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The Effect of Grape Seed Extract and Vitamin C Feed Supplements Carcass Characteristics, Gut Morphology and Ileal Microflora in Broiler Chickens Exposed to Chronic Heat Stress

Research Article

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ABSTRACT

The effects of dietary supplementation of grape seed extract (GSE) and vitamin C on carcass characteristics, gut morphology and ileal microflora in broiler chickens exposed to chronic heat stress were examined. Experimental diets included a control diet (without additive), 3 levels of GSE (150, 300, 450 mg/kg of diet), and vitamin C (300 mg/kg of diet). Each of the five diets was fed to 5 replicates of 12 male Cobb-500 chicks, from 1 to 42 d of age. The birds were exposed to chronic daily heat stress of 34 ± 1 °C with 65-70% relative humidity for 5 hours per day from 29 to 42 d of age. Addition of GSE or vitamin C to the basal diet did not affect the percentage by weight of the edible carcass, breast, drumstick + thighs, liver, empty gizzard, or abdominal fat. Furthermore, in heat-exposed broiler chickens the relative length of duodenum, jejunum and ileum did not show significant alteration when compared to the control group before and after chronic heat stress. Dietary GSE and vitamin C caused differences in jejunum muscle layer thickness, villus height, villus width and crypt depth of birds before heat stress. Addition of the GSE or vitamin C did not affect the jejunum muscle layer thickness, villus width, crypt depth and villus: crypt ratio of broilers under heat stress (42 d). However, broilers fed a diet containing GSE had higher villus compared to the control group at 42 d (P<0.05). Adding GSE or vitamin C reduced ileal coliforms and the *Escherichia coli* population of broilers before heat stress condition (28 d). Under chronic heat stress conditions, broilers fed diet supplemented with GSE had lower ileal coliforms and *Escherichia coli* populations compared to control birds (P<0.05). Thus, GSE can be recommended as a new herbal extract supplement to the diet of broiler chickens in order to improve jejunum villus height and decrease detrimental bacteria in the ileum of birds before and during chronic heat stress condition.

KEY WORDS carcass, grape seed extract, gut morphology, heat stress, microflora.

INTRODUCTION

Heat stress is one of the main problems in hot seasons or hot areas of the world that can cause huge economic losses to the poultry industry. Furthermore, global warming is occurring due to factors such as deforestation, and increased green house gas emissions (CO_2, N_2O, CH_4), with the data suggesting that temperatures are rising at a rate of 0.2 °C per decade, a rate that is likely to increase in the future (US EPA, 2010). It is well known that poultry are warm-blooded animals (body temperature is about 40.5-41.5 °C) (Etches *et al.* 2008), with almost all parts of their bodies covered by feathers and with no sweat glands. They are therefore susceptible to the danger of heat stress because of difficulties in releasing their body heat to the environment (Tamzil *et al.* 2013). Animals have known zones

of thermal comfort that vary by species, physiological status, relative humidity, the velocity of ambient air and the degree of solar radiation. When the environmental temperature exceeds the upper critical temperature of the thermoneutral zone, thermoregulatory reactions are limited and animals are considered to be in a heat stress condition (Sahin et al. 2009). These adverse environmental condition lead to oxidative stress associated with increased oxidative damage and lowered plasma concentrations of antioxidant vitamins (Sahin et al. 2009). Tao and Xin (2003) proposed a temperature humidity index (THI) for poultry, using wind speed as a variable, and called this index the temperature-humidity-velocity index (THVI). They also established several stages of thermal comfort values including: normal ≤ 70, alert from 70 to 75, danger values are those from 76 to 81 and emergency values were ≥ 82, based on variations in the bird's body temperature. Thus, the value of 70 was established as the standard threshold value for THVI in poultry (Ajakaiye et al. 2011). Also, the intestinal tract of poultry harbors a complex and dynamic microbial ecosystem (or microbiome), which may be affected by a variety of factors (Wei et al. 2013). Very little has been published on the effects of environmental stressors (particularly, heat stress) on the intestinal microbial ecosystem of poultry. Altered morphology, as well as changes in the microbial community structure in the intestinal tract of broilers subjected to heat stress has been reported (Burkholder et al. 2008; Laudadio et al. 2012). Under heat stress conditions, increased concentrations of reactive oxygen species (ROS) occur leading to increased intestinal permeability, which in turn facilitates the translocation of bacteria from the intestinal tract (Lara and Rostagno, 2013). Increased inflammation and translocation of Salmonella enteritidis in broilers subjected to heat stress has been reported (Quinteiro-Filho et al. 2010; Quinteiro-Filho et al. 2012) and this resulted in increased levels of the pathogen in spleen samples. Strategies such as zone-cooling by air conditioners are not cost efficient and may not be practical for many farms. Thus, there is an increasing trend to search for natural nutrients, especially those of plant origin, that are capable of supporting birds' body homeostasis with minimal undesirable side effects.

Grape, one of the world's largest fruit crops, with more than 60 million tons harvested per year, is cultivated mainly as Vitis vinifera for wine production (Chedea et al. 2011). It is estimated that around 13% of the total weight of grapes used for the wine making results in grape pomace, which is a by-product in this process (Chedea et al. 2011). Grape (Vitis vinifera) seeds are considered as rich sources of polyphenolic compounds that show antioxidant or antimicrobial effects (Furiga et al. 2008). Among the different parts of the grape plant, grape seeds exhibit highest antioxidant ac-

tivity followed by the skin and the flesh (Pastrana-Bonilla et al. 2003). The antioxidant potential of GSE is twenty and fifty fold greater than those of vitamins E and C, respectively (Shi et al. 2003). Previous studies have shown an increase in the antioxidant activity of broiler diets, excreta, and meat as a result of the dietary administration of grape pomace concentrate (Goni et al. 2007; Brenes et al. 2008). Also, phenolic compounds of grapes have shown inhibitory effects on bacteria (Perumalla and Hettiarachchy, 2011). The increasing order of the antimicrobial activity reported for grape plants was flesh, whole fruit grape extracts, fermented pomace, skin, leaves and seeds (Xia et al. 2010). Thus, the objective of this study was to evaluate the effect of GSE and vitamin C feed supplementation on carcass characteristics, gut morphology and ileal microflora in broiler chickens exposed to chronic heat stress.

MATERIALS AND METHODS

Grape Sseed analysis
Black grape (Vitis vinifera) samples were collected during September of 2012 from Sari, Mazandaran, Iran. Berries were snipped from the cluster. The seeds from berries were manually separated from pulp, washed with tap water and air dried. The proximate composition of grape seeds was measured by AOAC procedures (AOAC, 1990).

Preparation of grape seed extract (GSE)
Grape seeds was ground and extracted with acetone: methanol: water (60:30:10 v/v/v) solution for 12 h using a shaker incubator. Solvents were removed by rotary evaporator. Then, the extract was dried in a vacuum oven and kept at -20 °C (Salari et al. 2009).

Grape seed extract analysis
GSE analysis was carried out in the Iran antibiotic company (Sari, Iran). The chromatographic analysis was carried out on a Knauer HPLC system (Berlin, Germany) equipped with a Triathlon auto sampler, a K-1001 pump and a UV–visdetector (K-2600). A reversed-phase C18 Nucleosil 100 (12.5 cm×5.0 mm×5.0 μm) column was used for the separation of sample components. Standards of catechein, epicatechin, procyanidin B1, B2, C1 were purchased from Sigma-Aldrich (St. Louis, USA). Analysis of catechein, epicatechin, procyanidin B1, B2, C1 was performed according to the method of Iacopini et al. (2008) with some modifications.

In vitro assay of antibacterial activity of grape seed extract
The extracts were tested for anti-microbial activity against E. coli and S. typhi bacteria using modified agar-well diffu-

sion procedures described by CLSI (2006). Five hour broth cultures of the test bacteria were adjusted to 10^8 CFU-1 and applied on the surface of nutrient agar. A sterile flamed cork borer of 8 mm diameter size was used to punch four wells into each of the plates and 0.5 ml of 150, 300, 450 ppm GSE was dispensed in each well. The plates were then incubated at 35 ˚C for 24 h. The assay was done in triplicate and the means of the diameters of the inhibition zones were calculated.

In vitro assays of antioxidant activity of grape seed extract

The antioxidant potencies of GSE and vitamin C were examined by ferric reducing / antioxidant power (FRAP) and 2, 2'-azino-bis (3-ethylbenzothiazoline-6-sulphonic acid) (ABTS) tests. The FRAP assay was performed according to the method of Benzie and Strain (1999) with slight modifications. The ABTS assay was performed according to the methods of Re *et al*. (1999).

Animals, diets and management

This experiment was carried out using a total of 300 Cobb-500 male broiler chicks. One-day-old chicks (initial weights (g), 36.28±0.38) were obtained from a local hatchery and divided into 25 groups of 12 birds each. All procedures for the use and the care of animals were conducted after approval by the Ferdowsi University of Mashhad.

The 5 dietary treatments were, control, control diet supplemented with 150, 300, 450 mg GSE/kg, and control supplemented with 300 mg vitamin C/kg. Each of the five diets was fed to 5 replicates of 12 male chicks each from 1 to 42 d of age.

The feeding program consisted of starter (1 to 10 d), grower (11 to 22 d) and finisher diets (23 to 42 d). The basal diet was fed in mash form and prepared with the same batch of ingredients for starter, grower, and finisher periods and was formulated to meet the nutrient requirements according to Cobb-500 rearing guidelines (Cobb-Vantress, 2012). The ingredients and chemical composition of the basal diets are shown in Table 1.

Each desired level of GSE or ascorbic acid was added to 100 mL distilled water, well mixed and then the premixes were sprayed on the basal diet. Feed was prepared weekly and stored in airtight containers.

All birds had free access to feed and water during the whole rearing period. Temperature was initially set at 34 ˚C on d 1 and decreased linearly by 0.5 ˚C per day up to 42 d. The birds were kept under chronic daily heat stress at 34 ± 1 ˚C with 65-70% relative humidity, THVI about 86, for 5 hours per day from 29 to 42 days of age. During the study, the birds received a lighting regimen of 23 L: 1 d from d 1 to 42.

Table 1 Ingredients and nutrient composition of the experimental diets			
Ingredient (%)	0-10 d	11-22 d	23-42 d
Corn, ground	56.2	59.9	63.34
Soybean meal	37.11	32.55	28.71
Soybean oil	2.26	3.3	3.94
Dicalcium phosphate	1.92	1.86	1.74
Oyster shell	1.16	1.12	1.06
Common salt	0.3	0.3	0.3
Vitamins mix[1]	0.25	0.25	0.25
Minerals mix[2]	0.25	0.25	0.25
DL-methionine	0.31	0.26	0.23
L-lysine hydrochloride	0.24	0.21	0.18
Calculated composition			
ME (kcal/kg)	3000	3105	3180
CP (%)	21.23	19.46	18
Ca (%)	1	0.96	0.9
AP (%)	0.50	0.48	0.45
Lysine (%)	1.32	1.19	1.06
Methionine + cystine	0.98	0.89	0.82

[1] Vitamins mix supplied the following per kg of diet: vitamin A: 18000 IU; vitamin D_3: 4000 IU; vitamin E: 36 mg; vitamin K_3: 4 mg; vitamin B_{12}: 0.03 mg; Thiamine: 1.8 mg; Riboflavin: 13.2 mg; Pyridoxine: 6 mg; Niacin: 60 mg; Calcium pantothenate: 20 mg; Folic acid: 2 mg; Biotin: 0.2 mg and Choline chloride: 500 mg.
[2] Mineral mix supplied the following per kg of diet: Cu: 20 mg; Fe: 100 mg; Mn: 100 mg; Se: 0.4 and Zn: 169.4 mg.
ME: metabolizable energy; CP: crude protein and AP: ????.

Carcass characteristics

At 28 or 42 d of age, two birds per pen close to the mean weight for the pen were selected and killed by cervical dislocation, to determine the carcass traits. The edible carcass (without viscera or feet), breast, drumstick + thigh, liver, empty gizzard and abdominal fat were weighed and expressed as percentages of live body weight.

Gut morphology

Intestinal tissues were obtained immediately after slaughter at 28 and 42 d. Segments were removed from the duodenum, jejunum and ileum as follows:1) intestine from the gizzard to pancreatic and bile ducts was referred to as the duodenum, 2) the jejunum was defined as the portion of intestine extending from the bile duct entrance to Meckel's diverticulum, 3) the ileum was defined as the region from Meckel's diverticulum to a point 40 mm proximal to the ileo-cecal junction. The relative length of duodenum, jejunum and ileum to 100 g live body weight was calculated. Jejunum samples (3 cm) were taken at the midpoint of each section and immersed in a phosphate-buffered formalin solution. Two portions per sample were cut perpendicular to the longitudinal axis of the intestine and embedded in paraffin wax. Transverse sections were cut (3 μm), stained with hematoxyline-eosin and analyzed under a light microscope (Model U-TV0.5 XC-2, Olympus corporatration, BX51, Japan) to determine morphometric indices using image-analysis software (DP2-BSW). Measurements for the villi height were taken from the tip of the villus to the villus-crypt junction.

The crypt depth was defined as the depth of the invagination between adjacent villi and the villus width was measured at the top and bottom of villi.

Ileal microflora

The ileums were excised and contents were collected by gently pressing the fingers to move the content into tubes at 28 and 42 d of age. Digesta of birds within a replicate were pooled and put on ice until they were transported to the laboratory for enumeration of microbial populations. One g of ileal content was homogenized in 9 mL sterile water. Each sample was serially diluted. Using these diluted sub-samples, *Lactobacillus* was enumerated on De Man-Rogosa-Sharpe (MRS) agar after incubation at 37 °C in an anaerobic chamber for 48 h (Guban *et al.* 2006) and coliforms and *E. coli* was counted on CHROM agar ECC (EF322-Paris France) after incubation at 37 °C in an aerobic chamber for 48 h (Sallam, 2007).

Statistical analysis

Data were analyzed by analysis of variance using GLM procedures (SAS, 2001). Means were compared using Duncan's new multiple ranges test (Duncan, 1955). The level of significance was reported at $P < 0.05$.

RESULTS AND DISCUSSION

Grape seed composition and extract analysis

The chemical composition (dry matter, crude fat, crude protein, nitrogen free extract, crude fiber, calcium, total phosphorus and ash) of the grape seed and the content of catechin, epicatechin and procyanidins of grape seed extract is shown in Table 2.

In vitro assay of antibacterial activity of grape seed extract

Results of the inhibition zone (mm) are shown in Table 3. The GSE at all concentrations were effective on *E. coli* whit inhibition zones ranging from 30 to 32 mm. GSE treatments had a bacteriostatic effect on *S. typhi* colonies, but did not show inhibition zones against *S. typhi* (Figure 1).

In vitro assays of antioxidant activity of grape seed extract

Standard curves of FRAP and ABTS assays are shown in Figure 2 (A, B). Results of the FRAP assays showed that GSE at the levels of 300 or 450 ppm had a higher antioxidant effect than vitamin C. The ABTS assay showed that there was no difference between the percentage inhibition of GSE and vitamin C at 300 ppm. Both FRAP and ABTS assays showed that GSE at 450 ppm had the highest antioxidant activity (Table 4).

Table 2 Composition of the grape seed analyzed by AOAC methods and analysis of the grape seed extract by HPLC method

Grape seed		Grape seed extract	
Dry matter (%)	90.93	Catechin (mg/100 g)	1420
Gross energy (kcal/kg)	3292	Epicatechin (mg/100 g)	1080
Crude fat (%)	24.83	Procyanidine B1 (mg/100 g)	830
Crude protein (%)	10.17	Procyanidine B2 (mg/100 g)	770
Nitrogen free extract (%)	17.86	Procyanidine C (mg/100 g)	530
Crude fiber (%)	35.39	-	-
Calcium (%)	0.56	-	-
Phosphorus (%)	0.31	-	-
Ash (%)	2.68	-	-

Table 3 Antimicrobial activity of different levels of grape seed extract

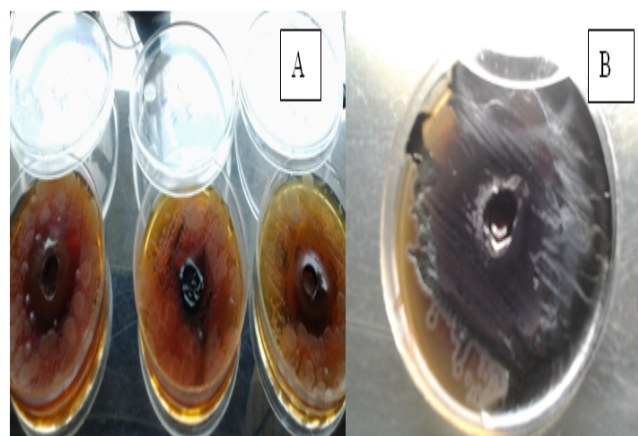|	Zone of inhibition (mm)		
	150 ppm	300 ppm	450 ppm
E. coli	30±0.58	30±0.58	32±0.61
S. typhi	0	0	0

Figure 1 Antimicrobial activity of different levels of grape seed extract against *E. coli* (A) and *S. typhi* (B)
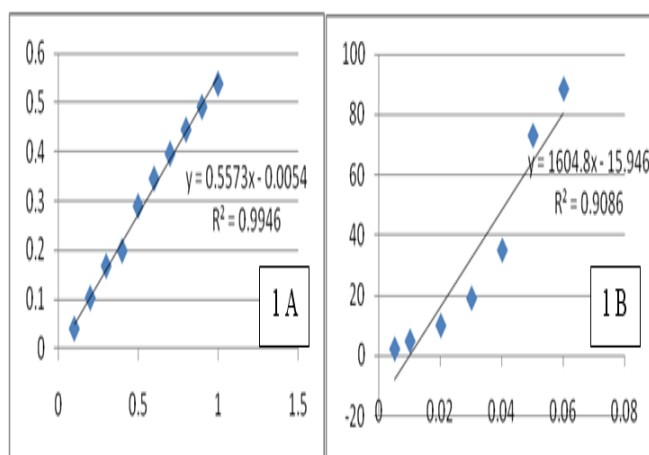

Figure 2 Standard curves of FRAP (A) and ABTS (B) assays

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Carcass characteristics
Dietary supplementation of GSE or vitamin C did not affect the percentage of edible carcass, breast, drumstick + thigh, liver, empty gizzard and abdominal fat of birds before and after chronic heat stress (Table 5).

Gut morphology
Addition of GSE or vitamin C to the diet did not affect the relative lengths of the duodenum, jejunum, or ileum before and after chronic heat stress (Table 6). In addition, GSE or vitamin C did not affect the relative lengths of large intestine, and ceca of the broilers under heat stress. Dietary GSE and vitamin C increased the jejunum muscle layer thickness of the broilers before heat stress. Dietary GSE increased the jejunum villus height and crypt depth, and GSE at 450 mg / kg diet or vitamin C increased jejunum villus width of the compared to the control group before heat stress. There was no difference among jejunum muscle layer thickness, villus width, crypt depth and villus height: crypt depth ratio of broilers after heat stress. However, broilers fed with GSE had higher jejunum villus heights compared to the control group after heat stress (P<0.05).

Ileal microflora
Birds fed diets supplemented with GSE or vitamin C had lower ileal coliforms and *E. coli* populations compared to the control group before heat stress condition. Different levels of GSE decreased ileal coliforms s after heat stress (P<0.05). Also, dietary supplementation of grape seed extract at 300 and 450 mg/kg decreased the ileal population of *E. coli*. However, there were no significant differences between populations of lactobacillus (Table 7).

***In vitro* assay of antibacterial activity of grape seed extract**
In the present study, GSE showed antibacterial properties. *E. coli* was more susceptible to the antibacterial effect of GSE than *S. typhi* bacterium. GSE at 450 ppm caused the largest inhibition zone (32 mm) against *E. coli*. Previous studies reported that the outer cell membrane or cytoplasmic membrane of a bacterium is essentially composed of a phospholipid bi-layer and proteins and is the major site of interaction with antimicrobial compounds (Perumalla and Hettiarachchy, 2011). Damage to this vital membrane results in death of bacterium and can occur in the following ways: (i) physical disruption of the membrane (Shimamura *et al.* 2007); (ii) dissipation of the proton motive force (PMF) (Juven *et al.* 1994) and (iii) inhibition of membrane-associated enzyme activity (Perumalla and Hettiarachchy, 2011). Functional hydroxyl groups and conjugated double bonds in the reactive groups of natural plant extracts may be involved in their binding to the cell wall components

(Mason and Wasserman, 1987). Catechins have deteriorating effects on the lipid bi-layer membrane that results in the loss of cell structure and function, eventually leading to bacterial death (Cox *et al.* 2001). Also, the presence of gallic acid esters in epicatechin, epigallocatechin gallate are responsible for their high affinity for lipid bi-layers and their effects on membrane structure (Hashimoto *et al.* 1999). On the other hand, major phenolic constituents like epicatechin, caffeic acid, benzoic acid and syringic acid may alter cell morphology by influencing the osmotic pressure of the cell, thus disrupting the cytoplasmic membrane and causing leakage of cell constituents (Sivarooban *et al.* 2008).

Ignat *et al.* (2013) reported that the gram-positive bacteria are more sensitive in general than gram-negative species to the phenolic compounds of plant extracts. This can be due to the lipophilic nature of phenolics that decreases their ability to diffuse across the outer membrane.

***In vitro* assays of antioxidant activity of grape seed extract**
FRAP assay showed that GSE at 300 or 450 ppm had higher antioxidant potency than vitamin C at 300 ppm. Also, the ABTS assay revealed that the percentage of inhibition of GSE at 300 ppm was not different from vitamin C at the same level. Jacob *et al.* (2008) reported that the antioxidant properties of GSE are primarily due to flavonoids that can perform scavenging action on free radicals (superoxide, hydroxyl and 1, 1-diphenyl-2-picrylhydrazyl (DPPH)), metal chelating properties, reduction of hydro peroxide formation, and their effects on cell signaling pathways and gene expression (Jacob *et al.* 2008). The presence of the functional group "-OH" in the structure and its position on the ring of the flavanoid molecule determines the antioxidant capacity (Arora *et al.* 1998). Addition of "-OH" groups to the flavonoid nucleus will enhance the antioxidant activity, while substitution by -OCH3 groups diminishes the antioxidant activity (Majo *et al.* 2008). Degree of polymerization of the procyanidins may also determine the antioxidant activity as the higher the degree of polymerization, the higher the antioxidant activity is (Spranger *et al.* 2008).

Carcass characteristics
Addition of GSE or vitamin C to the basal diet did not affect the carcass traits of birds before and during chronic heat stress. This is in agreement with the results of Akbarian *et al.* (2013) who reported that three plant extracts, i.e. lemon peel extract, orange peel extract and Curcuma xanthorrhiza essential oil, caused no difference in body composition traits of broilers exposed to daily increased temperature.

Table 4 Antioxidant activity of grape seed extract and vitamin C in FARP and ABTS assays

	GSE (mg/kg)			Vitamin C (300 mg/kg)	SEM	Pr > F
	150	300	450			
FRAP (mM FeSO$_4$.7H$_2$O Equivalents)	0.384[d]	0.551[b]	0.575[a]	0.500[c]	0.0168	< 0.0001
ABTS (% inhibition)	31.078[d]	69.276[b]	76.228[a]	67.388[b]	0.3480	< 0.0001

The means within the same row with at least one common letter, do not have significant difference (P>0.05).
GSE: grape seed extract; FRAP: ferric reducing / antioxidant power and ABTS: 2, 2'-azino-bis (3-ethylbenzothiazoline-6-sulphonic acid).
SEM: standard error of the means.

Table 5 Effects of grape seed extract and vitamin C on the carcass characteristics of broilers at 28 or 42 d

	Control	GSE (mg/kg)			Vitamin C	SEM	Pr > F
		150	300	450			
28 d							
Edible carcass (%)	59.42	61.18	62.27	60.01	61.02	0.274	0.1835
Breast (%)	20.71	21.64	22.38	21.14	22.10	0.244	0.4117
Drumstick + thigh (%)	17.26	17.55	17.73	17.49	17.60	0.143	0.7065
Liver (%)	2.17	2.14	2.05	2.08	2.11	0.069	0.2555
Gizzard (%)	1.38	1.31	1.35	1.37	1.42	0.051	0.1220
Abdominal fat (%)	0.989	0.944	0.977	0.951	1.01	0.071	0.9196
42 d							
Edible carcass (%)	64.09	68.35	66.16	67.65	66.39	0.336	0.1973
Breast (%)	22.83	24.34	23.05	24.93	22.92	0.300	0.4838
Drumstick + thigh (%)	17.35	17.79	17.92	17.90	17.73	0.179	0.8063
Liver (%)	2.84	2.27	2.16	2.10	2.22	0.1280	0.0644
Gizzard (%)	1.52	1.31	1.43	1.51	1.49	0.070	0.0727
Abdominal fat (%)	2.16	2.26	2.27	2.09	2.18	0.133	0.2718

The means within the same row with at least one common letter, do not have significant difference (P>0.05).
GSE: grape seed extract.
SEM: standard error of the means.

Cross *et al*. (2007) compared the effects of various herbs on broiler performance and they concluded that the quality as well as the quantity of active chemicals in plant extract determines the response. Thus, it seems that the quantity of supplements in the present study were not sufficient to have an effect on carcass traits.

Gut morphology

The main functions of the gastrointestinal tract are digestion and absorption of nutrients as well as to maintain immunity and a barrier function (Burkey *et al*. 2009). Brenes *et al*. (2010) reported a significant reduction of the relative length of the jejunum and ileum in chickens fed GSE (0.6, 1.8, 3.6 g/kg diet) at 21 days of age. Thomas *et al*. (2007) also observed a reduction of intestinal length in birds fed diets containing 0.5% green tea rich in polyphenolic flavonoids, mainly catechins. Sehm *et al*. (2007) reported an inhibitory effect on the jejunum villus growth in piglets fed red-wine pomace rich and flavan-3-ol and proanthocyanidins. Nyamambi *et al*. (2007) observed a decrease in small intestinal weight, duodenal villus height and crypt depth in broiler chicks fed sorghum based diets differing in condensed tannin (syn. proanthocyanidins) levels at 21 days of age. However, Brenes *et al*. (2010) reported a significant increased ileum and ceca relative length in chickens fed with 3.6 g/kg GSE at 42 days of age. Chamorro *et al*. (2013) examined the effect of inclusion of GSE at the levels of 0.025, 0.25, 2.5 and 5.0 g/kg in a control diet. They concluded that incorporation of GSE in chicken diets up to 2.5 g/kg had no adverse effect on growth performance or protein and amino acid digestibility. They reported increased feed conversion ratio and retarded growth rate of chickens fed 5 g GSE/kg. On the other hand, previous studies on pigs indicated that blood flow to the gastrointestinal tract was reduced during heat stress condition (Pearce, 2011). This can lead to tissue hypoxia which depletes ATP stores, intracellular acidosis, changes ion pump activity and increased permeability of tight junctions of the intestinal epithelium, which can cause the translocation of bacterial and endotoxins across the membrane and into the circulation. This may lead to severe illness, endotoxemia or death (Lambert, 2009).

Table 6 Effects of grape seed extract and vitamin C on gut characteristics of broilers at 28 or 42 d

	Control	GSE (mg/kg)			Vitamin C	SEM	Pr > F
		150	300	450			
28 d							
Intestinal length (cm/100 g of BW)							
Duodenum length	2.177	2.201	2.260	2.415	2.270	0.100	0.6158
Jejunum length	4.332	4.096	4.213	4.439	4.245	0.126	0.7201
Ileum length	4.038	3.685	3.805	4.135	3.795	0.124	0.3544
Jejunum morphology (μm)							
Muscle layer thickness	129.04[b]	208.30[a]	204.83[a]	194.41[a]	197.03[a]	1.069	0.0015
Villus height	626.17[b]	815.60[a]	772.94[a]	753.92[a]	722.12[ab]	1.763	0.0127
Villus width	79.63[b]	72.45[b]	96.85[b]	123.56[a]	123.27[a]	0.864	0.0005
Crypt depth	97.90[b]	130.12[a]	126.47[a]	135.06[a]	119.91[ab]	0.886	0.0586
Villus: crypt	6.518	6.301	6.282	5.793	6.098	0.218	0.9006
42 d							
Intestinal length (cm/100 of BW)							
Duodenum length	1.259	1.408	1.469	1.409	1.318	0.0846	0.3938
Jejunum length	2.821	2.893	2.875	3.175	2.831	0.101	0.2061
Ileum length	2.949	3.041	2.979	3.400	2.977	0.102	0.0688
Large intestine	1.368	1.614	1.482	1.506	1.264	0.097	0.2140
Cecum	0.792	0.763	0.797	0.815	0.808	0.059	0.9059
Jejunum morphology (μm)							
Muscle layer thickness	189.62	192.92	196.23	212.33	232.09	1.125	0.2246
Villus height	851.93[c]	1043.41[ab]	1087.97[a]	1081.05[ab]	931.49[bc]	2.078	0.0086
Villus width	92.79	92.72	109.24	123.47	139.29	1.118	0.1218
Crypt depth	121.67	142.04	147.94	157.26	134.60	0.875	0.0769
Villus: crypt	7.124	7.432	7.362	6.894	7.087	0.199	0.9117

The means within the same row with at least one common letter, do not have significant difference (P>0.05).
GSE: grape seed extract.
SEM: standard error of the means.

Table 7 Effects of grape seed extract and vitamin C on ileal microbial population (log CFU/g of digesta) of broilers at 28 or 42 d

	Control	GSE (mg/kg)			Vitamin C	SEM	Pr > F
		150	300	450			
28 d							
Coliforms	5.89[a]	5.86[a]	5.54[b]	5.47[b]	5.47[b]	0.065	< 0.0001
E. c oli	5.83[a]	4.97[b]	4.92[b]	4.78[b]	5.17[b]	0.139	0.0234
Lactobacillus	5.45	5.48	5.60	5.58	5.51	0.118	0.9500
42 d							
Coliforms	5.34[a]	4.55[bc]	4.47[c]	4.38[c]	5.00[ab]	0.120	0.0018
E. coli	5.56[a]	5.23[abc]	5.08[bc]	4.97[c]	5.41[ab]	0.109	0.0346
Lactobacillus	5.61	5.75	5.92	6.14	5.91	0.108	0.0933

The means within the same row with at least one common letter, do not have significant difference (P>0.05).
GSE: grape seed extract.
SEM: standard error of the means.

Heat stress can induce cytokine release. Major cytokines associated with heat stress are IL-1ß, IL-6, IL-10, IFNy and TNFa (Pearce, 2011). Increased gut permability occurs due to elevated nitric oxide levels induced by cytokine stimulation. In the periphery, cytokine release causes systemic inflammation, leading to multi-organ damage, organ failure and finally death of animal (Leon, 2007). Garriga *et al.* (2006) showed that acute heat stress reduced jejunum fresh weight by 22% and jejunum length by 5%. It also reduced villous length. Moreover, chickens submitted to acute heat stress (30 ˚C/24 h) showed ileal crypt depth reduction, but no changes in villus height or in the villus/crypt ratio (Burkholder *et al.* 2008). Differences in villus height and villus / crypt ratio were not observed after long-term heat stress (Quinteiro-Filho *et al.* 2010).

In our study, grape seed extract (150, 300 or 450 mg/kg) and vitamin C supplementation of diet did not affect the relative length of duodenum, jejunum and ileum of birds before and after heat stress. GSE or vitamin C increased jejunum muscle layer thickness of birds before heat stress (P>0.05). This is in agreement with the findings of Viveros *et al.* (2011), who reported that muscularis thickness was increased in birds fed avoparcin (AVP) (50 mg/kg of avoparcin), grape pomace concentrate (GPC) (60 g/kg grape pomace concentrate), and GSE (7.2 g/kg). The highest villus height: crypt depth ratio corresponded to birds fed GPC and AVP diets. The researchers stated that lengthening of the villus and a short crypt can lead to better nutrient absorption, decreased secretion in the gastrointestinal tract, increased disease resistance and greater overall perform-

ance (Viveros *et al*. 2011). In the present study, broilers fed with GSE had higher villus compared to the control group before and after heat stress. GSE at the level of 450 mg/kg or vitamin C increased villus width of the broilers before heat stress.

It is assumed that an increased villus height is paralleled by an increased digestive and absorptive function of the intestine which results in an increase in absorptive surface area, expression of brush border enzymes, nutrient transport systems, and an increased body weight gain (Viveros *et al*. 2011).

In contrast to the present study, Laurent *et al*. (2005) observed a decrease in the size of the microvilli by the addition of GSE in human intestinal Caco-2 cells. In our study there were no significant differences in the jejunum villus height: crypt depth ratio of birds before and under heat stress. However, the GSE increased the crypt depth of broilers before heat stress condition. A stimulating effect on crypt colon size in piglets fed red wine pomace was reported by Sehm *et al*. (2007). This diversity in the effects of GSE supplementation on gastrointestinal morphology may be due to the method of extraction and conservation of GSE, which can affect the effective substances in GSE, as well as the different levels of GSE in the diet.

Ileal microflora

Heat stress changes the commensal microbiota of the chickens and also the host mucosal enterocyte resistance against intestinal pathogens. Indeed, alterations in intestinal microbiota balance are known to reduce the protector mechanisms against pathogen invasion, such as by Salmonella species (Burkholder *et al*. 2008).

Moreover, the commensal chicken microbiota strongly contributes to the innate and adaptive immune responses (Brisbin *et al*. 2008). In diseased chickens (such as those infected with avian *Mycoplasma gallisepticum* or *Eimeriatenella*), (Guo *et al*. 2004a; Guo *et al*. 2004b; Guo *et al*. 2004c) demonstrated that plants and their extracts could improve the growth performance, reduce the populations of coliforms and / or *C. perfringens* and enhance both cellular and humoral immune responses of chickens. Jamroz and Kamel (2002) reported that the dietary herbal treatment results in lower *E. coli* counts comparing to the control group. It has been shown that phytogenics modulated the intestinal microflora composition via the reduction of coliforms at 14 days of age and the beneficial fortification of gut microflora with purportedly beneficial members such as the lactobacillus and bifidobacterium at 42 day of age (Mountzouris *et al*. 2011). Also, it has been reported that a mixture of thymol and carvacrol increases the population of Lactobacillus in the ileum (Akyurek and Yel, 2011). In our study the GSE decreased the ileal coliform population of

chickens at 42 d. Also, dietary supplementation of GSE at the levels of 300, and 450 mg/kg diet decreased the ileal population of *E. coli*. Polyphenols could have bacteriostatic or bactericidal actions or act to inhibit adhesion of infection-causing bacteria within cells of the intestinal tract (Viveros *et al*. 2011). As the pH in the gastrointestinal tract may be decreased by the active components of the herbal derivatives, the components can prevent the growth of pathogenic bacteria and promote the population of non-pathogenic ones like Lactobacillus and bifidobacterium (Khaksar *et al*. 2012). On the other hand, Goni and Serrano (2005) reported that intestinal bacteria showed a high capacity to degrade extractable polyphenols in rats. Deprez *et al*. (2000) and Ward *et al*. (2004) also reported that major polyphenolic constituents of grape polyphenols (polymeric proanthocyanidins) were degraded by human colonic microflora into smaller compounds including phenolic acids that could be absorbed and metabolized. The present study showed that there were no significant differences between populations of lactobacillus in broilers fed diets containing GSE or vitamin C compared to control fed birds, however, dietary GSE or vitamin C increased the population of lactobacillus numerically. Viveros *et al*. (2011) reported that in the ileal content of birds fed control and GSE diets had the highest populations of Lactobacillus compared to birds fed diets containing AVP or GPC. They also stated that animals fed GPC and GSE diets showed a higher biodiversity than those fed control diets and the frequency of detection of several potential phenol-degrading bacteria as well as unidentified and uncultured organisms was increased in animals fed GPC and GSE diets. Some microorganisms are able to use GPC and GSE compounds as nutritional substrates (Viveros *et al*. 2011). In the particular case of lactobacilli, these bacteria have the ability to metabolize phenolic compounds, thereby supplying energy to cells and positively affecting the bacterial metabolism (García-Ruíz *et al*. 2008). Isolation of tannin-degrading lactobacilli in human gut microflora has been reported by Osawa *et al*. (2000).

CONCLUSION

The present study showed that the GES had antioxidant activity comparable to vitamin C. Also, GSE had antibacterial activity in *in vitro* and *in vivo* assays. Indeed, the GSE had beneficial effect on jejunum morphology of birds before and after heat stress. Since it is the first report of the beneficial effects of dietary GSE on jejunum morphology and gut microbial ecology of birds exposed to chronic heat stress, it seems that further study is needed to introduce grape seed extract as a new herbal additive to improve health and welfare of poultry, especially during periods of chronic heat stress.

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