PAPER

Effects of verbascoside supplemented diets on growth performance, blood traits, meat quality, lipid oxidation and histological features in broiler chickens

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Abstract

A trial has been performed to evaluate the effects of dietary verbascoside (VB) on the growth performance, blood traits, meat quality and histological features of broiler chickens. Seventy-two male broiler chickens (Ross 708) were divided into three experimental groups (8 replicates): a control group (C; basal diet), a low VB group (LVB; basal diet+2.5 mg VB/kg feed) and a high VB group (HVB; basal diet+5.0 mg VB/kg feed). The animals were kept in cages, three birds per cage. The trial lasted 35 days. Growth performance and carcass yield were recorded. Blood samples were collected at day 35 in order to evaluate the blood, serum and plasma parameters. As a result of this study, growth and slaughter performances were not affected by the dietary treatments. The LVB and HVB groups showed significantly lower concentrations of total protein (P=0.000), albumin (P=0.000), α-globulin (P=0.001) and β-globulin (P=0.023) than C group. The heterophil to lymphocyte ratio was higher in HVB group than in LVB and C groups (P=0.005). The thiobarbituric acid reactive substances results indicated lower lipid oxidation in the LVB group than in C group. The most relevant histological feature was the lymphoid depletion observed in the thymus and *bursa of Fabricius* in LVB and HVB. Other studies are still needed to identify the optimal dosage and exposure time of VB dietary supplementation and to further investigate the effects of VB on lymphoid tissue as well as to establish the VB antioxidant effect on broiler chickens.

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Introduction

Feed additives are used in animal nutrition to improve feed quality and the performance and health of animals. The European Union introduced the complete ban of growth promoters for broilers in January 2006, allowing only 4 antibiotics that are not associated with human treatment (Hernández *et al.,* 2004). This has had significant consequences on the growth performance of animals, especially for the poultry industry (Attia *et al*., 2011).

The dietary strategies adopted to improve the nutritional value, oxidative stability and sensory properties of poultry products have been reviewed by Bou *et al.* (2009), and these include plant derived or phytogenic additives. These compounds, including polyphenols, are characterized by low-molecular-weight reactive oxygen species-scavenging activity (Pastorelli *et al.,* 2012), and are thought to be potential growth promoters in animal diets (Hashemi and Davoodi, 2011). Hernández *et al.* (2004) showed that *Salvia officinalis*, *Thymus vulgaris* and *Rosmarinus officinalis* extracts, improved feed digestibility in broilers. Phenylpropanoid glycosides (PPGs) are an important chemical group of plant extracts, that belong to the largest group of secondary metabolites produced by plants. Phenylpropanoid glycosides are water-soluble derivates of phenylpropanoids (Korkina, 2007) with antioxidant properties (Paola *et al.,* 2011). Verbascoside belongs to the PPGs group. It is structurally characterized by caffeic acid (phenylpropanoid moiety) and 4,5-hydroxyphenylethanol (phenylethanoid moiety) bound to a β-(d)-glucopyranoside (Perron and Brumaghim, 2009). The PPGs verbascoside (VB) has been suggested to be efficient in attenuating oxidative stress and hence in ameliorating health because of its antitumoral, antiviral, anti-inflammatory, antibacterial, antioxidative, hepatoprotective and free radical scavenging activity, among others (Korkina, 2007).

Lippia citriodora (Ort.) HBK (*Verbenaceae*) is a herbal species that grows spontaneously in South America, and is cultivated in northern Africa and southern Europe. In these countries, it is used as a spice and medicinal plant. The leaves of this species are reported to possess digestive, antispasmodic, antipyretic, sedative, and stomachic properties as well as strong antioxidant properties (Valentão *et al.,* 2002). Phenylpropanoids, and above all mainly VB, are the most abundant compounds in *Lippia* extracts. In animals, the antioxidant properties of VB have been demonstrated in suckling lambs (Casamassima *et al.,* 2009), weaned piglets (Pastorelli *et al.,* 2012) and hares (Casamassima *et al.,* 2013).

The present study was designed to evaluate the effects of a *Verbenaceae* extract rich in phenylpropanoid glycosides and its potential application as a dietary supplement in chicken diets. Hence, the aim of the study was to investigate the influence of two different levels of dietary VB supplementation on the growth and slaughter performances, blood and histological traits and meat quality of broiler chickens.

Materials and methods

Animals, diets and experimental design

The study was performed in the animal farm of the Department of Veterinary Science of the University of Turin (Italy). The experimental

protocol was designed according to the guidelines outlined in European and Italian law for the care and use of experimental animals (European directive 86/609/EEC, introduced in Italy with D.L. 116/92; European Commission, 1986).

Seventy-two day-old [41.3±0.5 g/body weight (BW)] male broiler chickens (Ross 708) were randomly allotted to 3 experimental groups and kept in heated two-floor cages (3 birds per cage/8 replicates), taking care to ensure that the chicks were randomly distributed between the treatments. Three dietary treatments were used, with differing levels of plant extract, standardized for VB added to the basal diet: 0 mg VB/kg to the control group (C), 2.5 mg VB/kg to the low verbascoside group (LVB), and 5.0 mg VB/kg to the high verbascoside group (HVB).

A commercial *Verbenaceae* (*Lippia spp./ Lippia citriodora*) extract obtained by hydroalcoholic extraction of leaves and devoided of essential oils, standardized with 20% (w/w) of VB (I.R.B. s.r.l., Altavilla Vicentina, Vicenza, Italy) was used. In order to avoid oxidation in the complete feed, the supplement was microencapsulated within a protective hydrogenated vegetable lipid matrix using spray-cooling (Sintal Zootecnica, Isola Vicentina, Vicenza, Italy). Diets were formulated to meet or exceed National Research Council (1994) requirements and adjusted according to Aviagen (2014) recommendations. The composition of the basal diet for both the starter/grower and finisher periods is given in Table 1. Feed and drinking water were provided *ad libitum* for the entire duration of the trial, until day 35, which corresponded to the slaughtering day. The lighting schedule was 23L:1D during the first 3 d, followed by 18L:6D until slaughtering. Ambient temperature was kept within the thermoneutral zone. The chicks were vaccinated at hatching against Newcastle disease, Marek disease, infectious bronchitis and coccidiosis. Health status and mortality were monitored daily throughout the whole experimental period.

Sampling and analytical determinations

Medium chicken weight were recorded at day 1 and day 35 while feed intake were recorded for the 1-35 days period using a high precision scale (Signum®; Sartorius, Göttingen, Germany) on the cage basis. Final BW (FBW) was recorded on day 35 and feed conversion ratio (FCR) was calculated for 1-35 day period. Feed samples were taken for proximate analysis (AOAC, 2005). At day 35 the birds were fasted over 6 hours before the blood sampling and slaughtering procedures. Blood samples were taken from one bird per cage and collected by means of venipuncture of the brachial vein, using a 21G needle. A 5 mL sample of blood was collected from each bird: 1.0 mL was placed in a litio eparine tube and 4.0 mL in a tube without anticoagulant. Serum samples (obtained by centrifugation for 15 min at 3000×g) were stored at -80°C, pending analysis. Lithium heparine-blood samples were used to evaluate the total red and white cells, the heterophil to lymphocyte ratio (H/L) and the total antiradical activity. The total red and white cell counts were determined in an improved Neubauer haemocytometer, after mixing with a Natt-Herrick solution in a 1 to 200 ratio (Natt and Herrick, 1952). Blood smears were stained with May-Grünwald and Giemsa-Romanowski stains (Campbell, 1995). One hundred white blood cells were evaluated per smear in order to determine the H/L, and the blood cell type number was determined according to Campbell (1995). Blood samples were stored at 4°C for the determination of the total blood antiradical activity, and analysed by means of the Kit Radicaux Libres (KRL) biological test (Laboratoires Spiral, Dijon, France), within 24 h of collection. The principle behind the KRL test is to submit whole blood to thermocontrolled free radical aggression in order to mobilize all the families of any free radical scavengers present in the blood so as to neutralize the oxidation processes (Stocker *et al.,* 2003). Whole blood and red blood cell (RBC) samples were submitted to an isotonic saline solution of organic free radicals produced at 40°C from the thermal decomposition of a solution of 2.20-azobis (2-amidinopropane) dihydrochloride (Kirial International, Dijon, France). Haemolysis was recorded, using a 96-well microplate reader, by measuring the optical density decay at 450 nm. Absorbance measurements were performed 75 times, once every 150 s, the results were expressed as the time (min) required to reach 50% of maximal haemolysis (half-haemolysis time – HT50 – in min), which reference to the whole blood and RBC resistance to free-radical attack. The following biochemical parameters were evaluated on the serum samples: total protein (biuret method, Bio Group Medical System kit; Bio Group Medical System,

Table 1. Ingredients and chemical composition of the basal diet (values as fed).

Item	Starter/grower	Finisher
Ingredients, g/kg		
Wheat	274.0	308.0
Corn	253.5	252.4
Soybean meal (48% CP)	376.7	332.4
Animal fat	56.0	68.0
Dicalcium phosphate	13.0	12.4
Calcium carbonate	11.5	11.2
Sodium chloride	2.3	2.2
Sodium bicarbonate	1.3	1.5
DL-methionine	3.9	3.8
L-lysine	2.0	2.0
Treonin	0.8	1.1
Vitamin-mineral premix	5.0°	5.0#
Analyzed composition		
Dry matter, g/kg	904.9	907.4
Crude protein, g/kg	230.5	195.0
Ether extract, g/kg	96.6	95.7
Crude fibre, g/kg	30.4	27.5
Ash, g/kg	52.9	50.4
DL-methionine§, g/kg	7.2	6.9
L-lysine§, g/kg	13.9	12.8
Treonin§, g/kg	9.3	8.9
Calcium§, g/kg	9.2	8.8
Phoshorus§, g/kg	6.1	5.8
Metabolizable energy§, MJ/kg	13.1	13.5

CP, crude protein. °Supplied per kilogram of diet: vitamin A, 12,500 U; vitamin D_3, 5000 U; vitamin E, 30 mg; iron, 10 mg; Endo-Beta-1,4-Xylanase, 20000 BXU; Endo-Beta-1,3-glucanasi, 5000 BU. #Supplied per kilogram of diet: vitamin A, 12,500 U; cholecalciferol, 2000 U; vitamin B_1, 1.5 mg; riboflavin, 3.0 mg; vitamin B_6, 1.5 mg; vitamin B_{12}, 15 μg; dl-α-tocopheryl acetate, 75 U; niacin, 25.0 mg; d-pantothenic acid, 8.0 mg; cobalt, 0.2 mg; iron, 30.0 mg; iodine, 1.4 mg; manganese, 80 mg; copper, 1.5 mg; zinc, 30.0 mg. §Based on the National Research Council (1994) ingredient composition.

Talamello, Italy), albumin, α-globulin, β-globulin, γ-globulin (semi-automated agarose gel electrophoresis system, Hydrasys®; Sebia, Norcross, GA, USA), α-1-acid-glycoprotein (AGP) (commercial radial immunodiffusion tray, Cardiotech Services, Inc.), lysozyme (Osserman and Lawlor, 1996), albumin-globulin ratio (A/G), alanine aminotransferase (ALT), aspartate aminotransferase (AST) and uric acid [enzymatic method using a clinical chemistry analyzer, Screen Master Touch, Hospitex diagnostics srl. Florence, Italy, based on Lowry *et al*.'s method (1951)].

One bird per cage was slaughtered by CO_2 gassing and immediately bled, plucked and the eviscerated carcasses were then weighed. Head, neck, feet, and abdominal fat were removed to obtain the carcass-for-grilling. The weight of the breast, thighs, heart, liver, spleen and *bursa of the Fabricius* were recorded and expressed as a percentage of live weight (LW). Breast samples were vacuum-packaged and kept frozen (-20°C) until the analyses were performed.

The chemical composition (water, ash, and crude protein and ether extract analyses) was determined on lyophilized samples of the breast (m. *pectoralis major*) and expressed on a fresh basis, according to the AOAC method (2005).

The oxidative stability of the m. *pectoralis major* samples was determined using a modification of the method described by Monahan *et al.* (1992). One gram of muscle homogenate was incubated with 9 mL of 1.15% KCl at 37°C in a 40 mmol Tris-maleate buffer (pH 7.4), containing 1 mmol $FeSO_4$ (to catalyze lipid peroxidation). A sample aliquot was collected at 0, 60 and 120 minutes of incubation with $FeSO_4$ for thiobarbituric acid reactive substances (TBARS) determination, and was expressed as mg malondialdehyde (MDA)/kg of muscle tissue.

The liver, kidneys, spleen, thymus and *bursa of Fabricius* samples of 24 animals (one each cage, eight for each experimental group) were collected for histological examination and fixed in 10% neutral buffered formalin. The tissues were paraffin embedded, sectioned at a thickness of 5 µm and stained with haematoxylin and eosin. All the samples were examined before under a light microscope by two pathologists and subsequently all discordant cases were reviewed at a multi-head microscope until a consensus was reached. The mean value of the apoptotic and mitotic cells in the lymphoid organs was counted in 10 follicles under 40x magnification (Pozzo *et al*., 2013).

Statistical analyses

All the statistical analyses were performed with SPSS 17 for Windows (SPSS, Inc., Chicago, IL, USA; 2008). The experimental unit was the cage for growth performance, while the individual bird was considered for the slaughtering performance, blood and histological traits. Before testing for group differences, normality of data distribution and homogeneity of variance were assessed using the Shapiro-Wilk test and the Levene test, respectively. Thiobarbituric acid reactive substances data were further evaluated by performing a repeated measurement analysis .

All the remaining data were analyzed by means of one-way ANOVA with the dietary treatment as the fixed effect. Post-hoc Dunnet's t-test was used to compare the treated groups (LVB and HVB) against the control group. The results are presented as mean values and pooled standard error of mean (SEM). Differences were considered statistically significant for $P<0.05$. A statistical trend was considered for $P<0.1$.

Results and discussion

The present study provides new *in vivo* information about the use of VB in broiler chicken nutrition.

The chickens remained healthy throughout the study. Health was based on the absence of clinical signs. No mortality occurred throughout the trial. This may be due to high hygienic

Table 2. Effect of dietary verbascoside on growth performance and carcass yield of broiler chickens.

Item	C	LVB (2.5 mg/kg)	HVB (5.0 mg/kg)	SEM	P
Growth performance (n=8 cages per treatment)					
Initial BW (d 1), g	41.3	41.1	41.5	0.5	0.941
Final BW (d 35), g	2013	2019	2009	23	0.986
Feed intake (d 1-35), g	3434	3426	3437	49	0.996
FCR (d 1-35)	1.74	1.73	1.75	0.019	0.959
Comparison for standard ROSS 708 performance, %					
Final BW, 2166 g (d 35)	-7.1	-6.8	-7.2	1.1	0.986
FCR, 1.516 (d 1-35)	+12.5	+12.0	+12.8	1.0	0.942
Slaughter performance (n=8 birds per treatment)					
Carcass for grilling, g	934	1002	1021	23	0.288
Carcass for grilling, % of LW	52.5	52.1	52.7	0.3	0.661
Breast, g	350	376	372	10	0.556
Breast, % of LW	19.7	19.6	19.2	0.3	0.782
Thighs, g	346	361	378	7.1	0.207
Thighs, % of LW	19.6	18.8	19.6	0.2	0.201
Heart, g	12.0	12.9	13.3	0.4	0.426
Heart, % of LW	0.67	0.68	0.69	0.02	0.935
Liver, g	31.8	34.5	34.5	0.8	0.294
Liver, % of LW	1.8	1.8	1.8	0.0	0.965
Spleen, g	1.9	2.0	1.7	0.1	0.378
Spleen, % of LW	0.08	0.11	0.10	0.01	0.241
Bursa of Fabricius, g	3.9	3.7	4.1	0.3	0.767
Bursa of Fabricius, % of LW	0.21	0.19	0.21	0.01	0.686

C, control group; LVB, low verbascoside group; HVB, high verbascoside group; BW, body weight; FCR, feed conversion ratio; LW, live weight. Values are expressed as mean and standard error of the mean.

condition for broilers reared in cages compared to broilers housed in floor pens (Attia *et al*., 2011). Growth performance (Table 2) was not influenced by the dietary treatments, and the FBW and FCR on average resulted -7.0% and +12.4% lower and higher, than the Ross 708 broiler performance objectives (Aviagen, 2014). However, the trial was not designed to test this effect, since the rearing conditions used in the present study (*i.e.*, housing in cages) did not reflect the commercial rearing practice. Conflicting results on growth performance have been found when dietary plant extracts are used in poultry diets. Javed *et al.* (2009) reported FCR optimization, due to the drinking of water supplemented with a mixture of medicinal plant extracts in commercial broiler chickens. Ciftci *et al*. (2005) found that anise essential oil supplementation in basal diets, fed to Ross broilers, resulted in a significant improvement in BWG and FCR. A significantly improvement in FCR (3.9%) was highlighted for a plant extract containing carvacrol, cinnamaldehyde and capsicum oleoresin added to a basal diet, fed to a Hubbard Hi-Y broiler hybrid (Jamroz *et al*., 2005). Jamroz and Kamel (2002) demonstrated that diet supplementation with a plant extract containing capsaicin, cinnamaldehyde, and carvacrol at 300 ppm improved the FCR in 17-d-old broilers by 7.7%. Other authors have shown that plant extract supplementation (with orange peel, lycopene, green tea, oregano, thymol, rosemary, cinnamon and pepper) as is – in the form of essential oils or as oleoresin – does not improve the FCR (Lee *et al*., 2003; Hernández *et al*., 2004; Marzoni *et al*., 2014). These contrasting results are mainly related to the different content of bioactive compounds in the plant extract used and the interaction between several components substances. In the present test, slaughtering yield and organ weight were not influenced by dietary treatments (Table 2). Others studies on broiler chickens have confirmed the lack of effects of dietary plant extract supplementation with dietary nettles (Ocak *et al*., 2008), peppermint and thyme extracts (Sarica *et al.,* 2005), oregano essential oil and thyme powder supplementation (Sarica *et al.,* 2005) or tomato, orange peel and green tea extract (Marzoni *et al*., 2014) on carcass yield.

Majority of blood parameters were not affected, but several blood parameters were influenced by the treatments (Table 3). Verbascoside treated groups showed decreased values of serum total protein (P<0.001), associated with a decreased value of albumin (P<0.001), α-globulin (P=0.001) and only as far as the HVB group, β-globulin (P=0.023). A trend effect (P=0.079) was observed for γ-globulin with the LVB and HVB groups displaying a lower concentration than the C group. This pattern could be associated with liver stress conditions (Lumeij, 2008) which could also be confirmed from the trend increase of ALT (P=0.076); no differences among groups was observed for hepatic histological lesions. Most serum proteins (albumin, globulins and total proteins) are synthesized in the liver and their total level in the blood reflects liver activity (Lumeij, 2008). The data obtained in the present study are in disagreement with previous studies in mammals fed dietary VB supplemented diets. Casamassima *et al.* (2012) showed a lower serum AST and bilirubin concentration in Lacaune ewes fed dietary VB, and demonstrated an improvement in the liver function. Similar result have also been found in hares (*Lepus corsicanus*) (Casamassima *et al.,* 2013).

Heterophil to lymphocyte ratio – another index of the physiological condition of birds – resulted higher in the HVB group than in the C group (P=0.005). Heterophils and lymphocytes constitute the majority of circulating immune cells in birds and H/L is particularly sensitive to natural stressors and its augmentation is associated with stress conditions (Davis *et al.,* 2008). Heterophil to lymphocyte ratio resulted higher in the HVB group than in the control group (P<0.05), suggesting a stress condition. This concurred with an increase in ALT and a decrease in serum protein and albumin which may reflect a negative effect on liver function or kidney disease. Although, histological examination of liver and kidney did not refer a signs of pathological effects. An increase in class A immunoglobulin has been observed in post-weaned piglets fed dietary VB (5.0 and 10.0 mg VB/kg diet), thus indicating positive immunomodulatory activity (Pastorelli *et al.,* 2012).

In the present experiment VB did not affect the total antiradical activity, measured through

Table 3. Effect of dietary verbascoside on blood traits of broiler chickens at the age of 35 days.

Item	C	LVB (2.5 mg/kg)	HVB (5.0 mg/kg)	SEM	P
Erythrocyte, $x10^{12}$/L	2.3	2.5	2.4	0.1	0.651
Leucocyte, $x10^{9}$/L	15.4	17.2	14.6	1.2	0.665
Total protein, g/dL	3.3	2.4*	2.1*	0.1	0.000
Albumin, g/dL	1.29	0.91*	0.85*	0.05	0.000
α-Globulin, g/dL	0.91	0.70*	0.63*	0.04	0.001
β-Globulin, g/dL	0.31	0.19	0.10*	0.03	0.023
γ-Globulin, g/dL	0.78	0.66	0.58	0.04	0.079
A/G	0.65	0.61	0.65	0.01	0.518
AGP	720	868	752	55	0.536
Uric acid, mg/dL	6.3	5.8	5.1	0.3	0.309
AST, UI/L	216	210	205	7	0.818
ALT, UI/L	6.1	7.8	11.7	1.0	0.076
AST/ALT	36.7	34.8	22.4	2.7	0.061
Lysozyme, µg/mL	2.4	3.0	2.7	0.3	0.673
H/L	0.09	0.12	0.20*	0.02	0.005
HT_{50}, blood, min	77.6	76.3	74.8	0.8	0.379
HT_{50}, RBC, min	64.9	65.6	67.3	0.6	0.286

C, control group; LVB, low verbascoside group; HVB, high verbascoside group; A/G, albumin-globulin ratio; AGP, α-1-acid-glycoprotein; AST, aspartate aminotransferase; ALT, alanine aminotransferase; H/L, heterophil to lymphocyte ratio; HT_{50}, blood, half-haemolysis time in whole blood; HT_{50}, RBC, half-haemolysis time in red blood cells. n=8 birds per treatment; values are expressed as mean and standard error of the mean. *Values within rows were significantly different (P<0.05) compared to the Control group.

the KRL test, in either whole blood (HT_{50} blood) or RBC (HT_{50} RBC). The KRL test is an alternative approach used to evaluate the total antioxidant defenses of blood and RBC (Blache *et al.,* 1991). In the KRL test, free radicals are generated in plasma and react with RBC membranes. To the best of the authors' knowledge no previous study reported the total antiradical activity, measured trough KRL tests in chickens. A higher total antioxidant activity of whole blood and RBC ($P<0.05$) was found in post-weaned piglets fed dietary vitamin E supplementation for 60 days, than in the control pigs. The total blood antioxidant activity in pigs receiving a long-term supplementation of plant antioxidant from weaning to slaughter, tended to be higher ($P=0.091$) than the controls (Rossi *et al*., 2013). In the present study, no difference among groups was observed for the total antiradical activity of the blood or RBC. Other authors have not observed any *in vivo* antioxidant effect in chicken fed different levels of grape pomace concentrate (Goni *et al*., 2007; Brenes *et al.,* 2008) or in piglets fed dietary vitamin E (90 g/kg feed) (Frankic *et al*., 2010). The majority of the breast meat trait parameters (moisture, ash, crude protein, ether extract and drip loss) were not affected by the dietary treatments (Table 4). The TBARS results of m. *pectoralis major* showed that VB supplemented at a 2.5 mg/kg diet significantly reduced lipid peroxidation for both the T60 ($P=0.007$) and T120 ($P=0.017$) incubation times compared to the C group, while 5.0 mg/kg of VB did not display any effect (Table 4). A time effect was also noted ($P<0.001$). Thiobarbituric acid reactive substances are frequently used to evaluate lipid oxidation. The TBARS values were determinated as a function of the forced oxidation of the breast meat. The TBARS formation increased as the time of incubation increased, as expected. In the present study, only the lowest dietary supplementation (LVB group, 2.5 mg VB/kg feed) produced an antioxidant effect. Similarly Smet *et al*. (2008) found higher TBARS values with increasing dose of green tea extracts (100 and 200 mg kg^{-1}) in broiler meat. On the contrary Tang *et al.* (2000), observed a clear antioxidant dose-response effect at levels of 100 to 300 mg of catechins kg^{-1} of feed. Differences between studies could be due to a different content of catechins present in the green tea extracts. However, caution is required when applying the results of the different antioxidant treatments, because commercial extracts used may contain different levels of active compounds. No other study reported the effects of dietary supplementation with VB on meat oxidative stability in broiler chickens. Dietary supplementation with polyphenols is recognized to increase oxidative stability in postmortem skeletal poultry muscle (Bou *et al.,* 2009). Liu *et al.* (2003) found a lower value of TBARS in the plasma of rabbits fed 0.8 mg/kg of VB twice a day. *In vitro* culture studies have shown both antioxidant and prooxidant effects of VB extracted from olive oil. Cardinali *et al*. (2012) found *in vitro* antioxidant properties of VB in the HT-29 cell line, while Dell'Aquila *et al*. (2014) have demonstrated a pro-oxidant short and long term effect of VB in *in vitro* fresh ovine oocystes. Dell'Aquila *et. al.* (2014) have underlined that a higher accumulation of VB occurs in ovine oocystes than in the HT-29 cell line. These authors stressed that a dose-dependent effect is involved in the balance between the antioxidant and prooxidant effects of VB in cell cultures. Further studies are required to obtain a clear explanation of the results of the present study, and of the lack of the antioxidant effect from the highest dietary VB supplementation (5.0 mg VB/kg feed), in order to clarify whether a dose-dependent effect mechanism is involved *in vivo*.

The macroscopical and histological aspects of both kidney and spleen was judged to be completely normal in all the birds. Focal to diffuse moderate degenerative lesions were detected in all the livers, but no difference among groups was observed. Five out of eight and six out of eight thymus samples from LVB and HVB, respectively, showed a moderate to severe lymphoid depletion of the medulla, associated with an increase in stromal/epithelial cells (Figure 1). In some cases, multifocal hemorrhages were also detected. Only two control birds showed moderate medullar lymphoid depletion. One out of eight and five out of eight *bursa of Fabricius* samples from the LVB and HVB groups respectively showed a moderate to severe and diffuse lymphoid depletion of the follicles, mainly of the medulla (Figure 2). Intrafollicular multifocal cysts of different sizes were also found in the *bursa of Fabricius* in one bird belonging to the HVB group and one bird belonging to the LVB group. No difference in the number of apoptotic or mitotic cells was reported in the lymphoid organs among groups.

Lymphoid depletion and lymphopenia in peripheral blood might be due to a reduced production of bone marrow, reduced proliferation in secondary lymphoid tissues, or due to an increased loss of lymphocytes in the bone marrow, peripheral blood, or secondary lymphoid tissues via necrosis or apoptosis, due to chemical or biological agents. No increase in lymphoid tissue necrosis or apoptosis has been detected in the present study. The activity of the bone marrow was not evaluated, but the possibility of the recruitment of lymphocytes from the lymphoid tissues and its transfer to other sites could be considered due to the anti-inflammatory and immunomodulatory effects of VB (Korkina, 2007), even though other studies are needed to further investigate the effects of VB on lymphoid cells and to correlate the histological features to

Table 4. Effect of dietary verbascoside on breast meat traits.

Item	C	LVB (2.5 mg/kg)	HVB (5.0 mg/kg)	SEM	P
Chemical composition, % on fresh matter					
Moisture	74.0	74.3	74.2	0.1	0.323
Ash	1.3	1.3	1.2	0.0	0.564
Crude protein	23.0	22.5	22.3	0.3	0.697
Ether extract	1.2	1.2	1.0	0.1	0.470
Drip loss	1.7	1.8	1.8	0.0	0.921
TBARS, mg MDA/kg meat					
0'	0.55	0.43	0.44	0.06	0.706
60'	1.21	0.64*	1.51	0.12	0.007
120'	3.40	1.53*	3.91	0.38	0.017

C, control group; LVB, low verbascoside group; HVB, high verbascoside group; TBARS, thiobarbituric acid reactive substances; MDA, malondialdehyde. Values are expressed as mean and standard error of the mean. *Values within rows were significantly different ($P<0.05$) compared to the Control group.

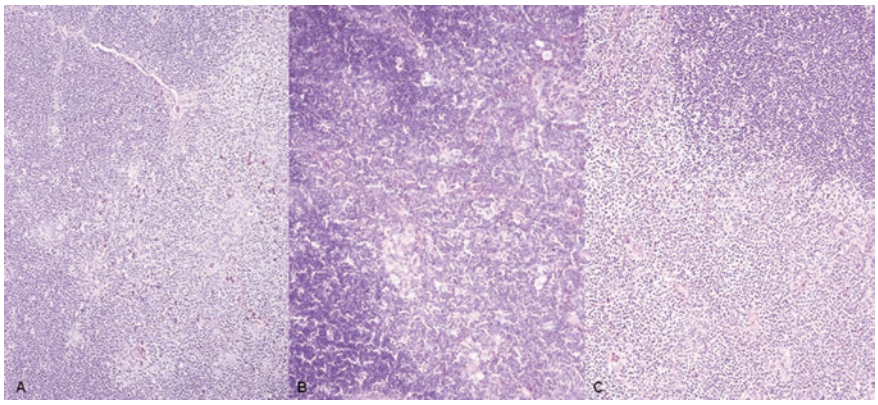

Figure 1. Thymus sample showing a lymphoid depletion of the medulla associated with an increase of stromal/epithelial cells. A) Control sample; B) moderate lymphoid depletion (low verbascoside group); C) severe lymphoid depletion (high verbascoside group). Haematoxilin and eosin, 200x.

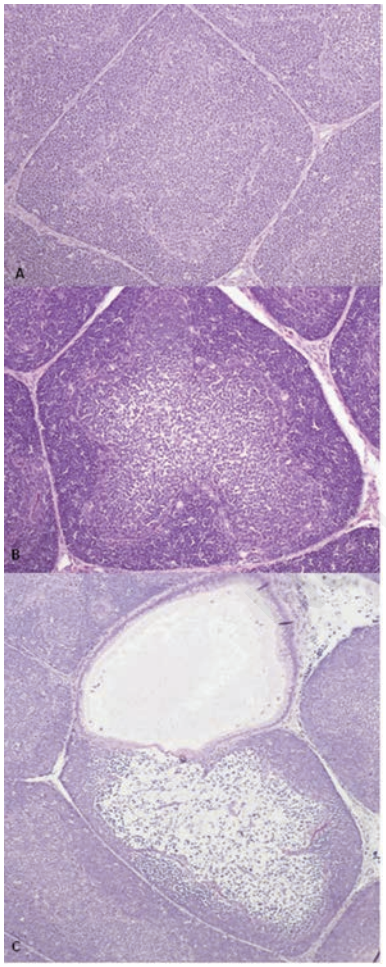

Figure 2. *Bursa of Fabricius* showing different grade of severity of lymphoid depletion of the follicles. A) Normal follicle; B) moderate depletion (low verbascoside group); C) severe depletion and intrafollicular cyst (high verbascoside group). Haematoxilin and eosin, 200x.

the blood parameters and particularly H/L. Dietary supplementation with VB has been shown to prevent intestinal damage in swine (Di Giancamillo *et al*., 2013). Experimental studies have been conducted with several animal inflammatory disease models (Hausmann *et al*., 2007; Esposito *et al*., 2010; Paola *et al*., 2011), but only Hausmann *et al*. (2007) examined the lymphoid tissues with an induced colitis mice model. Despite the anti-inflammatory effect of VB, no evidence of altered dimension or distribution of the lymphoid follicles was observed and no significant differences were detected in their number (Hausmann *et al*., 2007).

Conclusions

In conclusion, VB confirmed its potential antioxidant activity even *in vivo*. However, further studies are needed to identify the optimal dosage and exposure time of VB dietary supplementation in order to establish the VB antioxidant effect in broiler chickens.

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