EFFECT OF GRAZING ON SAINFOIN OR ALFALFA DURING LACTATION ON LAMBS' MUSCLE METABOLITES. A HPLC-ESI-QTOF MS APPROACH

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Abstract – Polyphenols are increasingly used in animal feeding in order to delay the lipid oxidation of meat and, eventually, increase the deposition of beneficial compounds for the consumer. Interestingly, the biological activities of polyphenols go beyond their widely known antioxidant capacity, being able to modify several physiological pathways. Therefore, the aim of this study was to evaluate the effect of grazing on sainfoin or alfalfa - legumes rich in polyphenols but with different amount of proanthocyanidins – during lactation in the metabolite profile of lambs' muscle, by using HPLC-ESI-QTOF MS analysis. Thirteen metabolites were modified in the polar fraction of muscle in Sainfoin lambs, as compared with Alfalfa lambs (P<0.05). Among them, 6 were related to fat metabolic fate (biliary acids, fatty acids, etc.), showing the wide variety of processed affected by polyphenols. Further studies should evaluate the effect of these strategies on the whole metabolome of lamb muscle, in particular considering their health implications.

Key Words – animal nutrition; metabolomics; metabolic fate

I. INTRODUCTION

There is an increasing interest for the incorporation of antioxidants into the diet of ruminants in order to obtain meats with high resistance to lipid oxidation and, eventually, with high concentrations of beneficial compounds for the consumer. Within this frame, polyphenols are a promising class of natural antioxidants, which have been successfully incorporated in the feeding of different animals [1]. Nevertheless, the biological activities of polyphenols go beyond their antioxidant capacity, being able to modify several physiological pathways [2]. Therefore, in order to properly evaluate the activity of polyphenols their effect on the whole metabolome profile should be assessed. Sainfoin (*Onobrychis viciifolia*) and alfalfa (*Medicago sativa*) are both legumes of high nutritive value for ruminants. They are rich in polyphenols, but Sainfoin has an important proportion of the class of proanthocyanidins [3], whereas Alfalfa has a low proportion [4].

Therefore, the aim of this study was to evaluate the effect of grazing in sainfoin or alfalfa during lactation, before weaning and fed concentrate in the metabolite profile of lamb's muscle, by using HPLC-ESI-QTOF MS analysis.

II. MATERIALS AND METHODS

II.I. Animal study

Twenty pairs ewe-lamb were randomly assigned to 1 of 2 feeding systems during lactation. Half of the pairs grazed on alfalfa and the other half grazed on sainfoin until the lambs reached 12-14 kg. Then lambs were weaned and stalled indoors, receiving a concentrate-based diet until slaughter at 22-24 kg. After slaughter, carcasses were kept at 4 ºC during 24 h in total darkness. Then a sample of the *Longissimus thoracis and lumborum* muscle was obtained from each carcass. Samples were vacuum-packed and kept at -80 ºC until analyses. Before the extraction, samples were lyophilized and minced through a 0.5 mm sieve.

II.II Sample preparation

A solid-liquid extraction, followed by purification by SPE (Solid Phase Extraction) was carried out in the samples in order to isolate the most polar fraction of muscle, expected to contain specially polyphenol metabolites [5]. Briefly, ascorbic acid, catechol (as internal standard) and phosphoric acid were added to the freeze –dried sample. This

mixture was subjected to three successive extractions with water/methanol/phosphoric acid 94:5:1. After centrifugation, the supernatant was processed by SPE Oasis HLB cartridges (Waters, Milford, MA, USA) previously activated with methanol and water. The washing step was performed with water and acetic acid, while elution was carried out with the extraction mixture described above. The eluates were concentrated with nitrogen, filtered at 0.45 µm and transferred to HPLC vials.

II.III Metabolites analysis

The extracts were subjected to HPLC-ESI-QTOF MS analysis (Agilent 1200 series LC coupled to a QTOF MSD 6530A, Agilent, Santa Clara, CA, USA) according to a procedure previously described [6,7] with slight modifications. Briefly, a C18 column (Luna, 50 × 2.1 mm i.d., 3.5-µm particle size, Phenomenex, Torrance, CA, USA) was employed, a gradient with 0.1% aqueous formic acid and 0.1% formic acid with acetonitrile was carried out and signals were detected in negative ionization mode up to m/z 1,000.

HPLC-MS signals were processed with the MassHunter software (Agilent, Qualitative Analysis, B30.0). For identification, exact mass was collected for all signals detected and the suggested molecular formulas with error below 10 ppm were stored. Molecular mass and formula were then searched in scientific databases [8-11]. For quantitative analysis, since the aim was to compare metabolite ranges in the Alfalfa and in the Sainfoin diet, peak areas were calculated and divided by the area of the internal standard. Results are expressed as mean value ± SEM.

II.IV Statistical analysis

Levene's test and the Kolmogorov–Smirnov test were applied to assess variance equality and normal distribution, respectively. One-way ANOVA followed by Tukey's post hoc significance test was used when the assumptions of normality and equal variance were met. Otherwise, non-parametric tests (Kruskal–Wallis and Mann–Whitney U rank-sum) were used to assess significance. Differences were considered significant at $P < 0.05$. All statistical analyses were performed using the statistical package SPSS IBM version 22 for Windows.

III. RESULTS AND DISCUSSION

A total of 25 ions were detected in the muscle samples. Thirteen of them were present at different concentrations in Alfalfa and Sainfoin meat ($P<0.05$), so further analysis was focused in these ions. The areas of the 13 ions detected in each group are shown in Table 1, ranked by molecular weight.

Table 1. Area[1] (mean ± standard error) of the metabolites detected at different concentrations in lamb muscle after grazing in Alfalfa or Sainfoin with their dams during lactation ($P < 0.05$).

ID	$[M-H]^-$ exp	Alfalfa	Sainfoin
1	112.9909	1.368 ± 1.964	n.d.
2	131.0352	n.d.	0.229 ± 0.478
3	161.0454	n.d.	0.043 ± 0.881
4	187.0976	0.214 ± 0.154	0.077 ± 0.048
5	225.1498	0.008 ± 0.007	n.d.
6	261.1348	0.015 ± 0.014	n.d.
7	329.2337	0.049 ± 0.081	n.d.
8	407.2811	n.d.	0.037 ± 0.069
9	549.2691	n.d.	0.013 ± 0.015
10	723.5036	0.433 ± 0.471	n.d.
11	909.4842	0.756 ± 1.696	n.d.
12	921.4439	n.d.	0.044 ± 0.049
13	933.3712	0.557 ± 0.852	n.d.

n.d., non detected; [1]Results are expressed as compound area/internal standard area

Among these 13 metabolites, 7 were only detected in the Alfalfa lambs, 5 were only detected in the Sainfoin lambs and only 1 was detected in both diets. Therefore, grazing sainfoin or alfalfa caused significant metabolic modifications as shown by significant differences in the metabolites present in the lambs' muscle.

An effort was made to identify the nature of these compounds, based on their exact molecular weight. Possible identities were assigned to compounds 2-8, as shown in Table 2.

Table 2 Tentative identification of compounds modified in lambs' muscle, in lambs grazing in Alfalfa or Sainfoin with their dams during lactation

ID	Compound	Molecular formula	Error (ppm)
2	2-C-Methyl-1,4-erythrono-D-lactone, dimethylmalonic acid, glutaric acid, 2-acetolactate	$C_6H_{12}O_3$	-1.51
3	2- or 3-Hydroxyadipic acid	$C_6H_{10}O_5$	1.06
4	2,4 Dimethylpimelic acid	$C_9H_{16}O_4$	-1.75
5	Aromatic compound	$C_{13}H_{22}O_3$	-0.72
6	Phaseolic acid (5,8(9),12-Trihydroxy-2-oxododecanoic acid)	$C_{12}H_{22}O_6$	-1.75
7	9,10,13-TriHOME	$C_{18}H_{34}O_5$	-1.17
8	Biliary acid	$C_{24}H_{40}O_5$	-2.04

Although sainfoin and alfalfa have a relevant polyphenol content, and sainfoin in particular of proanthocyanidins [3], none of the modified signals corresponded to metabolites of these compounds. This may be due either to the absence of these compounds in the samples or to their presence below the limit of detection. Both hypotheses are plausible, since information on the accumulation of polyphenol in tissues, especially in the long-term is still quite limited [12]. It has to be taken into account that since ewes ingested the forages, metabolites of polyphenols would be transfer to the lambs through the milk and after digestion and absorption, the possible metabolites would be deposited in the muscle of the lambs [13]. However, grazing in sainfoin clearly had an effect on physiological metabolism, since 6 out of the 7 suggested compounds belonged to metabolic routes of fats. In particular, the detected compounds are involved in many metabolites routes, such as 9,10,13-TriHOME, reported to participate in the synthesis of prostaglandins [11]. The biological relevance of the changes observed in this study remains to be elucidated. Additionally, an aromatic compound was detected, with molecular mass and formula similar to aromatic structures described in wine or fruits [14, 15].

It should be remarked that these data correspond exclusively to the analysis carried out in the polar fraction of muscle and after performing MS analysis in negative mode- this analytical approach was selected in order to improve the detection of polyphenol metabolites. Therefore, it would be expected that grazing in sainfoin caused more modifications in the rest of the metabolome, i.e, compounds present in the polar fraction but detected with positive mode as well as in the whole of metabolites present in the apolar fraction.

IV. CONCLUSION

Grazing in sainfoin of ewe and lamb as compared grazing in alfalfa during lactation induced metabolic modifications, as shown by significant differences in the metabolites present in the polar fraction of lambs' muscle, detected by HPLC-ESI-QTOF MS analysis (negative mode). In particular, modifications in fats metabolic fate were observed. Despite the relevant proanthocyanidin content in sainfoin, none derived metabolite was detected. Further studies should evaluate the effect of this feeding system during lactation on the whole metabolome of lamb muscle, in particular considering their health implications.

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