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Antioxidant effects of ryegrass phenolics in lamb liver and plasma

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Sixteen lambs were divided into two groups and fed two different diets. Eight lambs were stall-fed with a concentrate-based diet (C), and the remaining eight lambs were allowed to graze on Lolium perenne *(G). The antioxidant status was measured in the liver and plasma samples before and after solid-phase extraction (SPE) to probe the antioxidant effects that grass phenolic compounds may have conferred onto the animal tissues. The liver and plasma samples from grass-fed lambs displayed a greater antioxidant capacity than the tissues from C lamb group, but only if samples had not been passed through SPE cartridges. Finally, the feed and animal tissues, which had been purified by SPE, were analysed by liquid chromatography combined with mass spectrometry (LC–MS) to identify phenolic compounds present in* L. perenne *and to evaluate the results from the antioxidant assays. It would appear that the improvement of the antioxidant capacity of lamb liver and plasma from lambs fed ryegrass was not related to the direct transfer of phenolic compounds from grass to the animal tissues.*

Keywords: phenolic, *Lolium perenne*, antioxidant, lamb, tissues

Implications

Feeding ruminants at pasture improves the antioxidant defences of the animal tissues. As phenolic compounds are natural antioxidants that occur largely in plants, we studied whether they can be transferred from herbage to the tissues of grazing lambs, and thus contribute to improving their antioxidant status. Grazing on *Lolium perenne* at pasture improved the antioxidant capacity of lamb liver and plasma. However, none of the phenolic compounds found in grass were detected in the lamb tissues. Therefore, it would appear that ryegrass phenolics had no direct effect on the animal antioxidant status compared with other antioxidant mechanisms associated with pasture feeding.

Introduction

The consumption of fresh herbage by ruminants can improve several quality traits of meat as compared with concentrate-based diets, with some of the most important ones being fatty-acid composition (French *et al*., 2000; Realini *et al*., 2004) and oxidative stability (Luciano *et al*., 2011a, 2012). In particular, the positive effect of pasture feeding on meat oxidative stability is attributed to the high content of antioxidant compounds in fresh herbage and to their deposition in the animal tissues. Yang *et al.* (2002) reported higher concentrations of α-tocopherol and β-carotene in the liver from pasture-fed compared with grain-fed cattle. Prache *et al*. (2003) found higher plasma carotenoid content in grass-fed than stall-fed lambs, and La Terra *et al.* (2010) showed that when cows were fed with increasing proportions of fresh forages in their diet, plasma α-tocopherol and β-carotene increased progressively. Finally, higher concentrations of vitamin E, carotenoids and ascorbic acid were extensively reported in the muscle from grass-fed compared with concentrate-fed animals (Descalzo and Sancho, 2008).

Phenolic compounds are widespread in the plant kingdom and their effectiveness as dietary antioxidants in animal feeding has received considerable attention in recent years (Vasta and Luciano, 2011). They are natural antioxidants that are ingested by pasture-fed animals and are believed to contribute towards an improved antioxidant status of the animal tissues (Wood and Enser, 1997). However, the bioavailability of dietary phenolic compounds in ruminants is still controversial. To date, only a few studies, in which animals were fed with high doses of phenolic compounds,

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showed that simple phenolics are partially bioavailable in ruminants. Gladine *et al.* (2007) detected monomeric phenolic compounds in the plasma of sheep receiving a ruminal infusion of galloylated condensed tannins or simple phenolics from grape or rosemary extracts. Moñino *et al.* (2008) reported that the muscle from lambs raised on milk from ewes fed with a rosemary-enriched concentrate contained several of the phenols from rosemary (i.e. rosmarinic acid, carnosol and carnosic acid), whereas other phenolics, that is, flavonoids, which were also present in rosemary extract, were not detected in the lamb tissues. Recently, we found improved antioxidant capacity of the liver and plasma from lambs supplemented with quebracho-condensed tannins; however, no phenolic compounds could be detected in these tissues (López-Andrés *et al.*, 2013). These studies suggest that only some dietary phenolic compounds are bioavailable in ruminants. It seems that the possible antioxidant mechanism of these phenolic molecules in the animal tissues is still unclear. To the best of our knowledge, no studies have attempted to assess whether polyphenols naturally occurring in common forages, when ingested, are delivered to the animal tissues.

In the light of the above, the aim of this study was to investigate whether phenolic compounds or their metabolites can be detected in the liver and plasma from lambs raised on concentrate feeds or on a ryegrass (*Lolium perenne*) pasture. To achieve this objective, feeds as well as liver and plasma samples were subjected to solid-phase extraction (SPE) to purify and concentrate possible phenolic compounds before LC–MS analysis. Besides, the antioxidant capacity of the SPE-treated and SPE-untreated liver and plasma samples was measured to assess, if possible, dietary effects on the antioxidant status that could be directly related to the presence of phenolic compounds in the tissues.

Material and methods

Animals and diets

The animal tissues used in this experiment were sourced from lambs used in a study aimed at assessing the effect of restricting the daily grazing duration on meat quality. Therefore, details about results pertaining to animal performances and meat quality can be found elsewhere (Luciano *et al.*, 2012; Vasta *et al.*, 2012). Briefly, Merinizzata Italiana lambs were assigned at 90 days of age to one of the two groups of eight animals each (average body weight: 16.0 kg ± s.d. 2.15 kg). One group (G) was allowed to graze every day from 0900 to 1700 h on a perennial ryegrass (*L. perenne*) sward. At the end of the day, the lambs were penned indoors as a group without receiving any feed supplementation but with *ad libitum* access to water. Herbage intake at pasture was estimated twice (on days 15 and 65) by equipping each lamb with a harness (as described by Avondo *et al.*, 2002). The average herbage dry matter (DM) intake measured for lambs in the G group was 485 g/day (Vasta *et al.*, 2012). The lambs in the other group (C) were raised in stalls for the duration of the trial and were fed a commercial pelletted concentrate comprising, on a DM basis, 38.4% barley, 16.7% maize, 30.0% alfalfa, 13.0% soya bean meal and 1.9% mineral–vitamin premix. The concentrate contained 50 mg/kg DM of α-tocopheryl acetate and 20.000 IU vitamin A/kg of DM. All animals were weighed weekly and the rations for the C group were adjusted to achieve comparable growth rates to those of the lambs in the G group. Water was always available in the pens.

Samples of concentrate feeds and herbage were collected during the trial, vacuum-packaged and stored at −30°C for analyses. On the last day of experimental feeding, after overnight fasting, blood samples were taken from the jugular vein of each lamb and collected in heparin tubes. Blood samples were centrifuged at 3000 × **g** for 20 min at 4°C and plasma was stored at −80°C. After 72 days of experimental feeding, lambs were slaughtered and the liver was immediately collected, vacuum-packaged and stored at −30°C for analyses.

Preparation of feed samples and purification of phenolic compounds by SPE

The extraction of phenolic compounds from feeds and their purification with SPE were performed as described by López-Andrés *et al.* (2013). Feeds (2.5 g) given to both C and G groups were finely chopped (<1 mm) and weighed into 50 ml centrifuge tubes. Samples were homogenised with 15 ml of acetone/water (70/30; v/v) for 60 s at 4000 r.p.m. using a Heidolph Diax 900 tissue homogeniser (Heidolph Elektro GmbH & Co. KG, Kelheim, Germany). Samples were then sonicated for 6 min (with a break of 2 min after the first 3 min of sonication) using a Bandelin Sonoplus HD2070 sonicator (cycle: 4 × 10%, power: 0.31%). Samples were kept in a water/ice bath during both homogenisation and sonication procedures. The sonicated homogenates were centrifuged at 3000 × **g** for 15 min at 4°C using a centrifuge tube (model IEC CL31R; Thermo Fisher Scientific, Milan, Italy). Then the supernatants were filtered through Whatman No. 541 filter paper before SPE purification. Phenolic compounds were isolated from feed samples on a reversed phase C18 Sep-Pak Vac 6 cc (500 mg) cartridge (WAT043395, WATERS SpA, Milan, Italy). Before use, cartridges were conditioned with methanol followed by distilled water. The filtered supernatant (10 ml) was acidified with 0.5M-H_2SO_4 before loading onto the cartridge to disrupt polyphenol–protein binding, as explained in Juan *et al.* (2010). Phenolic compounds were eluted with 2 ml of methanol; this final eluate was kept in a freezer at −30°C. As shown by López-Andrés *et al.* (2013), these procedures of extraction and SPE purification resulted in an efficient recovery of phenolic compounds with losses of about 13%.

Preparation of the lamb tissues and purification of phenolic compounds by SPE

The extraction of phenolic compounds from the liver and plasma and their purification by SPE followed the procedure described by López-Andrés *et al.* (2013). The liver (5 g) was weighed into 50 ml centrifuge tubes. Preparation steps of the liver samples and SPE purification of the liver extracts were carried out in the same way as for feed samples. For the liver

samples, phenolic compounds were eluted with 3 ml ethyl acetate. The collected eluate was evaporated to dryness under nitrogen and then dissolved in 3 ml of methanol and kept at −30°C.

For the plasma samples, 500 μl of the plasma was acidified with glacial acetic acid and applied to a reversed phase C18 Sep-Pak 1 cc (100 mg) cartridge (WAT023590, WATERS SpA). Before use, the cartridges were conditioned with methanol followed by distilled water. The acidified plasma sample was completely loaded onto the cartridge followed by 1 ml of distilled water. Phenolic compounds were eluted with 2 ml of methanol. Aqueous ascorbic acid (10 μl; 15%, w/v) was added to the final eluate to avoid any possible oxidation in the plasma samples. This eluate was evaporated to dryness under nitrogen and then dissolved in 3 ml of methanol and stored at −30°C.

Antioxidant status of the liver and plasma samples

Liver and plasma antioxidant status was determined with the FRAP (ferric reducing antioxidant power) and the Folin–Ciocalteu assays. Both assays were applied to samples either treated with SPE (SPE samples) or not treated with SPE (RAW samples).

Folin–Ciocalteu assay of RAW samples. For the preparation of RAW liver for the Folin–Ciocalteu assay, 2 g of the liver was placed into 50 ml centrifuge tubes and homogenised with 10 ml of distilled water. Homogenisation, sonication, centrifugation and filtration steps were performed as described above. A 1 : 4 dilution of the extract (3 ml of distilled water added to 1 ml of liver extract) was chosen. The assay was performed as described by Luciano *et al.* (2011b). Briefly, 100 μl of the diluted RAW-liver extract was transferred into 15 ml centrifuge tubes and 900 μl of distilled water were added. The Folin–Ciocalteu reagent was diluted to 1 N and 500 μl were added to the tubes followed by 2.5 ml aqueous solution of sodium carbonate (20%; w/v). The mixture was vortex mixed for 30 s and incubated for 40 min in the dark at room temperature. The samples were centrifuged at 2700 × **g** for 10 min at 4°C to remove any sodium carbonate precipitates. A double-beam spectrophotometer (model UV-1601, Shimadzu Corporation, Milan, Italy) was used to measure the absorbance of the samples at 725 nm. A vial containing all reagents, except the tissue extract, was used as blank. Aqueous solutions of gallic acid were used for the calibration curve, which covered 0 to 80 μg/μl of gallic acid. The results were expressed as mg of gallic-acid equivalents (GAE)/g of liver.

For the RAW-plasma samples, 100 μl of the plasma were transferred to 15 ml centrifuge tubes and diluted with 900 μl of distilled water. The Folin–Ciocalteu assay was carried out as described for the RAW-liver samples. The results were expressed as mg of GAE/ml of plasma.

Folin–Ciocalteu assay of SPE-treated samples. SPE-treated liver and plasma samples were subjected to the Folin–Ciocalteu assay as follows. The purified extract obtained after the SPE step was evaporated to dryness under nitrogen and then dissolved in 1.5 ml of methanol/distilled water (1/1; v/v). The SPE-liver samples (500 μl) were transferred into a 15 ml centrifuge tubes and 500 μl of methanol/distilled water (1/1; v/v) was added. Then the Folin–Ciocalteu assay was performed as described above for RAW liver. Solutions of gallic acid in methanol/distilled water (1/1; v/v) were used for the calibration curve.

The SPE-plasma samples were obtained as described above; however, ascorbic acid had not been added to these samples to avoid its interaction with the Folin–Ciocalteu reagent (Georgé *et al*., 2005). The SPE-plasma extracts were similarly evaporated to dryness under nitrogen and then dissolved in 3 ml of methanol/distilled water (1/1; v/v). An aliquot of this sample (500 μl) was placed into a 15 ml centrifuge tube and 500 μl of methanol/distilled water (1/1; v/v) was added. The Folin–Ciocalteu assay was performed as described above for RAW-liver samples. The results were expressed as mg of GAE/ml of plasma.

FRAP assay of RAW samples. The method described by Luciano *et al.* (2011b) was used for measuring FRAP values. Briefly, for RAW-liver samples, the liver (0.5 g) was extracted 10 ml of distilled water. Homogenisation, sonication, centrifugation and filtration steps were performed as described above. Liver extract, distilled water and FRAP reagent (74 μl, 220 μl and 3.0 ml, respectively) were mixed and incubated in a water bath at 37°C for 4 min, and then the absorbance was recorded at 593 nm. The assay was calibrated using standard $FeSO_4{\cdot}7H_2O$ solutions and results were expressed as μmoles of Fe^{+2} equivalents/g of the liver.

For the RAW-plasma samples, 74 μl of plasma, 220 μl of distilled water and 2.2 ml of FRAP reagent were added into a glass test tube. Tube contents were mixed and incubated for 4 min at 37°C. The absorbance was immediately recorded at 593 nm. Results of the FRAP assay were expressed as μmoles of Fe^{+2} equivalents/ml of plasma as explained for the liver samples.

FRAP assay of SPE samples. The same method described above was also applied to the SPE samples. However, as the SPE samples were dissolved in methanol/distilled water (50/50; v/v), the same solvent was used instead of distilled water for this assay. Methanol/distilled water was also used to prepare the standard $FeSO_4{\cdot}7H_2O$ solutions. The assay was performed by adding in a glass test tube 200 μl of the SPE-liver samples or SPE-plasma samples (without ascorbic acid) to 200 μl of methanol : distilled water and 3 ml of FRAP reagent. Incubation time, absorbance measurement and calculations were performed as described above.

Identification of phenolic compounds in feed and tissue extracts by LC–MS

The feed and lamb tissue extracts were isolated and then analysed by HPLC–MS as described by López-Andrés *et al.* (2013), using an ACE 5 2.1 × 150 mm C18 column (Hichrom Ltd, Theale, Berkshire, UK) fitted to an Agilent 1100 liquid chromatograph with diode array detector. A binary mobile

phase system was used where solvent A was HPLC-grade water + 0.1% formic acid and solvent B was HPLC S-grade acetonitrile + 0.1% formic acid (Rathburn Chemicals Ltd, Walkerburn, UK). A solution (5 µl) of a catechin standard (10 ng/µl) was first injected separately with each batch of samples to check system integrity and performance.

Statistical analysis
To test the effects of the SPE method and of the dietary treatment, data from the Folin–Ciocalteu and FRAP assays were analysed using the MIXED procedure of SAS (version 9). The model adopted was: $Y_{ijk} = \mu + SPE_i + Diet_j + (SPE_i \times Diet_j) + lamb_k + \varepsilon_{ijk}$: where μ is the overall mean, Y_{ijk} is the dependent variable, SPE_i is the effect of the SPE purification treatment (RAW *v.* SPE samples), $Diet_j$ is the effect of the dietary treatment (G *v.* C), $(SPE_i \times Diet_j)$ is the interaction between the SPE treatment and the dietary treatment, $lamb_k$ is the random effect of the individual lamb and ε_{ijk} is the error.

Multiple comparisons of the least squares means (LSM) were made using the Tukey's adjustment.

Results

Folin–Ciocalteu assay of lamb tissues
As shown in Table 1, the SPE purification procedure strongly reduced the values of the Folin–Ciocalteu assay in both the liver and plasma ($P < 0.0005$). The dietary treatment had an effect in tendency on the values of the Folin–Ciocalteu assay performed in the liver samples, whereas no significant effect was found in plasma samples ($P = 0.056$ and 0.322, respectively; Table 1). However, in both the liver and plasma samples, there was a Diet × SPE interaction ($P = 0.050$ and 0.030, respectively; Table 1). This showed that, in both the liver and plasma, the effect of the dietary treatment on the Folin–Ciocalteu values was dependent on the SPE purification treatment of the samples. Indeed, the Folin–Ciocalteu values of samples that had *not* been SPE-treated (i.e. RAW samples) were higher in the liver of pasture-fed lambs compared with the tissues of concentrate-fed animals (+13.13%, $P = 0.029$). Data of the Folin–Ciocalteu values of RAW-plasma samples had a higher variability (higher pooled standard error; PSE) compared with RAW-liver samples (Table 1). Nevertheless, a trend similar to that observed in the liver was evident for RAW plasma, with the Folin–Ciocalteu values being higher in tendency in samples from lambs in the G group compared with the C group (+4.27% $P = 0.088$). Conversely, when the liver and the plasma samples were treated with SPE, the Folin–Ciocalteu assay showed no differences between the experimental treatments (Table 1).

FRAP assay of the lamb tissues
Table 1 also shows that the SPE purification procedure strongly reduced the FRAP assay values in both the liver and plasma ($P < 0.0005$). Dietary treatment had a significant effect on the values of the FRAP assay performed in the liver and plasma samples ($P = 0.001$ and 0.009, respectively; Table 1). Furthermore, in both the tissues, the Diet × SPE interaction was significant ($P = 0.001$ and 0.014; Table 1). This showed that, in both the liver and plasma, the effect of the dietary treatment on the FRAP values was affected by SPE treatment of samples. Indeed, as shown in Table 1, the values of the FRAP assay performed on the RAW samples were much higher in both the liver and plasma from pasture-fed lambs compared with the tissues from concentrate-fed animals (+20.03%, $P < 0.0005$ for the liver samples and +12.27%, $P = 0.002$ for the plasma samples). However, when the liver and plasma samples had been treated with SPE, the FRAP assay showed no difference between the experimental treatments (Table 1).

Analysis of feed samples and animal tissues by LC–MS
Representative chromatograms of grass and concentrate diets are given in Supplementary Figure S1. Under the LC–MS

Table 1 *Effect of the dietary treatment (C = concentrate; G = grass) and of the SPE treatment on the Folin–Ciocalteu and FRAP values of the liver and plasma samples (least square mean values and PSE)*

	RAW samples		SPE samples			*P*-values[1]		
	C	G	C	G	PSE	Diet	SPE	Diet × SPE
Number of lambs	8	8	8	8				
Liver								
Folin–Ciocalteu[2]	1.409^{b}	1.622^{a}	0.012^{c}	0.012^{c}	0.051	0.056	<0.0005	0.050
FRAP[3]	5.309^{b}	6.639^{a}	0.062^{c}	0.065^{c}	0.020	0.001	<0.0005	0.001
Plasma								
Folin–Ciocalteu[2]	1.614^{a}	1.686^{a}	0.078^{b}	0.048^{b}	0.152	0.322	<0.0005	0.030
FRAP[3]	0.786^{b}	0.896^{a}	0.119^{c}	0.120^{c}	0.018	0.009	<0.0005	0.014

SPE = solid-phase extraction; FRAP = ferric-reducing antioxidant power; PSE = pooled standard error; GAE = gallic-acid equivalents.
[1] *P*-values of the test of the fixed effects performed with the MIXED procedure statistical analysis. The fixed effects tested were dietary treatment (Diet; C or G), SPE treatment (SPE; RAW samples or SPE samples) and their interaction (Diet × SPE).
[2] Expressed as mg of GAE/g of the liver, or as mg of GAE/ml of the plasma.
[3] Expressed as mmol of Fe^{2+} equivalents/g of the liver, or as mmol of Fe^{2+} equivalents/ml of the plasma.
[a,b,c] Within a row, different superscript letters indicate significant differences between least squares means ($P < 0.05$) tested using the Tukey's adjustment for multiple comparisons.

Table 2 *LC–MS analysis of grass diet (see Supplementary Figure S1)*

Peak number	RT (min)	*m/z* (M-H$^-$) Observed	*m/z* (M-H$^-$) Calculated	Formula	Proposed assignments[1]
1	10.8	755.2110	755.2022	$C_{33}H_{40}O_{20}$	Kaempferol-3-*O*-glucosyl-rhamnosyl-galactoside
2	11.2	563.1438	563.1397	$C_{26}H_{28}O_{14}$	Kaempferol-xylosyl-rhamnoside
3	11.7	609.1506	609.1451	$C_{27}H_{30}O_{16}$	Rutin
4	12.0	593.1549	593.1502	$C_{27}H_{30}O_{15}$	Kaempferol-3-*O*-rutinoside
5	12.5	489.1054	489.1031	$C_{23}H_{22}O_{12}$	Kaempferol-3-*O*-acetyl-glucoside
6	12.7	285.0430	285.0401	$C_{15}H_{10}O_6$	Kaempferol

LC–MS = liquid chromatography–mass spectrometry.
[1]Although not confirmed by other means, it is likely that the flavonols are glycosylated at carbon 3.

conditions used in the present study, phenolic compounds should elute between 5 and 13 min (Wright *et al.*, 2010). In grass samples, only a few peaks and with relatively low intensity were observed in the range where phenolic compounds tend to elute (see Supplementary Figure S1). These compounds were flavonols and most were present as glycosides (Table 2). The mass *m/z* (M-H$^-$) of 755.211 ($C_{33}H_{45}O_{20}$) could be assigned to kaempferol-3-*O*-glucosyl-rhamnosyl-galactoside, 563.1438 ($C_{26}H_{28}O_{14}$) to kaempferol-xylosyl-rhamnoside, 593.1549 ($C_{27}H_{30}O_{15}$) to kaempferol-3-*O*-rutinoside and 489.1054 ($C_{23}H_{22}O_{12}$) to kaempferol-3-*O*-acetyl-glucoside. The *m/z* value (M-H$^-$) at 285.043 was assigned to kaempferol and *m/z* (M-H$^-$) 609.1506 to rutin, which is a glycoside of quercetin. None of these compounds could be detected in LC–MS chromatograms from the concentrate diet.

The liver and plasma extracts, which had been treated with SPE, were analysed for the same phenolic compounds as found in the grass diet or for possible metabolites arising from their degradation. No masses corresponding to phenolic compounds could be detected in the liver or plasma samples from the grass-fed lambs (see Supplementary Figures S2 and S3). Similarly, liver and plasma chromatograms from the concentrate-fed lambs also contained no detectable phenolic compounds.

Discussion

Several previous studies have demonstrated that pasture feeding can positively affect the antioxidant status of the animal tissues. For instance, Descalzo *et al.* (2007), found that the muscle from the cattle raised on pasture had a higher overall antioxidant status, in terms of FRAP values, than meat from the grain-fed animals. Furthermore, using the same lambs in the present study, Luciano *et al.* (2012) showed an improved oxidative stability of the muscle from lambs raised on pasture. In agreement with these observations, in the present study, we found that the total antioxidant status (FRAP and Folin–Ciocalteu values) of the liver and plasma samples, which had not been subjected to SPE purification (RAW samples), were increased by pasture feeding. It is known that diets based on the consumption of herbage at pasture result in much higher intakes of natural antioxidants and can also positively affect the endogenous antioxidant defences of the animal tissues compared with conventional concentrate-based diets (Descalzo and Sancho, 2008). Both FRAP and Folin–Ciocalteu assays measure the overall antioxidant status and are not specific for particular antioxidants, but react with a wide spectrum of antioxidant compounds (Benzie and Strain, 1996; Georgé *et al.*, 2005). Therefore, the improved FRAP and Folin–Ciocalteu values found here in the RAW samples from grass-fed animals simply suggest a positive effect of pasture on the animal antioxidant status, which is likely to be due to pasture feeding improving several different antioxidant defences and thus confirming previous findings.

However, the specific objective of the present study was to assess whether phenolic compounds present in ryegrass could contribute to improving the antioxidant status of the liver and plasma of grazing lambs. Therefore, we have used the FRAP and Folin–Ciocalteu assays also for the liver and plasma samples, which had been subjected to an SPE purification treatment to selectively isolate and purify phenolic compounds, while removing other interfering compounds (López-Andrés *et al.*, 2013). This explains the much lower FRAP and Folin–Ciocalteu values found in the SPE-treated liver and plasma extracts compared with the RAW samples. Interestingly, after the SPE purification treatment, differences in the overall tissue antioxidant status between pasture- and concentrate-fed lambs disappeared, as similar Folin–Ciocalteu and FRAP values of the liver and plasma were found between G and C lambs. On the basis of these results, it would appear that the increased antioxidant capacity of the liver and plasma consequent to pasture feeding was not because of the phenolic compounds that might have been transferred from grass to the animal tissues.

To further test our hypothesis, we first analysed the composition of phenolic compounds in the herbage grazed by the animals. We then analysed lamb liver and plasma samples for the same compounds and for possible metabolites. Ryegrass (*L. perenne*) is a member of the *Poaceae* family and is one of the most important pasture grass species for animal feeding (Cai *et al.*, 2011). There is surprisingly little information available on the polyphenols that are present in this plant. Several studies analysed simple phenolic acids in ryegrass (Hartley and Jones, 1976, 1977) and two recent reports also described the presence of flavonoids (Tu *et al.*, 2010; Qawasmeh *et al.*, 2012). As plants belonging to the *Poaceae* family appear to have a narrower range of polyphenolic compounds than plants belonging to the *Fabaceae* family (Harborne, 1967;

Marais *et al*., 2000; Regos *et al*., 2009; Reynaud *et al*., 2010), therefore it would be interesting to evaluate whether feeding of forage legumes would lead to larger antioxidant effects in the lamb tissues.

The chromatogram of the grass sample (Supplementary Figure S1a) showed only few peaks and with relatively low intensities between 5 and 13 min. These compounds were identified mainly as kaempferol glycosides (Table 2), which occur in many plants (Chopin and Dellamonica, 1988). Tu *et al.* (2010), recently identified several flavonols in *L. perenne*, including some of the compounds identified in the present study, that is, kaempferol-3-*O*-rutinoside, rutin and kaempferol-3-*O*-glucosyl-rhamnosyl-galactoside. However, these authors did not report the presence of kaempferol, kaempferol-*O*-acetyl-glucoside and kaempferol-xylosyl-rhamnoside found in the present study (Table 2).

It is likely that when *L. perenne* is ingested phenolic compounds are metabolised first in the rumen, then in the liver and finally they may be excreted as conjugated compounds in urine (Martin, 1982). Indeed, it is known that some phenolic acids of forage cell walls can be degraded to hippurate, which is excreted in the urine, and this compound has been used as an indicator of detoxification load (Foley *et al*., 1999). It is also known that absorbed, low molecular weight phenolics, such as orcinol and quinol, can contribute to the digestible energy content of the diet without necessarily contributing to its nutritional value (Iason and Murray, 1996). Although it has been reported that dietary flavonoids (quercetin, genistein, catechin) are absorbed by humans (Paganga and Rice-Evans, 1997; Day *et al*., 2001; Manach *et al*., 2005), no phenolic compounds or any of the above metabolites could be detected, in our study, in the liver or plasma from lambs fed the grass diet. Three possible explanations may be offered to explain these findings. First, the phenolic compounds from ryegrass were not absorbed in the digestive tract of lambs or they were quickly excreted in the urine. Indeed, the bioavailability of these polyphenols in ruminants is as yet unknown and, in monogastric animals, it has been reported that most dietary polyphenols are quickly eliminated in both the urine and bile after ingestion (Scalbert *et al*., 2002). Second, flavonoids from grass could have been absorbed in the gastrointestinal tract, but had already been excreted by the time the sampling was carried out. In the present study, the blood and liver samples were taken at least 12 h after the last grass feeding and no information is available on the turnover of polyphenols in ruminants. Third, the concentration of phenolic compounds in *L. perenne* was too low for appreciable deposition into the animal tissues.

However, it is noteworthy that that the results of the present study are not directly comparable to those of other authors who studied the bioavailability of dietary phenolic compounds in ruminants and reported that some phenolic compounds were transferred from the diet to the animal tissues. For example, Gladine *et al.* (2007), administered a single acute dose of plant extracts (rosemary, grape, citrus or marigold) directly into the rumen of sheep. The authors found that, after administration, epicatechin was detected in the plasma of animals dosed with grape extract, whereas naringenin was detected in the plasma of lambs dosed with citrus extract. However, some phenolic compounds commonly found in rosemary (rosmarinic acid) or in marigold (vanillic, caffeic, chlorogenic, p-coumaric and ferulic acids) were not detected in the plasma of sheep. A study by Moñino *et al.* (2008) found that the muscle of lambs receiving the milk of ewes fed with a rosemary-rich concentrate contained some of the phenols that were present in the diet of the ewe. However, of the numerous phenolic compounds present in rosemary leaves, only three were actually found in lamb meat (rosmarinic acid, carnosol and carnosic acid). Clearly, these plant extracts and aromatic plants contained relatively higher concentrations and different phenolic compounds (Zheng and Wang, 2001) than ryegrass.

Conclusions

In conclusion, the results of this study confirm the positive effect of a pasture-based diet on the overall antioxidant status of ruminant tissues compared with a concentrate-based diet. However, it was not possible to attribute this effect to the transfer of phenolic compounds from herbage to the animal tissues, as suggested by the fact that when the liver and plasma extracts were concentrated to isolate phenolics, there was no difference in the overall antioxidant capacity between the pasture- or concentrate-fed animal tissues. Furthermore, with the analytical procedures adopted in the present study, none of the phenolic compounds present in *L. perenne* or their metabolites could be detected in the liver or plasma samples from grass-fed lambs. Therefore, it appears that the improvement of the tissue antioxidant status in lambs consequent to a grass-based diet could not be linked to the intake of polyphenols from grass. Therefore, the phenolic compounds present in *L. perenne* appear to have a negligible impact on the animal antioxidant status relative to other antioxidant mechanisms induced by grass feeding. Further studies are needed to understand the bioavailability of flavonoids and to identify their role in the global antioxidant status of ruminants. Furthermore, it would be of interest to study the possible antioxidant effects of a wide range of dietary polyphenols, especially if present at high concentrations in other commonly used forages, such as forage legumes.

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Supplementary material

To view supplementary material for this article, please visit http://dx.doi.org/10.1017/S1751731113001821

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