, the level of the antioxidant enzyme, catalase (CAT), which is normally depleted under oxidative stress, increased (Kempaiah and Srinivasan, 2004). Taniguchi *et al*. (2000) too showed that sufficient amounts of methionine and cystine in a diet were important to protect the liver of rats from oxidative damage after N-nitrosodimethylamine administration. Gawain *et al*. (1960) showed that while birds consuming TSAA-adequate diets exhibited the expected severe growth depression when given the same dose of oocysts, an unexpected infection × TSAA interaction occurred when they were fed diets deficient in TSAA, and that *Eimeria acervulina* infection produced a marked growth response. Métayer *et al*. (2008) reported that garlic cystine improved oxidative injuries of the intestine.

Data concerning the effects of organosulfur compounds on the antioxidant status of broilers under oxidative stress is limited. The principal objective of this study was thus, to evaluate the antioxidant status of uninfected and *Eimeria*-infected broilers fed diets supplemented with garlic powder (GP) and TSAA.

Materials and Methods

Birds and experimental design

This experiment was designed as a split-plot-factorial (2 × 2 × 2) arrangement of treatments with two levels of garlic (0 and 0.5%), two levels of total SAA (TSAA requirement based on Ross 2009 recommendations and 50% more TSAA by adding extra DL-methionine) and two oocyst levels; non-infected and infected.. Using a plastic barrier, the rearing room was partitioned to two plots and the 480-day-old male broiler chicks (Ross 308) were divided equally between each. Chickens in each plot were randomly assigned to 4 treatments with 5 replicates of 12 birds per cage. In each plot, chickens were fed one of four diets. Diet 1 was a basal diet without any supplements. In diet 2, 0.5% garlic powder (GP) was added to the basal diet and diet 3 contained 50% more TSAA added to the basal diet by supplementing DL-methionine. Diet 4 was a combination of diets 2 and 3. The TSAA content of diets 3 and 4 was 1.61%, supplied by the addition of 0.87% DL-methionine to the basal diet and given to chickens 0-10 days of age. The content of TSAA was 1.43%, supplied by adding 0.74% DL-methionine to the basal diet and given during 10-24 days of age. The content of TSAA was 1.3%, supplied by adding 0.65% DL-methionine and given during 24-49 days of age. An equal amount (0.5%) of wheat bran was added to diets 1 and 3 that had no GP. All diets were isocaloric and isonitrogenous. At 34 days of age, 240 birds were infected with 7.5×10^2 mixed sporulated oocysts (suspended in 1 ml of distilled water) by oral inoculation. Chicks were reared in wire cages to prevent contact with faeces. Strict precautions were taken to avoid cross contamination between infected and uninfected birds. To meet the nutrient requirements of the broiler chickens during the experimental period (1-49 days), complete mashed corn-soybean basal diets were formulated to meet or exceed the nutrient requirements of the Ross 308 recommendation for broiler chickens (Ross manual, 2009). The composition of the basal diets for 0-10, 11-24 and 25-49 days of age is shown in Table 1.

Eimeria oocyst prepartion and bird infection

The *Eimeria* species used in this experiment were provided according to the Soulsby (1982) method. 0.5×10^2 mixed sporulated oocysts of *Eimeria* (*E. maxima*, *E. acervulina*, *E. necatrix* and *E. tenella*) were inoculated orally to all 10 coccidia-free 14-day-old chickens in one cage. After 5 to 10 days, all faecal samples were collected and oocysts were isolated and counted according to the Soulsby (1982) method. Finally, at 34 days of age, 240 birds were infected with 7.5×10^2 mixed sporulated oocysts (suspended in 1 ml of distilled water) by oral inoculation.

Table 1: Composition of the basal diet

Ingredient (%)	(Starter) 0-10 days	(Grower) 11-24 days	(Finisher) 25-49 days
Corn, grain	51.4	53.3	58
Soybean meal-44%	40.6	36.5	32.2
Poultry fat	3.63	5.7	5.7
Limestone	1.24	0.9	0.8
Dicalciumphosphate	1.81	1.7	1.6
Common salt	0.39	0.3	0.3
Vit/Min premix	0.5	0.5	0.5
L-Lysine HCl	0.15	0.15	-
DL-Methionine	0.32	0.25	0.20
Wheat bran	0.5	0.5	0.5
Garlic powder[1]	-	-	-
Nutrient content (calculated)			
ME kcal/kg	3020	3160	3210
CP (%)	22.21	21.11	19.47
Calcium (%)	1.02	0.93	0.85
Available phosphorus (%)	0.5	0.45	0.42
Lysine (%)	1.42	1.33	1.11
Cystin (%)	0.39	0.36	0.34
Methionine (%)	0.68	0.59	0.52
TSAA (%)	1.07	0.95	0.86

[1] The experimental diets for each feeding period were provided by replacing the wheat bran with equal amounts of (0.5%) garlic powder to all basal diets (treatment 2) and treatment 3 and 4 were prepared by the addition of 0.87%, 0.74% and 0.65% DL-methionine to starter, grower and finisher basal diets, respectively. Treatment 4 was made by the addition of 0.5% garlic powder to treatment 3

Assessment of faecal oocyst output

Faecal samples in each treatment were collected 5 to 10 days post infection. Oocysts were isolated and counted per gram of faecal samples (OPG) using a modified MacMaster method (Soulsby, 1982).

Assessment of oxidative stress markers and antioxidant status

At 42 days of age, 5 birds per treatment were randomly selected and killed for blood and liver sampling. To obtain the serum, blood was centrifuged at 3,000 rpm for 15 min and stored at -20°C until analysis for nitric oxide. Measurements of nitrite and nitrate were based on the reduction of nitrate to nitrite by cadmium, and the produced nitrite level was determined by Griess method (Navarro-Gonzalvez *et al*., 1998). Liver samples were used to measure MDA and antioxidant enzyme activities. The samples were taken immediately after killing, were frozen by immersion in liquid nitrogen and were stored at -80°C until analysis. Lipid peroxidation, an indicator of tissue injury induced by reactive oxygen species, was measured as thiobarbituric acid reactive substance (Buege and Aust, 1978). Liver samples were homogenized in a cold PBS solution with a homogenizer at 2800 rpm for 2 min. Tissue homogenates were centrifuged at 1500 rpm for 15 min at 4°C. In the obtained supernatants, SOD and GPX activities were assessed using commercial kits [Ransod (SD125) and Ransell (RS504); Randox Co., Ireland] in an autoanalyzer (Biolis 4I, Tokyo Boeki Medical System). Enzyme activities were expressed as unit per gram of liver.

Statistical analysis

Data were subjected to ANOVA using SAS. Differences among treatments were tested by Duncan's multiple range tests using MsTaT-C software (version 1.42). P-values less than 0.05 were considered significant.

Results

The effect of coccidiosis infection, GP and TSAA on ADG

Average daily gain (ADG) was not affected by garlic powder or total sulfur containing amino acid supplementation from days 1 to 34 (Table 2). After inoculation with 7.5×10^2 oocysts of *Eimeria* species, ADG significantly reduced (P<0.01) 9 days post infection (PI) (94.67 vs 74.44 g/bird/d). Coccidial infection reduced ADG by about 21%. All birds fed with any supplement (GP and 50% more TSAA individually or in combination) had significantly higher ADG (P<0.05) compared to those fed with the basal diet. Infected birds fed with the diet containing GP or TSAA had significantly higher ADG (P<0.05) compared to the control birds. Uninfected birds did not show any significant difference in ADG for 9 days post infection (9 d PI) and for the whole period of study. In other words, diets supplemented with GP and TSAA improved the ADG of infected birds by 29% and 25%, respectively, compared to birds fed with the basal diet (81.11 and 77.55 vs 57.78 g/bird/d).

The effects of GP and TSAA on coccidial oocysts output

The anticoccidial effect of GP and TSAA was assessed using two parameters; weight gain and faecal oocyst output (OPG). In the infected birds, OPG increased (Fig. 1) at days 5 to 6 PI and the peak level of OPG was observed at days 8 to 9, sharply decreasing at day 10. Maximum OPG (12×10^3 oocysts/gram faecal content) was observed in birds fed with the basal diet at day 9 PI. Birds fed with GP plus 50% more TSAA had the lowest OPG (7×10^3 oocysts/gram faecal content). A significant difference was observed among treatments at day 9 PI (P<0.05). Supplementing the basal diet with GP and TSAA significantly reduced OPG by about 35% and 18.33%, respectively (P<0.05).

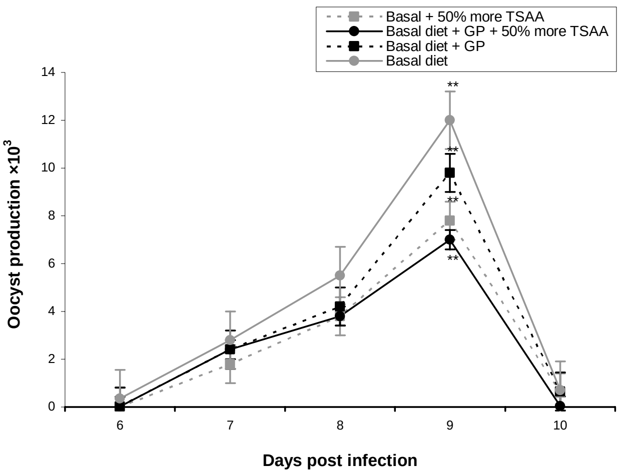

Fig. 1: Effects of garlic powder and total sulfur amino acid supplementation on OPG faecal contents. Bars represent the means ± SD of 36 chickens (three replicates from each treatment). Asterisks indicate significantly different OPG compared to other groups (**=P<0.05)

Table 2: Effects of diets on ADG of broiler chicks before and post infection with *Eimeria* spp

Plots	Treatments	Average daily gain (g/bird/d)			
		Challenge day (34)	9 d PI[1]	1-49 d	n[2]
Infected	Basal diet	31.35	57.78^c	42.65^d	5
	Basal diet + 0.5% GP	31.76	81.11^b	49.75^{bc}	5
	Basal diet + 50% more TSAA	30.59	77.55^b	48.65^c	5
	Basal diet + 0.5% GP + 50% more TSAA	31.59	81.33^b	49.42^{bc}	5
Uninfected	Basal diet	31.24	93.11^a	51.59^{ab}	5
	Basal diet + 0.5% GP	31.35	98.45^a	52.94^a	5
	Basal diet + 50% more TSAA	30.82	93.78^a	51.18^{ab}	5
	Basal diet + 0.5% GP + 50% more TSAA	31.70	93.33^a	52.04^a	5
	SEM	0.731	3.863	0.771	
P-values					
Oocyst		-	***[3]	***	
GP		NS	***	***	
TSAA		NS	NS	*	
GP*TSAA		NS	*	***	
Oocyst*GP		-	*	**	
Oocyst*TSAA		-	*	***	
Oocyst*GP*TSAA		-	NS	**	

[a-d] Mean values with different superscripts are significantly different (P≤0.05). [1] 9 d PI: 9 days post infection. [2] n: number of observations in each treatment. One bird from each replicate was randomly selected, slaughtered and weighed. [3] *, *** and NS are significant, highly significant and non-significant, respectively. SEM: Standard error of means

Table 3: Effects diets on oxidative stress and antioxidant status markers of chicks infected with *Eimeria* species

Main plot	Treatments	Oxidative stress and antioxidant status markers				
		NO[1] (µmol/L)	MDA[1] (µmol/g liver)	SOD[1] (U/g liver)	GPX[1] (U/g liver)	n[2]
Infected	Basal Diet	70.83[a]	0.96[a]	402.74	1151.40	5
	Basal Diet + 0.5% GP	60.01[bc]	0.77[bc]	413.60	1214.20	5
	Basal Diet + 50% more TSAA	64.96[b]	0.84[b]	413.48	1312.40	5
	Basal Diet + 0.5% GP + 50% more TSAA	61.82[b]	0.83[b]	415.28	1144.60	5
Uninfected	Basal Diet	56.60[c]	0.65[de]	411.02	994.40	5
	Basal Diet + 0.5% GP	55.72[c]	0.60[e]	410.76	1159.60	5
	Basal Diet + 50% more TSAA	60.43[bc]	0.72[cd]	413.56	1251.80	5
	Basal Diet + 0.5% GP + 50% more TSAA	55.95[c]	0.66[de]	416.84	1145.80	5
	SEM	1.561	0.0322	5.41	99.673	
P-values						
Oocyst		***[3]	***	NS	NS	
GP		***	***	NS	NS	
TSAA		NS	NS	NS	NS	
GP*TSAA		NS	NS	NS	NS	
Oocyst*Garlic		NS	NS	NS	NS	
Oocyst*TSAA		NS	*	NS	NS	
Oocyst*GP*TSAA		***	NS	NS	NS	

[a-e] Mean values with different superscripts are significantly different ($P \leq 0.05$). [1] MDA: Malonyldialdehyde, NO: Nitric oxide, SOD: Superoxide dismutase, and GPX: Glutathione peroxidase. [2] n: number of observation in each treatment. One bird from each replicate was randomly selected and slaughtered. [3] *, *** and NS are significant, high significant and non-significant, respectively. SEM: Standard error of means

Oxidative stress markers

The effects of GP and TSAA on NO markers

The summation of serum nitrite/nitrate (No_2/No_3) at day 9 PI (Table 3) as NO showed that inoculation of birds with *Eimeria* oocysts significantly increased serum NO production ($P<0.01$). Infected birds fed with GP and 50% more TSAA individually or in combination had lower serum NO ($P<0.05$) compared to the control birds in the infected plot. Uninfected birds did not show any significant differences compared with the control birds. In addition, the NO in all birds (infected and uninfected) fed with 50% more TSAA (diet 3) and GP plus 50% more TSAA (diet 4) was significantly higher ($P<0.05$) than those of birds fed with basal diet (diet 1) or basal diet supplemented with GP (diet 2).

The effects of GP and TSAA on MDA markers

Inoculation of birds with *Eimeria* oocysts significantly increased hepatic MDA ($P<0.01$). Infected birds fed with GP and 50% more TSAA had lower hepatic MDA compared with other birds in the infected group. In other words, the hepatic MDA in infected birds fed with diets containing GP and 50% more TSAA decreased by 20% and 12%, respectively. Uninfected birds did not show any significant difference compared to the control birds in the uninfected plot. In addition, hepatic MDA in all birds (infected and uninfected) fed with basal diet supplemented with GP was significantly higher than those of birds fed with the basal diet only or those fed with the basal diet supplemented with 50% more TSAA solely or together with GP ($P<0.05$).

The effects of GP and TSAA on antioxidant parameters

Inoculation of birds with *Eimeria* oocysts (Table 3) did not change hepatic SOD and GPX activities. No specific trend was observed for hepatic SOD activity; however, birds fed with the basal diet without any supplement had lower hepatic SOD activities compared with the other treatments in the infected birds. GPX-activity was not affected by *Eimeria* infection or the GP or 50% more TSAA diets.

Discussion

The approximate 21% reduction in ADG in infected birds in comparison with uninfected birds 9 d PI could be attributed to coccidiosis infection. Similar results have been reported by other researchers (Allen, 1996; Patricia and Allen, 1997; Wang *et al*., 2008). It is well known that coccidial infection reduces the growth rate of birds (Champan, 1998; Lillehoj and Lillehoj, 2000; Guo *et al*., 2007). Higher ADG in infected birds fed with the basal diet supplemented with GP and 50% more TSAA (29% and 25%, respectively) could be attributed to the many beneficial properties related to the bioactive compounds in garlic (Banerjee *et al*., 2003). Garlic is rich in organosulfur compounds (OSC) whose precursors (allicin, diallyl sulfide and diallyl trisulfide) are believed to play key roles in antioxidant and anti-inflammatory effects. Therefore, garlic probably eliminated the negative effects of coccidial infection and improved ADG in infected birds. The beneficial results of garlic supplementation are reported by the other researchers. According to khan *et al*. (2012), the effects of garlic on the production of healthy broilers are different depending on variations, doses, processing and duration of feeding. However, numerous reports indicate the efficacy and integrity of garlic in the alleviation of diseases. For instance, Worku *et al*. (2009) reported that garlic extract contributes to the treatment of gastrointestinal infections in goats by reducing oocysts output and may improve performance in adult goats. Our results were consistent with these reports.

Faecal oocyst output following *Eimeria* infection was used as an assessment for anticoccidial activity and resistance to Eimerian infection. At day 9 PI, the highest and lowest OPG was seen in birds fed with the basal diet and those fed with basal diet supplemented with GP plus 50% more TSAA, respectively. Lower OPG in birds fed with the basal diet supplemented with GP and TSAA in comparison to the birds fed with the basal diet alone may indicate that garlic and TSAA impair the development of parasites in the host before oocysts are formed and released, the same as what occurs with most anticoccidial drugs (Dkhil *et al*., 2011). These results are in agreement with the findings of Nidaullah *et al*. (2010) on broilers and Dkhil *et al*. (2011) on murines regarding the anticoccidial effect of garlic extracts.

Serum NO concentration and liver MDA increased in broiler chickens infected with *Eimeria* oocysts 9 d PI. This could be attributed to enhanced immune cell activity causing the overproduction of free radicals (Allen, 1996) and an increased ROS production resulting in lipid peroxidation. Higher NO and MDA levels in infected birds are probably due to oxidative stress occurring after the coccidial infection. Similar results on oxidative stress in parasitic diseases have been reported by others (Allen, 1996; Allen, 1997; Guarrera, 1999; Georgieva *et al*., 2006; Wang *et al*., 2008).

Adding garlic and TSAA to the diet decreased concentrations of NO and MDA by about 12% and 30%, respectively, compared to infected birds fed with the basal diet. This result is similar to the findings of Dkhil *et al*. (2011) who showed that garlic treatment lowered MDA and NO. However, the oxidative stress markers of uninfected birds fed with GP and TSAA did not change compared to those fed with the basal diet.

Coccidiosis infection and supplemental GP and SAA in the diets did not have any effect on SOD activity. However, SOD activity in infected birds fed with the basal diet was slightly lower than that of uninfected birds. GPX activity was not affected by coccidial infection. In other words, infection and extra supplementation of TSAA did not induce a stress high enough to change GPX activity. Contrary to our results, some reports have mentioned changes of antioxidant enzyme activities after infection. Wang *et al*. (2008), for instance, studied the effect of grape seed proanthocyanidin extract on the antioxidant status of chickens infected with *E. tenella* and found that infection (without supplementation) increased NO and MDA and decreased SOD activities. However, grape extract supplementation increased SOD activity and reduced NO and MDA. Georgieva *et al*. (2006) showed a decrease in SOD and CAT activities in birds infected with *E. tenella* compared to control birds. Discrepancy regarding the results of previous studies could be related to the differences in oocysts dose, supplementation, sampling day post infection and the *Eimeria* species itself. Our data indicated that the supplementation of GP and/or 50% more TSAA than required might have alleviated the adverse effects of coccidiosis, significantly improved ADG and decreased OPG, MDA and NO, but not changed antioxiant enzymes in birds infected with *Eimeria*. Further studies are needed to determine the optimal ratio of sulfur compounds (TSAAs and garlic) in a diet to minimize the adverse effects of oxidative stress. The precise mechanism by which OSC sulfur reacts with thiol groups to reduce oxidative stress also needs to be explored.

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