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Addition of fermented and unfermented grape skin in broilers' diets: effect on digestion, growth performance, intestinal microbiota and oxidative stability of meat

M. Nardoia[1,2], C. Romero[3], A. Brenes[1], I. Arija[4], A. Viveros[4], C. Ruiz-Capillas[1] and S. Chamorro[1†]

[1]*Instituto de Ciencia y Tecnología de Alimentos y Nutrición (ICTAN-CSIC), José Antonio Novais, 10, 28040 Madrid, Spain;* [2]*Department of Agricultural, Environmental and Food Sciences, University of Molise, 86100 Campobasso, Italy;* [3]*Facultad de Ciencias y Artes, Universidad Católica de Ávila, Canteros s/n, 05005 Ávila, Spain;* [4]*Departamento de Producción Animal, Facultad de Veterinaria, Universidad Complutense de Madrid, 28040 Madrid, Spain*

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*Grape skin is a source of polyphenols with antioxidant and antimicrobial properties. Little information is available regarding its application in animal feeding. The present study investigated the effect of inclusion of fermented (**FS**) and unfermented (**UFS**) grape skin at two different doses (30 g/kg, FS30 and UFS30, and 60 g/kg, FS60 and UFS60) and 200 mg/kg vitamin E (α-tocopheryl acetate) in a corn–soybean diet on growth performance, ileal protein digestibility, ileal and excreta total extractable polyphenols content and digestibility, intestinal microbiota and thigh meat oxidation in broiler chickens. Growth performance was depressed in chickens fed UFS and FS diets. A reduction in ileal protein digestibility was also observed in birds fed UFS, being this effect more pronounced in those fed 60 g/kg. The dietary inclusion of grape skin increased both ileal and excreta polyphenols contents, being higher in birds fed UFS than in those fed FS. Excreta moisture content increased in birds fed UFS and FS diets. No effect of dietary inclusion of grape skin was observed on ileal counts of lactic-acid bacteria and* Clostridium, *but UFS inclusion in the diet reduced ileal count of* Escherichia coli *as compared with FS dietary inclusion. After 7 days of refrigerated storage, values of thiobarbituric acid reactive substances (**TBARS**) were lower in chicken meat when grape skin was added in the diet at 60 g/kg instead of 30 g/kg, and meat from birds fed 60 g/kg of grape skin reached TBARS values similar to those of birds supplemented with vitamin E. In conclusion, high doses of grape skin polyphenols depressed growth performance and protein digestibility, and increased excreta moisture content. Unfermented grape skin contained more polyphenols than FS, and its inclusion in the diet led to higher ileal and excreta polyphenols contents and to a lower ileal count of* E. coli. *Furthermore, the antioxidant potential of the polyphenols present in grape skin was observed after 7 days of meat storage, with the dose of 60 g/kg of grape skin being as effective as vitamin E supplementation in maintaining oxidative stability of meat.*

Keywords: polyphenols, grape byproducts, antioxidant, intestinal bacteria, chickens

Implications

Grape byproducts (grape pomace, seeds and skin) contain a wide range of phenolic compounds with antioxidant and antimicrobial properties and are now being revaluated for its potential use in animal nutrition. In the present study with broiler chickens fed grape skin, the diets with the higher polyphenols content (unfermented skin) showed greater antimicrobial and antioxidant potential but were also linked to a reduction in ileal protein digestibility and to a worsening of growth performance. Hence, the dietary dose of grape skin to be used should be adjusted in accordance with the objectives targeted in commercial conditions.

Introduction

Grape, which is one of the world's largest fruit crop, is a rich source of polyphenols. Either for wine or juice production, grapes processing generates huge amounts of a byproduct/waste termed pomace, which consists of pressed skin and seeds. The valorization of grape byproducts could

† E-mail: susana.chamorro@ictan.csic.es

offer ways to reduce its environmental impact. Actually, grape byproducts can become potential functional ingredients rich in bioactive constituents, namely dietary fibre and phenolic compounds with antioxidant properties (Pérez-Jiménez and Saura-Calixto, 2008).

During the winemaking process, the extracted juice along with the remaining pulp, skin and seeds are fermented together. After fermentation, the juice is removed with only the pomace remaining. On the contrary, pomace from unfermented grapes is obtained immediately after the grapes are pressed.

Grape byproducts are significant sources of a wide range of polyphenols, mainly anthocyanins and flavanols, ranging from the monomeric to the oligomeric and polymeric forms (Ky *et al.*, 2014). Grape seed presents highly polymerized procyanidins, while grape skin contains both procyanidins and delphinidins (Vivas *et al.*, 2004).

The polyphenol composition of grape varies depending on the grape variety, the growing conditions, climate and maturity (Rodríguez-Montealegre *et al.*, 2006). Furthermore, winemaking process and its fermentation conditions also affect the polyphenol composition of grape byproducts (Vergara-Salinas *et al.*, 2013).

Polyphenols were historically considered anti-nutritional factors since dietary incorporation of tannin-rich ingredients, such as sorghum and faba bean, negatively affected nutrient efficiency and animal performance (Ortiz *et al.*, 1993; Nyachoti *et al.*, 1997). However, current scientific evidence suggests that the dietary addition of moderate amounts of grape byproducts in monogastric diets may turn out to be a strategy to improve health, antioxidant status and animal product quality (Brenes *et al.*, 2016).

In chickens, the incorporation of fermented grape pomace (**GP**) to diets promoted the proliferation of beneficial intestinal bacteria (Viveros *et al.*, 2011) and maintained the antioxidant status (Chamorro *et al.*, 2017). In addition, meat from birds fed this grape byproduct showed higher α-tocopherol and polyunsaturated fatty acid contents and a lower susceptibility to lipid oxidation (Goñi *et al.*, 2007). The potential functional effect of grape seed has also been explored in chickens using grape seed extracts (**GSE**). In this sense, the effect of dietary incorporation of commercial GSE on intestinal barrier and antioxidant status has also been reported (Viveros *et al.*, 2011; Abu Hafsa and Ibrahim, 2018; Chamorro *et al.*, 2019). However, the presence of a high amount of grape polyphenols in the diet might also have some adverse effects. Accordingly, high levels of GP and GSE were associated with lower efficiency of some nutrients, particularly fat, protein and amino acids (Goñi *et al.*, 2007; Chamorro *et al.*, 2013).

Regarding grape skin, there is no information about the individual effect of its dietary inclusion on productive performance, health and meat quality in chickens. Thus, in order to establish the optimal and practical inclusion level of this grape byproduct in broiler chicken diets, the present study was designed to evaluate the effect of dietary addition of fermented and unfermented grape skin (**FS** and **UFS**) on performance, protein and polyphenols digestibility, intestinal microbiota and meat oxidative stability in broiler chickens.

Table 1 *Proximate composition, expressed as g per 100 g of DM, of the FS and UFS included in the chickens' diets*

	FS	UFS
DM	89.5 ± 0.4	85.1 ± 0.3
Crude fibre	14.4 ± 1.9	12.2 ± 0.4
Protein	16.3 ± 0.0	10.0 ± 0.1
Gross energy (cal/g)	4 500 ± 19.8	4 933 ± 17.5

FS = fermented grape skin; UFS = unfermented grape skin.

Material and methods

Tested product

Fermented and unfermented grape skins were obtained from Explotaciones Hermanos Delgado S.L. Socuéllamos (Ciudad Real, Spain). Unfermented grape skin originated from grapes (*Vitis vinifera* L.) pressed for juice production, whereas FS was obtained from grapes used for winemaking and it was taken after the fermentation process had finished. However, in both cases, grape skin resulted from the same red grape variety (var. Cencibel). Grape skins were added to the experimental diets after having been dried in a convection oven at 60°C. Proximate composition of FS and UFS is shown in Tables 1 and 2. The vitamin E (α-tocopheryl acetate) used in the diets was provided by DSM Nutritional Products Iberia S.A. (Alcalá de Henares, Madrid, Spain).

Birds and diets

A total of 150 1-day-old male broiler Cobb chicks were obtained from a commercial hatchery. The birds were housed in electrically heated starter battery brooders in an environmentally controlled room with 23 h of constant overhead fluorescent lighting during 3 weeks. The chicks were allocated to 30 pens, each pen containing five chicks, to receive six dietary treatments during 21 days with five replicates per treatment. Diets in mash form and water were provided *ad libitum*. The diets were stored in a dark and cool dry location during the experimental period. Ingredients and nutrient composition of diets are shown in Table 3. Celite (Celite Corp., Lompoc, CA, USA), a source of acid-insoluble ash (**AIA**), was added at 10 g/kg to all diets as an indigestible marker. All diets were formulated to meet or exceed the minimum nutrient requirements for broiler chickens of the National Research Council. Experimental procedures were approved by the University Complutense of Madrid Animal Care and Ethics Committee in compliance with the guidelines for the Care and Use of Animals for Scientific Purposes of the Ministry of Agriculture. Experimental diets were as follows: (1) Control corn–soybean diet (**C**); (2) C + Vitamin E (200 mg/kg); (3) C + 30 g/kg FS; (4) C + 60 g/kg FS; (5)'C + 30 g/kg UFS; (6) C + 60 g/kg UFS. At the end of the

Table 2 *Main phenolic compounds (expressed as mg per 100 g of DM) identified in the FS and UFS included in the chickens' diets*

		FS	UFS
Flavanol monomers	Catechin	6.09 ± 0.28	20.7 ± 2.53
	Epicatechin	9.68 ± 0.73	42.9 ± 7.0
	Epicatechin 3-*O*-gallate	0.71 ± 0.01	3.66 ± 0.10
Flavanol dimers	Procyanidin B1	3.81 ± 0.14	13.7 ± 1.11
	Procyanidin B2	4.26 ± 0.16	13.1 ± 1.14
	Procyanidin B3	1.51 ± 0.29	4.16 ± 1.82
	Procyanidin gallate 1[1]	3.04 ± 0.15	7.81 ± 0.84
	Procyanidin gallate 2[1]	4.67 ± 0.32	16.1 ± 3.13
Flavanol trimers	Procyanidin C1	2.92 ± 0.23	9.93 ± 1.52
	Trimer 2[2]	1.16 ± 0.09	2.88 ± 0.52
	Trimer 3[2]	2.29 ± 0.23	7.35 ± 1.61
	Trimer 4[2]	1.25 ± 0.09	3.93 ± 0.58
Flavanol tetramers	Cinnamtannin A2	5.20 ± 0.14	11.3 ± 0.69
	Procyanidin tetramer[3]	3.55 ± 0.20	10.3 ± 1.13
Total extractable polyphenols, g GAE/100 g DM		2.3 ± 0.1	7.11 ± 0.2

FS = fermented grape skin; UFS = unfermented grape skin; GAE = gallic acid equivalents.
[1]Identified by prediction of chemical formula from accurate ion mass measurement. Quantified by using the calibration curve of procyanidin B2.
[2]Identified by prediction of chemical formula from accurate ion mass measurement. Quantified by using the calibration curve of procyanidin C1.
[3]Identified by prediction of chemical formula from accurate ion mass measurement. Quantified by using the calibration curve of cinnamtannin A2.

Table 3 *Ingredients and nutrient composition (g/kg as fed) of the experimental diets fed to the broiler chickens*

Item	C	C + VitE	C + FS30	C + FS60	C + UFS30	C + UFS60
Ingredients						
Corn (8.1% CP)	422.90	422.90	411.13	399.35	410.38	397.85
Soybean (48% CP)	375.00	375.00	366.78	358.55	368.75	362.50
Sunflower oil	100.00	100.00	100.00	100.00	100.00	100.00
Salt	3.00	3.00	3.00	3.00	3.00	3.00
Monocalcium phosphate	17.90	17.90	17.90	17.90	17.90	17.90
Calcium carbonate	14.20	14.20	14.20	14.20	14.20	14.20
Vitamin–mineral premix[1]	5.00	5.00	5.00	5.00	5.00	5.00
DL-Methionine	2.00	2.00	2.00	2.00	2.00	2.00
Straw	50.00	50.00	40.000	30.00	38.75	27.50
Grape skin	0.00	0.00	30.00	60.00	30.00	60.00
Vitamin E (mg/kg)	0.00	200.00	0.00	0.00	0.00	0.00
Celite[2]	10.00	10.00	10.00	10.00	10.00	10.00
Analysed composition						
Total extractable polyphenols	2.08	1.90	1.98	2.27	2.42	3.13
CP	207.00	207.00	206.00	205.00	205.00	204.00
Ether extract	122.00	122.00	125.00	127.00	124.00	126.00
Crude fibre	45.00	45.00	44.00	44.00	44.00	43.00
Calculated composition						
AME (kcal/kg)	3 131	3 131	3 089	3 048	3 093	3 056
Lysine	12.00	12.00	12.00	12.00	12.00	12.00
Methionine + cystine	9.00	9.00	9.00	9.00	9.00	9.00
Calcium	10.00	10.00	11.00	11.00	11.00	11.00
Available *P*	5.00	5.00	4.00	4.00	5.00	4.00

C = control; FS = fermented grape skin; UFS = unfermented grape skin; AME = apparent metabolizable energy.
[1]Vitamin–mineral mix supplied the following per kilogram of diet: vitamin A, 8 250 IU; cholecalciferol, 1 000 IU; vitamin E, 11 IU; vitamin K, 1.1 mg; vitamin B12, 12.5 μg; riboflavin, 5.5 mg; Ca panthothenate, 11 mg; niacin, 53.3 mg; choline chloride, 1020 mg; folic acid, 0.75 mg; biotin, 0.25 mg; ethoxiquin, 125 mg; DL-methionine, 500 mg; amprol, 1 g; Mn, 55 mg; Zn, 50 mg; Fe, 80 mg; Cu, 5 mg; Se, 0.1 mg; I, 0.18 mg; and NaCl, 2500 mg.
[2]Celite Corp., Lompoc, CA, USA.

experimental period, birds were weighed and feed consumption was recorded for feed efficiency computation.

Collection of samples and measurements

At 19 days of age, clean stainless steel collection trays were placed under each pen, and excreta from the birds were collected for 48 h. A subsample of excreta was collected in polyethylene bags and freeze-dried (Telstar, Tarrasa, Spain) for subsequent determination of celite and polyphenols content.

At 21 days of age, 15 birds per treatment (3 birds of each replicate of the treatments) were euthanized with carbon dioxide (100%). Immediately after, the ileum was quickly dissected out and the content expressed by gentle manipulation into a plastic container. Digesta were pooled from the three birds of each replicate within the same treatment. Samples of fresh digesta (five samples per treatment) were used for the microbiological analysis. Ileal contents were then freeze-dried and ground (1 mm screen) and used to determine celite, protein and total extractable polyphenols (**TEP**) content. Carcasses from 42 birds (7 birds per treatment) were also immediately trimmed for thigh meat, and tissues were individually sampled and used to assess lipid oxidation (1 bird per replicate). For lipid oxidation study, tissues samples were wrapped in transparent oxygen-permeable polyvinyl chloride film (13 500 cm^3/m^2 per day), frozen and stored at −20°C for 1 week. Thereafter, meat samples collected for lipid oxidation assessment were thawed, and subsequently the progress of lipid oxidation in the samples was determined after 1 and 7 days of storage in a no illuminated refrigerated cabinet at 4°C.

Chemical analysis

Dry matter (930.15), CP (976.05) and crude fibre (978.10) were analysed in tested products and diets according to the methods of the Association of Official Analytical Chemists (1995). Gross energy was measured in tested products using an adiabatic bomb calorimeter (model 356; Parr Instrument Company, Moline, IL, USA). Crude fat was determined in diets by extraction in petroleum ether after acidification with 4 N HCl solutions (Wiseman *et al.*, 1992). The AIA contents of diet, ileal content and excreta were measured after ashing the samples and treating the ash with boiling 4 M HCl (Siriwan *et al.*, 1993). Chemical analyses were performed by triplicate.

Total extractable polyphenols content

Total extractable polyphenols content were determined by Folin–Ciocalteau procedure (Montreau, 1972) after the extraction with methanol/acetone/water (Panreac, Barcelona, Spain) in FS and UFS (four extracts) and in diets, ileal digesta and excreta (three extracts). Briefly, a mixture of 0.5 ml of extract, 0.5 ml of Folin–Ciocalteau reagent (Sigma-Aldrich, St. Louis, MO, USA) and 10 ml of Na_2CO_3 1 M were introduced in a 25 ml volumetric flask. After reacting for 1 h, absorbance was measured at 750 nm using an ultraviolet–visible spectrophotometer (Hitachi U-2000; Hitachi, Ltd, Tokyo, Japan). Absorbance values were compared against a standard curve made with gallic acid (Sigma-Aldrich) ranging from 0.05 to 0.5 mg of GA/ml (mg GA/ml = 0.519 × absorbance − 0.0231; $R^2 = 0.994$). Results were expressed as grams of gallic acid equivalents (**GAE**) per 100 g of DM.

The analysis of phenolic compounds present in FS and UFS was performed by HPLC-QTOF-MS in two different FS and UFS extracts. For separation, the HPLC apparatus (Agilent 1200; Agilent Technologies, Santa Clara, CA, USA) was coupled with DAD (Agilent G1315B) and an Agilent 6530 Accurate-Mass Quadrupole Time of Flight (**QTOF**) LC/MS with ESI-Jet Stream Technology (Agilent Technologies, Waldbroon, Germany). Separation was performed on a Zorbax Eclipse Plus C18 100 mm × 3.5 μm × 4.6 mm column (Agilent) with a pre-filter (Sigma-Aldrich). A gradient composed of solvents A (water:formic acid, 99.9:0.1, v/v) and B (acetonitrile:formic acid, 99.9:0.1, v/v) was applied at a flow rate of 0.5 mL/min as follows: 10% B at 0 min, 30% B at 15 min and 30 min, 80% B at 32 min, and 10% B at 35 min and 45 min. The ESI parameters were as follows: drying gas flow, 8 l/min; nebulizer pressure, 45 psi; gas drying temperature, 350°C; sheath gas temperature, 325°C; sheath gas flow, 11 l/min; capillary voltage, 3500 kV; nozzle voltage, 500 V; and fragmentator, 100 V. The ESI was operated in negative mode. Data were collected in Extended Dynamic Range, 100 to 1000 m/z. Data acquisition and processing were carried out with the Masshunter Data Acquisition B.05.01 and Masshunter Qualitative Analysis B.07.00 SP2 software. Compounds were identified by comparing mass spectra with the corresponding standard if available and confirmed by comparison with the retention time of the standard. The following standard compounds were purchased from Sigma-Aldrich or Extrasynthese (Genay, France): catechin, epicatechin, epicatechin gallate, procyanidins B1, B2, B3 and C1, and cinnamtannin A2. Liquid chromatography–mass spectrometry grade solvents were purchased from ThermoFischer Scientific (Waltham, MA, USA). In the case of compounds with standards that were not available, identification was based on prediction of chemical formula from accurate ion mass measurement, and quantification was performed by interpolation into the calibration curves of some structurally related compounds: procyanidin gallates with procyanidin B2; procyanidin trimers with procyanidin C1 and procyanidin tetramer with cinnamtannin A2 (Muñoz-González *et al.*, 2019).

Microbiological analysis

Digesta (0.1 to 0.2 g) from the ileum were collected aseptically in preweighed 20-ml sterilized plastic tubes. The samples for the microbiological determinations were weighed and diluted in 9 ml of peptone water. All blended samples were vortexed and further diluted tenfold down to 10^{-10} dilution before inoculation onto Petri dishes of sterile agar. Lactic-acid bacteria were grown on de Man, Rogosa and Sharpe agar (Difco Laboratories, Detroit,

MI, USA). *Escherichia coli* was grown on Coli ID agar (bioMérieux España, Madrid, Spain). The agar used to grow *Clostridium* spp. was sulfite polymyxin sulfadiazine (Difco Laboratories). The plates were incubated at 37°C anaerobically (73% N, 20% CO_2 and 7% H_2) for *Clostridium* and lactic-acid bacteria, and aerobically for *E. coli*. Incubation of plates lasted 24 h for *Clostridium* and *E. coli*, and 72 h for lactic-acid bacteria. After incubation, all colonies appearing on the plates were observed and counted. Colony-forming units were defined as being distinct colonies measuring at least 1 mm in diameter.

Meat lipid oxidation

The extent of lipid oxidation of meat was determined by measuring the thiobarbituric acid reactive substances (**TBARS**) after 1 and 7 days of refrigerated storage using the procedure described by Botsoglou *et al.* (1994) with minor modifications. Briefly, 5 g of ground meat were homogenized with 25 ml of 5% trichloroacetic acid in an Ultraturrax at 21 280 × *g* for 1 min. Butylated hydroxytoluene (Sigma-Aldrich) was added prior to homogenization at a level of 125 µg/mg fat. Samples were centrifugated (5 min at 3000 × *g* at 4°C) and the supernatant was filtered through Whatman number 2V filter (Whatman International Ltd, Maidstone, England) and made to 25 ml volume with 5% trichloroacetic acid. Then, 2.5 ml of the filtrate were mixed with 1.5 ml of 0.8% thiobarbituric acid in distilled water in capped test tubes. Tubes were vortexed, incubated at 70°C for 30 min and absorbance was determined at 532 nm using an ultraviolet-visible spectrophotometer Hitachi U-2000 (Hitachi, Ltd). Results were expressed as mg of malondialdehyde (**MDA**) per kg of muscle after the preparation of a standard curve (ng MDA/ml = 10.83 × absorbance + 0.018; $R^2 = 0.996$) with 1,1,3,3-tetraethoxy propane (Sigma-Aldrich) ranging from 2.39 to 19.12 ng/ml.

Calculations and statistical analysis

Apparent ileal (**AID**) and excreta digestibility of CP (only ileal) and TEP (ileal and excreta) was determined by using the AIA content and calculated with the following formula:

$$100\% - (100\% \times ((\text{AIA concentration in feed}/\text{AIA concentration in ileal content or excreta}) \times (\text{CP or TEP concentration in ileal content or excreta}/\text{CP or TEP concentration in feed})))$$

Data were subjected to a one-way analysis of variance by using the general linear model procedure of SAS (Version 9.4; SAS Institute Inc., Cary, NC, USA). When the effect was declared significant ($P < 0.05$), treatment means were compared using a Duncan's multiple-range test. Non-orthogonal contrasts were used to test differences between the combined means of several groups. Pen served as experimental unit for growth performance, ileal and excreta contents and digestibilities and microbial counts, whereas the experimental unit used for TBARS determination was the bird.

Results

Growth performance

Growth performance of broiler chickens is summarized in Table 4. The inclusion of UFS and FS in chicken diets reduced ($P < 0.05$) daily weight gain and increased ($P < 0.01$) feed conversion ratio as compared with the control and vitamin E diets. A significant ($P < 0.01$) decrease in daily weight gain ($P < 0.01$) and daily feed intake ($P < 0.05$) was observed in birds fed 60 g/kg of grape skin, either FS or UFS, in comparison with those fed 30 g/kg.

Ileal and excreta total extractable polyphenols contents

The total ileal and excreta TEP polyphenols contents are reported in Table 5. A higher ileal TEP content was found ($P < 0.05$) in birds fed UFS and FS diets than in birds fed the control diet (0.470 *v.* 0.426 g GAE/100 g). Besides, the ileal TEP content was higher ($P < 0.001$) in birds fed UFS than in those receiving FS (0.501 *v.* 0.439 g GAE/100 g). On average, TEP content decreased by 21.3% in the excreta as compared with the mean value found in the ileal contents of chickens. In the excreta, TEP was higher for birds fed UFS

Table 4 *Growth performance of broiler chickens (1 to 21 days) fed diets containing FS and UFS and vitamin E*

									P-value of contrasts[3]			
Item	C	C + vit E	C + FS30	C + FS60	C + UFS30	C + UFS60	SEM [1]	*P*-value[2]	1	2	3	4
Daily weight gain (g/day)	38.6[a]	40.2[a]	38.3[ab]	34.3[b]	37.1[ab]	34.5[b]	1.15	**	*	**	ns	**
Daily feed intake (g/day)	51.2	54.4	53.3	51.6	55.2	51.8	1.23	ns	ns	ns	ns	*
Feed conversion ratio	1.33[b]	1.35[b]	1.40[ab]	1.51[a]	1.49[a]	1.50[a]	0.037	**	**	**	ns	ns

C = control; FS = fermented grape skin; UFS = unfermented grape skin; ns = no significant effect.
[1]Each value represents the mean of five replicates (five birds per replicate).
[2]ns ($P > 0.05$).
[3]Non-orthogonal contrasts were: (1) effect of C diet *v.* FS + UFS diets; (2) effect of vitamin E diet *v.* FS + UFS diets; (3) effect of FS diets *v.* UFS diets; (4) effect of FS30 + UFS30 diets *v.* FS60 + UFS60 diets.
[a,b]Means in a row with different superscripts differ significantly ($P < 0.05$).
*$P < 0.05$;**$P < 0.01$.

Table 5 *Ileal and excreta TEP (g GAE/100 g) contents in broiler chickens (21 days) fed diets containing FS and UFS and vitamin E*

Item	C	C + vit E	C + FS30	C + FS60	C + UFS30	C + UFS60	SEM [1]	*P*-value[2]	*P*-value of contrasts[3] 1	2	3	4
TEP												
Ileal	0.426^{c}	0.442^{c}	0.430^{c}	0.448^{c}	0.480^{b}	0.522^{a}	0.011	***	*	ns	***	ns
Excreta	0.341^{c}	0.330^{c}	0.338^{c}	0.357^{bc}	0.380^{ab}	0.405^{a}	0.009	***	*	*	***	**

C = control; FS = fermented grape skin; UFS = unfermented grape skin; GAE = gallic acid equivalents; TEP = total extractable polyphenols; ns = no significant effect.
[1]Ileal and excreta values represent both the mean of five replicates, with three birds per replicate.
[2]ns ($P > 0.05$).
[3]Non-orthogonal contrasts were: (1) effect of C diet *v.* FS + UFS diets; (2) effect of vitamin E diet *v.* FS + UFS diets; (3) effect of FS diets *v.* UFS diets; (4) effect of FS30 + UFS30 diets *v.* FS60 + UFS60 diets.
[a,b,c]Means in a row with different superscripts differ significantly ($P < 0.05$).
*$P < 0.05$; **$P < 0.01$; ***$P < 0.001$.

Table 6 *Ileal digestibility of protein and ileal and excreta digestibility of TEP in broiler chickens (21 days) fed diets containing FS and UFS and vitamin E*

Item	C	C + vit E	C + FS30	C + FS60	C + UFS30	C + UFS60	SEM [1]	*P*-value[2]	*P*-value of contrasts[3] 1	2	3	4
Ileal protein digestibility (%)	80.3^{a}	79.7^{a}	77.6^{a}	78.2^{a}	76.9^{ab}	74.7^{b}	0.932	**	*	*	**	*
Ileal TEP digestibility (%)	47.5^{a}	49.9^{a}	44.1^{ab}	44.2^{ab}	40.6^{bc}	36.9^{c}	1.53	**	**	**	**	ns
Excreta TEP digestibility (%)	65.2^{a}	64.6^{a}	63.8^{ab}	63.6^{ab}	60.8^{bc}	58.0^{c}	0.979	***	**	**	***	ns
Excreta moisture content (%)	26.6	26.6	29.7	28.4	30.0	30.3	1.27	ns	*	*	ns	ns

C = control; FS = fermented grape skin; UFS = unfermented grape skin; TEP = total extractable polyphenols; ns = no significant effect.
[1]Ileal and excreta values represent both the mean of five replicates, with three birds per replicate.
[2]ns ($P > 0.05$).
[3]Non-orthogonal contrasts were: (1) effect of C diet *v.* FS + UFS diets; (2) effect of vitamin E diet *v.* FS + UFS diets; (3) effect of FS diets *v.* UFS diets; (4) effect of FS30 + UFS30 diets *v.* FS60 + UFS60 diets.
[a,b,c]Means in a row with different superscripts differ significantly ($P < 0.05$).
*$P < 0.05$; **$P < 0.01$; ***$P < 0.001$.

and FS diets than for control chickens (0.370 *v.* 0.341 g GAE/100 g, $P < 0.05$). Moreover, the excreta TEP content was also greater when UFS was included in the diet instead of FS (0.392 *v.* 0.347 g GAE/100 g, $P < 0.001$) and when grape skin was added at 60 g/kg in the diet rather than at 30 g/kg (0.381 *v.* 0.359 g GAE/100 g, $P < 0.01$).

Protein and polyphenols digestibility

Ileal protein digestibility and ileal and excreta TEP digestibilities are reported in Table 6. Ileal protein digestibility was reduced by 4.3% ($P < 0.05$) in birds fed UFS and FS diets in comparison with those fed the control diet and by 1.0% ($P < 0.05$) in birds fed 60 g/kg UFS and FS diets as compared with those fed 30 g/kg diets. Furthermore, also dietary inclusion of UFS led to a protein digestibility lower (by 2.7%, $P < 0.01$) than that of birds fed FS diets. Ileal TEP digestibility was lower in birds fed UFS than in those receiving FS (38.7% *v.* 44.1%, $P < 0.01$). Likewise, also excreta digestibility of TEP was reduced with UFS inclusion in the diet as compared with birds fed FS diets (59.4% *v.* 63.7%, $P < 0.001$). Both for ileal and excreta digestibility of TEP, values were lower in birds fed diets containing grape skin than in those fed the control diet (41.4% *v.* 47.5%, $P < 0.01$, and 61.5% *v.* 65.2%, $P < 0.01$, for ileal and excreta digestibility of TEP, respectively). Finally, excreta moisture content increased by 11.3% ($P < 0.05$) in birds fed UFS and FS diets as compared with those fed the control diet.

Microbiological counts

The effect of including UFS and FS in chicken diets on the microbiological ileal count of different bacterial species is reported in Table 7. In the current study, no effect was detected on the count of lactic-acid bacteria or on that of *Clostridium* species with the addition of grape skin in the diets. Nevertheless, a decrease ($P < 0.01$) in ileal count of *E. coli* was observed in birds fed UFS diets as compared with those fed FS diets.

Meat lipid oxidation

The extent of meat lipid oxidation, measured as TBARS formation in thigh meat, is reported in Figure 1. Thiobarbituric acid reactive substances increased with storage time in all of the treatments. The dietary supplementation with vitamin E reduced TBARS values in meat both at day 1 and 7 ($P < 0.05$) of refrigerated storage, whereas significant differences ($P < 0.05$) between control birds and those fed the diets including FS or UFS were only detected at day 7 of storage. Moreover, after 7 days of refrigerated storage, TBARS values were lower ($P < 0.05$) in meat of chickens fed diets containing grape skin at 60 g/kg rather than at 30 g/kg. Furthermore,

Table 7 *Effect of dietary inclusion of FS and UFS and vitamin E on growth of lactic-acid bacteria,* E. coli *and* Clostridium *spp. in the ileal contents of 21-days broiler chickens*

Item	C	C + vit E	C + FS30	C + FS60	C + UFS30	C + UFS60	SEM [1]	*P*-value[2]	*P*-value of contrasts[3] 1	2	3	4
Lactic-acid bacteria (log cfu/g)	7.72	6.51	7.42	7.96	8.13	7.03	0.613	ns	ns	ns	ns	ns
Escherichia coli (log cfu/g)	6.75	6.22	7.10	6.60	5.56	5.74	0.429	ns	ns	ns	**	ns
Clostridium (log cfu/g)	6.54	6.08	6.73	6.39	8.03	7.26	0.650	ns	ns	ns	ns	ns

C = control; FS = fermented grape skin; UFS = unfermented grape skin; ns = no significant effect.
[1]Each value represents the mean of five replicates, with three birds per replicate.
[2]ns ($P > 0.05$).
[3]Non-orthogonal contrasts were: (1) effect of C diet *v.* FS + UFS diets; (2) effect of vitamin E diet *v.* FS + UFS diets; (3) effect of FS diets *v.* UFS diets; (4) effect of FS30 + UFS30 diets *v.* FS60 + UFS60 diets.
** $P < 0.01$.

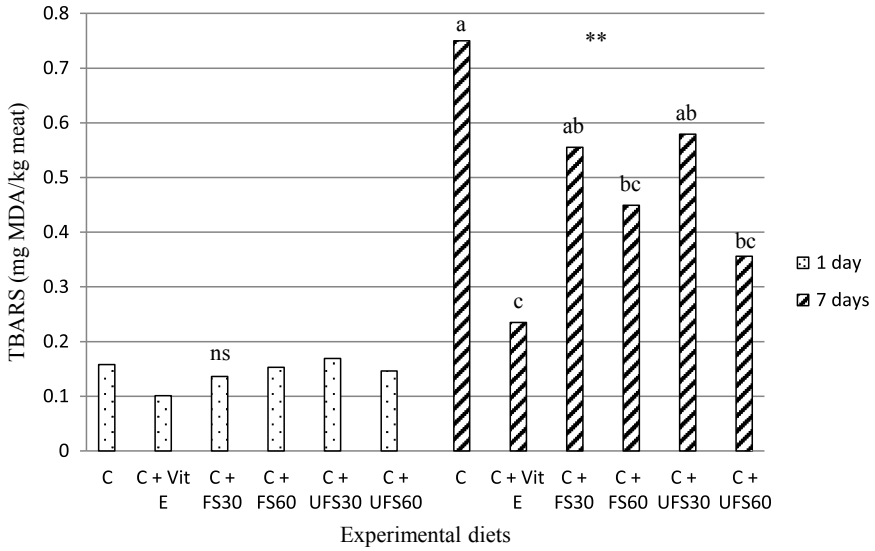

Figure 1 Lipid oxidation (**TBARS**) during refrigerated storage of thigh meat in broiler chickens (21 days) fed diets containing FS and UFS and vitamin E. C = control; FS = fermented grape skin; UFS = unfermented grape skin; TBARS = thiobarbituric acid reactive substances; MDA = malondialdehyde. Data are means of seven chicks for each treatment (one bird per replicate). SEM = 0.017 and 0.084 at 1 and 7 days, respectively. ns = no significant effect ($P > 0.05$); * $P < 0.05$; ** $P < 0.01$. Non-orthogonal contrasts were: (1) effect of C diet *v.* FS + UFS diets (ns at 1 day and * at 7 days); (2) effect of vitamin E diet *v.* FS + UFS diets (* at 1 and 7 days); (3) effect of FS diets *v.* UFS diets (ns at 1 and 7 days); (4) effect of FS30 + UFS30 diets *v.* FS60 + UFS60 diets (ns at 1 days and * at 7 days). [a,b,c] Means with different superscripts differ significantly ($P < 0.05$).

after 7 days of storage, TBARS value did not differ significantly between chickens fed grape skin at 60 g/kg and those supplemented with vitamin E.

Discussion

Growth performance, protein and polyphenol utilization

Previous studies have been carried out on the dietary use of GP and GSE in poultry but no data are hitherto available on the dietary effect of grape skin. The present study showed that the inclusion of grape skin (either FS or UFS) at 60 g/kg in the diet negatively affected the growth performance of chickens, reducing daily weight gain and increasing feed conversion ratio (Nardoia *et al.*, 2017a). When this byproduct was incorporated at a lower concentration (30 g/kg), no effect on weight gain was observed. Previous research shows that the effect of grape polyphenols on chicken's growth performance depends on the dietary dose used. In this sense, growth performance of chickens fed GP was not affected when GP was added to diet up to 60 g/kg (Goñi *et al.*, 2007; Brenes *et al.*, 2008). Regarding seeds, an improvement in chicken growth rate was reported with dietary addition of 5 g/kg of grape seed (Pascariu *et al.*, 2017). However, when seeds were incorporated as GSE, a detrimental effect on growth performance was detected at 5 g/kg, with no negative effect observed up to 3.6 g/kg (Brenes *et al.*, 2010; Chamorro *et al.*, 2013).

Grape skin contains a significant amount of insoluble fibre and condensed tannins (Deng *et al.*, 2011). In general, in the grape berry the greatest amounts of tannins are found in the skin, and these tannins differ from those of the other

grape fractions by having a higher polymerization degree (Souquet *et al.*, 1996). In the fermentation process, the pomace cell structure is modified with the release of polysaccharides and some polyphenols. Thus, TEP content and phenolic composition are expected to be different in FS and UFS. In this experiment, chemical analysis showed that UFS contained a higher amount of gross energy and TEP and a lower concentration of dietary fibre and protein in comparison with FS composition. In parallel, the content of phenolic extractable compounds identified and quantified in grape skins were lower in FS than in UFS (Table 2). Taking into account that diets of the current study were formulated to contain the same amounts of fibre, protein and energy, differences in the chickens' responses when feeding FS or UFS might be attributed to differences in the dietary polyphenols content and profile. Fermentation process increases the release of numerous compounds such as polysaccharides, mannoproteins and polyphenols (Vergara-Salinas *et al.*, 2013).

The structure and molecular weight of polyphenols play an important role in protein–polyphenol interactions. High-molecular-weight polyphenols are able to bond more strongly to proteins before the breakdown of the latter by pancreatic enzymes (Frazier *et al.*, 2010). These interactions might influence digestibility and availability of certain amino acids. In the present study, the inclusion of the highest level of UFS negatively affected protein digestibility of chickens, probably due to the higher concentration of TEP in UFS and to the interaction between these polyphenols and dietary protein. Similarly, Chamorro *et al.* (2013) observed, in parallel to the worsening of growth performance, that the dietary inclusion of 5 g/kg of GSE in chickens reduced AID of CP and that of some essential (arginine, histidine and phenylalanine) and non-essential (cystine, glutamic acid and proline) amino acids. Likewise, dietary inclusion of GP at 100 g/kg reduced ileal protein digestibility (Chamorro *et al.*, 2017), whereas at 30 g/kg no effect on protein and amino acids AID was reported (Goñi *et al.*, 2007).

There are many references in the literature dealing with the composition and the antioxidant properties of grape polyphenols (Brenes *et al.*, 2016), but there are few studies available on the intestinal digestibility of grape polyphenols. Monomeric and some oligomeric polyphenols have been found to be directly absorbed at the small intestine with no prior chemical modification, while oligomeric or polymeric forms are not absorbed in their native forms and must be hydrolysed by the intestinal microbiota (Tsang *et al.*, 2005; Monagas *et al.*, 2010). Consequently, polyphenols are metabolized by gut bacteria, producing thereby new phenolic compounds *in situ*, which could have better bioavailability and higher biological activity than their parent compounds and may be involved in both body systemic and local action (Requena *et al.*, 2010). As concerns chickens, recent studies (Chamorro *et al.*, 2017 and 2019) with dietary GP and GSE have also demonstrated the intestinal utilization of monomeric and dimeric catechins, showing similar microbial metabolism for grape catechins.

In the current experiment, ileal TEP content was higher in chickens fed the diets containing grape skin than in control birds, which resulted in a lower ileal TEP digestibility for chickens receiving grape skin than for those fed the control and vitamin E diets. For both ileal and excreta TEP digestibilities, the lowest value was obtained with the diet containing UFS at 60 g/kg, which was actually the diet presenting the highest dose of TEP (more than 57% of TEP with respect to the control and vitamin E diets). The differences reported for the TEP digestibilities between UFS and FS (lower for UFS both at the ileum and in the excreta) could also be due in part to the different composition of polyphenols in these skins. Results found in the present work for polyphenols digestibilities fall within the range of values obtained, also with broiler chickens fed grape byproducts, in our previous studies (Brenes *et al.*, 2008 and 2010; Chamorro *et al.*, 2015 and 2017), where polyphenol digestibility was also reduced by increasing dietary doses of GP and GSE. However, to the authors' knowledge, no references are available for the specific intestinal use of dietary grape skin in chickens. Finally, in the present work, excreta moisture content increased by 11.3% with dietary inclusion of grape skin, compared with the control corn–soybean diet. Higher excreta moisture content is regarded as a drawback since this could directly lead to a wetter litter, which is a major concern in commercial poultry production. The increase in the excreta moisture content found in the current study could be related to the reduction in protein digestibility resulting from the dietary inclusion of grape skin. Actually, Van der Hoeven-Hangoor *et al.* (2013) found that the protein content in the excreta was positively associated with excreta moisture content in broiler chickens.

Microbiological counts

A considerable number of *in vitro* studies have shown that flavonoids present in grape byproducts have the capacity to inhibit the growth of certain microorganisms (Özkan *et al.*, 2004). However, literature dealing with the *in vivo* effects of grape polyphenols on the intestinal microbiota is much less abundant. In the current experiment, the ileal count of *E. coli* was reduced in chickens fed UFS, which is richer in polyphenols than FS, whereas no dietary effect was detected for lactic-acid bacteria and *Clostridium* spp. ileal counts. Dietary grape polyphenols have been associated with both promoting and suppressing bacteria effects in the ileum of broiler chickens. For instance, the ileal population of *Lactobacillus* was increased with the dietary inclusion of grape seed at doses ranging from 10 to 40 g/kg (Abu Hafsa and Ibrahim, 2018), whereas neither Viveros *et al.* (2011) with a dietary dose of 7.2 g/kg of GSE nor Chamorro *et al.* (2017) with 50 g/kg of GP in the diet found any effect on the ileal count of *Lactobacillus*. Neither did the latter authors with those doses detect any effect on the ileal count of *E. coli*. Nevertheless, other authors (Hajati *et al.*, 2015; Chamorro *et al.*, 2019) did report a reduction in the ileal population of *E. coli* with doses of GSE ranging from 0.45 to 5 g/kg. Concerning *Clostridium* ileal populations,

Viveros *et al.* (2011) observed a reduction in the ileal population of *Clostridium* with both 7.2 g/kg of GSE and 60 g/kg GP. Also a dietary inclusion of 50 g/kg of GP was found to exert an antimicrobial effect on *Clostridium perfringens* in the ileum (Chamorro *et al.*, 2017), but 5 g/kg of GSE in the diet caused no effect on the ileal count of *Clostridium perfringens* in the study of Chamorro *et al.* (2019).

The different dietary amount and composition in polyphenols between skin and grape byproducts used in other experiments might contribute to explain the differences observed on the intestinal populations of bacteria. Besides, it has been observed in broiler chickens by using T-RFLP techniques (Viveros *et al.*, 2011) that dietary grape polyphenols increased mainly the frequency of unknown bacteria groups rather than that of known groups. The intestinal ecosystem of chickens remains largely unknown and, despite the advances made in the field of microbial metabolism of phenolics compounds in human beings (Braune and Blaut, 2016), the specific bacterial species able to metabolize grape polyphenols in the gastrointestinal tract of chickens, the intermediate products and the enzymes involved are yet to be elucidated.

Meat lipid oxidation

Lipid oxidation is one of the primary processes in the quality deterioration of meat. In the present study, the addition of vitamin E in the diet reduced TBARS values in chickens' meat both at day 1 and day 7 of refrigerated storage of meat. The protective effect of dietary supplementation with vitamin E on meat quality in male broiler chickens has recently been highlighted in a meta-analysis including 51 scientific papers (Pompeu *et al.*, 2018).

Nutritional interest in polyphenolic compounds has increased greatly in light of their antioxidant activity. Thus, it has also been shown that the inclusion of grape polyphenols in animal diets enhances oxidative stability in chicken and turkey meat (Rababah *et al.*, 2006). Previous studies (Goñi *et al.*, 2007; Brenes *et al.*, 2008) reported a protective effect of grape polyphenols, similar to that of vitamin E, with dietary addition of up to 60 g/kg of GP in terms of enhanced oxidative stability in stored meat. However, in the present study dietary FS and UFS did not reach, on average, a protective effect in delaying meat lipid oxidation similar to the one observed with the addition of vitamin E. It is true, nonetheless, that after 7 days of storage, meat from birds fed grape skin at 60 g/kg showed TBARS values lower than those of birds fed grape skin at 30 g/kg, and, additionally, TBARS values in the meat of chickens fed 60 g/kg of grape skin did not differ significantly from those of chickens receiving vitamin E. Although these results suggest an antioxidant potential of grape skin when added at high doses, its applicability in practical nutrition remains unclear due to its negative impact on growth performance of chickens. The different results among studies obtained with GP, grape seed and grape skin might be partially explained by differences in the phenolic compounds present in the various grape byproducts (Ky *et al.*, 2014). Actually, in grape skin a higher mean degree of polymerization of proanthocyanidins, the presence of prodelphinidins and a lower amount of galloylated derivatives have been observed (Souquet *et al.*, 1996; Vivas *et al.*, 2004). Furthermore, GP consists of a mixture of seeds and skin, and Yilmaz *et al.* (2015) reported a higher polyphenol content and antioxidant capacity in grape seed than in grape skin. In this sense, the direct addition of grape seed to chicken thigh patties resulted more effective in retarding lipid oxidation during storage than grape skin (Nardoia *et al.*, 2017b). Differences in the intestinal use of phenolic compounds present in the different grape fractions, along with the importance of intestinal microbiota in the metabolism of grape polyphenols could contribute to explain the discrepancies observed among *in vitro* and animal studies.

The byproducts of the wine industry (GP, skin and seeds) contain a wide range of bioactive compounds. In the present experiment, we have focused on the extractable polyphenols fraction of grape skins. However, it should be pointed out that a significant fraction of the polyphenols present in the food matrix is linked to polymeric molecules like dietary fibre and remains in the corresponding residues after the extraction; these are the so-called non-extractable polyphenols (Pérez-Jiménez *et al.*, 2013). After ingestion, this polyphenol fraction remains almost unchanged along the intestinal tract and might be transformed by the intestinal microbiota into metabolites with biological activities (Mateos-Martín *et al.*, 2012). Studies dealing with the quantification and characterization of this non-extractable fraction in raw materials are still scarce and have not been included in the present study, but might also contribute to explain the different responses observed between UFS and FS treatments.

In conclusion, high doses of grape skin polyphenols depressed growth performance and protein digestibility, and increased excreta moisture content. Unfermented grape skin contained more polyphenols than FS, and its inclusion in the diet led to higher ileal and excreta polyphenols contents and to a lower ileal count of *E. coli*. Furthermore, the antioxidant potential of the polyphenols present in grape skin was observed after 7 days of meat storage, with the dose of 60 g/kg of grape skin being comparable to vitamin E supplementation in maintaining oxidative stability of stored meat. Thus, further research on the use of grape skin in broiler chicken diets would be helpful to assess the optimal doses of grape byproducts that ensure the beneficial potentials without impairing growth performance.

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S. Chamorro 0000-0002-3136-833X
C. Romero 0000-0001-6909-7048

Declaration of interest

There is no conflict of interest.

Ethics statement

Experimental procedures were approved by the University Complutense of Madrid Animal Care and Ethics Committee in compliance with the guidelines for the Care and Use of Animals for Scientific Purposes of the Ministry of Agriculture.

Software and data repository resources

None of the data were deposited in an official repository.

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