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Condensed tannin changes along the digestive tract in lambs fed with sainfoin pellets or hazelnut skins

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Abstract

The variable anthelmintic efficacy of condensed tannins (CT) against gastrointestinal nematodes may depend on CT concentration, composition or fate along the digestive tract. We analyzed CT concentration and composition by acetone-HCl-butanol and thiolysis coupled to HPLC-MS in digesta and feces of lambs. Lambs had been infected with *Haemonchus contortus* and *Trichostrongylus colubriformis* and received sainfoin pellets and hazelnut skins of contrasting prodelphinidin/procyanidin ratios. The digesta and feces had lower CT concentrations than the original feeds, but similar concentration patterns across the digestive compartments. The changes in assayable CT concentrations between rumen, abomasum and small intestine may be due to complex formation between CT and other dietary components. However, the large CT disappearance (61-85%) from feed to feces could also indicate that CT may have been structurally modified, degraded or absorbed during digestion. Interestingly, there were no changes in the structural features of assayable CT in the digesta.

Keywords: condensed tannins, nematode, *Onobrychis viciifolia*, *Corylus avellana*, flavan-3-ols, acetone-HCl-butanol, thiolysis, HPLC-MS

Introduction

Tannins are polyphenolic plant compounds and can confer beneficial effects on animal nutrition and health, with anthelmintic (AH) effects being of particular interest.[1,2] Therefore, tannin-containing resources represent a model to explore the concept of nutraceuticals for controlling gastrointestinal nematodes in ruminants.[1] Proanthocyanidins or condensed tannins (CT) are oligomeric or polymeric flavan-3-ols, where (epi)catechin and (epi)gallocatechin are the most widespread subunits and these give rise to procyanidin (PC) and prodelphinidin (PD) tannins, respectively. In addition, a few plants also contain CT with galloylated flavan-3-ol subunits.[3-5]

It is often assumed that many of the positive effects of CT in terms of animal health and nutrition are based on their protein binding capacity and possibly also on their antioxidant activities.[2,6] Formation of CT-protein complexes is thought to cause a shift from urinary to fecal N-excretion, but with a few CT-containing diets this shift can also lead to better dietary protein utilization and, therefore, animal production.[5,7,8] In addition, dietary CT can also decrease ruminal methanogenesis[9-11] and exert anthelmintic activities.[1,2,12,13]

Our interests focus on the anthelmintic (i.e. antiparasitic) activity of CT against gastrointestinal nematodes both *in vitro* and *in vivo.*[1,13] Although some *in vitro* and *in vivo* results suggest that CT act via a dose-dependent anthelmintic response,[14-18] CT quantity is not always related to anthelmintic activity.[19,20] Indeed, recent evidence indicates that CT structural compositions are important for understanding their anthelmintic activities against parasites from cattle,[21] small ruminants[22] and pigs.[23] Of particular interest are polymer size in terms of mean degree of polymerization (mDP) and the composition of monomeric flavan-3-ol subunits (*i.e.* PD/PC ratio), which can modulate their anthelmintic effects.

Recent evidence from both *in vitro* and *in vivo* studies suggests that anthelmintic effects vary against gastrointestinal nematode species[24] and depend on whether they inhabit the abomasum or the small intestine. Variations with regard to gastrointestinal nematode species have been described *in vitro*. For example, Moreno-Gonzalo et al.[18,25] evaluated the anthelmintic effect of heather (Ericaceae) extracts on the exsheathment process of *T. circumcincta, H. contortus* and *T. colubriformis* infective L3 larvae using the larval exsheathment inhibition assay (LEIA). The EC_{50} results showed a higher susceptibility for the intestinal *T. colubriformis* than for the two abomasal species.

On the other hand, the effects on gastrointestinal nematodes seem to depend also on the local conditions related either to the host species and/or the local digestive conditions, *e.g.* whether the worms inhabit the stomach or the small intestine. For example, experimentally infected sheep showed a strong anthelmintic effect with quebracho CT against two intestinal species (*Nematodirus battus* and *Trichostrongylus colubriformis*) in terms of lower adult worm burden and female fecundity; however, there was no anthelmintic effect against two abomasal species (*Teladorsagia circumcincta* and *Haemonchus contortus*).[14] In contrast, the same CT (*i.e.* quebracho) fed to goats reduced the *T. colubriformis* worm burden and *H. contortus* fecundity but there were no changes for *T. circumcincta.*[26,27]

To explain these variations against gastrointestinal nematodes, two hypotheses can be proposed: i) anthelmintic activity stems from a species-specific response or ii) there are differences in CT activity along the digestive tract and the local environmental conditions (*e.g.* pH).[28,29]

For example, with regard to the first hypothesis, when purified CT fractions from 15 different plants were evaluated *in vitro* with the LEIA, Quijada et al.[22] observed that nematode species

showed different *in vitro* susceptibilities to CT since lower EC_{50} were recorded for *H. contortus* (more susceptible) than *T. colubriformis*. This also depended on the CT composition. Namely, anthelmintic activity against *H. contortus* (an abomasal species) could be linked to two structural features, mDP-values and PD/PC ratios, whereas for the small-intestinal worm, *T. colubriformis*, only the PD/PC ratio was important. Similar findings on differences in susceptibility between abomasal and intestinal species have also been obtained *in vitro* with gastrointestinal nematodes of cattle.[20]

Up to now, very few studies have addressed the second hypothesis by measuring CT concentrations or activities along the ruminant gut,[28-30] and no study has compared the effects of CT quality along the gut. Therefore, the present study sought to evaluate the changes of two different CT types from sainfoin plant pellets and hazelnut skins during their passage along the digestive tract of sheep. This study focused i) on CT quantity (concentration) and ii) on CT quality (composition in terms of PD/PC ratios and mDP) in order to assess whether these could explain their *in vivo* anthelmintic activities in lambs, which were experimentally infected with *H. contortus* and *T. colubriformis*.

Materials and Methods

Trial site

The experiment was carried out at ENVT (National Veterinary School of Toulouse) in the southwest of France (43°35'59'' N, 1°22'41'' E). The facilities hosting the animals and trial performance met and was approved by the French ethical and welfare rules (*Comité d'éthique en expérimentation animale* agreement, *Science et Santé Animales SSA N° 115* of December 15, 2014). Each group was housed in experimental facilities with concrete floors that had separated boxes of ca. 12 m^2 each. All animals had ready access to water.

Animals

Twenty-seven 4-month-old lambs of Tarascon breed were used. They had been raised under helminth-free conditions and tests were negative for strongyle nematode infections (by McMaster technique according to Raynaud, 1970) before the start of the study. Diclazuril (Vecoxan®, 2.5 mg/mL, Lilly-France, Neuilly-sur-Seine, France) was used, twice at three weekly intervals, at the recommended dose of 1 mg/kg of live weight to prevent coccidian infection. The study was conducted indoors.

Infective larvae

The isolates of either *H. contortus* or *T. colubriformis* were susceptible to anthelmintics. The infective larvae (L3) were cultured from feces of monospecifically infected donor sheep. Larvae were recovered with the Baermann technique and then stored at 4 °C for 1 month (*H. contortus*) or 4 months (*T. colubriformis*).

Experimental design

On day 0 (D0), all lambs were orally infected with a single dose of 2000 L3 *H. contortus* and 2000 L3 *T. colubriformis*. They had access to *ad libitum* grass hay, mineral block and water and a ration of commercial (tannin-free) pellets. On day 21 (D21) after parasite infection was confirmed by fecal examination, the animals were allocated into three groups of nine lambs, based on experimental diets [hazelnut skin; sainfoin pellets; control (tannin-free) pellets]. The groups were balanced according to sex, live body weight (mean 29.19 ± 2.71 kg), packed cell volume (PCV% = 39.11 ± 2.38) and fecal egg counts (EPG = 1124.1 ± 370.8). From D24 to D28, lambs were allowed to adapt to their diets. During the experimental period (D28 – D57), the rations were adjusted once based on body weight (D34), to meet animal growth requirements. Therefore, from

D37 to D44 a second adjustment period was used for the three diets in order to reach an optimal intake level of the two CT-containing diets and to maintain isoproteic and isoenergetic levels in all groups. The condition of the animals was monitored on a daily basis after the infection by checking their feeding and movement behavior and by looking for diarrhea symptoms. Once a week the anemia level was measured (*i.e.* packed cell volume or hematocrit). None of the lambs got severely ill or died during the trial. All lambs were humanely sacrificed under anesthesia, by intravenous injection (3.6 g/lamb) of pentobarbital sodium (Doléthal®, 182.2 mg/mL, Vétoquinol S.A., Magny-Vernois, France) on day D57.

Experimental feeds

Lambs in the experimental group were allocated three different diets. The first group (hazelnut skin) received commercial feed pellets (tannin free) + hazelnut endocarps; the second group (sainfoin) was fed with sainfoin pellets; the third group was the control group and received only commercial, CT-free feed pellets (Passio Ovi Primeur®, Sud Ouest Aliment SOAL, France). During the whole study period (*i.e.* 57 days), all groups received a fattening (total mixed) ration diet, which was isoproteic, isoenergetic and balanced for Ca, P and the Ca:P ratio. Additionally, the two CT-diets (*i.e.* sainfoin pellets and hazelnut skin groups) were fed at equal CT concentrations.

Preparation of digesta and fecal samples

At necropsy, individual digesta samples were retrieved from five lambs (out of nine) per experimental group (*i.e.* sainfoin pellet; hazelnut skin; control). Whole digesta (200 mL) were taken directly from each organ, *i.e.* rumen, abomasum or small intestine (ileum) and fecal samples were

collected from the rectum. Each sample was transferred to a 500 mL container and stored at -20 °C.

The frozen digesta or feces were cooled to -40 °C (-0.5 °C/min) for 2 h (Cryotec, MUT PCCPLS1.5 001, France) and freeze-drying was carried out in two phases. Samples were first subjected to a progressive freeze-drying process using the following temperature and pressure program: -30 °C (0.1 °C/min, 0.1 mbar), then at -10 °C (0.2 °C/min, 0.3 mbar) for 19 h 45 min, and finally at -5 °C (0.2 °C/min, 0.15 mbar) until reaching -2 °C. The second phase started when samples had reached -2 °C. They were then kept at 20 °C with a pressure of 0.05 mbar for 15 to 20 h until dry. The freeze-dried digesta or feces were ground in a Retsch impeller SM1 cutting mill (Haan, Germany) to pass a 1 mm sieve and stored at -20 °C until CT analysis.

Condensed tannin analyses

Chemicals

Hydrochloric acid (37%, analytical reagent grade), acetone (analytical reagent grade), butan-1-ol (standard laboratory reagent grade), acetonitrile (HPLC grade), formic acid (HPLC grade), methanol (HPLC grade) were obtained from Fisher Scientific (Loughborough, UK); benzyl mercaptan (BM) from Sigma-Aldrich (Poole, UK), and ultrapure water (MQ H_2O) from a Milli-Q Plus system (Millipore, Watford, UK).

Tannin analysis by acetone-HCl-butanol assay

The acetone-HCl-butanol assay was described by Grabber et al.[31] and used with a slight modification as described.[28] All samples (sainfoin pellets, control pellets or hazelnut skin, digesta and feces) and a freeze-dried sainfoin sample, which served as an internal laboratory control, were run in triplicate with each batch of samples. . After adding the reagent (10 mL) to the samples (10

147 mg), the tubes were left at room temperature for 1 hour to check for the possibility of flavan-4-ol
148 or flavan-3,4-diol interference. The tubes were then heated at 70 °C for 2.5 hours in the dark. After
149 cooling to room temperature and centrifugation spectra were recorded between 450 and 650 nm on
150 a Jasco V-530 spectrophotometer (Jasco UK, Dunmow, UK). The acetone-HCl-butanol reagent
151 was used as a blank. The absorbance at the peak maximum was determined and converted to CT
152 concentration based on calibration curves derived from a purified prodelphinidin standard, isolated
153 from *Lespedeza cuneata* plants, for sainfoin samples and a purified procyanidin standard, isolated
154 from Tilia flowers, for hazelnut samples.[22] The CT concentration was reported as g CT/100 g on a
155 dry weight (DW) basis.

156 **Tannin analysis by thiolysis**

157 The thiolysis reaction was carried out as described previously.[32] The reaction products were
158 identified by HPLC-MS analysis[23,28] and quantified based on peak areas at 280 nm using published
159 flavan-3-ol response factors against taxifolin.[3,32] This provided information on CT concentration
160 (% CT) and size (mean degree of polymerization, mDP), molar percentages of prodelphinidins
161 (PD) and procyanidins (PC) within CT, and molar percentages of *trans*- vs *cis*-flavan-3-ols (*trans*
162 and *cis*).[3] Samples were also analyzed for free flavan-3-ols, but none were detected.

163 **Statistical Analyses**

164 Non-parametric analysis (Kruskal-Wallis and Kolmogorov-Smirnov test) was applied to CT values
165 (CT concentration, mDP, PC, PD, *cis*, *trans*) per sample type (*i.e.* digesta or feces) as determined
166 by each CT assay (acetone-HCl-butanol or thiolysis) and flavan-3-ol terminal and extension units.
167 Comparisons were made between 1) the different diet treatments, and 2) the different segments of
168 the digestive tract within each diet treatment group. All statistical analyses were performed using
169 Systat® 9 software (SPSS Ltd).

Results

Condensed tannin concentrations in digesta and feces

According to the acetone-HCl-butanol assay, there were no differences ($P > 0.05$) in the CT-concentrations of sainfoin feed pellets and hazelnut skins, *i.e.* 6.5 and 5.1 g CT/100 g DW, respectively (Table 1). As expected the control pellets had no CT. Digesta and fecal samples had significantly lower CT concentrations than the feeds in both the sainfoin- and hazelnut-fed lamb groups (Table 1), *i.e.* from 1.0 to 2.1 g CT/100 g DW. For the lambs of the sainfoin group, these values represented reductions of 84.6 %, 67.7%, 72.4% and 69.2% and for the lambs of the hazelnut group, these CT losses were 78.5%, 66.7%, 76.5% and 60.8% for ruminal, abomasal, small intestinal and fecal samples, respectively. Overall, the CT concentrations showed similar patterns in both groups: slightly higher values were measured in the abomasal and fecal samples, and lower values in the ruminal or small intestinal samples. There were no differences in CT concentrations between the sainfoin and hazelnut groups ($P > 0.05$) but differences were found between the digesta or feces samples within each feed group ($P < 0.05$).

In contrast to the acetone-HCl-butanol assay, the thiolysis reaction gave quite different CT concentrations ($P < 0.01$) for the sainfoin pellets (1.7 ± 1.01 g CT/100 g DW) and hazelnut skins (6.3 ± 1.01 g CT/100 g DW) (Table 1). The sainfoin group had the highest CT value in the abomasal digesta (0.7 ± 0.1 g CT/100 g DW), and the hazelnut group in the abomasal and fecal samples (approx. 0.7 ± 0.1 g CT/100 g DW). Thus, apparent CT losses were 85.3%, 58.8%, 76.5% and 76.5% in the sainfoin group, and 92.1%, 88.9%, 93.7% and 88.9% in the hazelnut group in the rumen, abomasum, small intestine and feces compared to the diets, respectively. Differences were found for the CT concentrations measured by thiolysis between the two types of feeds and between the digesta and fecal sample within each feed-group ($P < 0.05$). No differences were recorded between

193 the feed groups when comparing the samples from the same organs ($P > 0.05$). Once, again thiolysis
194 also did not detect any CT in the samples from the control animals.

195 **CT structural features in digesta and feces**

196 Thiolysis also afforded information on the CT composition in terms of molar percentages of
197 prodelphinidins, procyanidins (or PD/PC ratios), *cis*- and *trans*-flavan-3-ols and mean degrees of
198 polymerization (Table 2). The CT in the sainfoin digesta and fecal samples had high percentages
199 of prodelphinidins (*i.e.* rumen 79.5, abomasum 84.1, small intestine 78.7, and feces 72.4%) and
200 *cis*–flavan-3-ols (*i.e.* rumen 87.9, abomasum 91.3, small intestine 87.5, and feces 88.9%), which
201 were similar to the original sainfoin pellets (*i.e.* PD 74.8 and *cis*–flavan-3-ols 85.3%). Due to the
202 low CT concentrations (Table 1), it was not possible to calculate the mDP values in these digesta
203 samples as the peaks of the terminal flavan-3-ol units were too small to be detected. In the hazelnut
204 group, the CT composition was also preserved: hazelnut skins, digesta and fecal samples had high
205 percentages of procyanidins, similar percentages of *cis*- and *trans*-flavan-3-ols and similar mean
206 degrees of polymerization (Table 2).

207 **Discussion**

208 This study was carried out to determine the changes in CT concentrations and compositions during
209 the transit of the sainfoin pellet and hazelnut skin diets in the digestive tract of lambs in order to
210 provide a basis for understating the anthelmintic effects of these diets. Our previous research
211 discovered that gastrointestinal parasites that reside in the abomasum tended to be more sensitive
212 to tannins (*i.e.* lower EC_{50}-values) than parasites that are found in the intestines.[22] Lambs were fed
213 with two diets that differed in CT compositions: sainfoin pellets had a high PD/PC ratio (75/25)
214 and hazelnut skins had a low PD/PC ratio (28/72). Samples were taken from along the digestive

tract to study CT concentration and compositional changes in the rumen, abomasum, small intestine (ileum) and feces and were compared with the feeds.

Given the absence of data on CT changes along the digestive tract, we decided to use two assays that employ different reagents and reaction conditions for the degradation of tannins: the acetone-HCl-butanol reaction uses harsher conditions and is carried out at 70 °C for 2.5 h with 5% HCl and 33% water, whereas the thiolysis reaction is milder and takes place at 40 °C for 1 h with <1% HCl in methanol. Previous studies demonstrated that the acetone-HCl-butanol assay can occasionally give higher CT concentrations than the thiolysis assay when plant materials are analyzed. [2,33,34]

Condensed tannin contents in digesta and feces

There are only a few studies so far that have evaluated changes in CT concentrations in small ruminants and these used a previous, less sensitive, version of the HCl-butanol assay.[29,30] One recent study also reported thiolysis results for CT concentrations and compositions in digesta from sainfoin-fed cattle, which had been infected with gastro-intestinal nematodes.[28] To the best of our knowledge, the current study, therefore, presents for the first time CT concentrations and composition in digesta and feces of lambs. The 60% to 80% decrease of CT concentrations (by acetone-HCl-butanol) from feeds to digesta or feces was comparable to the ^{14}C-labelled CT losses in sheep of 71.1 - 98.5%.[29] Similarly, large decreases in digesta or fecal samples were also described in post-rumen losses in sheep (85 – 86%) and goats (83%).[29,30]

The relatively mild conditions during thiolysis reaction compared to the acetone-HCl-butanol assay may not release all CT from the sample matrix.[32] In addition it has also been shown that some CT polymers are resistant to degradation with thiols,[34,35] which may explain the lower CT concentrations detected by thiolysis than by acetone-HCl-butanol in digesta and feces (Table

237 1).[28,33,36] Thiolysis also measured much lower CT concentrations than the acetone-HCl-butanol
238 method for the sainfoin pellets (1.7 vs 6.5 g CT/100 g DW) but surprisingly not for the hazelnut
239 skins (6.3 vs 5.1 g CT/100 g DW). The reason for this discrepancy is not clear and will need further
240 investigation; this finding also illustrates the need for using more than one analytical technique
241 when dealing with unusual matrices in order to probe the biological effects of CT.[2]

242 Despite these differences, both assays revealed a similar pattern (Table 1): the highest CT
243 concentrations were measured in the abomasal samples in both the sainfoin and the hazelnut groups
244 and also in the feces from the hazelnut lamb group. Interestingly, another study that fed sainfoin
245 pellets to cattle also found that CT concentrations were higher in the abomasum (acetone-HCl-
246 butanol: 5.8%; thiolysis 2.3%) than the rumen (acetone-HCl-butanol: 3.0%; thiolysis: 0.5%).[28] It is
247 well known that CT bind dietary Rubisco protein optimally at a pH that is close to neutral.[2] Thus,
248 we hypothesize that dietary proteins are complexed by CT in the rumen (pH 6-7) and released
249 under the acid conditions in the abomasum (pH < 3.5).[29] Indeed, the results support this
250 explanation: measured concentrations were highest in the abomasum (Table 1) and a possible
251 explanation could be that these CT were not complexed by proteins and thus remained more
252 accessible and reactive in both assays. In fact, Ramsay et al.[34] also noted that benzyl mercaptan in
253 the thiolysis reagent appeared to react preferentially with extractable rather than tightly bound CT.
254 The increased CT concetrations in feces could be due to the combined action of matrix digestion
255 plus bile acids and pH (> 7) that can disrupt CT-protein complexes.[30] However, there are also
256 numerous other matrix components with which CT can interact, such as carbohydrates, lipids and
257 intestinal mucosa[5,37-39] and further work will be needed to establish the interactions between CT
258 and dietary matrix components. Whilst thiolysis appears to preferentially detect extractable CT,[28,34]
259 the acetone-HCl-butanol assay appears better able to detect bound CT.[34]

However, these results also point to considerable CT modification or degradation in the digestive tract of sheep as pointed out previously with sheep, goat, cattle and pig feeding trials.[28,29,30,40] If CT were inert, CT concentrations would be expected to increase progressively throughout the tract as dietary matrix components are digested and only the undigestible and non-absorbed components would remain.[41] Mean dry matter digestibilities in sheep are 58% according to a meta-analysis[42] and, therefore, the CT concentration in feces of sainfoin-fed sheep should have been close to 15%. However, as we could only detect 2% by the acetone-HCl-butanol assay, it would appear that 87% of the CT could no longer be detected. A cattle study that used the same sainfoin diet and acetone-HCl-butanol assay estimated that ca 50% of the CT had disappeared.[28] Considerable losses of CT, 29% by thiolysis and 17% by acetone-HCl-butanol, were also reported after fermentation of silages[34] and from the human digestive tract, where the gut microflora caused extensive losses due to CT metabolism.[43]

CT structural features in digesta and feces

The CT compositions in Tables 2 and 3 of sainfoin, (mostly prodelphinidins), and hazelnut skins (mostly procyanidins), agree with literature reports.[32,44,45] Table 3 lists the monomeric subunits that give rise to prodelphinidins (gallocatechin and epigallocatechin) and to procyanidins (catechin and epicatechin). Once again, there were no significant changes in these flavan-3-ol compositions between the digesta and the sainfoin feed pellets. The molar composition of these flavan-3-ols decreased as follows: EGC > EC > GC > C, which was in line with the literature.[45] The flavan-3-ol compositions in the hazelnut skins and the corresponding digesta and fecal samples were also not significantly different (Table 3). However, ca. 5% of the subunits in the hazelnut skins were galloylated, *i.e.* epicatechin gallate (ECg) and epigallocatechin gallate (EGCg), but none of these

galloylated subunits could be detected in the digesta or feces, which indicated that the esterified gallic acid may have been cleaved from the CT either by esterases or acids in the gut.

It can be concluded that the CT compositional features of PD/PC and *cis/trans* ratios, mean degrees of polymerization, and molar percentages of individual flavan-3-ol subunits were preserved during the digestion in lambs. A similar conclusion was reached after examining the CT composition of ensiled sainfoin.[34] These results suggested that CT structures *per se* were not modified during fermentation and digestion - with the exception of esterified gallic acids, which appeared to be cleaved. However, the acetone-HCl-butanol assay measured CT reductions of up to 85% and thiolysis up to 94% in digesta and feces (dry weight basis) compared to the original feeds. These CT decreases suggested that there may be similar processes taking place in the ruminant digestive tract as in the colon of monogastric animals.[43,47,48] In addition, abomasal digesta samples tended to have the highest levels of assayable CT, which could be due to a matrix effect, as CT tend to bind less strongly at acid pH-values to most proteins.

These findings lend support to the hypothesis that CT activity is higher in the abomasum than the intestine, which could explain why CT are more effective against abomasal than intestinal parasite species.[49] However, our data do not provide support for a species-specific response to CT, despite such evidence from *in vitro* studies with *Haemonchus contortus* (an abomasal species) and *Trichostrongylus colubriformis* (an intestinal species).[22] Our results have now revealed that the CT flavan-3-ol subunit composition was preserved along the digestive tract, hence the higher *in vitro* biological activity of prodelphinidins can be expected to be maintained under *in vivo* conditions as long as the overall CT concentration remains sufficiently high.

Abbreviations Used

CT, condensed tannins; PD, prodelphinidins; PC, procyanidins; mDP, mean degree of polymerization; BM, benzyl mercaptan; C, catechin; EC, epicatechin; ECg, epicatechin gallate; EGC, epigallocatechin; EGCg, epigallocatechin gallate; GC, gallocatechin.

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Supporting information. Feed nutritional analyses results for each experimental group are shown in regard to composition, fiber content and nutrition values.

Author's contribution

JQ and HH designed and performed the animal experiments. IMH designed the chemical analyses. EG and REK helped in the animal experiment. JQ, HH and IMH analyzed the data and prepared the manuscript. CD, EG, REK contributed reagents, materials and analysis tools. All authors critically read and approved the final manuscript.

Competing interest

The authors declare that they have no competing interests.

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Table 1. Mean (±SD) Concentrations of Condensed Tannin (g CT/100 g DW) Measured Either With the Acetone-HCl-butanol or the Thiolysis Assays in Feeds, Digesta and Fecal Samples from Each Experimental Group (n= 5 lambs).

	Feed	Rumen	Abomasum	Small Intestine	Feces
Acetone-HCl-butanol assay					
Sainfoin pellets group	6.5±0.3^{a}	1.0±0.1^{b}	2.1±0.3^{c*}	1.8±0.3^{b}	2.0±0.4^{b}
Hazelnut skin group	5.1±0.1^{a}	1.1±0.1^{b}	1.7±0.2^{bc}	1.2±0.1^{bc}	2.0±0.3^{c*}
Thiolysis assay					
Sainfoin pellets group	1.7±0.1^{a**}	0.3±0.1^{b}	0.7±0.1^{c*}	0.4±0.1^{bc}	0.4±0.1^{b}
Hazelnut skin group	6.3±0.1^{a**}	0.5±0.1^{b}	0.7±0.1^{b}	0.4±0.1^{b}	0.7±0.1^{b}

** ($P < 0.01$) indicates significant differences between sainfoin pellets and hazelnut skin feeds; * ($P < 0.05$)

^{a,b,c} different superscripts within rows indicate significant differences depending on the digestive organs or feces; ± indicates standard deviations

Table 2. Condensed Tannin Compositions in Digesta or Fecal Samples from Lambs (n= 5) Fed with either Sainfoin Pellets or Hazelnut Skins.

	mDP	**PD/PC %**	***cis/trans*-flavan-3-ols *%***
Sainfoin pellets	11.5±0.3	74.8/25.2 (±0.5)	85.3/14.7 (±0.1)
Rumen	-	79.5/20.5 (±0.9)	87.9/12.1 (±0.7)
Abomasum	-	84.1/15.9 (±0.50	91.3/8.7 (±0.5)
Small intestine	-	78.7/21.3 (±1.1)	87.5/12.5 (±0.3)
Feces	-	72.4/27.6 (±1.6)	88.9/11.1 (±1.3)
Hazelnut skin	13.3±0.1	27.9/72.1 (±0.2)	58.4/41.6 (±0.2)
Rumen	14.8±0.7	34.3/65.7 (±1.5)	46.3/53.7 (±1.2)
Abomasum	13.9±0.3	33.4/66.6 (±0.7)	51.3/48.7 (±0.6)
Small intestine	13.8±1.2	33.4/66.6 (±1.7)	46.9/53.1 (±2.3)
Feces	13.2±0.3	18.9/81.1 (±2.4)	48.4/51.6 (±0.6)

Note: there were no significant differences between the different organs.

Abbreviations: mean degree of polymerization (mDP); % refers to molar percentages of procyanidins (PC), prodelphinidins (PD), *cis*- or *trans*- flavan-3-ols (*cis* or *trans*); ± refers to standard deviations

Table 3. Molar Percentages (%) of Terminal and Extension Flavan-3-ol Subunits within CT from Digesta and Fecal Samples Collected from Lambs that Had Been Fed with Sainfoin Pellets or Hazelnut Skins.

	Terminal units (%)				**Extension units (%)**					
	GC	EGC	C	EC	GC-BM	EGC-BM	C-BM	EC-BM	ECg-BM	EGCg-BM
Sainfoin pellets	2.4±0.1	1.8±0.1	1.9±0.1	2.7±0.1	9.5±0.3	61.2±0.5	0.9±0.1	19.7±0.3	0.0	0.0
Rumen	0.0	0.0	0.0	0.0	10.3±0.5	69.2±1.5	1.7±0.1	18.8±0.9	0.0	0.0
Abomasum	0.0	0.0	1.3±0.0	1.2±0.0	8.3±0.3	75.7±0.7	0.6±0.0	15.4±0.5	0.0	0.0
Small intestine	0.0	0.0	0.0±0.0	1.9±0.0	11.9±0.3	66.7±1.5	0.5±0.0	19.8±1.9	0.0	0.0
Feces	0.0	0.0	2.5±0.2	2.3±0.2	10.1±0.7	62.3±2.2	0.0	24.8±1.1	0.0	0.0
Hazelnut skins	0.0	0.0	7.5±0.1	0.0	12.1±0.1	15.1±0.1	21.2±0.1	39.4±0.3	0.8±0.1	3.9±0.1
Rumen	0.0	0.0	6.8±0.2	0.0	20.1±1.5	14.3±0.3	26.8±0.6	31.9±1.1	0.0	0.0
Abomasum	0.0	0.0	6.5±0.1	0.7±0.1	16.3±0.6	17.2±0.2	25.9±0.6	33.5±0.4	0.0	0.0
Small intestine	0.0	0.0	7.5±0.8	0.0	17.8±0.5	15.6±1.4	27.8±1.7	31.4±1.1	0.0	0.0
Feces	0.0	0.0	7.6±0.2	0.0	10.1±1.7	8.8±0.9	34.0±1.0	39.5±1.3	0.0	0.0

Abbreviations: Gallocatechin (GC), epigallocatechin (EGC), catechin (C), epicatechin (EC), epicatechin gallate (ECg), epigallocatechin gallate (EGCg), benzyl mercaptan adduct (-BM); ± refers to standard deviations

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