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Growth performance, serum biochemical parameters and microbial profile of broiler chickens fed on silver nitrate chelated with chitosan

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ABSTRACT

Objectives: This study was designed to evaluate the chelating effect of silver nitrate with chitosan on growth performances of broiler chickens.

Methodology and results: Experimental diets consisted of a negative control diet without any supplement (R_0^-) and three other diets obtained from the negative control ration by adding 0.1% doxycyclin antibiotic considered as positive control (R_0^+), 10mg/kg of unchelated silver nitrate (R_{Ag}) and 10mg/kg of silver nitrate chelated with chitosan (R_{Ag+Cs}). The result revealed that chelating silver nitrate with chitosan induced a significant ($P<0.05$) decrease in feed intake compared to the positive control diet. Both unchelated and chelated silver nitrate had no significant ($P>0.05$) effect on weight gain and feed conversion ratio (FCR). Silver nitrate chelated or not induced a decrease in the relative weight of liver as compared to the negative control diet. Feeding broiler with unchelated or chelated silver nitrate resulted in a significant ($P<0.05$) decrease in the ileal and caecal lactic acid bacteria count. Feeding with unchelated silver nitrate markedly ($P<0.05$) increase the serum albumin and globulin contents with a marked decrease in the albumin/globulin (A/G) ratio indicating a better disease resistance.

Conclusions and application of findings: Unchelated or chelated silver nitrate has no significant effect on growth performances. However, unchelated silver nitrate can be used to stimulate and reinforce the immune system of broiler chickens.

Key words: Broiler chickens, chelation, chitosan, feed additive, gut microbiota, plant charcoal, serological parameters, silver nitrate.

INTRODUCTION

Emergence of antibiotic resistant bacteria has created the necessity of antibiotic growth promoter substitution with non-therapeutic compounds such as organic acids, probiotics, prebiotics, essential oils (Gunal *et al*., 2006), plant charcoal (Kana *et al*., 2010; 2011) and metallic compounds like silver (Lok *et al*., 2006; Dongwei *et al*., 2009; Pineda *et al*., 2012). There has been a recent renewed interest in the use of silver as an antimicrobial agent. At the nano-scale, silver exhibits biological properties and broad-spectrum bactericidal activity against Gram-negative and Gram-positive bacteria (Lok *et al*.,

2006; Ahamed *et al*., 2010). Thus, silver is toxic to microorganisms (Percival *et al*., 2005) and may modulate the digestive microbiota. With the view that silver-containing antimicrobials should combine desirable attributes such as potent bacterial efficacy, environmental safety, low toxicity and easy fabrications, there has been an attempt to prepare antibacterial silver ion with chitosan (Dongwei *et al*., 2009). Chitosan is a natural biodegradable, biocompatible and non-toxic biopolymer (Vimal *et al*., 2013) extracted by alkaline deacetylation of chitin from crustaceans shell (Rabea *et al*., 2003). It has been documented that chitosan itself has antimicrobial activity (Dongwei *et al*., 2009) and the published researches about the antimicrobial activity include especially strong effects against bacteria and fungi (Rabea *et al*., 2003; Dutta *et al*., 2009). Despite the beneficial properties mentioned above, silver ion (Ag+) is toxic (Dongwei *et al*., 2009). Using silver as feed additive needs it to be reduced from the toxic form Ag+ to the non-toxic and environmental friendly form Ag0 using chelating agents like chitosan. Chelating of silver with chitosan is possible only in a solution and incorporating chelated silver solution in poultry feed is very problematic due to it liquid aspect. Absorption of silver solution by a dry and stable matrix can overcome the technical issues leading to better handling and enhance availability of silver in the digestive tract. Plant charcoal has the ability to bind a variety of substances (Kana *et al*., 2010; 2011). This property can be exploited to bind chelated silver ions in solution in order to facilitate their incorporation in animal feed, their transport and release in the target sites along the digestive tract. The present study will provide information on the effect of the fore mentioned properties of silver chelated with chitosan in poultry. The present study is proposed to give an overview on the potential of chitosan-based silver nitrate as silver ion source towards support of a positive gut bacteria growth and production performances of broiler chickens.

MATERIALS AND METHODS

Study site: This study was conducted at the poultry unit of the Teaching and Research Farm of the University of Dschang, Cameroon. This farm is located at 5°26' North and 10°26' EST and at an altitude of 1420 m above sea level. Annual temperatures vary between 10°C and 25°C. Rainfall ranges from 1500-2000 mm per annum over a 9 months rainy season (March to November).

Charcoal: Black fruit seeds (*Canarium schweinfurthii* Engl.) were collected in the market place in the Dschang town. They were burnt on a wire netting using firewood and quenched with water to obtain charcoal. After sun-dried, the chaocoal was ground and sieved to pass a 1-mm mesh and used to absorb unchelated and chelated silver nitrate in solutions as feed additive in the experimental rations.

Chitosan and silver nitrate solutions preparation: Analytical grade silver nitrate ($AgN0_3$) and water-soluble chitosan 0820a® used in this experiment were provided by Sigma Aldrich company and Shandong Guanghao biological product Co.Ltd (Shandong, China), respectively. Silver nitrate solution was prepared as described by Dongwei *et al*. (2009) by dissolving 8.85 g of silver nitrate in 1000 ml of distilled water using magnetic stirrer. Chitosan solution was prepared according to a method modified from the procedure reported by Chi *et al.* (2006). Briefly, chitosan stock solution (1% (w/v)) was prepared under magnetic stirring by dissolving 10.875g of chitosan in 1000ml of distilled water at ambient temperature overnight. Chelation or reduction of Ag^+ ions to Ag^0 was obtained by mixing 435 ml of $AgN0_3$ solution with 1087.5 ml of chitosan solution under magnetic stirrer for 2 hours. The homogeneous solution obtained was kept in a sealed transparent bottle at ambient temperature and the creation of the complex between chitosan and $AgNO_3$ or the bioreduction of Ag^+ to Ag^0 was effective when the colour of the solution changes from transparent to yellowish-brown as reported by Sougata *et al.* (2012). To the silver nitrate-chitosan solution, 768g of charcoal powder were introduced, homogenized by hand shacking to allow charcoal to absorb all the solution. This charcoal was finally dried in an oven at 60^0C for 72 hours and used as feed additive during the starter and the grower phases in this experiment. The concentration of $AgNO_3$ was 10mg per kg of feed.

Birds, dietary treatments and experimental design : In this study, 256 day-old Cobb 500 strain broiler chicks acquired from a local hatchery were randomly assigned to four experimental diets replicated four times each. Each replicate contained 16 chicks (8 males and 8 females). Chicks were randomly fed *ad-libitum* from day 1 to the end of the experiment on a negative control diet (Table 1) with no supplement (R_0^-), and three other diets obtained

from the control ration by adding 0.1% doxycyclin antibiotic and considered as positive control (R_0^+), 10mg/kg of unchelated silver nitrate (R_{Ag}) and 10mg/kg of silver nitrate chelated with chitosan (R_{Ag+Cs}).

Table 1: Composition and nutritive value of the experimental diets

Ingredients (%)	Starter phase	Finisher phase
Maize	54	64
Wheat bran	5	1
Cotton seed cake meal	5	5
Fish meal	5	5
Soybean meal	22	16
Oyster shell	1	1
Bone powder	1	1
Palm oil	2	2
Premix 5%*	5	5
Total	100	100
Calculated chemical composition		
Metabolisable energy (kcal/kg)	2928.66	3008.45
Crude protein (%)	23.00	20.40
Calcium (%)	1.17	1.35
Phosphorous (%)	0.53	0.56
Calcium/Phosphorous	2.19	2.19
Lysine (%)	1.39	1.19
Methionine (%)	0.48	0.44

*Premix 5%: Crude protein=40%, Lysine= 3.3%, Methionine=2.40%, Calcium=8%, Phosphorous=2.05%, Metabolisable energy =2078kcal/kg.

Growth and serum biochemical parameters: Growth of chicks and feed intake were recorded each week starting from week 1 until the end of the experiment. At 49 days of age, 10 chickens (5 males and 5 females) per treatment were randomly selected, fasted for 24 hours and slaughtered for the evaluation of the carcass characteristics. From each slaughtered chicken, blood was collected in test tube and serum obtained after centrifugation was preserved at -20°C for the evaluation of biochemical parameters. Biochemical parameters consisted of total protein, albumin, globulin, aspartate aminotransferase (ASAT), alanine aminotransferase (ALAT), total cholesterol, HDL-cholesterol, LDL-cholesterol, triglyceride, urea and creatinin using colorimetric method as prescribed by the Chronolab® commercial kits (Barcelona, Spain).

Microbial count: After slaughtering, the ileum and the cæcum from four birds were sampled per treatment and pooled by intestinal segment. The numbers of lactic acid bacteria, *Escherichia coli* and salmonella were counted in appropriate specific culture medium (MRS Agar for lactic acid bacteria, Mac Conkey AGAR for *E. coli* and SS AGAR for salmonella respectively) as proceeded by Pineda *et al*. (2012).

Statistical analysis: Results recorded on growth, microbial and serological parameters were subjected to the analysis of variance test by general Linear Model Procedure using the Statistical Package for Social Sciences (SPSS 20.0) computer software. Significant differences between treatment means were separated using Duncan's multiple Range test at 5% threshold significance (Vilain, 1999).

RESULTS

Growth performances: From Table 2, it can be observed that silver nitrate chelated with chitosan decreases feed intake during the entire period of the study (days 1 to 49). During the starter phase (days 1 to 21) of the experiment, there was no significant ($P>0.05$) effect of chelated-silver nitrate on feed intake meanwhile during the finisher phase (days 22 to 49) feeding broiler with chitosan-based silver nitrate resulted in a marked ($P<0.05$) decrease in feed intake. Chelated or unchelated silver nitrate had no marked ($P>0.05$) effect on live body weight, weight gain and feed conversion ration irrespective to the study phase. However, during finisher phase and throughout

the experimental period, the average live body weight and the weight gain had an upward trend with unchelated and chelated silver nitrate as compared to the negative control diet.

Table 2: Growth performances of broilers as affected by chitosan-chelated and unchelated silver nitrate

Study periods (days)	Treatments					
	R_0^-	R_0+	R_{Ag}	R_{Ag+Cs}	SEM	*P value*
Feed intake (g)						
1-21	1086.61[a]	1127.86[a]	1147.78[a]	1072.58[a]	16.22	0.356
22-49	4560.51[ab]	4589.22[a]	4545.35[ab]	4410.11[b]	28.94	0.106
1-49	5647.00[ab]	5717.00[a]	5693.00[a]	5483.00[b]	37.95	0.098
Live body weight (g)						
1-21	655.17	708.22	669.69	646.03	12.83	0.368
22-49	2589.33	2707.33	2663.33	2674.67	31.45	0.662
Weight gain (g)						
1-21	612.17	665.22	626.69	603.03	12.83	0.368
22-49	1934.23	1999.08	1993.67	2028.72	27.83	0.739
1-49	2546.33	2664.33	2620.33	2632.00	31.44	0.661
Feed conversion ratio						
1-21	1.79	1.70	1.83	1.79	0.047	0.806
22-49	2.36 [a]	2.29[ab]	2.28[ab]	2.18[b]	0.029	0.158
1-49	2.22	2,14	2.17	2.09	0.025	0.322

a, b: Means on the same row with different superscripts are significantly different (P < 0.05).
R_0^- = control diet, R_0^+= R_0 + 0.1% antibiotic, R_{Ag}= R_0 + unchelated silver nitrate, R_{Ag+Cs}= R_0 +chitosan-base silver nitrate

***Carcass characteristics*:** Carcass characteristics of broiler chickens as affected by chitosan chelated-silver nitrate are presented in table 3. It can be observed that chelating silver nitrate with chitosan had no significant (P>0.05) effect on the carcass yield of broilers. A part for the relative weight of liver which significantly (P<0.05) decreased with antibiotic and silver nitrate, the relative weight of organs and cut-out were not markedly affected by the dietary treatments.

Table 3: Carcass yield and relative weight of organs of broilers as affected by chelated and unchelated silver nitrate

Carcass traits (%BW)	Treatments					
	R_0^-	R_{0+}	R_{Ag}	R_{Ag+Cs}	SEM	*P value*
Carcass yield	73.86	74.06	74.7	74.22	0.28	0.824
Head	2.02	1.99	2.02	1.96	0.026	0.891
Legs	3.50	3.42	3.42	3.36	0.060	0.932
Liver	1.77[a]	1.56[b]	1.59[b]	1.56[b]	0.024	0.009
Heart	0.46	0.44	0.47	0.47	0.012	0.831
Abdominal fat	1.77	1.81	2.05	2.00	0.069	0.560

a, b: Means on the same row with different superscripts are significantly different (P < 0.05).
R_0^- = control diet, R_0^+= R_0 + 0.1% antibiotic, R_{Ag}= R_0 + unchelated silver nitrate, R_{Ag+Cs}= R_0 +chitosan-base silver nitrate

Digestive organs: The development of digestive organs of chickens as affected by the chelated silver nitrate is presented in table 4. Apart for the relative weight of the pancreas which significantly (P<0.05) increased with chelated silver nitrate as compared to antibiotic, chitosan-based silver nitrate had no marked (P>0.05) effect on gizzard, intestine weight, and intestine length and density.

Table 4: Development of digestive organs of broilers as affected by chitosan-based silver nitrate

Parameters	Treatments					
	R_0^-	R_{0+}	R_{Ag}	R_{Ag+Cs}	SEM	*P value*
Pancreas (% BW)	0.17 ^{ab}	0.15 ^{b}	0.17 ^{ab}	0.20 ^{a}	0.006	0.120
Gizzard (% BW)	1.37	1.38	1.29	1.31	0.023	0.527
Weight of the intestine (g)	82.50	85.08	82.00	86.17	1.60	0.794
Length of the intestine (cm)	190.75	196.92	190.75	191.33	3.35	0.920
Density of the intestine	0.44	0.43	0.43	0.46	0.01	0750

a, b: Means on the same row with different superscripts are significantly different (P < 0.05).
R_0^- = control diet, R_0^+= R_0 + 0.1% antibiotic, R_{Ag}= R_0 + unchelated silver nitrate, R_{Ag+Cs}= R_0 +chitosan-base silver nitrate

Microbial count: As shown in table 5, the average lactic acid bacteria count in the ileum significantly (P<0.05) increase with chitosan chelated-silver nitrate as compared to other treatments. Apart for the lactic acid bacteria count which markedly (P<0.05) increase with the chelated silver nitrate in the ileum and decrease with the silver nitrate and antibiotic in the cæcum, *E. coli* and salmonella populations were not markedly affected by the dietary treatments both in the ileum and the cæcum.

Table 5: Gut microbiota of broilers as affected by chelated and unchelated silver nitrate

Bacteria count (Log10CFU)	Treatments					
	R0	R0+	RAg	RAg+Cs	SEM	P value
			Ileum			
Lactic acid bacteria	8,11bB	8,24bB	8,26bA	8,89aB	0.13	0,004
E. coli	9,35aA	9,06aA	8,37aA	9,48aA	0.19	0,152
Salmonella	8,18aB	8,25aB	8,45aA	9,15aAB	0.15	0,349
SEM	0.24	0.17	0.19	0.10		
P value	0,035	0,051	0,937	0,038		
			Cæcum			
Lactic acid bacteria	8,08a	7,65b	7,80bB	7, 70bB	0.13	0,010
E. coli	8,27a	8,24a	9,11aA	8,67aA	0.17	0,395
Salmonella	7,75a	7,47a	8,11aB	7,98aB	0.16	0,424
SEM	0.16	0.21	0.24	0.16		
P value	0,488	0,347	0,032	0,013		

a, b: Means on the same row with different superscripts are significantly different (P< 0.05).
A, B: Means on the same column with different superscripts are significantly different (P< 0.05)
R_0^- = control diet, R_0^+= R_0 + 0.1% antibiotic, R_{Ag}= R_0 + unchelated silver nitrate, R_{Ag+Cs}= R_0 +chitosan-base silver nitrate

Serological parameters: As reported in table 6, chelating silver nitrate with chitosan significantly (P<0.05) increased serum content in total protein as compared to the negative control ration. The serum contents in albumin and albumin markedly (P<0.05) increased with unchelated silver nitrate as compared to the negative control diet, and the diet supplemented with chitosan-based silver nitrate. Unchelated silver nitrate also induced a marked (P<0.05) decrease in albumin/globulin ratio in this study.

Table 6: Variation of the serological parameters of broilers as affected by chitosan-chelated and unchelated silver nitrate

Biochemical parameters	Treatments					
	R_0	R_{0+}	R_{Ag}	R_{Ag+Cs}	SEM	*P value*
Total Protein (g/dl)	0.98^{b}	1.93^{a}	1.52^{ab}	1.94^{a}	0.13	0.040
Albumin (g/dl)	1.40^{b}	1.72^{ab}	1.94^{a}	1.46^{b}	0.08	0.041
Globulin (g/dl)	0.48^{b}	0.38^{b}	0.98^{a}	0.46^{b}	0.06	0.000
Albumin/ Globulin	2.91^{b}	4.52^{a}	1.98^{b}	3.17^{ab}	0.50	0.019

a,b: Means on the same row with different superscripts are significantly different (P < 0.05).
R_0^- = control diet, R_0^+= R_0 + 0.1% antibiotic, R_{Ag}= R_0 + unchelated silver nitrate, R_{Ag+Cs}= R_0 +chitosan-base silver nitrate.

DISCUSSION

The significant improvement in feed intake with the antibiotic in this study could be attributed to the healthy digestive tract of the chickens. The present result is in agreement with the earlier findings of Kana *et al.* (2009) who reported that antibiotic feed additive improved feed intake, growth and reduces mortality rate in chicken. This improvement could be associated with the beneficial effects of antibiotic, which suppress present infections or prevent eventual diseases and act as a growth factor (Devie *et al.,* 2006). Live body weight and weight gain did not significantly differ among the treatments. This result agreed with the earlier findings of Fondevila *et al.* (2009), Sawoz *et al.* (2009) and Pineda *et al*. (2012). In fact, according to Pineda *et al.* (2012) the provision of silver nanoparticles in drinking water did not affect the growth performance of broilers. Although an interaction effect between silver nanoparticles concentration and period was noted on body weight, significance was not sustained. The present result contradict the earlier findings of Kana *et al*. (2011), who showed that live body weight and weight gain were significantly improved with diets supplemented with 0.2% charcoal from Canarium fruit seeds. FCR did not significantly differ among treatments. This result agrees with the findings of Sawoz *et al*. (2007), Pineda *et al.* (2012) and Kana *et al.* (2011) who respectively reported that the provision of silver nanoparticles in drinking water and Canarium charcoal as feed additive did not affect FCR in broilers. Carcass characteristics did not differ significantly between treatments. The present result agrees with the findings of Emadi and Kermanshi (2006) and Kana *et al.* (2011) who respectively mentioned that turmeric feed additive and Canarium charcoal has no effect on carcass yield and relative weight of organ. Contrary to the findings of these authors, this study revealed that the relative weight of the liver markedly increased with the negative control diet as compared to all other treatments. The decrease in liver weight with chelated and unchelated silver nitrate could be related to the prevention in silver ion (Ag^{+}) toxicity by chitosan and charcoal. In fact, charcoal is a detoxifier or toxin binder (Mégarbane *et al*., 2006; Rizoug, 2006; Hazourli *et al*., 2007) and the chelation or reduction of silver (Ag+) to it non toxic form (Ag^{0}) by chitosan (Dongwei *et al*., 2009) could have suppressed toxic-induced effects of silver nitrate on the liver. The density of intestine (weight/length) which is an indication of the intestinal villi size of mucosa layer and the relative weight of gizzard did not vary significantly between the treatments. This result contradicted the findings of Kana *et al*. (2012) who reported that adding above 0.6% Canarium charcoal to diet depressed the intestine density in broilers. The present result is in agreement with the findings of the same author (Kana *et al*., 2011) who revealed no significant effect on the gizzard weight of broiler fed on diets supplemented with charcoal from Canarium fruit seeds. The present result also revealed that feeding chitosan-based silver nitrate to broilers markedly increased the relative weight of the pancreas. This result is in agreement with the findings of the above-mentioned authors (Kana *et al*., 2010; 2011; 2012) who reported that 0.2% of Canarium charcoal increased the relative weight of pancreas. The increase in relative weight of pancreas recorded in the present study might be due to this same charcoal, which was used to absorb and facilitated the incorporation of chelated silver ions in the experimental rations. Feeding broilers with chitosan chelated-silver nitrate markedly improve the lactic acid bacteria growth in the ileum. This result contradicted the earlier findings of Pineda *et al*. (2012) who reported that silver nanoparticles had no effect on the most dominant ileal species, in particular lactic acid bacteria. The significant growth of lactic acid bacteria observed in this study may also be due to the difference in silver nitrate concentrations, mode of administration or dietary ingredients as compared to the previous findings. Apart for the lactic acid bacteria count which markedly ($P<0.05$) increase with chelated silver nitrate in the ileum and decrease with silver nitrate and antibiotic in the cæcum, the *E. coli* and salmonella populations were not markedly affected by the dietary treatments in both the ileum and the cæcum. This result contradicted the earlier findings of Fondevila *et al*. (2009) and Pineda *et al.* (2012) who reported that silver nanoparticles had no effect on lactic acid bacteria count in the cæcum. In this study, *E. coli* population was also significantly higher than the lactic acid bacteria and salmonella counts. This result contradicted the earlier findings of Dongwei *et al.* (2009) and Wen-Ru *et al*. (2010) who reported that silver nanoparticles could inhibit the growth and reproduction of *E.coli* and chitosan-based silver nitrate showed high antibacterial activity towards *E. coli*. Lower albumin/globulin ratios indicate better disease resistance and immune response of birds as reported by Abdel-Fattah *et al.* (2008). In the present study, the serum content in albumin and globulin were higher and the albumin/globulin ratio was lower with unchelated silver nitrate suggesting that silver nitrate as Ag+ ions source reinforce the immune system of the broilers. This result is in agreement with the findings of Pineda *et al.* (2012), Bovera *et al*. (2015) and Sugiharto *et al.* (2016). In fact,

Pineda *et al.* (2012) recorded a positive effect of silver nanoparticles on immunoglobulin concentration meanwhile, Bovera *et al*. (2015) and Sugiharto *et al.* (2016) reported a lower albumin/globulin ratio in broilers fed yellow mealworm larvae and fermented dried cassava respectively.

CONCLUSION

Chelating silver nitrate with chitosan decreased feed intake with no significant effects on body weight gain and feed conversion ration. Unchelated silver nitrate increased serum content in albumin and globulin, and decreased albumin/globulin ratio indicating a better disease resistance of broiler chickens. Unchelated silver nitrate could be used as feed additive to stimulate and reinforce the immune system of broiler chickens.

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