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Growth performance and gut response of broiler chickens fed diets supplemented with grape (*Vitis vinifera* L.) seed extract

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ABSTRACT

To evaluate the effects of the dietary supplementation with a grape seeds extract (GSE) on growth performance, gut morphology and immune response, and meat quality, 800 chickens (8 pens/group) were fed a control diet or the same diet added with 0.1%, 0.2%, or 0.4% GSE from hatching to 42 d of age. Growth performance did not differ among dietary treatments: final live weight averaged at 3,179 g, which corresponded to a daily growth rate of 76.1 g/d; feed intake averaged 113 g/d, for a feed conversion ratio at 1.49. On average of samplings at 14 d and 28 d of age, the density of CD45+ cells in the jejunal mucosa was higher (2,497 vs. 1,931 cells/10,000 μm^2; $p < .001$) in chickens fed diet 0.2% GSE compared to chickens fed the other diets; jejunum villi height tended to be lower in chickens fed diet 0.2% GSE compared to those fed the other diets (965 μm vs. 1,054 μm; $p = .07$). Slaughter results and carcase traits, occurrence of myopathies at breast and meat quality after 24 h or 11 d of refrigerated storage were not affected by GSE inclusion or level. Under the conditions of the present study, no relevant effect of the dietary inclusion of GSE was observed on performance and health, but a pro-inflammatory immune response at the level of jejunum, based on which a positive response of chickens fed GSE can be expected under challenging conditions.

HIGHLIGHTS

- Dietary supplementation with grape seed extract from 0.1% to 0.2% and 0.4% were tested in broiler chickens.
- Growth performance, meat oxidation and myopathy occurrence were not affected.
- A pro-inflammatory immune response at the level of jejunum was recorded with 0.2% inclusion level.

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Tannins; immune response; myopathies; lipid oxidation; sex

Introduction

At a world level, among livestock, poultry production stands out as a sustainable and low-carbon footprint system (Jeswani et al. 2019) which provides high-quality protein food and contributes to achieve 'Zero Hunger', Goal 2 of Sustainable Development Goals of the United Nations. On the other hand, in the context of the 'antibiotic-free' farming urged by European consumers and policies within the Green Deal and the Farm2Fork strategies (European Parliament 2019), the main challenge for poultry producers is to ensure gut health for improving broiler welfare and guaranteeing productivity without the use of antimicrobial substances. Moreover, breast muscle abnormalities (white striping, WS; wooden breast, WB; spaghetti meat, SM) have recently emerged as meat quality problems (Soglia et al. 2021), which have been associated to a reduced vascularity and ischaemia, consequent hypoxia and lack of blood flow (Mutryn et al. 2015), and thus metabolic oxidative stress, inflammation, regeneration, glucose metabolism, lipidosis, fibrosis, and proteoglycan synthesis (Zambonelli et al. 2016).

Overall, this scenario opens new opportunities to research in the use of phytochemicals in poultry feeding (Quiroz-Castañeda and Dantán-González 2015), such as by-products from *Vitis vinifera*, which fruits are among the most abundant ones all over the world (about 78 million tons) (OIV 2019). These by-products are rich in nutrients (proteins, fibres, sugars, fats, minerals, vitamins) (Alonso et al. 2002; Pop et al. 2014)

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and, importantly, in bioactive substances such as polyphenols (flavonoids and tannins) (Saito et al. 1998; Yilmaz and Toledo 2004). Several grape by-products can be used in poultry feeding, from pomace as such (i.e. the residual obtained after pressing grapes, which contains grape seed, skins and/or stems in different proportions) (Pascariu et al. 2017; FEDNA 2019). Alternatively, extracts from grape by-products can be included as feed additives, such as grape seed extracts (GSE) (Pascariu et al. 2017), grape pomace extracts (Sayago-Ayerdi et al. 2009) or grape polyphenolic extracts (Pascariu et al. 2017).

These extracts are especially used for their content of tannins, water-soluble polyphenolic compounds (Spencer et al. 1988) which have shown several properties in living organisms, i.e. antioxidant, antimicrobial, and anti-inflammatory (Liu et al. 2012; Redondo et al. 2014). Tannin concentration and type greatly vary depending on the plant species and the part considered (seed, leaves, roots, flowers, etc.), the harvest season and geographical origin, besides the treatment used (cold, distillation, soaking, etc.) for extraction or maceration (Kim et al. 2007; Windisch et al. 2008). As for grapes, higher to lower contents of tannins are respectively found in skins, seeds, and stems (Vivas et al. 2004). Condensed tannins are found in the stem, skin, flesh and seeds of the berries (Souquet et al. 2000); hydrolysable tannins can be found in the stem and skin (Narduzzi et al. 2015).

In poultry, tannins from grape by-products have been supplemented for promoting growth performance through a better gut health, with not consistent results among studies (Brenes et al. 2010; Viveros et al. 2011; Chamorro et al. 2013). In fact, the intestinal epithelial cells play a key role in nutrient absorption, besides providing a physical barrier where immune cells work for the organism defence (Göbel et al. 2001). Based on their antioxidant properties, grape tannins have been also used for increasing the oxidative stability of meat (Goñi et al. 2007; Brenes et al. 2008; Sayago-Ayerdi et al. 2009) and, more recently, to reduce myopathies occurrence by controlling the oxidative damage in the muscle (Erinle et al. 2022). Thus, the present trial aimed to evaluate the effect of the dietary supplementation with different levels of a grape seed extract (GSE) compared to a control diet on growth performance, carcase and meat quality during storage in broiler chickens, besides the effect on myopathies occurrence. Investigations included the effects at the gut level as for morphology and immune response to elucidate mechanisms of actions of grape seed tannins.

Materials and methods

Experimental facilities

The study run at the poultry barn of the Experimental Farm of the University of Padova (Legnaro, Padova, Italy). The poultry house was equipped with a cooling system, forced ventilation, heating system, windows with complete darkening, programmed lightening with dawn-sunset effect.

Thirty-two pens (2.5 m × 1.2 m; 3 m^2) with 1.2 m-high wire-net walls were available in the barn, each equipped with five nipple drinkers and a circular feeder for manual distribution of feed. Each pen had a concrete floor covered with 5-cm wood shaving and chopped wheat straw litter. A total of 24 h of light was provided during the first 2 d after the arrival of the chicks. Then, the hours of light were progressively reduced until an 18 L:6D photoperiod was achieved, which was then maintained from 13 d of age onward.

Animals, experimental groups and in vivo recordings

A total of 800 broiler chicks (1-d old; 400 males and 400 females; Ross 308, Aviagen, USA) were delivered by a commercial truck to the experimental facilities. All chicks were vaccinated against Marek's disease, infectious bronchitis, and avian pseudopestis disease at the hatchery. They were randomly allocated in 32 pens (25 birds per pen) according to a bi-factorial arrangement, that is, two sexes × four dietary treatments, with 4 pens per experimental group. The following diets were used: the basal control diet (diet GSE0); the basal control diet supplemented with 0.1% (diet GSE01), 0.2% (diet GSE02), and 0.4% (diet GSE04) of GSE.

Chicks were individually weighed on the day of their arrival, identified by a plastic band at the leg, and then weighed once a week and at the end of the trial, i.e. 42 d of age. The chickens were fed *ad libitum*. The pen feed consumption was measured daily through a computerised weighing system connected to all feeders. Birds were slaughtered at 42 d of age. By the end of the trial, 8 chickens were found dead and 23 chickens were excluded from the trial because of lameness.

Grape seed extract, diets and feeding plans

The GSE was produced by Tampieri Financial Group (Faenza, RA, Italy) and the batch used in the trial had the following composition: water, 5.2%; tannin, 23.0%;

nontannin, 66.7%; insolubles, 5.10%. Tannin percentage was obtained by gravimetric analysis of vegetable tanning agents by using the filter Freiberg-Hide powder method (Küntzel 1954).

Three commercial diets were formulated as the control diet (GSE0) to be fed during three periods, i.e. from arrival to 14 d (D1), from 15 to 31 d (D2); and from 32 d until slaughtering (D3) (Table 1). Control diets (GSE0) were produced by an industrial feed mill (Nuova Padana Mangimi, Piove di Sacco, PD) in a mash form.

The inclusion of GSE in the diets was performed at the experimental farm by thoroughly mixing the diet GSE0 with the dried extracts using an electric concrete mixer (Suncoo 4/5HP Concrete mixer, 140 L, 600 W; SUNCOO, China). A total of 3 kg of mash diet were progressively added with the extract in two steps (starting with 1 kg) and mixed by hand in a box prior mixing them with other 47 kg of mash diet in the electric concrete mixer.

All diets were analysed in the laboratory of DAFNAE to determine their dry matter content, ash, crude protein, and starch (amyloglucosinade-α-amilase method) using AOAC (2000) methods. The ether extract was analysed after acid hydrolysis (EC 1998).

Table 1. Ingredient composition and chemical analysis of the diets.

Diet	D1	D2	D3
Period of administration	1–13 d	14–31 d	32–42 d
Ingredients			
Maize (%)	56.60	59.20	63.15
Soybean meal 48% Crude protein (%)	34.50	30.05	24.60
Full fat soybean (%)	3.00	5.00	7.00
Animal fat (%)	2.50	2.50	2.50
Dicalcium phosphate (%)	1.00	0.50	0.25
Calcium carbonate (%)	0.92	1.33	1.30
Liquid methionine, 40% L-methionine (%)	0.31	0.28	0.23
Sodium chloride (%)	0.26	0.27	0.27
Vitamin mineral supplement[A] (%)	0.25	0.25	0.25
Liquid lysine, 50% L-lysine (%)	0.25	0.24	0.16
Phytase (Ronomix hiphos) (%)	0.20	0.20	0.20
L-threonine (%)	0.11	0.08	0.04
Biotin[a] (%)	0.05	0.05	0.05
Coccidiostat[B] (%)	0.05	0.05	–
Determined analysis (as-fed)			
Moisture (%)	11.5	11.8	11.8
Ether extract (%)	5.75	5.80	5.61
Crude protein (%)	21.4	20.5	19.3
Total ash (%)	5.63	5.17	4.86
Starch (%)	31.1	33.9	36.2
Calculated values[C]			
Calcium (%)	0.81	0.80	0.71
Phosphorous (%)	0.58	0.47	0.42
Digestible phosphorous (%)	0.34	0.24	0.18
Digestible lysine (%)	1.32	1.23	1.05
Digestible methionine + cysteine (%)	0.91	0.85	0.76
Digestible threonine (%)	0.83	0.76	0.66
Apparent metabolizable energy (kcal/kg)	2,982	3,045	3,087

[A]Premix provided per kg of feed: vit. A, 10000 IU; vit. D_3, 3500 IU; vit. E acetate, 90 mg; vit. K_3, 6 mg; Biotin, 0.38 mg; Thiamine, 3.75 mg; Riboflavin, 8 mg; vit. B_6, 5.75 mg; vit. B_{12}, 0.1 mg; Niacin, 70 mg; Pantothenic acid, 17.5 mg; Folic acid, 2.25 mg; Fe, 45 mg; Cu, 10 mg; Mn, 70 mg; Zn, 65 mg; Se, 0.25 mg.
[B]Sodium Monensin, 100 mg/kg feed.
[C]Values calculated according to FEDNA (2019).

Sampling of gut tissues for morphometric analysis

At 14 d and 28 d after hatching, 32 chickens per age (one chick per pen with body weight corresponding to the average body weight of the pen) were selected and euthanized with CO_2 asphyxiation, prior to gut tissue collection. One sample of approximately 2 cm was taken from the jejunum, at the midpoint between the end of the duodenal loop and the location of the Merckel's diverticulum (Wang et al. 2016), and washed in phosphate-buffered saline (PBS). Sections (approximately 1-cm thick) were fixed in paraformaldehyde in PBS (0.1 M, pH 7.4), dehydrated, embedded in paraffin at the laboratory, and later submitted to the histological analyses and immunohistochemistry as detailed below.

Histological analyses and immunohistochemistry

Two serial 4-μm sections per jejunum sample were obtained using a microtome and stained with hematoxylin/eosin for morphometric evaluation and by the Alcian blue (pH 2.5)-PAS method for quantitative analysis of goblet cells. Two further serial sections were used for CD3+ and CD45+ immunohistochemical analyses.

The villi length and crypt depth were collected by a slide scanner (D-Sight, A. Menarini Diagnostics, Firenze, Italy) and measured using image analysis software (DP-soft, Olympus Optical, Co., Hamburg, Germany), according to the procedure described by Hampson (1986). The goblet cells positive for Alcian blue (pH 2.5)-PAS staining were counted using NIH ImageJ software (Rueden et al. 2017) on 10 different villi per animal along 300 μm of the villus surface.

Immunohistochemical analyses to identify CD3+ intraepithelial T-cells and CD45+ intraepithelial leukocytes in broiler jejunal mucosa were performed following the procedure described by Röhe et al. (2017). Intraepithelial leukocytes were counted in the epithelium using a reference rectangle with the short side at 100 μm and expressed as the density of CD45+ and CD3+ cells (cells/10,000 μm^2).

Commercial slaughtering and carcase and meat quality recordings

At 42 d of age, 705 remaining chickens (23 per pen without losses) were slaughtered in a commercial slaughterhouse. The chickens were weighted

individually on farm before crating after 4-h fasting. Loading took approximately 1 h, transport from the experimental facilities to the commercial slaughterhouse took approximately 15 min, and lairage before slaughtering took approximately 3 h. Ready-to-cook carcases were recovered after 2 h of refrigeration at 2 °C and individually weighed to measure the slaughter dressing percentage (World's Poultry Science Association, Working Group 5 1984).

A total of 128 carcases (four per pen), previously selected on the basis of the final live weight as corresponding to the mean body weight within a pen, were subjected to gross examination to evaluate the occurrence (presence or absence) in the *pectoralis major* muscles of white striping (WS) (Kuttappan et al. 2012), wooden breast (WB) (firm upon palpation, prominent ridge like bulge on caudal area of fillet, clear viscous fluid cover and/or petechia multifocal lesions on the fillet surface) (Sihvo et al. 2014), and spaghetti meat (SM) (exhibiting an overall impaired integrity and tendency towards separation of the muscle fibre bundles especially within the cranial part of the fillet) (Baldi et al. 2018).

Then, carcases were dissected for the main cuts (breast, wings, thighs, and drumsticks) (Petracci and Baéza 2011) and two sets of 64 breast (two per pen) were selected to be analysed at 1 and 11 days after slaughtering and stored in plastic bags at 2 °C. On the sampling day, *pectoralis major* muscles were separated from the breasts for meat quality analyses. The pH values of the *pectoralis major* muscles were measured in triplicates on their ventral side, with a pH metre (Basic 20, Crison Instruments Sa, Carpi, Italy) equipped with a specific electrode (cat. 5232, Crison Instruments Sa, Carpi, Italy). The L*a*b* colour indexes were measured in triplicate on the ventral side of the same muscles using a Minolta CM-508 C spectrophotometer (Minolta Corp., Ramsey, NJ, USA) (Petracci and Baéza 2011). After measuring the pH and colour indexes, one meat portion (8 cm × 4 cm × 3 cm) was separated from the cranial side of the *pectoralis major* muscle, parallel to the direction of the muscle fibres, and stored under vacuum in plastic bags at −18 °C until meat analyses. Thawing and cooking losses were measured in this cut (Petracci and Baéza 2011). After thawing, the meat portion was placed in a plastic bag and cooked in a water bath until an internal temperature of 80 °C was achieved. After 40 min of cooling, another meat portion (4 cm × 2 cm × 1 cm) was separated to assess the maximum shear force using an LS5 dynamometer (Lloyd Instruments Ltd, Bognor Regis, UK) using the Allo-Kramer (10 blades) probe (load cell: 500 kg; distance between the blades: 5 mm; thickness: 2 mm; cutting speed: 250 mm/min) (Mudalal et al. 2015).

The *pectoralis major* muscles from the left side were dissected and used to assess the meat oxidation level as for the thiobarbituric acid reactive substances (TBARs) (Botsoglou et al. 1994) using spectrophotometric measurements (Jasco Mod. 7800 UV/VIS) at 532 nm. The results were expressed as mg of malondialdehyde (MDA)/kg.

Statistical analysis

Individual data of live weights, daily growth, slaughter yield, carcase dissection, and meat quality traits were submitted to analysis of variance (ANOVA) with diet, sex, sampling time (only for meat quality) and their interaction as the main factors of variability and pen as a random effect, using the PROC MIXED procedure of SAS (SAS Institute 2013). Individual data related to gut morphology, goblet cells, and CD3+ and CD45+ cell densities were submitted to ANOVA using the PROC GLM (SAS Institute 2013) with diet, age, sex, and their interactions as the main effects. Pen data of feed intake and feed conversion were subjected to ANOVA, with diet, sex, and their interactions as main factors of variability, using the PROC GLM procedure. The PROC CATMOD was used to test differences in losses of animals and rate of myopathies according to diet and sex.

Adjusted means were compared using Bonferroni t-test. Differences between the means with $p \leq .05$ were considered statistically significant.

Results

Growth performance, slaughter yield, carcase traits and meat quality

The mortality (1.0%) and losses due to exclusions (2.9%) were low and not related to the experimental treatments. Final live weight averaged at 3,179 g, which corresponded to a daily growth rate of 76.1 g/d. Feed intake averaged 113 g/d, for a feed conversion ratio at 1.49, without significant differences due to the dietary GSE supplementation or level (Table 2). Consistently, slaughter yield and carcase traits (Table 3), the occurrence of myopathies at breast (Table 4) and meat quality (Table 5) did not differ among dietary treatments. Only the b* index was higher in meat of chickens fed the diet GSE01 and the diet GSE04 compared to those fed the control diet (19.5 and 19.6 vs. 18.5; $p = .026$). A trend to a linear decrease of meat TBARs was observed when the dietary inclusion of

Table 2. Growth performance (LS means) and mortality of broiler chickens until slaughter.

	Diet (GSE)				Sex (S)		*p*-Value			
Items	GSE0	GSE01	GSE02	GSE04	Females	Males	GSE	S	GSE × S	RMSE
Chickens (n)	176	176	175	178	360	345				
Pens (n)	8	8	8	8	16	16				
Live weight[A] (g)										
Initial (1 d)	57.1	57.2	56.8	57.0	57.2	56.8	.788	.146	.285	4.00
Final (42 d)	3,174	3,181	3,193	3,168	2,837	3,521	.673	<.001	.176	196
Whole trial (1–42 d)										
Daily weight gain[A] (g/d)	76.0	76.2	76.5	75.9	67.8	84.5	.665	<.001	.170	4.76
Daily feed intake[B] (g/d)	112	111	113	113	103	122	.303	<.001	.498	2.05
Feed conversion[B]	1.49	1.48	1.50	1.51	1.53	1.45	.071	<.001	.546	0.02
Total losses[C] (%)	4.4	4.4	4.9	3.3	2.2	6.2	.987	.984	.959	–

RSME: root mean square error.
GSE0: control diet. GSE01, control diet supplemented with 0.1% grape seed extracts. GSE02, control diet supplemented with 0.2% grape seed extracts. GSE04, control diet supplemented with 0.4% grape seed extracts.
[A]Individual data.
[B]Pen data.
[C]Dead and excluded chickens.

GSE increased (linear component of variance, $p = 0.089$).

As expected, at the end of the trial, males were heavier than females ($p < .001$) which corresponded to a higher daily weight gain (+24.6%) and feed intake (+18.4%) and a lower feed conversion ratio (-5.22%) ($p < .001$) (Table 2). Males also had heavier carcases (+19%, $p < .001$) and higher proportion of legs (+1.1%; $p < .001$), while lower breast and *pectoralis major* muscle proportions were recorded (-1.8% and −0.6%, respectively; $p < .001$) (Table 3). As for myopathies, males displayed higher rate of WB (39.1% vs. 12.5%; $p < .001$) compared to females that, conversely, showed higher rates of SM (31.2% vs. 1.5%; $p = .001$), without differences in WS rates between sexes (Table 4). As for meat quality, males displayed a higher breast meat pH (5.95 vs. 5.91; $p = .044$) and lightness (L*) (49.4 vs 48.4; $p < .001$) besides higher cooking losses (21.5% vs. 19.7%; $p < .001$) than females (Table 5).

Finally, lightness (L*) and yellowness (b*) indexes of refrigerated meat were higher in samples stored for 1 day compared to those kept for 11 days ($p < .001$) (Table 5). Thawing losses (12.6% vs. 9.90%; $p < .001$) and shear force (2.93 vs. 2.50 kg/g; $p < .001$) were significantly higher in breasts that had been refrigerated for 1 d compared to those refrigerated for 11 d (Table 5), whereas meat lipid oxidation was lower in the former compared to the latter (0.031 vs. 0.083 mg MDA/kg; $p < .001$) (Table 5).

Gut morphology and immuno-histochemical analyses

On average of the two slaughtering ages (14 and 28 d), villi height tended to decrease when chickens were fed diet GSE02 compared to those fed diets GSE0, GSE01 and GSE04 (965 μm vs. 1,046 μm, 1,059 μm and 1,058 μm, respectively; $p = .067$). Moreover, the density of CD45+ cells was higher (2,497 vs. 1,931 cells/10,000 μm^2; $p = .018$) in chickens fed the diet GSE02 compared to chickens fed the other diets (Table 6).

As age increased from 14 to 28 d, villi height (968 vs. 1,096 μm; $p < .001$), density of goblet cells (18.4 vs. 20.1 cells/300 μm; $p = .042$) and density of CD3+ cells (1,805 vs. 2,180 cells/10,000 μm^2; $p = .020$) increased (Table 6).

Discussion

Previous studies tested the use of by-products or extracts from *Vitis vinifera* in diets for monogastrics. These products, containing different quantity and type of tannins, can be effective in improving animal performance and health by increasing feed intake and modulating gut response (Huang et al. 2018), reducing avian protozoa in broiler chickens (e.g., *Eimeria*) (Wang et al. 2008), and suppressing inflammatory responses (Gessner et al. 2017). However, tannins can also exert anti-nutritional effects at the gut level, which can reduce nutrients digestibility and, in turn, growth performance (Smulikowska et al. 2001; Redondo et al. 2014). Quality and quantity of tannins in the different by-products and extracts could account for differences in results among studies.

Regarding available data about GSE, few studies are available which report no effects on broiler chicken performance with dietary inclusion levels ranging from 0.25% (until 21 d of age, Chamorro et al. 2013) to 0.36% (Brenes et al. 2010) or 0.40% (present study). With higher GSE levels (0.50% in Chamorro et al 2013; 0.72% in Viveros et al. 2011), feed conversion has been found to increase while growth and apparent

Table 3. Carcase traits (LS means) in chickens slaughtered at 42 d.

	Diet (GSE)				Sex (S)		*p*-Value			RMSE
	GSE0	GSE01	GSE02	GSE04	Females	Males	GSE	S	GSE × S	
Chickens (n)	32	32	32	32	64	64				
Cold carcases (CC) (g)[A]	2,289	2,322	2,316	2,293	2,059	2,551	.577	<.001	.872	114
Dressing percentage (%)	73.1	73.6	73.3	73.3	73.5	73.2	.388	.200	.238	1.2
Breast yield (% CC)[B]	41.1	41.5	41.3	40.9	42.1	40.3	.400	<.001	.879	1.6
P. major (% CC)	12.7	12.9	12.9	12.7	13.1	12.5	.606	<.001	.602	0.7
Wings (% CC)	9.82	10.0	9.87	10.0	9.86	10.0	.198	.068	.871	0.49
Thighs + drumsticks (% CC)	29.1	28.4	28.6	28.8	28.2	29.3	.183	<.001	.809	1.2

RMSE: root mean square error.
GSE0, control diet. GSE01, control diet supplemented with 0.1% grape seed extracts. GSE02, control diet supplemented with 0.2% grape seed extracts. GSE04, control diet supplemented with 0.4% grape seed extracts.
[A]Carcasses without feet. [B]With bone and skin.

Table 4. Myopathy rates (means and number of carcases in brackets) at gross examination in chickens slaughtered at 42 d.

	Diet (GSE)				Sex (S)		*p*-Value	
	GSE0	GSE01	GSE02	GSE04	Females	Males	GSE	S
Carcases (n)	32	32	32	32	64	64		
White striping[A] (%)	75.0 (24)	71.9 (23)	84.4 (27)	62.5 (20)	68.7 (44)	78.1 (50)	.273	.224
Wooden breast[A] (%)	21.9 (7)	28.1 (9)	34.3 (11)	18.7 (6)	12.5 (8)	39.1 (25)	.458	<.001
Spaghetti meat[A] (%)	15.6 (5)	9.38 (3)	21.9 (7)	18.7 (6)	31.2 (20)	1.5 (1)	.517	.001

GSE0: control diet. GSE01, control diet supplemented with 0.1% grape seed extracts. GSE02, control diet supplemented with 0.2% grape seed extracts. GSE04, control diet supplemented with 0.4% grape seed extracts.
[A]Not exclusive myopathy, i.e. white striping, wooden breast and/or spaghetti meat can be associated in the same breast.

Table 5. Rheological traits and lipid oxidation status (TBARs) of the *pectoralis major* muscle in chickens slaughtered at 42 d of age.

	Diet (GSE)				Sex (S)		Storage time (T)		*p*-Value						
	GSE0	GSE01	GSE02	GSE04	Females	Males	1 d	11 d	GSE	S	T	GSE × S	GSE x T	S x T	RMSE
P. major (n)	32	32	32	32	64	64									
pH	5.95	5.92	5.92	5.94	5.91	5.95	5.93	5.94	.590	.044	.453	.565	.797	111	0.10
L*	48.7	49.1	48.9	48.8	48.4	49.4	47.1	50.7	.898	.001	<.001	.641	.340	.237	2.7
a*	1.89	2.22	2.20	2.31	2.16	2.15	2.08	2.22	.063	.973	.215	.722	.176	.618	0.65
b*	18.5[a]	19.5[b]	19.0[ab]	19.6[b]	19.2	19.1	18.3	20.0	.026	.654	<.001	.709	.838	.187	1.8
Thawing losses (%)	11.5	11.7	11.6	11.8	11.3	12.0	12.7	10.6	.970	.122	<.001	.113	.307	.642	4.4
Cooking losses (%)	20.2	20.6	20.7	20.9	19.7	21.5	21.0	20.2	.747	<.001	.096	.120	.211	.434	2.7
Shear force (kg/g)	2.61	2.69	2.80	2.80	2.65	2.80	2.93	2.50	.462	.127	<.001	.621	.231	.476	0.57
TBARs (mg MDA/kg)	0.064	0.058	0.054	0.051	0.060	0.054	0.031	0.083	.399	.287	<.001	.705	.261	.341	0.03

RMSE: Root mean square error; TBARs: thiobarbituric acid reactive substances.
GSE0: control diet. GSE01, control diet supplemented with 0.1% grape seed extracts. GSE02, control diet supplemented with 0.2% grape seed extracts. GSE04, control diet supplemented with 0.4% grape seed extracts.
[a,b]Values with different superscript letters within the same line and effect are significantly different ($p < .05$).
[A]Linear component of variance, $p = 0.089$; quadratic component of variance, $p = 0.835$.

Table 6. Jejunum mucosa morphometry, number of goblet cells and densities of CD45+ and CD3+ cells at 14 and 28 d of age.

	Diet (GSE)				Age (A)		Sex (S)		*p*-Value						
	GSE0	GSE01	GSE02	GSE04	14	28	F	M	GSE	A	S	GSE × A	GSE × S	A × S	RMSE
Broilers (n)	16	16	16	16	32	32	32	32							
Villi height (μm)	1,046	1,059	965	1,058	968	1,096	1,004	1,060	.067	<.001	.057	.746	.820	.272	112
Crypt depth (μm)	137	143	140	139	140	140	138	141	.847	.994	.529	.173	.943	.287	18.6
Villi/Crypt ratio	7.64	7.56	6.95	7.67	7.01	7.90	7.32	7.59	.130	<.001	.270	.055	.934	.864	0.95
Goblet cells (n/300 μm)	18.7	19.4	19.7	19.4	18.4	20.1	19.1	19.5	.832	.042	.687	.836	.987	.564	3.3
CD3+ cells (n/10,000 μm^2)	1,868	1,955	2,228	1,936	1,805	2,189	2,015	1,978	.424	.020	.818	.934	.800	.232	591
CD45+ cells (n/10,000 μm^2)	1,867[a]	2,065[ab]	2,497[b]	1,861[a]	2,076	2,069	2,102	2,042	.018	.965	.695	.721	.512	.755	579

RMSE: Root mean square error.
GSE0: control diet. GSE01, control diet supplemented with 0.1% grape seed extracts. GSE02, control diet supplemented with 0.2% grape seed extracts. GSE04, control diet supplemented with 0.4% grape seed extracts.
[a,b]Values with different superscript letters within the same line and effect are significantly different ($p<.05$).

ileal digestibility decreased. Viveros et al. (2011) also observed a decrease in villi height and crypt depth in broiler chickens fed diets supplemented with 0.72% GSE, besides changes in the gut microflora biodiversity. In fact, since the gut is responsible for nutrients absorption and digestion, observations about gut morphology can explain differences in growth performance (Baurhoo et al. 2007; Qaisrani et al. 2014). Finally, the depression in growth of broiler chickens was severe when dietary inclusion levels of GSE reached 2.6% and 5.2% (Lau and King 2003).

The intestinal epithelium includes enterocytes, goblet cells, immune cells like T cells (CD3+), intraepithelial leukocytes (CD45+) and molecules (cytokines and chemokines) (Trowbridge and Thomas 1994; Göbel et al. 2001). The density of the goblet cells in the epithelium can provide a measure of the gut protection against any challenges (Romero et al. 2011). In our trial, GSE supplementation did not affect the density of jejunal goblet cells, while we observed some changes in the immune gut response. To our knowledge, few studies are available about the effect of the dietary supplementation with grape by-products on the gut associated immune reactions. In rats, the supplementation of proanthocyanins extracted from grape seed has been found to reduce pro-inflammatory cytokines at the gut level (Li et al. 2001). In broiler chickens, tannins included in the raw materials (soybean vs. processed peas) of the diet, acting as anti-nutritional factors, could prepare the intestinal mucosa for a fast-immune response in jejunum increasing the density of CD45+ cells (Röhe et al. 2017). Moreover, the dietary supplementation with tannic acid (0.005% and 0.30%) was found to affect T cells by cytokines, stimulating the immune response at the lowest dose and supressing it at the highest dose in broiler chickens (Ramah et al. 2020). This latter result could explain the increase of CD45+ cells we observed in chickens fed the diet supplemented with 0.20% GSE compared to those fed the control diet and the diet with 0.40% GSE, which however was also associated to a decrease in villi height under our conditions. Finally, when age increased, the higher number of goblet and CD3+ cells we recorded was supposed to be associated, respectively, to a defence mechanism and to a triggered local gut immune response, consistently with previous results (Röhe et al. 2017; Kogut et al. 2018; Pascual et al. 2020a).

As for meat quality, oxidation processes lead to a deterioration in flavour, texture and nutritional value to which poultry meat is especially sensitive during storage (Morrissey et al. 1998; Galarz et al. 2016). Since polyphenols of grape products have shown an antioxidant potential four to five times higher than vitamin E and C (Shi et al. 2003), GSE polyphenols were expected to exert a protective role on meat quality during storage. In fact, the dietary supplementation with grape by-products (Goñi et al. 2007; Brenes et al. 2008; Sayago-Ayerdi et al. 2009) and GSE (see Brenes et al. 2016 for a review) has been reported to successfully protect meat of swine, turkey, besides broiler chickens. However, we observed only a weak effect of GSE supplementation on lipid meat oxidation and no beneficial effect on the occurrence of breast myopathies. As for lipid meat oxidation during storage, the inclusion of vitamin E in all diets (i.e. 90 mg vit. E acetate) could have hindered the potential antioxidant effects of GSE. As for myopathies, since these meat defects have been associated to an oxidative stress at the muscle level (Mutryn et al. 2015; Zambonelli et al 2016; Abasht et al 2016), previous authors hypothesised that the dietary supplementation with antioxidants could be a useful strategy for their control. However, the present study showed that such strategy is not effective, as previously found also with the dietary inclusion of selenium and vitamin E (Kuttappan et al. 2021), arginine and vitamin C (Bodle et al. 2018), or grape pomace (Erinle et al. 2022). In fact, on one side, antioxidant levels in the diets might have been not sufficient to mitigate muscle oxidation; on the other side, based also on literature (Zambonelli et al. 2016; Kuttappan et al. 2017; Soglia et al. 2021), we can argue that reduced vascularity and ischaemia are the triggering causes for hypoxia and no mitigation is possible on the consequence. Finally, physiopathological mechanisms other than hypoxia, such as changes in energy metabolism, inflammation and degeneration in the muscle, are likely to play a major role in myopathies occurrence (Soglia et al. 2021).

As an additional result, the present study confirmed a difference between sexes as for myopathies occurrence, i.e. a higher rate of WB in males (Trocino et al. 2015; Pascual et al. 2020b; Erinle et al. 2022) and a higher rate of SM in females (Pascual et al. 2020b).

Conclusions

Under the conditions of the present study, i.e. broiler chickens in good health, no relevant effect of the dietary inclusion of GSE was observed on growth performance. On the other hand, the study proved an effect on gut morphology and, importantly, on gut immune response based on which a positive response of chickens fed GSE is likely expected under challenging conditions. Also, a dose-response effect was highlighted

based on which a 0.2% GSE inclusion might be suggested. Finally, the study confirmed that a mitigation strategy based on the use of antioxidant products alone is not sufficient for the reduction of myopathy occurrence that has to be addressed by other nutritional or management approaches.

Ethical approval

The study was approved by the Ethical Committee for Animal Experimentation (Organismo Preposto al Benessere Animale) of the University of Padua (project 92/2019–prot. No. 469509, approved on 15/11/2019). All animals were handled according to the principles of EU Directive 2010/63/EU regarding the protection of animals used for experimental and other specific purposes. The research staff involved in animal handling were animal specialists (PhD or MS in Animal Science) and veterinary practitioners.

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Data availability statement

The datasets analysed in the current study are available from the corresponding author upon reasonable request.

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