Romanian Biotechnological Letters *Vol. 23, No. x, 2018*

ORIGINAL PAPER

The use of ascorbic acid and *Artemisia annua* powder in diets for broilers reared under heat stress

Received for publication, December, 14, 2017
Accepted, April, 23, 2018

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Abstract

A feeding trial was conducted to determine the effects of supplementing the diets for broilers (14-35 days) reared under heat stress (32^0 C) with ascorbic acid and Artemisia annua powder on broiler performance, internal organ weight and gut microflora. A total of 96 Cobb 500 broilers, aged 14 days, assigned to 3 groups (C, E1 and E2) were housed in an experimental hall under a constant temperature of 32^0C. The premix of the conventional diet for the control group (C) was supplemented with monensin and contained 2000 mg ascorbic acid /kg premix. Compared to C diet, the premix for diet E1 contained 8000 mg ascorbic acid /kg premix, while the diet for group E2 contained 2% Artemisia annua powder. Diets E1 and E2 didn't have monensin in their premix. In the end of the feeding trial, six broilers per group were slaughtered and measurements were performed to determine the relative weight of internal organs of broilers and samples of intestinal content were collected for bacteriological examination. The experimental results showed that the heat stress affected adversely the intake and gain of the all three groups. The total Enterobacteriaceae count was significantly ($P \leq 0.05$) lower in the intestine of the experimental groups than in group C, but E.coli count was significantly ($P \leq 0.05$) higher in group E2 than in group C. The lactobacilli concentration in the intestinal content of the experimental groups was significantly ($P \leq 0.05$) higher than in group C.

Keywords: chicks, growing phase, stress, performance, organs, gut microflora

1. Introduction

Heat stress is of great concern in the poultry industry (BelhadjSlimen & al. [1]; Wang & al. [2]; Niu & al. [3]) since it can cause major economic losses (Gursu & al.[4]; Ryder & al. [5]). Their higher production performance and feed conversion efficiency make today's chickens more susceptible to heat stress than ever before (Lin & al. [6]). Rafiee & al. [7] showed that heat stress results in reduced feed intake, body weight gain, carcass yield and increased mortality and feed conversion ratio of broilers. Furthermore, Fuller [8] shows that young animals under stressful conditions suffer from changes in the composition and activity of the gut microbiota. Suzuki & al. [9] demonstrated that heat stress resulted in a marked change of bacterial composition in chicken intestine, which was subsequently associated with depression of body-weight gain. The gastro intestinal tract is particularly responsive to stressors like heat stress, which modify the normal and protective microbiota (Bailey & al. [10]) and decreased integrity of the intestinal epithelium (Lambert [11]) which in turn can affect its barrier function and the absorption of nutrients, impairing productive performance of animals (Liu & al. [12]).

Nutritional manipulation is one of the methods used in poultry production to alleviate the adverse effects of heat stress (Sahin & al. [13]). Several authors consider that antioxidants can be supplemented into the feed in order to alleviate the adverse effects of high ambient

temperature (Lohakare & al. [14]; Hussein [15]). At high temperature, broiler chicken seems to have a special appetite for ascorbic acid and tends to consume more diet supplementing of ascorbic acid (Kutlu and Forbes [16]). On the other hand, the same authors noticed that the supplementation with ascorbic acid in the absence of heat stress tends to decrease broiler performance. Feeding trials with supplemental ascorbic acid given to broilers reared under heat stress showed the beneficial effects for the performance of broilers (Mckee and Hurrison [17]; Pardue & al. [18]; Pardue and Thaxton [19]). Different dietary ascorbic acid levels (alone or next to other supplements), given to broilers reared under heat stress, showed beneficial effects on broiler performance and metabolism: 200-250 mg/kg diet (Rafiee & al. [7] ; Ghazi & al. [20]; Kutlu and Forbes [16]); 500 mg/kg diet (Celik and Oztürkcan [21]); 150 or 400 mg/kg (McKee & al. [22]).

Bartlett and Smith [23] consider that besides dietary supplements of vitamins and minerals, herbal antioxidants can be used to defeat the heat stress in poultry. Other studies too confirmed that the plants have active components that act as possible antioxidants (Wang & al. [24]; Dragland & al. [25]; Halvorsen & al. [26]). The interest for the possibility of using new natural material instead of antibiotics in diets increased because of potential development of antibiotic resistant human pathogenic bacteria after long-term use (Acikgoz & al. [27]; Jang & al. [28]), which resulted in the ban on the dietary use of antibiotics in animal feeds in The EU (EC regulation No. 1831/20031 of January 1, 2006). Diet supplemented with phytogenic feed additives, which contain abundant phytochemicals and can be used as growth promoters and antioxidants, is a satisfactorily feasible approach that has been developed to ameliorate the detrimental effects of animals under challenging conditions (Akbarian & al. [29]).

Artemisia annua L. (A. annua) is one of the typical herbs that belongs to Artemisia species of Asteraceae (Wan & al. [30]). Plants belonging to the Asteraceae family have been used in poultry and in small ruminants as anticoccidial and antiparasitic agents (Cherian & al. [31]). The antiparasitic effects of A. annua have been attributed to artemisinin, a sesquiterpene lactone that contains an endoperoxide bridge (Ferreira and Janick [32]; Brisibe & al. [33]). In addition to artemisinin, A. annua is a storehouse of different bioactive compounds including 40 different flavonoids, phenolics, purines, lipids, and other aliphatic compounds (Brisibe & al. [33]). Whereas the anticoccidial effect of A. annua in poultry has been documented (Allen & al. [34]; Brisibe & al. [35]), less or no information is available on other health-promoting effects of A. annua in poultry (Cherian & al. [31]). Wan & al. [30] conducted an experiment on broilers reared under heat stress (34 ± 1 °C, 8 hours / day and 22 ± 1 °C, 16 h / day), to investigate the effects of a dietary enzymatic preparation of Artemisia annua on broiler performance and blood parameters. They noticed that the concentrations of 1.00 and 1.25 g enzymatic preparation of *Artemisia annua* / kg cancelled the adverse effects of the heat stress on the broilers.

Within this context, we conducted a feeding trial to assess the effects of supplementing the diets for broilers (14-35 days) reared under heat stress (320 C) with ascorbic acid or *Artemisia annua* powder on broiler performance, internal organ weight and gut microflora.

2. Materials and Methods

The feeding trial was conducted in the experimental halls of The National Research-Development Institute of Animal Biology and Nutrition (IBNA-Balotesti, Romania) according to a protocol approved by the Commission of Ethics of the institute.

Table 1. Composition of the diets

Ingredient	Diet formulations (broilers 14 - 35 days)		
	C	E1	E2
Corn, %	59.8	59.8	57.81
Soybean meal, %	28.2	28.2	27.48
Gluten, %	5	5	5
Plant oil, %	3.3	3.3	3.96
Lysine, %	0.24	0.24	0.27
Methionine, %	0.22	0.22	0.24
Choline, %	0.05	0.05	0.05
Calcium carbonate, %	0.54	0.54	0.54
Monocalcium phosphate, %	1.33	1.33	1.33
Salt, %	0.32	0.32	0.32
Premix (PVM) with coccidiostat (50 g sodium monensin/kg), %	1	-	-
Premix (PVM) with 8000 mg ascorbic acid /kg, %	-	1	-
Premix (PVM), %	-	-	1
Artemisia, %	-	-	2
Total	100	100	100
Calculated			
ME, kcal/kg	3108.06	3108.06	3108.21
Crude Protein, %	20.00	20.00	20.00
Crude Fat, %	5.54	5.54	6.19
Crude Fibre, %	3.61	3.61	3.51
Calcium, %	0.84	0.84	0.84
Phosphorous total, %	0.78	0.78	0.77
- av. Phosphorous, %	0.42	0.42	0.42
Lysine, %	1.23	1.23	1.23
Methionine, %	0.56	0.56	0.57
Meth+Cys, %	0.92	0.92	0.92
*1kg PVM contains: = 1100000 IU/kg vit. A; 200000 IU/kg vit. D3; 2700 IU/kg vit. E; 300 mg/kg Vit. K; 200 mg/kg Vit. B1; 400 mg/kg Vit. B2; 1485 mg/kg pantothenic acid; 2700 mg/kg nicotinic acid; 300 mg/kg Vit. B6; 4 mg/kg Vit. B7; 100 mg/kg Vit. B9; 1.8 mg/kg Vit. B12; 2000 mg/kg Vit. C; 8000 mg/kg manganese; 8000 mg/kg iron; 500 mg/kg copper; 6000 mg/kg zinc; 37 mg/kg cobalt; 152 mg/kg iodine; 18 mg/kg selenium;			

A total of 96, Cobb 500 broiler chicks, aged 14 days, weighed individually, were housed in an experimental hall under a constant temperature of 32^0C, humidity 36% and 23 h light regimen. During the 3 experimental weeks (14-35 days) the broiler chicks were assigned to 3 groups (C, E1 and E2), with free access to the feed and water. The premix of the conventional diet for the control group (C) was supplemented with monensin (COXIDIN with 20% monensin, supplied by HUVEPHARMA, Sofia, Bulgaria) as coccidiostat and contained 2000 mg ascorbic acid /kg premix (Table 1). Compared to C diet, the premix for E1 diet contained 8000 mg ascorbic acid /kg premix, while the diet for group E2 contained 2% *Artemisia annua* powder. Diets E1 and E2 didn't have monensin in their premix (Table 1). *Artemisia annua* plant material used was harvested when plants were in the late vegetative stage in Livezeni, Târgu-Mureş (46.55° N, 24.63° E). Plants were dried for three weeks under shade at ambient temperature (20°C) and ground finely to obtain *Artemisia annua* powder.

Diets formulations were calculated in agreement with the feeding requirements (NRC [36]) and the nutritional requirements of Cobb 500 hybrid.

The following parameters were monitored throughout the experimental period: body weight (g), average daily feed intake (g feed/chick/day), average daily weight gain (g/chick/day) and feed conversion ratio (g feed/g gain).

In the end of the feeding trial (35 days broilers), six broilers per group were slaughtered, according to the working protocol, and measurements were performed to determine the relative weight of internal organs of broilers. Samples of intestinal content were collected, in sterile tubes, from the slaughtered chicks, for bacteriological examination (determination of the *Enterobacteriaceae– E. coli* and *Salmonella* – and lactobacilli).

Samples of *Artemisia annua* powder and feeds were collected and assayed for the basic chemical composition: dry matter, crude protein, ether extractives, crude fibre, ash and phosphorus, using the chemical methods from Regulation (CE) no. 152/ 2009 (Methods of sampling and analysis for the official inspection of feeds). The calcium concentration in the *Artemisia annua* samples was determined according to the titrimetric method SR ISO 6490-1/1996. High performance liquid chromatography (RP-HPLC) was used for the quantitative determination of the amino acids from the Artemisia samples. As instruments, we used HPLC Surveyor Plus (Thermo Electron Corporation, Waltham), fitted with PDA detector. Separation was done in chromatographic column Hypersil BDS (Base Deactivated Silica) C18, with silica gel, with dimensions 250 × 4.6 mm and particle size 5 μm. Trace minerals (Cu, Zn, Mn) concentrations in the *Artemisia annua* powder were determined by flame atomic absorption spectrometry after microwave digestion. The used equipment was: Atomic absorption spectrometer Thermo Electron – SOLAAR M6 Dual Zeeman Comfort (Cambridge, UK), with deuterium lamp for background correction and air-acetylene flame and microwave digestion system with remote temperature measurement, BERGHOF, Speedwave MWS-2 Comfort (Eningen, Germany).

The *Enterobacteriaceae* and *E. coli* were determined using a classical isolation medium, G.E.A.M. or Levine. The samples were first soaked in medium with lauryl-sulphate (enrichment medium), homogenized and left for 20-30 minutes are room temperature (23-24^{o}C). Decimal dilutions were made up to 10^{-5} in the medium with lauryl-sulphate. The dilutions of $10^{-2} – 10^{-5}$ were used to seed 2 Petri dishes each per dilution, on Levine medium. The Petri dishes were incubated for 48 h at 37^{o}C and the colonies were count. *E. coli* formed characteristic colonies on this medium (dark violet with metallic shine). The other *Enterobacteriaceae* formed either dark red opaque colonies (lactic-positive species) or pale pink semi-transparent or colourless colonies (lactic-negative species). The lactobacilli were determined on selective mediums (MRS broth and MRS agar), characteristic for the isolation and counting of these bacteria. The colony counter Scan 300, INTERSCIENCE (France) was used to determine the colony count of *Enterobacteriaceae*, *E. coli* and lactobacilli.

The effects of treatments were tested by analysis of variance using the GLM procedure of the Minitab software (version 17, Minitab® Statistical Software), with treatment as fixed effect, according to the model $Yi = Ti + ei$, where Yi was the dependent variable, Ti is the treatment and ei is the error. When overall F-test was significant, differences between means were declared significant at $p<0.05$ using the test of Tukey.

3. Results and Conclusions

Table 2 shows the basic chemical composition, amino acids profile of the protein and the mineral content of the *Artemisia annua* powder (whole plant dried and ground).

Table 2. Nutrient characterization of *Artemisia annua* powder

Item	Value
Gross energy (kcal/kg)	3876.7
Metabolisable energy (kcal/kg)	1511.39
Dry matter (%)	88.30
CP (%)	18.24
Total fat (%)	3.04
Cellulose (%)	27.61
Ash (%)	8.90
Amino acids (%)	
Aspartic acid	1.77
Glutamic acid	1.74
Serine	0.74
Glycine	0.79
Threonine	1.26
Arginine	1.02
Alanine	0.97
Tyrosine	0.53
Valine	0.89
Phenylalanine	0.78
Isoleucine	0.70
Leucine	1.32
Lysine	0.93
Cystine	0.129
Methionine	0.166
Minerals	
Ca (%)	0.58
P (%)	0.38
Cu (mg/kg)	17.50
Mn (mg/kg)	76.30
Zn (mg/kg)	24.32

The protein level (18.24%) determined in the *Artemisia annua* powder (Table 2) is close to that reported (19.66 %) by Brisibe & al. [35]) but lower than that reported (27.8 %) by Cherian & al. [31]), the latter being for dry *Artemisia annua* leaves. As expected, Table 2 data show a high fibre (27.61%) content of the *Artemisia annua* powder.The amino acids from the *Artemisia annua* powder (Table 2) were in lower amounts than those reported by Cherian & al. [31]), for the dry leaves. The concentration of minerals was different from those reported by the authors mentioned above.

Table 3. The effect of the dietary ascorbic acid and *Artemisia annua* powder on the broilers performance

Specification	Days	Group C	Group E1	Group E2	SEM	p value
Body weight (g/broiler)	14	412	410.727	406.190	3.738	0.2096
	35	2008.105b	1866.970a	1879.970a	28.684	0.0156
Average daily fed intake (g/broiler/day)	14-35	110.554	100.224	100.210	2.086	0.2566

Average daily weight gain (g/broiler/day)	14-35	76.005	69.345	70.180	2.425	0.7409
Feed conversion ratio (g feed/g gain)	14-35	1.459	1.445	1.425	0.039	0.8327

*Where: SEM: standard error of the mean; means in the same row with no common superscript are significantly different ($p \leq 0.05$)

The gains and the feed conversion ratio recorded for the entire experimental period (14-35 days) were not significantly ($P \leq 0.05$) different between the three groups (Table 3). At 35 days, the body weight of the broilers from groups E1 and E2 was significantly ($P \leq 0.05$) lower than that of group C broilers. The Cobb 500 broilers used in this feeding trial had a final weight at 35 days higher than the final weight obtained by Ross 308 broilers reared at a temperature of 32^0 C (Jang & al. [37]) or by male Shaver broilers (Geraert & al. [38]). The high temperature (32°C) caused a lower bodyweight of the broilers than the values mentioned in the Management guide for Cobb 500 broilers for normal environmental temperatures. The high temperature also decreased the average daily feed intake. These result are in agreement with those reported by Baziz & al. [39]; Geraert & al [38]; Imik & al. [40]; Jang & al. [37], etc.

Austic [41] considers that the main consequence of heat exposure is a reduction in feed intake in order to reduce metabolic heat production and this reduction is approximately 17 % per 10^0C increase in ambient temperature above 20^0 C. In our study, compared to the Cobb 500 management guide, group C had a feed intake lower by 19.62%, close to that reported by Austic [41], while the experimental groups E1 and E2 had a feed intake lower by 25 %. Unlike the results reported by other authors (Kutlu and Forbes, [16]; Alba & al [42]; Ghazi & al. [20]) the performance of E1 broilers (with ascorbic acid supplement) was not improved, under heat stress, compared to group C. All of these authors used 250 ppm ascorbic acid in the broilers diets so it might seem that the dietary level of ascorbic acid (80 mg/ kg of diet) used in our feeding trial was not enough to alleviate the adverse effects of the heat stress. On the other hand, the performance of E1 broilers group is in agreement with that reported by Zeferino & al. [43], on Cobb 500 broilers reared under heat stress from 28 to 42 days.These authors showed that diet supplementation with ascorbic acids (257 to 288 mg/kg) and vitamin E (93 to 109 mg/kg) simultaneously was not able to neutralize or reduce any of the negative effects of the exposure of chickens to heat stress, in the grower finishing phases, on performance, carcass and meat physical quality traits.

Although several authors showed that dry *Artemisia annua* leaves can be used as botanic coccidiostat (Almeida & al. [44]; Allen & al. [34]; Arab & al. [45]), the performance of E2 broilers (2% Artemisia) was generally poorer than that of C broilers. The performance of E2 broilers (Table 3) are not in agreement with that reported by Wan & al. [30] following a study on the effects of dietary enzymatically treated A*rtemisia annua* L. supplementation on growth performance of broilers reared under heat stress, who concluded that this diet alleviated growth depression.

Table 4. The effect of the dietary ascorbic acid and *Artemisia annua* powder on relative weight (% BW) of selected internal organs

Specification	Group C	Group E1	Group E2	SEM	p value
Gizzard %	2.593 [a]	2.797[b]	2.530 [a]	0.050	0.0418
Heart %	0.497	0.463	0.430	0.023	0.5568
Liver %	1.563	1.730	1.557	0.059	0.4607

Spleen %	0.088	0.097	0.091	0.007	0.8866
Bile %	0.100	0.085	0.090	0.012	0.3192
Bursa of Fabricius %	0.168	0.216	0.222	0.017	0.3866

* Where % BW =the weight expressed as % of body weight (BW); SEM: standard error of the mean; means in the same row with no common superscript are significantly different ($p \leq 0.05$)

The measurements performed after slaughter (35 days) show that the relative weight of the gizzard was significantly ($P \leq 0.05$) higher in the group with ascorbic acid (E1) than in the other two groups (Table 4). The relative weight of the spleen was also higher in group E1, but the difference was not statistically significant. These findings are in contradiction with the data reported by other authors. Bashir & al. [46] consider that the presence of ascorbic acid leads to higher spleen weight in the chicken reared under heat stress, while Naseem & al. [47] show that ascorbic acid supplementation during heat stress had beneficial effects on ratio of weight of bursa, thymus and spleen to body weight of the birds. Jang & al. [37] consider that there was no significant difference in relative organ weight, except the thymus, in response to dietary ascorbic acid given to chicks (0-35 days) exposed to summer diurnal heat stress at average daily fluctuations of temperature between 32^0C to 34^0C at day to 27^0C to 29^0C at night for the entire feeding periods. The results on the weight of the selected internal organs of E2 broilers (with *Artemisia annua*) are in agreement with the results reported by Cherian & al. [31], who conducted a study on Cobb broilers (14-42 days) reared under normal temperature conditions, noticing that the supplemental dietary *Artemisia annua* didn't produce significant differences in the weight of the liver, heart and spleen.

Table 5 shows the results of the bacteriological determinations in the intestine. We evaluated the populations of *Enterobacteriaceae* and lactobacilli, which are competing permanently for food and space, whose balance is essential for gut health. Among the *Enterobacteriaceae* we focused on *E. coli* and *Salmonella* which are the cause of most gastrointestinal disorders.

Table 5. The effect of the dietary ascorbic acid and *Artemisia annua* powder on the composition of broiler intestinal microbiota (log10 CFU*/g wet intestinal digesta)

Specification	Group C	Group E1	Group E2	SEM	P value
Enterobacteriaceae, log10	7.252^a	7.206^b	7.211^b	0.008	0.0139
E. coli, log 10	5.600^a	5.612^a	5.680^b	0.056	0.0383
Lactobacillus sp., log 10	6.312^a	6.776^b	6.693^b	0.095	0.1168
Salmonella	Absent	Absent	Absent		

Where: * colony forming units; SEM : standard error of the mean; means in the same row with no common superscript are significantly different ($p \leq 0.05$)

The concentration of the analysed microorganisms (Table 5) was within normal limits (Gournier-Chateau & al. [49]). It can be noticed that the total count of *Enterobacteriaceae* decreased significantly in the experimental groups compared to the control group. It seems that both ascorbic acid (E1) and *Artemisia* (E2) have a positive effect ($P \leq 0.05$) in limiting the development of the *Enterobacteriaceae* during the heat stress. On the other hand, *E.coli* was significantly ($P \leq 0.05$) higher in E2 than in C. This is not in agreement with the data reported by Khalaji & al. [50] who, using *Artemisia sieberi* leaves in the feeds for broilers reared under normal temperature conditions, noticed that the caecal populations of *Escherichia coli* decreased significantly. A study by Suzuki & al. [9] noticed that the heat

stress affects the intestinal flora of the broilers (7-28 days) reared at 35 ˚C, the count of aerobe bacteria from the small and large intestines increasing. They also noticed that *E. coli* and the streptococci also increased, but lactobacilli and *C. perfringens* did not change.
The higher count of lactobacilli in the intestinal content of the experimental groups E1 (with ascorbic acid) and E2 (with *Artemisia annua*) can be accounted due to the positive effect of these dietary additives in reducing the local pH, as compared to group C (Table 5). Indirect effects of plant extracts have been reported due to reducing the ileal pH value, while increasing the number of lactic acid bacteria and decreasing the coliform counts in the ileum and caecal contents of broiler chickens (Vidanarachchi & al. [51]). Murugesan & al. [52] too, noticed that the supplementation of Vencobb 400 broilers reared under normal temperature conditions with a commercial phytoadditive supported the establishment of a favourable gut microbiota composed of higher numbers of *Lactobacillus* spp.

Conclusions: The performance of Cobb 500 broilers (14-35 days) reared under heat stress were not improved by diet supplementation with ascorbic acid (80 mg/kg of diet) or with *Artemisia annua* powder (2%). The heat (32°C) decreased the body weight compared to the Cobb 500 Management guide for broilers (35 days) reared under normal environmental temperatures. No significant differences were recorded for the weight of the internal organs of the broilers (35 days), except for the gizzard, which was significantly ($P \leq 0.05$) heavier in the group with 80 mg ascorbic acid /kg of diet compared to C group and E2 group (2% *Artemisia annua* powder). The dietary inclusion of ascorbic acid (E1) or *Artemisia annua* (E2) had a positive effect ($P \leq 0.05$) compared to group C in limiting the development of the *Enterobacteriaceae* under heat stress. On the other hand, *E.coli* concentration was significantly ($P \leq 0.05$) higher in group E2 than in group C. The lactobacilli concentration in the intestinal content of the experimental groups E1 (80 mg ascorbic acid /kg of diet) and E2 (2 % *Artemisia annua*) was significantly ($P \leq 0.05$) higher than in group C.

4. Acknowledgements

This work was supported by a grant of the Romanian Ministry of Education and Research (Project PN 16. 41-0401).

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