PHENOLIC METABOLITES IN PLASMA AND THIGH MEAT OF CHICKENS SUPPLEMENTED WITH GRAPE BYPRODUCTS

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ABSTRACT

Grape byproducts are rich sources of polyphenols with powerful antioxidant and health-promoting effects. The impact of supplementing chicken diets with grape byproducts on plasma and thigh meat concentrations of phenolic metabolites was evaluated by analyzing samples by HPLC-QTOF-MS. Chickens were fed three experimental diets: Control diet, Control+8% grape pomace and Control+0.1% grape seed extract. In plasma, 32 phenolic metabolites were identified being some, such conjugated catechin/epicatechin metabolites, exclusively identified in chickens fed diets enriched in grape byproducts. Also, these chickens showed significantly higher plasmatic concentrations of 21 phenolic metabolites. In thigh meat, 14 phenolic metabolites were identified but no differences were found between diets.

Higher plasmatic tocopherol was found when supplementing diets with grape by products, while no changes were observed in meat. Thus, supplementing chicken diets with grape byproducts leads to a significant increase in the circulation of phenolic metabolites and tocopherol.

KEYWORDS: polyphenols, grape pomace, grape seed, chicken, plasma, thigh meat.

INTRODUCTION

Agro-industrial byproducts represent a promising means through which to improve animal welfare and growth, as well as meat quality. The winemaking industry produces large volumes of waste consisting mainly of solid byproducts such as grape pomace (GP). The valorization of such waste could offer ways to reduce the environmental impact of winery activity, as well as providing potential functional ingredients, due to their high content of bioactive constituents such as dietary fiber and phenolic compounds, when used as a whole (*1*), or to the high polyphenol content when used as grape seed extracts (GSE). The latter obtained from seeds removal after wine production. In particular, both GSE and GP are a significant source of a wide range of polyphenols, mainly anthocyanins and flavanols, ranging from the monomeric forms, catechin and epicatechin, to oligomeric and polymeric forms also known as proanthocyanidins (PAs) (*2*, *3*).

One of the potential applications of these grape byproducts is in animal nutrition. The dietary inclusion GP and GSE in monogastric diets has been demonstrated as a strategy to improve health status and animal product quality (Brenes et al. 2016) (4). In this sense, the incorporation of these sources of grape polyphenols to diets promoted the proliferation of beneficial intestinal bacteria and maintained the plasma antioxidant status of chickens (viveros et al., 2011, *chamorro et al., 2013, Chamorro 2017, Lichovnikova et al., 2015; Hafsa and Ibrahim, 2018)* . In addition, meat from birds fed GP showed a higher α-tocopherol and polyunsaturated fatty acid content and a lower susceptibility to lipid oxidation (Goñi et al., 2007; Chamorro et al. 2015). The potential of GSE as an alternative antioxidant in chickens (Brenes et al., 2010;*Hafsa et al., 2018)*has also been reported. These studies suggest therefore that grape polyphenols or their metabolites might be absorbed reaching and remaining active in tissues.

Most of the studies cited were based on the determination of overall markers of oxidative stress or antioxidant levels; but they did not measure specific polyphenol or polyphenol-derived compounds. However, these more precise determinations are needed to establish the exact presence of polyphenols in plasma and meat when they are used in animal nutrition. Indeed, it has been suggested that the beneficial health effects of polyphenols can mainly be attributed to their metabolic products rather than to the precursors themselves (Del Rio et al., 2013) . Therefore studies that improve our knowledge of the metabolism and bioavailability of dietary polyphenols in animal diets as supplements are essential to understand the beneficial effects reported above.

The metabolic fate of PAs has been widely studied in humans. Briefly, once consumed, monomeric flavanols and procyanidin dimers may be absorbed in the small intestine and later transformed in enterocytes or in liver by phase II enzymes into water-soluble methyl, glucuronide or sulfate conjugates. These are then rapidly released into systemic circulation and further distributed to tissues and organs, finally to be excreted in urine (*4*). In contrast, larger PAs are not absorbed in their native forms. These compounds, together with phase II metabolites that reach the colon by enterohepatic recirculation, are catabolized by the colonic microbiota before being absorbed, thus generating metabolites such as aromatic acids (Monagas et al., 2010; Selma et al. 2009; Sanchez-Patan 2012) . All these microbial-derived phenolic metabolites may be absorbed, entering portal system circulation and being transported to the liver, where they can be further subjected to phase II enzymes before entering circulation (Monagas et al. , Gonthier 2003); or they may be excreted in the feces (Muñoz-Gonzalez 2013). Several studies have also dealt with the metabolism of PAs in animals, but they have largely been orientated towards application for human nutrition, mostly using rats (*5*-*7*), and not towards animal nutrition. In chickens, only few studies have

explored the presence of phenolic metabolites in plasma or meat after supplementation with polyphenol-rich products such as tea or olive, respectively (*8*). Regarding grape polyphenols, recent studies have reported the intestinal digestibility of catechins and identified microbial-derived phenolic metabolites characteristic from PAs in the excreta of chickens supplemented with GP or GE (Chamorro et al. 2015 and Chamorro et al. 2019). However, the absorption of these compounds hasn't been yet evaluated. Since it has been reported that different species may transform PAs differently (*28*), it is important to know the metabolic fate of these compounds in chickens.

Therefore, the aim of this paper was to evaluate the impact of supplementing chicken diets with grape byproducts (GP and GSE) on plasma and thigh meat concentrations of phenolic metabolites and tocopherols in order to gain further understanding on the whole metabolic fate of these compounds in chicken with a specific approach on animal nutrition.

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MATERIALS AND METHODS

Test Products

Grape pomace (GP) from red grapes (*Vitis vinifera* var. Bobal) and grape seed extract (GSE) were kindly provided by Out Put Trade S.L (Girona, Spain). The total polyphenol content (TPC), expressed as gallic acid equivalents (GAE) and determined following the procedure described by Chamorro et al. (2012) was 4.5±0.2 gGAE/100g DM (dry material) for GP and 30.2±0.5 gGAE/100g DM for GSE. The main phenolic constituents of GP and GSE (Table 1) were analyzed by HPLC-QTOF-MS (*description in section "HPLC-QTOF-MS analysis of phenolic compounds"*).

Chemicals and reagents

The following standard compounds were purchased from Sigma-Aldrich Chemical Co. (St. Louis, MO) or Extrasynthese (Genay, France): catechin, epicatechin, epicatechin gallate (ECG), procyanidins b1, b2, b3 and c1, cinnamtannin a2, gallic acid, vanillic acid, salicylic acid, pyrogallic acid, 4-hydroxyphenylacetic acid, 2,3-dihydroxybenzoic acid, 3-(2-hydroxyphenyl)propionic acid, 3,4-dihydroxyhydrocinnamic acid, 4-ethylcatechol, trans-ferulic acid, 3-hydroxybenzoic acid, DL-3-phenyllactic acid, 2-hydroxyphenylacetic acid, 2,4-dihydroxybenzoic acid, p-coumaric acid, catechol, 3-hydroxytyrosol, 4-hydroxybenzoic acid, 3-hydroxyphenylacetic acid, protocatechuic acid ethyl, 3-(3-hydroxyphenyl)propionic acid, trans-cinnamic acid, homovanillic acid, vanillyl alcohol, protocatechuic aldehide, ethyl

gallate, 3,5-dihydroxybenzoic acid, 4-methylcatechol, 4-hydroxyphenyl acetic acid, 3-(4-hydroxyphenyl)-propionic acid.

Standards were individually weighed and dissolved in a solution methanol:water (1:1, v/v). For HPLC-QTOF-MS analysis, *a* multistock solution was prepared from the mixture of the individual standard stock solutions. Standard stock solutions were stored in dark-glass flasks at −20 °C. LC-MS grade solvents were purchased from ThermoFischer Scientific (Waltham, MA).

Chickens and diets

A total of 36 1-day-old broiler Cobb chickens were obtained from a commercial hatchery (Madrid, Spain). The chickens were housed in electrically heated starter battery brooders in an environmentally controlled room with 23 h of constant overhead fluorescent lighting for 35 days. The chickens were randomly allocated to cages according to regulations. Feed and water were provided *ad libitum*. The chickens were fed with a starter diet from day 1 to day 21 and, at the end of this period, they were weighed, moved to grower-finisher batteries and fed with a finisher diet from day 22 to day 35 (Table 2). Experimental diets in both starter and finisher periods were as follows: control corn-soybean diet (C diet); C + GP 8% (GP diet); C + GSE 0.1% (GSE diet). GP and GSE were incorporated into the diet at different percentages, according to the dose previously reported effective for both, health status and productive performance ((viveros 2011, Chamorro 2013, Chamorro 2015)). All diets were formulated to meet or exceed the minimum requirements for broiler chickens of the National Research Council (*9*)

Experimental procedures were approved by the Universidad Complutense de Madrid (UCM, Spain) Animal Care and Ethics Committee in compliance with Ministry of

153 Agriculture, Fishery and Food requirements for the Care and Use of Animals for Scientific
154 Purposes.

155

156 **Plasma Sampling and preparation**

157 Before slaughtering (35 days of age) and without previous fasting, blood samples were
158 obtained from 36 chickens (12 from each diet) by cardiac puncture. Blood was collected in
159 BD™ Vacutainer™ Lithium Heparin Plasma Tubes (Franklin Lakes, NJ), maintained on ice,
160 and then centrifuged at 2500 *g* for 15 minutes at 4ºC. After removing the supernatant to obtain
161 plasma, the samples were pooled in twos in order to increase sample volume, thus obtaining 6
162 samples per diet, and then acidified with acetic acid (1%, v/v). The samples were stored at -
163 80ºC until analysis. Before HPLC-QTOF-MS analysis, the samples were filtered through a
164 0.22 μm pore-size filter and diluted (1:1, v/v) with methanol.

165

166 **Thigh meat sampling and preparation**

167 After blood sample extraction, 36 chickens (12 per diet) were slaughtered using
168 carbon dioxide and exsanguinated. The carcasses were trimmed to obtain thigh meat. The
169 chicken thighs from each dietary treatment were then pooled in twos (n=6) to increase sample
170 quantity, vacuum packed and stored at -20ºC until analysis.

171 Before HPLC-QTOF-MS analysis, thigh meat samples were thawed, ground and
172 homogenized to obtain a representative sample. For the extraction of phenolic compounds, 5
173 g of ground thigh meat was suspended in 25 mL of MeOH:H2O (80:20, v/v) and sonicated (3
174 cycles of 30-second bursts with 30-second cooling intervals). After 120 minutes of agitation,
175 the samples were centrifuged at 1000 rpm for 5 minutes at 20ºC (Sorvall RTB600B, DuPont,

176 USA) , and evaporated to dryness. Residues were then reconstituted in 5 mL MilliQ water and
177 subjected to SPE with Supel™-Select HLB SPE cartridges (Sigma-Aldrich, St. Louis, MO).
178 The SPE procedure was performed as follows: cartridges were firstly pre-conditioned using 2
179 mL of methanol and equilibrated with 2 mL of MilliQ water (pH 2). Then, samples (5 mL)
180 were loaded in two steps (3 mL + 2 mL) and the cartridges were washed with 2 mL of MilliQ
181 water (pH 2). The elution step was assessed with 3 mL of MeOH and the eluate was filtered
182 through a 0.22 μm pore-size filter and diluted (1:1, v/v) with MilliQ water in order to keep an
183 accurate aqueous:organic proportion for further analysis by HPLC-QTOF-MS.

184

185 **HPLC-QTOF-MS analysis of phenolic compounds**

186 For separation, the HPLC apparatus (Agilent 1200, Agilent Technologies, Santa Clara,
187 CA) was coupled with DAD (Agilent G1315B) and an Agilent 6530 Accurate-Mass
188 Quadrupole Time of Flight (QTOF) LC/MS with ESI-Jet Stream Technology (Agilent
189 Technologies, Waldbroon, Germany). Separation was performed on a Zorbax Eclipse Plus
190 C18 100 mm x 3.5 μm x 4.6 mm column (Agilent) with a pre-filter (Sigma-Aldrich, St. Louis,
191 MO). A gradient composed of solvents A (water:formic acid, 99.9:0.1, v/v) and B
192 (acetonitrile:formic acid, 99.9:0.1, v/v) was applied at a flow rate of 0.5 mL/min as follows:
193 10% B at 0 min, 30% B at 15 min, 30% B at 30 min, 80% B at 32 min, 10% B at 35 min and
194 10% at 45 min. The volume of samples injected was 10 μl. The ESI parameters were as
195 follows: drying gas flow, 8 L/min; nebulizer pressure, 45 psi; gas drying temperature, 350ºC;
196 sheath gas temperature, 325ºC; sheath gas flow, 11 L/min; capillary voltage, 3500 kV; nozzle
197 voltage, 500 V; and fragmentator, 100 V. The ESI was operated in negative mode. Data were
198 collected in Extended Dynamic Range, 100-1000 m/z. For the identification and
199 quantification of components, MS and MS/MS experiments were performed. For MS/MS

200 experiments, a quite generic collision energy of 20 V was used, as a compromise, in order to
201 simplify development of the method and ensure good fragmentation of the majority of
202 targeted compounds. Data acquisition and processing was carried out with the Masshunter
203 Data Acquisition B.05.01 and Masshunter Qualitative Analysis B.07.00 SP2 software.
204 Compounds were identified by comparing mass spectra with the corresponding standard if
205 available, and confirmed by comparison with the retention time of the standard. In the case of
206 conjugated compounds or compounds with standards that were not available, identification
207 was based on prediction of chemical formula from accurate ion mass measurement and
208 characteristic isotopic pattern (*10*) and confirmed by comparing tandem mass spectrometry
209 fragmentation spectra (MS/MS) with data provided by relevant reference literature or
210 databases. Since there was not a commercial standard for all the compounds searched for, in
211 some cases quantification was performed by interpolation into the calibration curves of some
212 structurally related compounds: conjugated compounds with their corresponding non-
213 conjugated standard; procyanidin gallates with procyanidin B2; procyanidin trimers with
214 procyanidin C1; procyanidin tetramer with cinnamtannin A2; 3,4-dihydroxybenzoic acid with
215 2,4-dihydroxybenzoic acid; 3,4-dihydroxyphenyllactic acid methyl ester with epicatechin;
216 vanillic acid-4-O-sulfate with vanillyl alcohol; homovanillic acid sulfate with 4-
217 dihydroxyphenylpropionic acid; dihydroxyphenyl-gamma-valerolactone sulfate with catechin.

218

219 **Alpha/Gamma tocopherol analysis**

220 The α- and γ-tocopherol concentration in plasma and thigh samples was quantified by
221 direct extraction as described by Rey et al. (*11*). Thus, plasma and thigh samples were mixed
222 with 0.054 M dibasic sodium phosphate buffer adjusted to pH 7.0 with HCl. After mixing
223 with absolute ethanol and hexane, the upper layer containing tocopherols was evaporated to

dryness and dissolved in ethanol prior to analysis. Tocopherols were analysed by reverse phase HPLC (HP 1100, equipped with a diode array, fluorescent detector and LiChrospher 100 RP-18 column) (Agilent Technologies, Waldbronn, Germany). The eluting solvent was methanol:water (97:3) at a flow rate of 2 mL/min. Identification and quantification were carried out by means of a standard curve built using the pure compound (Sigma, Alcobendas, Madrid). All samples were analyzed in duplicate.

Statistical analysis

After checking for normality, means were compared using one-way analysis of variance (ANOVA) followed by Tukey's multiple comparison test, or the Kruskal-Wallis test followed by the non-parametric Mann-Whitney multiple comparison test, as appropriate. Statistical analyses were performed using IBM SPSS Statistics for Windows, version 24 (IBM Corp., Armonk, N.Y., USA). Differences were considered to be significant at $P<0.05$. Data are reported as mean ± standard deviation

RESULTS AND DISCUSSION

Phenolic compounds in plasma

The interest of including grape polyphenols in monogastric diets has recently been reviewed (Brenes et al. 2016). In poultry, polyphenols were traditionally considered as anti-nutritional factors, since dietary incorporation of high doses of tannin-rich ingredients (e.g., sorghum or faba bean) negatively affected nutrient efficiciency and animal performance (Jansman et al., 1989, Ortiz et al., 1993, Nyachioti et., 1997). However, current scientific evidence suggests that moderate amount of raw ingredients (such as GP up to 10%) or extracts (as GSE, up to

0.25%) rich in grape polyphenols might improve health status and animal product quality without compromising productive performance. Since each one of these approaches (raw ingredients or extracts) have pros and cons (price, presence of other constituents, etc.), in this study we evaluated doses of GP and GSE similar to those previously shown to be successful on health status and productive performance ((viveros 2011, Chamorro 2013, Chamorro 2015), and both with similar polyphenol profile (Table 1).

The phenolic compounds identified in the plasma of chickens fed the different diets tested are shown in Table 3. Since MS analysis was applied in negative mode, intact anthocyanins potentially present in grape products would not have been detected; nevertheless, these phenolic compounds also give rise to benzoic acids via microbial transformation (*12*), detected in negative mode. In total, 32 different phenolic compounds were identified in plasma. As explained, for those for which no commercial standard was available, identification was based on the combined analysis of exact mass and MS/MS fragments, as detailed above, such as those derived from the loss of conjugation substituents or decarboxylation. For instance, (epi)catechin sulfate (*m/z* 369) produced a fragment at *m/z* 289 corresponding to epicatechin, and another one at *m/z* 245 due to the decarboxylation of this compound.

Among the compounds detected, we found significant differences between the diets for 21 of them (Table 4). Eleven of these metabolites were detected exclusively in samples from chickens fed with grape polyphenols, while the others were found at higher concentrations in one or both of the groups fed with grape polyphenols than in the control group. Interestingly, neither the flavanol monomers, catechin and epicatechin, nor their different conjugated forms were detected in samples from the control group; this makes it clear that their presence in the plasma samples from the GP and GSE groups was due to the flavanols present in the grape products these diets were supplemented with. In a similar way, Serra et al.(*5*), found

remarkable increments in plasma catechin and epicatechin conjugates after long-term feeding of grape seed phenolic extract to rats, with those increments being greatest in the case of glucoronides, as well as in methyl glucoronides. Flavonoids are typically transformed into glucuronates by liver enzymes. After that, they can be metabolized to sulfonated derivatives or methoxylated, making them water soluble and thus more prone to being released into systemic circulation and to be distributed to tissues and organs (*6*). In the samples analyzed here, sulfate forms were more abundant than glucuronidated forms, thereby indicating an advanced metabolization process.

Although monomers and dimers of flavanols can be absorbed in the small intestine, more polymerized PAs cannot be absorb unless they are previously transformed by the microbiota, which plays an essential role in their bioconversion (*13, 14*), into a wide range of microbial-derived metabolites. One of these compounds, the sulfated form of dihydroxyphenyl-gamma-valerolactone, previously described as a flavanol metabolite (*15*), was detected in the GP and GSE groups but not in the C group. Interestingly, we previously reported 5-(3',4'- dihydroxyphenyl)-gamma-valerolactone as the most relevant metabolite in the excreta of chickens fed grape extract (Chamorro et al., 2019) . In a similar way, the non-conjugated form of this compound has been detected after different interventions with flavanol-rich products such as cocoa or red wine in humans (*16, 17*). This compound can be further metabolized via different pathways, resulting in benzoic acid, phenylpropionic acid and phenylacetic acid derivatives (*3, 18, 19*). Some of these metabolites were also quantified in our study; always at higher concentrations in the GP or GSE groups than in the C group. Therefore, although some of these compounds may be common to other metabolic pathways, such as catechol derivatives from other aromatic compounds -thus explaining why we detected them in the C group- their greater presence after supplementation with grape products means that at least a fraction of the flavanols is transformed by the intestinal

297 microbiota in chicken. Among them, and in agreement with the present findings, some
298 benzoic (salicylic acid, gallic acid) and phenylpropionic (3-3-(hydroxyphenyl) propionic)
299 acids were also previously detected at higher concentration in excreta of chickens fed grape
300 extract (Chamorro et al., 2019). Indeed, 3-3-(hydroxyphenyl) propionic acid, is also
301 considered one of the major metabolites of the phenolic degradation products of
302 proanthocyanidins by intestinal microbiota in humans (*20*).

303 Plasma concentrations of some metabolites as methyl(epi)catechin sulfate,
304 (epi)catechin glucuronide, salicylic acid, 3-(3-hydroxyphenyl)propionic acid and vanillyl
305 alcohol sulfate were higher in samples from the GP diet than in those from the GSE diet. In
306 contrast, concentrations of others as 3,4-dihydroxybenzoic acid, 4-methylcatechol sulfate and
307 ethylcatechol sulfate were higher in samples from chickens fed the GSE diet (Table 4).
308 Overall, it seems that the bioconversion of flavanol monomers into catechol derivatives was
309 enhanced in chickens fed GSE diet. These metabolites have been reported to be increased
310 after the consumption of some flavanol-rich products (*21*), although there were not the most
311 common ones. These differences between the two grape products could be due to the fact that
312 in GSE, flavanols are free; while in GP, a high proportion of them is present as the so-called
313 non-extractable PAs (*22*). Moreover, the high dietary fiber content of GP, a constituent not
314 present in GSE, may also affect the metabolic fate of flavanols (*23, 24*).

315 One limitation in the interpretation of our results from this study is the huge inter-
316 individual variability observed. This may be due to individual differences in the metabolic
317 fate of flavanols or, especially, to the fact that grape products were fed to chickens in their
318 normal diets, so the plasma samples correspond to different times since their last feed intake.
319 Nevertheless, this approach has a higher physiological relevance than acute supplementation
320 (*25*). So, the results obtained here show that, in a real situation, supplementing chickens with

grape products leads to a sustained increase in circulating phenolic metabolites, mostly microbial-derived ones.This increase might support the improvement on the plasma antioxidant status of the birds previously reported after dietary supplementation with GP and GSE (*26, 27*).

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Phenolic compounds in thigh meat

In thigh meat, a total of fourteen phenolic metabolites were identified (Table 3). Since the relation between an increment of circulating phenolic metabolites and their further deposit in muscle is still unknown, phenolic metabolites were quantified in thigh meat samples. Results showed that, in all cases, the concentrations of phenolic metabolites in thigh meat samples were lower than in plasma samples, as expected. However, no significant differences in the concentration of phenolic metabolites in thigh meat between groups were found, except

345 in the case of 3-(3-hydroxyphenyl)propionic acid, which was the only compound that showed
346 significantly higher concentrations in GP (2,69±3.12 ng/g meat, *P*=0.004) and GSE
347 (0.96±0.66 ng/g meat, *P*=0.03) groups compared to the control group (n.d.).

348 Previous studies have shown an improvement in several meat parameters after
349 supplementing chicken diets with grape polyphenols. For example, Goñi et al.(*28*) found a
350 reduction of lipid oxidation in breast and thigh meat when adding increasing amounts of GP
351 (0.5, 1.5 and 3%) to chicken diets. Brenes et al.(*29*) tested a higher dose of GP (6%) in diets
352 and found a protective effect on lipid peroxidation of the chicken meat during storage. In a
353 similar way, Chamorro et al., (*30*) demonstrated that chickens fed GP diets (5 and 10 %)
354 showed a protective effect of α-tocopherol by reducing the susceptibility of meat to lipid
355 oxidation and keeping high level of polyunsaturated fatty acids in meat sample. However,
356 there is scarce evidence regarding how polyphenols can reach tissues and whether they
357 accumulate or not. Some studies, mainly in rats, have shown that polyphenols are capable of
358 penetrating tissues, particularly those in which they are metabolized such as intestine and liver
359 (*31, 32*). However, again there is a lack of evidence regarding how polyphenols accumulate
360 once they reach the different tissues. Thus, studies that help to gain an understanding of
361 polyphenol bioavailability and their mode of action at the cellular level are essential if we
362 wish to understand their potential antioxidant properties. To the best of our knowledge, only
363 one previous study has reported phenolic metabolites in chicken meat after supplementation
364 of diets with olive polyphenols (*33*). However, the results obtained here suggest that, in spite
365 of the increment of circulating phenolic metabolites and therefore the continuous contact
366 between them and tissue, supplementation of chicken diets with grape polyphenols does not
367 lead to an increment in the accumulation of phenolic metabolites in muscle- at least not at this
368 level of supplementation. Anyway, it should not be ignored that these metabolites, during its
369 prolonged circulation through the body, might also exert a local antioxidant effect, even if not

accumulated in tissues. So, further studies should evaluate the association between the increment in circulating metabolites and the reported antioxidant effects on meat reported in the literature

Alpha and gamma tocopherol content in plasma and meat

As previously indicated, an increase in plasma tocopherol after supplementing broiler chickens with grape products has been reported (Chamorro et al. 2017). Thus, the effect of dietary GP and GSE on α- and γ-tocopherol concentrations in plasma and thigh meat is shown in Table 5. Chicks fed GP and GSE showed a significantly higher plasma α- and γ tocopherol concentration than those fed the C diet (table 5). Although grape seed is a source of tocopherols (Ben et al. 2016,), these increments could not be attributed to this fact since the amount provided by these byproducts is small regarding total dietary vitamin E. Consequently, these differences might be explained by the ability of polyphenols to spare/regenerate tocopherols as shown in previous animal studies (Frank et al., 2006; Luehring et al., 2011).

Concerning α-tocopherol accumulation in thigh meat, its concentration in the C group was similar to data presented in the literature according to supplementation time and the dietary fat (*34*). However, as occurred with most phenolic metabolites**,** changes detected on plasmatic tocopherols between different dietary treatments were not clearly observed in meat, what could be explained by the retention of tocopherols in transport lipoproteins for longer time, or a lower transference to muscle tissue. In addition, bioavailability of tocopherols and its utilization by different tissues could be influenced by the presence of other antioxidants (Phan et al. 2018, Neunert et al., 2015, Espin et al. 2007) Thus, although the effect of GP supplementation on plasmatic tocopherol seems to be consistent between experiments

performed under different experimental conditions, the effect of these phenolic combinations provided by grape ingredients on meat tocopherols accumulation seems not be so clear .

CONCLUSIONS

An extensive range of flavanol metabolites was found at higher concentrations in plasma from chickens supplemented with grape products than in the control group. They included both metabolites derived from intestinal absorption and from microbial transformation, some of them followed by hepatic conjugation. Therefore, chickens are capable of metabolizing grape polyphenols in a similar way to other species, leading to a significant increase in the circulation of phenolic metabolites. Dietary grape products also increased plasmatic α- and γ-tocopherol concentrations suggesting than the observed increment in circulating phenolic metabolites might be related with an improvement on the antioxidant status though a regeneration of these compounds. However, in this study, the supplementation with grape byproducts did not lead to an increment of phenolic metabolites (except in the case of 3-(3-hydroxyphenyl)propionic) nor α- and γ-tocopherols in thigh meat..

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TABLES

Table 1. Main phenolic constituents of test products (mg/100g DM)

		GP		GSE	
		Content	%	Content	%
Phenolic acids	Gallic acid	20.77±0.40	41.8	889.96±0.53	43.9
Flavanol monomers	Catechin	4.25±0.20	8.6	292.51±57.38	14.5
	Epicatechin	7.00±0.52	14.1	141.44±15.47	7.0
	Epicatechin gallate	0.76±0.02	1.5	19.05±2.38	0.9
Flavanol dimers	Procyanidin B1	2.54±0.09	5.1	151.23±7.32	7.5
	Procyanidin B2	2.38±0.08	4.8	100.57±21.78	5.0
	Procyanidin B3	2.34±0.25	4.7	91.70±9.66	4.5
	Procyanidin gallate 1[a]	1.31±0.08	2.6	54.03±0.66	2.7
	Procyanidin gallate 2[a]	1.63±0.16	3.3	61.26±3.15	3.0
Flavanol trimers	Procyanidin C1	1.97±0.15	4.0	97.71±6.05	4.83
	Trimer 2[b]	0.86±0.06	1.7	7.87±1.33	0.4
	Trimer 3[b]	1.37±0.13	2.8	68.90±2.33	3.4
	Trimer 4[b]	1.00±0.07	2.0	26.21±0.78	1.3
Flavanol tetramers	Cinnamtannin A2	0.78±0.01	1.6	12.38±3.67	0.6
	Procyanidin tetramer[c]	0.70±0.03	1.4	8.52±1.09	0.4

GP: Grape Pomace; *GSE*: Grape Seed Extract, *DM*: dry matter

[a] Identified by prediction of chemical formula from accurate ion mass measurement. Quantified by use of the calibration curve of procyanidin B2.

[b] Identified by prediction of chemical formula from accurate ion mass measurement. Quantified by use of the calibration curve of procyanidin C1.

[c] Identified by prediction of chemical formula from accurate ion mass measurement. Quantified by use of the calibration curve of pinnamtannin A2.

Table 2. Ingredients and nutrient composition of experimental diets

Ingredients, %	Day 1 to day 21			Day 22 to day 35		
	C diet	GP diet	GSE diet	C diet	GP diet	GSE diet
Corn	37.38	37.10	37.38	43.05	43.80	43.05
Soya vean	41.15	39.5	41.15	37.80	35.50	37.80
Sunflower oil	11.10	11.3	11.10	8.80	8.75	8.80
Straw	6.20	0	6.20	6.30	0	6.30
Grape pomace (GP)	0	8.00	0	0	8.00	0
Grape seed extract (GSE)	0	0	0.10	0	0	0.10
Monocalium phosphate	1.54	1.50	1.54	1.45	1.46	1.45
Calcium carbonate	1.57	1.54	1.57	1.60	1.50	1.60
Salt	0.30	0.30	0.30	0.30	0.30	0.30
Vitamin-mineral premix[a]	0.50	0.50	0.50	0.50	0.50	0.50
DL-Methionine	0.26	0.26	0.26	0.18	0.19	0.18
L-Lysine 50	0.00	0.00	0.00	0.02	0.00	0.02
Calculated composition, %DM						
AME (kcal/kg)	3049	3052	3049	3001	3001	3001
Crude protein	21.0	21.1	21.0	20.0	20.0	20.0
Crude fat	13.2	13.9	13.2	11.1	11.5	11.1
Crude fiber	5.4	5.4	5.4	5.4	5.4	5.4
Lysine	1.26	1.24	1.26	1.19	1.13	1.19
Methionine + Cysteine	0.90	0.90	0.90	0.80	0.80	0.80
Calcium	1.02	1.02	1.02	1.00	1.00	1.00
Available P	0.46	0.46	0.46	0.43	0.43	0.43

C diet: control corn-soybean diet; *GP diet*: control diet + grape pomace 8%; *GSE diet*: control diet + grape seed extract 0.1%; *AME*: apparent metabolizable energy. *DM*: dry matter.

[a] Vitamin-mineral mix supplied the following per kilogram of diet: vitamin A, 8250 IU; cholecalciferol, 1000 IU; vitamin E, 11 IU; vitamin K, 1.1 mg; vitamin B12, 12.5 g; 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; delquin, 125 mg; DL-Met, 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.

Table 3. Identification of phenolic compounds in plasma and thigh meat of chickens fed different diets

	No.	Compound	Sample	Formula	$[M-H]^-$	Identification	Error (ppm)	MS/MS ions	Reference
Flavanols and derivatives	**1**	Catechin	P	C15H14O6	289.0718	STD	-0.13		
	2	Epicatechin	P	C15H14O6	289.0718	STD	-0.13		
	3	Epicatechin gallate	P	C22H18O10	441.0820	STD	1.63		
	4	(Epi)catechin Sulfate	P	C15H14O9S	369.0286	MS/MS PUB	-0.06	289, 245	(*19*)
	5	(Epi)catechin Sulfate	P	C15H14O9S	369.0286	MS/MS PUB	-0.06	289, 245	(*19*)
	6	Methyl(epi)catechin-O-sulfate	P	C16H16O9S	383.0442	MS/MS PUB	0.07	303, 245	(*19*)
	7	Methyl(epi)catechin-O-sulfate	P	C16H16O9S	383.0442	MS/MS PUB	0.07	303, 245	(*19*)
	8	(Epi)catechin glucuronide	P	C21H22O12	465.1038	MS/MS PUB	0.11	289, 203	(*19*)
	9	(Epi)catechin glucuronide	P	C21H22O12	465.1038	MS/MS PUB	0.11	289, 203	(*19*)
Benzoic acids and derivatives	**10**	Protocatechuic aldehide	P, T	C7H6O3	137.0244	STD	0.13		
	11	4-Hydroxybenzoic acid	P, T	C7H6O3	137.0244	STD	0.13		
	12	3-Hydroxybenzoic acid	P, T	C7H6O3	137.0244	STD	0.13		
	13	Salicylic acid	P, T	C7H6O3	137.0244	STD	0.13		
	14	2,4-Dihydroxybenzoic acid	P, T	C7H6O4	153.0193	STD	0.21		
	15	Gallic acid	P, T	C7H6O5	169.014	STD	1.45		
	16	Vanillic acid-4-O-sulfate	P, T	C8H8O7S	246.9928	MS/MS PUB	-4.04	167	(*35*)
	17	n-Dihydroxybenzoic acid	P	C7H6O4	153.0193	MS	0.21		
	18	Homovanillic acid sulfate	P	C9H10O7S	261.0066	MS	3.23		
Phenylpropionic acids and derivatives	**19**	3-(4-Hydroxyphenyl)propionic acid	P	C9H10O3	165.0557	STD	0.11		
	20	3-(3-Hydroxyphenyl)propionic acid	P, T	C9H10O3	165.0557	STD	0.11		
	21	DL-3-Phenyllactic acid	P, T	C9H10O3	165.0557	STD	0.11		
	22	3,4-Dihydroxyphenyllactic acid methyl ester	P	C10H12O5	211.0609	MS	1.4		
Cinnamic acids and derivatives	**23**	p-Coumaric	P, T	C9H8O3	163.0401	STD	-0.20		
	24	Ferulic acid	P, T	C10H10O4	193.0506	STD	0.17		
	25	Ferulic acid-4-O-sulfate	P	C10H10O7S	273.0089	MS/MS PUB	-5.3	193	(*35*)
Valerolactones	**26**	Dihydroxyphenyl-gamma-valerolactone sulfate	P	C11H1207S	287.0238	MS/MS PUB	-2.44	207, 163	(*16*)
Phenols and derivatives	**27**	4-Hydroxyphenylacetic acid	P	C8H8O3	151.0401	STD	-0.21		
	28	2-Hydroxyphenylacetic acid	P	C8H8O3	151.0401	STD	-0.21		
	29	Catechol-O-sulfate	P, T	C6H6O5S	188.9863	MS/MS PUB	0.09	109, 79	(*35*) (*36*)
	30	Ethylcatechol sulfate	P, T	C8H10O5S	217.0176	MS/MS PUB	0.08	137, 122	(*37*)
	31	4-Methylcatechol-O-sulfate	P, T	C7H805S	203.0020	MS/MS PUB	-0.16	123	(*35*)
Others	**32**	Vanillyl alcohol sulfate	P	C8H10O6S	233.0110	MS/MS PUB	6.55	214, 153	(*36*)

P: identified in plasma samples
T: identified in thigh meat samples
STD: Identification by comparing mass spectra and retention time to available standard.
MS/MS PUB: Tentative identification by prediction of chemical formula from exact mass and confirmation by comparing fragmentation mass spectra with literature.
MS: Tentative identification by prediction of chemical formula from exact mass. Not confirmed by comparing fragmentation mass spectra- generic fragmentation conditions were used.

Table 4. Plasma concentration of phenolic metabolites that showed statistically significant differences between diets (ng/mL).

		Concentration in plasma (ng/mL)		
	Metabolite	**C diet**	**GP diet**	**GSE diet**
Flavanols and derivatives	**Catechin**	n.d.	2.42±1.42^{a}	1.51±1.15^{a}
	Epicatechin	n.d.	1.70±0.85^{a}	1.51±1.01^{a}
	Catechin glucuronide	n.d.	0.45±0.32^{a}	0.22±0.19^{a}
	Catechin Sulfate	n.d.	2.14±0.98^{b}	1.36±0.67^{a}
	Epicatechin gallate	n.d.	1.09±1.16^{a}	0.98±1.02^{a}
	Methylcatechin sulfate	n.d.	1.39±0.61^{b}	0.34±0.23^{a}
	(Epi)catechin sulfate	n.d.	6.62±2.28^{b}	2.85±1.00^{a}
	Methyl(epi)catechin sulfate	n.d.	0.84±0.20	n.d.
	(Epi)catechin glucoronide	n.d.	1.81±0.91^{b}	0.53±0.14^{a}
Benzoic acids and derivatives	**3-Hydroxybenzoic acid**	13.5±4.45^{a}	21.7±3.83^{b}	17.3±13.6^{b}
	Salicylic acid	7.95±3.00^{a}	15.5±7.82^{b}	11.9±4.44^{a}
	n-Dihydroxybenzoic acid	110±37.1^{ab}	99.4±33.4^{a}	179±60.8^{b}
	Gallic acid	7.99±5.60$^{a(4/6)}$	39.2±19.2^{b}	25.8.7±20.5^{b}
Phenylpropionic acids and derivatives	**3-(4-Hydroxyphenyl)propionic acid**	13.2±1.72^{a}	67.8±24.6^{b}	60.0±20.0^{b}
	3-(3-Hydroxyphenyl)propionic acid	9.00±7.23^{ab}	44.6±9.60^{c}	23.7±26.6^{b}
Valerolactones	**Dihydroxyphenyl-gamma-valerolactone sulfate**	n.d.	2.56±1.05$^{a(3/6)}$	5.08$^{a(1/6)}$
Phenols and derivatives	**Catechol-*O*-sulfate**	565±366^{a}	773±435^{ab}	107±84.8^{b}
	Ethylcatechol sulfate	41.1±17.0$^{a(5/6)}$	14.1±2.19$^{b(5/6)}$	107±84.8^{a}
	4-Methylcatechol-*O*-sulfate	8.38±4.75^{a}	14.5±6.98^{a}	41.7±31.8^{b}
	4-Hydroxyphenylacetic acid	94.5±27.8^{a}	186±47.2^{b}	182±58.9^{b}
Others	**Vanillyl alcohol sulfate**	n.d.	7.68±2.36^{b}	6.38±4.77^{b}

C diet: control corn-soybean diet; *GP diet*: control diet + grape pomace 8%; *GSE diet*: control diet + grape seed extract 0.1%. Each value is the mean of 6 samples (or x when indicated as x/6) per dietary treatment ± Standard Deviation. Different letters in the same row (a, b, c) indicate significant differences ($P<0.05$) after application of non-parametric Mann-Whitney test.

Table 5. Effect of dietary supplementation of grape pomace (GP) and grape seed extract (GSE) on plasma (µg/mL) and thigh meat (µg/g) alpha and gamma tocopherol content.

		C Diet	GP Diet	GSE Diet
Plasma (µg/mL)	**Alpha-tocopherol**	10.57±1.52[a]	15.24±2.11[b]	14.08±2.96[b]
	Gamma-tocopherol	0.83±0.09[a]	1.31±0.22[b]	1.13±0.31[ab]
Thigh (µg/g)	**Alpha-tocopherol**	8.96±3.24[a]	8.09±2.83[a]	8.16±2.29[a]
	Gamma-tocopherol	0.77±0.26[a]	0.66±0.27[a]	0.64±0.12[a]

C diet: control corn-soybean diet; *GP diet*: control diet + grape pomace 8%; *GSE diet*: control diet + grape seed extract 0.1%.
Each value is the mean of 6 samples per dietary treatment ± standard deviation. Different letters in the same row (a, b, c) indicate significant differences (P<0.05) after application of one-way analysis of variance (ANOVA) followed by Tukey's multiple comparison test.