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Condensed Tannins in the Gastrointestinal Tract of Cattle after Sainfoin (*Onobrychis viciifolia*) Intake and their Possible Relationship with Anthelmintic Effects

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ABSTRACT

The fate of condensed tannins (CTs) along the digestive tract of ruminants is not well known and may account for the variable efficacy of CTs against gastrointestinal nematodes in different locations. Here, we analyzed sainfoin CTs in the digesta of cattle from two separate experiments. When using the acetone-butanol-HCl assay, the total CTs concentrations in the digestive tract were close to those in the diets (6.3 and 1.5% of DM in Expt. 1 and 2, resp.) indicating that CTs remained largely undegraded and unabsorbed. Yet with the thiolysis assay in Expt. 1, CTs concentration was much higher in the abomasum (2.3 ± 0.4 % of DM) compared with the rumen, small and large intestines, along with increases of mean size and percentage of prodelphinidins within CTs. This corroborates the anthelmintic efficacy reported only against *Ostertagia ostertagi* in the abomasum. In Expt. 2, no anthelmintic effect was observed against the larval exsheathment in the rumen, probably because the dietary level of CTs was too low. Overall, the level of CTs accessible to thiolysis in the gut appears to be critical for anthelmintic activity, which is favored under the acidic conditions of the abomasum.

KEY-WORDS: proanthocyanidins; diet; helminth parasite; *Ostertagia ostertagi*; *Cooperia oncophora;* digesta; feces

INTRODUCTION

The control of gastrointestinal nematodes in cattle still relies extensively on the use of anthelmintic drugs to reduce production losses and diseases.[1] As farmers may have to face increasing numbers of drug-resistant nematode populations in cattle,[2] the use of bioactive natural compounds with anthelmintic properties may help to achieve more sustainable parasite control. Research has focused on condensed tannins (CTs; syn. proanthocyanidins), especially those found in temperate forage legumes such as sainfoin (*Onobrychis viciifolia*), which is also recognized for its high feeding values.[3]

Belonging to the family of flavonoids (a group of polyphenols), CTs occur in plants as mixtures of flavan-3-ol polymers and are usually described by their mean degree of polymerization (mDP). In addition, each constitutive flavan-3-ol subunit can be characterized by the number of hydroxyl groups in both the A- and B-rings and the relative stereochemistry of the substituents on the C-ring (i.e. *cis*- (epi) or *trans*-configurations). Hence, the flavan-3-ols catechin and epicatechin have two OH groups adjacent to each other (on carbons 3 and 4) on the B-ring and give rise to procyanidins (PCs), while gallocatechin and epigallocatechin have three OH groups adjacent to each other (on carbons 3, 4 and 5) on the B-ring and give rise to prodelphinidins (PDs).

The bioactivity of CTs, which is mostly explained by binding to other macromolecules, such as proteins, but also to polysaccharides and lipids, can be greatly influenced by various factors such as the structural diversity of CTs, solution conditions and protein characteristics.[4] For instance, increases in tannin size (mDP) and PDs (molar percentage) have shown greater capacity to bind to proteins[4] and exhibit higher *in vitro* anthelmintic activities.[5-8] Moreover, CTs encounter a large variety of macromolecules and are subjected to various conditions (e.g. pH, temperature) from plant harvest to digestion, which can

45 determine the nature and strength of the interactions between CTs and proteins. In fact,
46 plant processing such as drying, pelleting or ensiling have been shown to increase the
47 fraction of protein-bound CTs[9,10] or partly degrade CTs.[11] Moreover, reversible interactions
48 (non-covalent links) were proposed for the formation of CTs-protein complexes in the
49 rumen at a favorable pH range of 5–7, and dissociation in the acidic abomasum, which
50 impedes CTs-protein complexation.[12] Also, the recovery by butanol-HCl of CTs from *Lotus*
51 *corniculatus* fed to sheep was low in the lower part of the digestive tract,[13] probably due to
52 irreversible interactions (covalent links) formed after the oxidization of polyphenols in such
53 alkaline conditions[12] or colonic fermentation.[14]
54 Studies in sheep have shown that CTs apparently are not absorbed.[13,15] However,
55 ruminant species may have different adaptations and tolerance to dietary CTs; secretion of
56 tannin-binding salivary proteins appears to be a putative defense mechanism,[16] and this
57 complicates the comparison of CTs effects across animal species. In regard of the
58 anthelmintic effects, CTs can be directly detrimental to the worms at various life stages.[17]
59 However, the anthelmintic activity of CTs may also vary according to the different hosts[18]
60 or gastrointestinal nematode species as shown *in vitro*.[7,8,19] Additionally, these nematodes
61 reside in different gut compartments, which may account for the reported variation in
62 anthelmintic activity. In fact, a greater effect against abomasal nematode species
63 compared with intestinal species has been noticed in feeding trials with sainfoin in
64 sheep[20,21] and cattle.[22] However, no studies have directly linked CTs concentrations and
65 structures along the gut with anthelmintic activity.
66 In this study, based on two separate experiments in which sainfoin was fed to cattle, we
67 aimed at 1) analyzing the concentrations and structural compositions of CTs in the feed
68 (dried pellets of cv. Perly in Expt. 1; silages of cv. Zeus and Esparcette in Expt. 2), the

digesta (rumen, abomasum, small and large intestines in Expt. 1; rumen in Expt. 2) and in feces, and 2) linking the results with parasitological findings from the same experiments. In Expt. 1 an overall anthelmintic effect resulting in a significant reduction of *Ostertagia ostertagi* counts by 50% in the abomasum was observed while there was no effect against *Cooperia oncophora* in the small intestine of young cattle, as previously described in details.[22] In Expt. 2 we studied the effect against the larval exsheathment in the rumen of fistulated cows.

To address this, we used two different analytical methods, namely, acetone-butanol-HCl and thiolysis, which depolymerize CTs without prior extraction and can provide complementary data. In fact, the acetone-butanol-HCl is a quantitative colorimetric assay that has been optimized for quantification of "total" CTs including free and bound CTs in fresh forages[23] and tends to give a higher color yield than the traditional butanol-HCl reagent. The thiolysis is less sensitive to CTs in fermented samples (e.g. silage), where it mainly detects "free" CTs.[24] In contrast to the acetone-butanol-HCl assay, thiolysis when coupled with HPLC-MS provides an insight into the structure of CTs in terms of subunits (flavan-3-ol) composition.

MATERIALS AND METHODS

Chemicals

Hydrochloric acid (36%, analytical reagent grade), acetone (analytical reagent grade), butan-1-ol (analytical reagent grade), methanol (HPLC grade) and formic acid were purchased from ThermoFisher Scientific Ltd. (Loughborough, UK). Ammonium iron (III) sulphate dodecahydrate was from Acros Organics Ltd (Geel, Belgium).

Feeding Trial with Calves Infected with Gastrointestinal Nematodes (Expt. 1)

This experiment was conducted in the fall 2013 at the Large Animal Facilities of University of Copenhagen, Tåstrup, Denmark, as a sub-project of a previous *in vivo* study of anthelmintic effects of dried pelleted sainfoin (third cut of pure-stand sainfoin cv. Perly) in cattle.[22] Briefly, fifteen 2–4 month-old Jersey male calves were divided into two groups and fed isoproteic and isoenergetic diets comprising ryegrass-clover hay in addition to either a commercially available concentrate (55–65% of the diet) [Group control (CO); n=6] or sainfoin pellets (90% of the diet in average; increasing to 96% during the last two days of the experiment) [Group sainfoin (SF); n=9]. The animals in each group were penned in subgroups of three, according to bodyweights, to avoid bullying behavior and to better estimate the feed consumption. The feed intake of each subgroup was recorded daily. Then, the calves were infected with 10,000 third-stage larvae (L3) of *O. ostertagi* and 66,000 L3 of *C. oncophora* after 16 days of feed adaption. The calves were euthanized 42 days post infection for recovery of worms and digesta samples. Immediately after evisceration, 50 mL plastic tubes (or 15 mL for the organs containing worms) were filled with digesta from the rumen, whole abomasum (poured into a bucket and mixed), whole small intestine (poured into a bucket and mixed), large intestine and feces from each animal and stored at –20 °C until use. Worms were recovered from the abomasum and small intestine as previously described.[22] Feed samples (500 g) were collected at the beginning of the study. The study was approved by the Animal Experiments Inspectorate, Ministry of Justice, Denmark (Ref. 2013-15-2934-00763).

Larval Exsheathment in the Rumen of Adult Cows (Expt. 2)

The second experiment was conducted in the spring 2014 at the Carus Research Facilities of Wageningen University & Research, The Netherlands. We assessed the effect of sainfoin silages on the exsheathment kinetics of infective third-stage larvae (L3) of *O. ostertagi* and *C. oncophora* in specially designed semi-permeable tubes placed into the rumen of fistulated cattle. Each test was performed between 0900 and 1200 h using three Holstein cows in late lactation or dry period and fitted with a rumen fistula. To prevent feed selection all diets were prepared as total mixed rations which were stored at 4 °C for 1–2 days prior to use. The feed was offered *ad libitum* and replaced twice daily with a new batch (0800 and 1600 h). First, we tested the larval exsheathment following feeding with a control diet (CT-free) containing grass silage, maize silage and concentrate. Secondly, we incorporated a mixture of sainfoin silages (80% cv. Zeus and 20% cv. Esparcette) which constituted 40% of the ration on DM basis for 3 days and performed another exsheathment test. Then, we increased the same mixture of sainfoin silages to 80% of the diet and performed L3 exsheathment tests after 1, 3 and 5 days. Sainfoin cultivars Zeus and Esparcette were separately grown, harvested (second cut) and ensiled as previously described.[25]

The L3 were obtained from feces cultured for 13 days at 20 °C, which were collected from donor calves mono infected with drug-susceptible isolates of *O. ostertagi* (ref label: OOSG10) and *C. oncophora* kindly provided by M. Fisher (Ridgeway Research Ltd., St Briavels, UK) and J. Demeler and G. von Samson-Himmelstjerna (Freie Universität Berlin, Germany), respectively. The batches of L3 were kept at 5 °C for 3 and 6 months, respectively, prior to use. Ensheathment was confirmed before inoculation. Approximately 200 L3 of each species were pipetted into a separate small plastic tube (3 × 1 cm) fitted

with nylon mesh (10 µm pore size) on both sides. The pore size corresponded to less than half of the width of L3 of these nematode species, which ensured that the L3 remained in the tube without perturbing the passage of rumen fluid. For each time point, one tube with L3 per nematode species was placed in a small nylon bag (40 µm pore size). The nylon bags were inserted in a net inside the rumen of each fistulated cow after 0, 40, 80, 120 and 160 min. To retain the net in the rumen juice at the bottom of the organ the net was connected to the fistula at one end and to a stainless steel weight at the other end. All bags were retrieved simultaneously after the last time point. Then, 100 L3 from each tube were placed on a slide and the exsheathment process was stopped by addition of Lugol solution (Sigma-Aldrich Ltd., NL). The L3 were observed under a microscope (×100) and counted as exsheathed when the larval sheath was broken or lost.

Moreover, pH and temperature of the rumen were recorded with a probe during all exsheathment tests. The pH-meter was calibrated each day prior to the test, using two calibration points: pH 7.0 and 4.0. Finally, we collected samples from four different places in the ventral and dorsal rumen sac, feces and all feed items on the last day of the trial and kept them at –20 °C until use. This experiment was approved by the Institutional Animal Care and Use Committee of Wageningen University & Research and executed in accordance with EU directive 2010/63/EU implemented by the Dutch legislation on the use of experimental animals.

Sample Preparation and CTs Analysis by Acetone-Butanol-HCl and Thiolysis Assays

The frozen samples of feed (only silage), digesta and feces were freeze-dried and all samples were ground (<1 mm). Then, the total CTs concentrations were analyzed using

the acetone-butanol-HCl method[23] with slight modifications as previously described.[25] Briefly, 10 mg of ground material was added in a glass tube in triplicate for every sample. To each tube, 10 mL of reagent was added, which contained 150 mg of ammonium iron (III) sulphate dodecahydrate, 3.3 mL of water, 5 mL of 12 M HCl, 42 mL of butan-1-ol and 50 mL acetone. The tubes were left for 1 h at room temperature and then heated at 70 °C for 2.5 h in the dark. The samples were then analyzed by spectrophotometry (V530 Spectrophotometer, Jasco, Dunmow, UK) by scanning between 450–650 nm. Purified CTs fraction of freeze-dried sainfoin was used for CTs calibration [CTs content=100%, assessed by liquid chromatography-mass spectrometry (LC-MS) after thiolysis].

In addition, *in situ* thiolysis was performed in duplicate according to Gea et al.[26] with slight modifications. In short, 200 mg of ground material was weighed into a screw-top glass tube and a reagent containing 2 mL of MeOH, 1 mL of 3.3% HCl in MeOH and 100 µL of benzyl mercaptan (BM) was added. The tubes were heated at 40 °C for 1 h under vigorous stirring. Then, 9 mL of 1% formic acid in water was added and the tubes were subsequently vortex mixed and centrifuged for 5 min before transfer to high performance liquid chromatography (HPLC) vials. The CTs analysis by HPLC and LC-MS was described in detail by Williams et al.[6] with taxifolin as an external standard. This provided data on the molar percentages of the different flavan-3-ol subunits of the CTs in terms of terminal and extension (BM-adduct) units. The results provide information on CTs concentration (g/100g dry matter), mean degree of polymerization (mDP), and molar percentages of PCs vs. PDs and *cis*- vs. *trans* flavan-ols subunits.[26,27]

Statistical Analysis

The statistical analyses were performed with R software (version 3.2). In Expt. 1, the replicated CTs concentrations were averaged for each sample. Thus, the mean CTs concentrations of digesta and feces of sainfoin fed calves (=experimental units), as analyzed by the acetone-butanol-HCl assay (n=9 calves) or thiolysis (n=8 calves), were compared using pairwise comparisons with Wilcoxon rank sum tests including sample type (rumen, abomasum, small intestine, large intestine and feces) and post-hoc Holm's test for multiple comparisons. The results for CTs structures were not subjected to statistical analysis due to low recovery of CTs in the small and large intestines. In Expt. 2, the effects of sainfoin on the larval exsheathment were analyzed with linear regression models run separately for each parasite species and included: response variable (% of exsheathed larvae in triplicates) and explanatory factors as fixed effects (diet and time point). The values of rumen pH and temperature were compared between diets by one-way ANOVA with Tukey post-hoc test. Effects were considered significant at $P < 0.05$.

RESULTS & DISCUSSION

Analysis of "Total" CTs in Feed and Digesta/Feces by Acetone-Butanol-HCl Assay

In Expt. 1, the total CTs concentration in the sainfoin pellets was 6.5 ± 0.2% of dry matter (DM) using the acetone-butanol-HCl method (Figure 1), corresponding to a dietary level of 6.3 ± 0.0% of DM after correction for a small proportion of feed without CTs. In calves of Group SF, CTs concentrations in digesta samples were lower in the rumen (mean% of DM ± SD; 3.0 ± 1.4; $P < 0.05$) and increased gradually along the digestive tract. The average values of CTs concentrations in the abomasum (5.8 ± 0.6% of DM), small intestine (6.2 ± 0.9% of DM) and large intestine (6.6 ± 1.1% of DM) were close to that found in the pellets,

and maximum values were found in feces (7.8 ± 1.4% of DM). No CTs were detected in any control feed or control digesta. In Expt. 2, CTs concentrations in sainfoin silages were low: 1.8 ± 0.05% and 2.5 ± 0.09% of DM for cv. Zeus and Esparcette, respectively. Thus, the dietary level of CTs was estimated to be 1.5 ± 0.0% of DM when the mixture of sainfoin silages constituted 80% of the ration. The CTs concentrations of rumen digesta or fecal samples from the 3 cows were on average 1.2 ± 0.3 and 1.8 ± 0.1% of DM, respectively, on day 5 with 80% sainfoin in the diet. Moreover, the increase in CTs concentration of the rumen and the feces was consistent for all animals.

Thus, the total CTs concentration was highest in the feces in both experiments. This was expected; while organic matter is digested in the intestinal tract, uncertainty remains regarding the extent to which CTs concentrations and compositions are affected in the gut of the different ruminant species.[28] The harsh reaction conditions in acetone-butanol-HCl (70 °C, 5% HCl, 2.5 h) are more likely to release free and most of the bound CTs from feed and digesta matrices. However, when we consider a realistic DM digestibility of 60%, we found that the average concentrations of CTs in feces should have been twofold higher in both experiments; therefore a large proportion of CTs was not accounted for in the current study with young and adult cattle. Possible reasons for CTs losses are microbial fermentation that lead to depolymerization into bioavailable oligomers or biotransformation.[14,29] These intestinal losses agree with reported CTs losses during silage fermentation.[24] In addition, CTs may be involved in reactions with digesta components that lead to covalent links at acid and alkaline pH values,[30] and these derivative products may not be detected by current analytical methods. Finally, there was no evident relationship between total CTs concentrations in the different gut compartments measured with the

acetone-butanol-HCl assay and anthelmintic activity against nematodes in these two separate experiments.

Analysis of "Free" CTs in Feed and Digesta/Feces in Expt. 1 by Thiolysis and LC-MS as Indicator of Anthelmintic Activity.

In this study, we have clearly established a relationship between the concentrations and compositions of "free" CTs, when using the thiolysis method, in various compartments of the gastrointestinal tract and anthelmintic activity against gastrointestinal nematodes in cattle fed with sainfoin.

Overall, CTs concentrations obtained by thiolysis were lower than those obtained by the acetone-butanol-HCl method and with a different pattern of CTs changes between gut compartments (Figure 1 and 2.A). In fact, CTs concentrations obtained by thiolysis (Figure 2.A) were much higher in sainfoin pellets (2.0 ± 0.0% of DM) and abomasum (2.3 ± 0.4% of DM) compared with other compartments. In contrast, mean levels in rumen and feces were below 0.5%, and CTs were only detected in the small intestine of four animals and in the large intestine of three. The higher level of assayable CTs in the abomasum is in accordance with the significantly higher anti-parasitic activity of this diet comprising mainly of sainfoin pellets against *O. ostertagi* compared to the control (mean worm burden ± SD: 1,331 ± 947 in Group SF versus 2,715 ± 894 in Group CO; $P<0.05$).[22] Conversely, the almost complete lack of CTs measured by thiolysis in the small intestine is linked to the lack of efficacy of sainfoin against *C. oncophora* (mean worm burden ± SD: 19,664 ± 22,496 in Group SF versus 22,447 ± 17,639 in Group CO; NS).[22] These findings support previous feeding trials with sainfoin in small ruminants, where *H. contortus*, residing in the abomasum, was generally more affected than intestinal species, e.g. *Cooperia curticei* and

257 *Trichostrongylus colubriformis*.[20,21] The conditions in the gut can vary from pH <3 to 8 and
258 appear to impact on the reactivity and thus recovery of "free" CTs after thiolysis (mild
259 reaction conditions; 40 °C, 1.1% HCl, 1 h). Thus, sainfoin CTs seemed to be bound to the
260 digesta matrix of the rumen and released from these CTs-macromolecule complexes in
261 the abomasum, which agrees with sainfoin CTs-protein complexes being stable only
262 between pH 3.5–7.0.[12] In the lower parts of the digestive tract, the presence of tightly
263 bound CTs could originate from irreversible reactions between CTs and feed matrix
264 components, digestive enzymes or gut microbes that lead to thiolysis-resistant complexes
265 at alkaline pH; more work is needed to identify these reaction products.[24] This study has
266 highlighted the difficulty of analyzing CTs in digested and fermented samples and the
267 results should be interpreted with caution as the number of CTs-containing samples was
268 limited for the small and large intestines. It is of interest that the inflamed conditions in the
269 abomasum of animal #4413, perhaps inducing a higher pH, resulted in noticeably different
270 CTs results, e.g. values in the abomasum were more than five SD away from the group
271 mean and considered as outliers (Figure 2). This inflammation was likely related to the
272 infection, edematous abomasitis as reported by Uzal et al.[31] and apparently happened at a
273 late stage of the study as no clinical signs were observed.
274 Further, the CTs compositional analysis showed highest levels of mDP and PDs in the
275 sainfoin pellets (mDP=11.1 ± 0.2 and PDs=81.3 ± 0.2%) and the abomasum (mDP=15.9 ±
276 1.0 and PDs=86.6 ± 0.5%). In all samples the molar percentages of *cis* flavan-3-ols were
277 within 74–85% (Figure 2.B-D). It can be seen that especially the larger PD-rich tannins
278 were released in the abomasum; and this is interesting because these tannin types tend to
279 be more difficult to extract.[24] In addition, the binding affinity of CTs towards
280 macromolecules is also positively correlated with mDP and PDs%,[4] thus confirming that

281 larger and PD-rich CTs were preferably bound in the rumen and released in the
282 abomasum (Figure 2.C-D). It is notable that mDP and PDs% are positively correlated
283 within sainfoin CTs.[5] An increase of these two structural parameters, as we observed in
284 the abomasum, has been linked to greater *in vitro* anthelmintic activity of CTs against
285 cattle nematodes.[5,8] Moreover, sainfoin CTs contain complex mixtures of flavan-3-ols,[32] as
286 illustrated by our findings, where all types of flavan-3-ol subunits were detected in
287 extension and terminal units in sainfoin pellets and most digesta/fecal samples (Figure 3).
288 Although the CTs composition can vary between different sainfoin accessions,[26]
289 epigallocatechin extension units tend to be the major flavan-3-ol unit in sainfoin CT.[24,33]
290 This was also evident in our sainfoin pellets and the samples from the abomasum (Figure
291 3.B). A greater anthelmintic activity of epigallocatechin as compared with catechin or
292 epicatechin was shown recently against cattle nematodes.[8] The importance of the CTs
293 composition on anthelmintic activity is now well recognized and was also highlighted in
294 studies with the warm season legume *Lespedeza cuneata*, which is particularly rich in
295 large PD-type CTs.[9,34] CTs have been shown to survive the acidic conditions of the human
296 stomach[14] and the present study found high mDP values for CTs in the abomasum. The
297 analytical techniques cannot provide information on whether some of the CTs were acid
298 cleaved in this organ, i.e. pH around 2 in the abomasum of parasite-free cattle.[35]
299 The exact mechanisms for the anthelmintic efficacy of the easily assayable CTs, i.e. not
300 tightly bound CTs,[24] in the abomasum remain to be uncovered. Most likely the acidic
301 environment of the abomasum facilitates the release of tightly bound CTs from complexes
302 within the digesta matrix and this enables better interactions with both the thiolysis reagent
303 and nematode proteins.[17] Indeed, Jones and Mangan[12] reported that the CTs-Rubisco
304 protein complex is unstable at pH ≤ 3 and, therefore, the abomasal conditions may allow

the CTs to exhibit their anthelmintic effects more readily. It is also of interest that heavy infections with abomasal nematodes are associated with higher pH values, which could in turn lower CTs activity. However, there are several factors that may influence the efficacy of CTs: i) the nematode cuticle is rich in collagen in particular at the adult stage,[36] and contains a high proportion of proline residues that favor interactions with CTs; ii) CTs are known to interact most strongly close to the isoelectric point of proteins,[37] which may differ between proteins from feeds, animals, and worms; iii) *O. ostertagi* adults are actively feeding and reside mainly in the mucus layer of the abomasum, thus, the reactivity of CTs may differ in the local micro-environment of the mucosa and the worm. It seems reasonable to assume that in our study (Expt. 1) abomasal pH was close to normal at the end of the trial, considering the low infection levels and the timing. In fact, the rise of abomasal pH seems to correspond with the emergence of nematodes from gastric glands, which can vary between nematode species, e.g. elevated pH was observed 20 days post infection with *O .ostertagi* in calves.[38] Although pH can reach neutral values in some cases,[38] the severity of such changes is likely related to the parasite load and will be transient. As an example, studies in sheep infected with *O. circumcincta* demonstrated that pH returns to normal within 25-30 days post infection.[39] It has also been suggested that this elevation of pH is directly induced by parasites through the release of chemicals, to increase their survival as they do not usually survive in acidic medium.[40] Despite the profound effect on worm numbers, the adult worms from the calves fed sainfoin in Expt. 1 showed only minor morphological changes (i.e. few aggregates and damage) by scanning electron microscopy as compared with worms isolated from calves fed a control diet.[22] In contrast, other *in vivo* studies with sainfoin[41] and *Lespedeza cuneata*[34] have reported pronounced damage of adult *H. contortus* (especially female worms). It is noteworthy that

the abomasum of the youngest calf, harboring the highest number of abomasal worms,[22] had a higher water concentration. This resulted in a much lower CTs concentration in the abomasum (g CTs/kg of wet digesta) with both analytical methods, whereas CTs concentration in DM varied only slightly.

Analysis of "free" CTs in Expt. 2 by Thiolysis and LC-MS and Kinetics of Larval Exsheathment in the Rumen

In accordance with the results of Expt. 1, the CTs concentrations were much lower when using the thiolysis method, i.e. 0.02 ± 0.0 and 0.67 ± 0.0% of DM for sainfoin silages of cv. Zeus and Esparcette, respectively. Processing such as pelleting[9] or ensiling[10] has shown to increase the percentage of bound CTs and this could explain the low recovery of CTs with thiolysis in sainfoin samples for both studies. However, we could only detect PC-type tannins in silage of cv. Zeus that was the main component of the diet, which were based on epicatechin as terminal and extension units with mDP of 4.0 ± 0.5. For silage of cv. Esparcette, measured terminal units were only of catechin and extension units were of all types but mainly epigallocatechin and epicatechin (summarized as PC%= 37 ± 0.1; *cis%*=88 ± 0.2; mDP=34 ± 1.9). These tannin features were also reflected in the rumen/fecal samples from the last day of the experiment, although with a large variation and low CTs concentrations (0.09 ± 0.06 and 0.14 ± 0.16% of DM in rumen and feces, respectively). Thus, PC-type tannins were found predominantly in the rumen and feces (70 ± 26 and 73 ± 31% of CTs, respectively), these PCs had *cis*-configuration (83 ± 12 and 84 ± 11% of CTs, respectively) and an mDP of 5.1 ± 1.7 and 6.6 ± 5.4, respectively. Moreover, terminal units were only of the PC-type (catechin and epicatechin) and

352 extension units were predominantly epigallocatechin and epicatechin in rumen and fecal
353 samples.
354 The exsheathment for *O. ostertagi* L3 occurred very rapidly, with 90–100% of the L3
355 exsheathed after 80 min of incubation in the rumen with the control diet (Figure 4.A), in
356 accordance with a previous study.[42] We have also confirmed *in vivo* that L3 exsheathment
357 of the intestinal species *C. oncophora* is triggered in the rumen of cattle, in a similar
358 manner as *O. ostertagi*. Although nematode species are usually thought to exsheath in the
359 organ just prior to the living site of the adult stage, *Cooperia* spp. seem to be an exception.
360 This was shown *in vitro* and *in vivo* for *C. curticei* in sheep,[43,44] and *in vitro* for *C.*
361 *oncophora* by using rumen digesta of sheep.[44] The inclusion of sainfoin silages even at the
362 highest level did not reduce the rate of larval exsheathment. Yet, the potency of CTs-
363 containing sainfoin silages on the larval exsheathment could not be conclusively evaluated
364 as the dietary level of CTs was apparently too low, i.e. 1.5% DM in the diet by the acetone-
365 butanol-HCl method and thiolysis only detected a marginal level of CTs.[45] A dose-
366 response effect in the exsheathment of *H. contortus* L3 was demonstrated in cannulated
367 sheep with fresh sainfoin containing 3.9% of CTs. At a concentration of 75–100% of
368 sainfoin in the diet a significant exsheathment delay was shown, whereas 25% dietary
369 sainfoin did not generate this effect.[46] To a lesser extent, other factors may also explain
370 some of the differences between our study and this previous study,[46] e.g. lower
371 accessibility of CTs in silage than in fresh sainfoin, different CTs structures due to sainfoin
372 accession, higher ruminal pH, and shorter length of CTs exposure. Furthermore,
373 compared to the control, the inclusion of sainfoin silage in our study resulted in a slightly
374 faster exsheathment of L3, which was significantly faster for *C. oncophora* when sainfoin
375 was included in the diet at a level of 80% for 3 or 5 days ($P < 0.05$). This was likely due to

different local conditions in the rumen caused by the various diets, and possibly unrelated to the presence of CTs. Thus, the rumen temperature was found slightly higher at the beginning of the experiment with the control diet (mean temperature (°C) ± SD: 41.4 ± 0.3) and gradually decreased following the inclusion of sainfoin silage in the diet: 40.7 ± 0.3 at 40% ($P < 0.1$); and 40.1 ± 0.3, 40.4 ± 0.1 and 40.1 ± 0.3 at 80% on day 1, 3 and 5, respectively ($P < 0.05$). More importantly, pH values in the rumen gradually increased with the inclusion of sainfoin in the diet, although this was not statistically significant ($P > 0.05$) and only measured once per trial. The mean pH values were: 6.25 ± 0.04 with the control diet; 6.34 ± 0.23 with 40% sainfoin silage in diet; and 6.55 ± 0.32, 6.66 ± 0.25, 6.69 ± 0.09 with 80% sainfoin silage in the diet for 1, 3 and 5 days, respectively. We know that different physiological conditions in the rumen are likely to influence the rate of the host signal needed for the initiation of exsheathment.[47] For example, a CT-free diet, which drastically reduced ruminal pH, was shown to delay significantly the larval exsheathment of *O. ostertagi* L3 *in vivo,*[42] and *C. curticei* could exsheath faster *in vitro* at pH 7–8.[43] The present study suggests that a certain dietary level of active CTs from sainfoin, as indicated with the thiolysis method, is essential for an anthelmintic effect in the first place. Although CTs seem to be mainly undegraded and unabsorbed in the digestive tract of cattle as shown with the acetone-butanol-HCl assay, the gut conditions appeared to influence the reactivity of CTs and therefore the anthelmintic activity. In conclusion, the low recoveries of CTs by thiolysis in the rumen and small intestine were associated with a lack of efficacy against the larval exsheathment and the worm burdens of adult *C. oncophora*, respectively. However, the apparent release of active CTs from sainfoin in the abomasum led to a significant reduction in worm burdens of adult *O. ostertagi.*

ABBREVIATIONS USED

CTs – condensed tannins; HPLC – high performance liquid chromatography; L3 – third-stage larvae; mDP – mean degree of polymerization; MS – mass spectrometry; NS – non significant; PCs – procyanidins; PDs – prodelphinidins; SD – standard deviation

AUTHOR CONTRIBUTIONS

OD, WFP, HLE and SMT designed the animal experiments. OD, SMT and IMH designed the chemical analyses. OD carried out the study and analyzed the data. OD wrote the manuscript with inputs from all the co-authors. All authors critically read and approved the final manuscript.

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NOTES

The authors declare no competing financial interest.

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571 **FIGURE CAPTIONS**

572 **Figure 1.** Concentrations of condensed tannins (CTs; % of dry matter) in sainfoin and
573 digesta/fecal samples of 9 calves in Experiment 1 using the acetone-butanol-HCl assay.
574 SF=sainfoin pellets; RU=rumen; AB=abomasum; SI=small intestine; LI=large intestine;
575 FE=feces. Error bars are standard deviations for digesta/fecal samples (n=9). No CTs
576 were detected in control feedstuffs. Dietary level of CTs is approximately 6.3% of dry
577 matter. Different letters indicate significant differences ($P < 0.05$).

578

579 **Figure 2.** Concentration and composition of condensed tannins (CTs) in sainfoin and
580 digesta or feces of calves in Experiment 1 using *in situ* thiolysis. (A) CTs concentration (%
581 of dry matter), dietary level of CTs is approximately 1.9% of dry matter; (B) *cis*-
582 configuration (molar percentage); (C) mean degree of polymerization; (D) % of
583 prodelphinidins (PDs; molar percentage). SF=sainfoin pellets; RU=rumen, AB=abomasum;
584 SI=small intestine; LI=large intestine; FE=feces. Error bars are standard deviations for
585 digesta/fecal samples (n=8 except for SI=4 and LI=2). Calf #4413 was an outlier and is
586 represented separately (Δ). No CT were detected in control feedstuffs. Different letters
587 indicate significant differences for CTs concentrations ($P < 0.05$).

588 **Figure 3.** Flavan-3-ol subunit (mmolar) composition of condensed tannins in sainfoin and
589 digesta/feces of calves in Experiment 1 using *in situ* thiolysis. (A) terminal units; (B)
590 extension units (BM-adducts). BM=benzyl-mercaptan. Flavan-3-ols occuring in
591 prodelphinidins (–): GC=gallocatechin, EGC=epigallocatechin; in procyanidins (▪▪▪):
592 C=catechin; EC=epicatechin. SF= sainfoin pellets; RU=rumen; AB=abomasum; SI=small
593 intestine; LI=large intestine; FE=feces. Error bars are standard deviations for digesta/fecal

samples (n=8). Calf #4413 was not included. No CTs were detected in control feedstuffs. Please note the differently scaled y-axis.

Figure 4. Kinetics of the exsheathment of third-stage larvae of (A) *Ostertagia ostertagi* and (B) *Cooperia oncophora* in the rumen of fistulated cows (n=3) in Experiment 2. Control feed (**▪▪▪**) without sainfoin. SF=sainfoin silage percentage included in the ration (40% for three days; 80% for 1, 3 and 5 days). Error bars are standard deviations.

FIGURE GRAPHICS

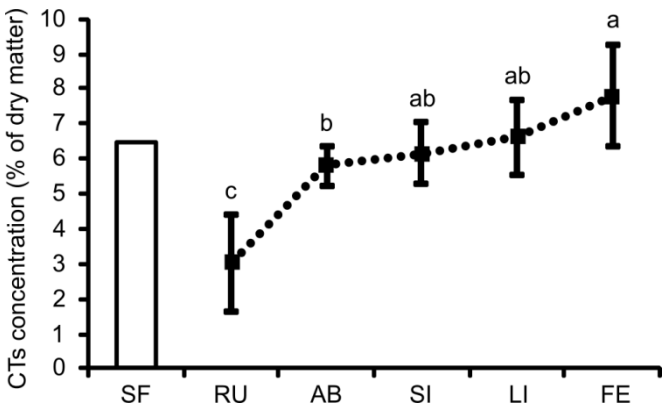


Figure 1.

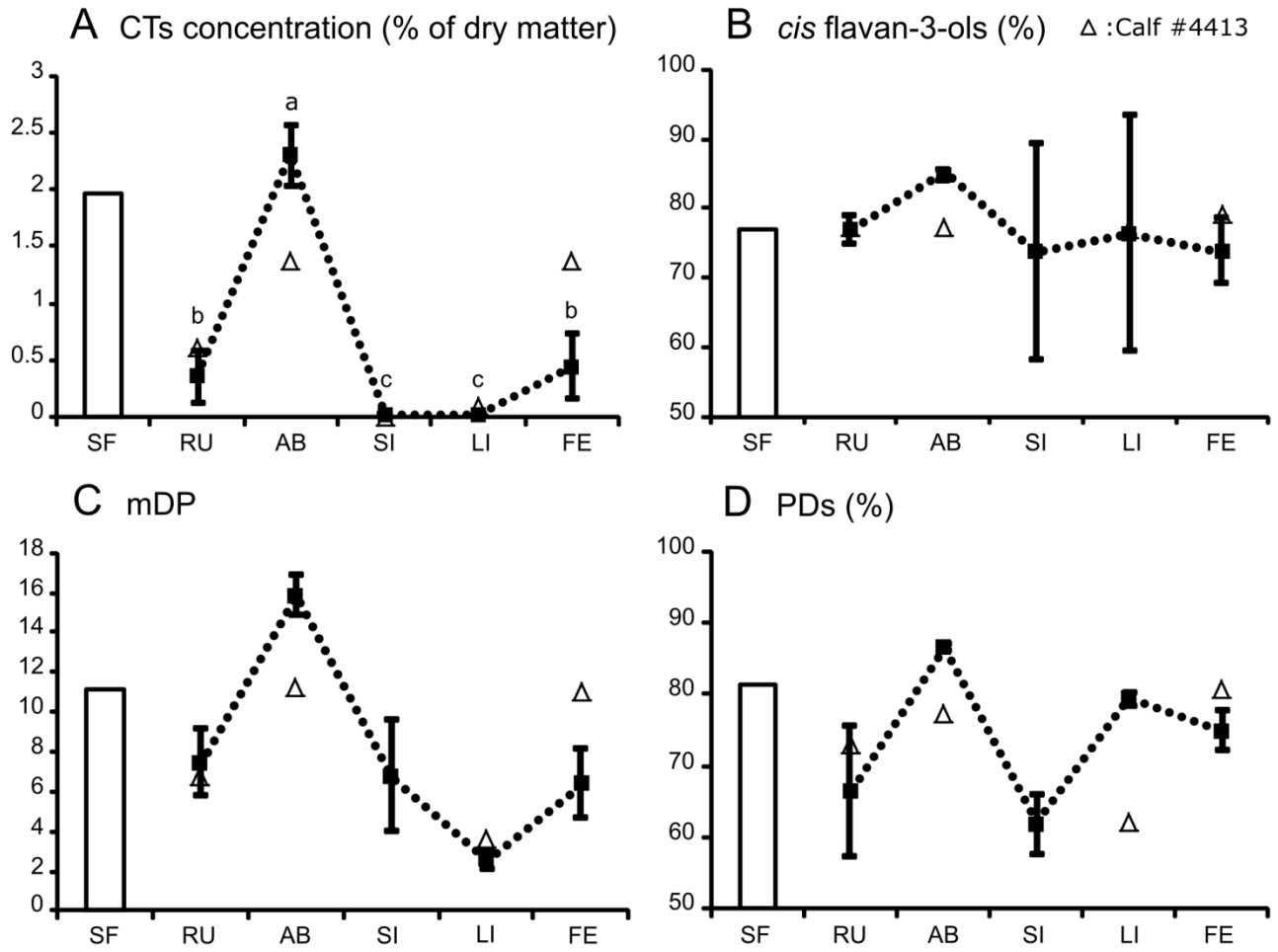

A CTs concentration (% of dry matter)
B cis flavan-3-ols (%)
△ :Calf #4413
C mDP
D PDs (%)
SF
RU
AB
SI
LI
FE
a
b
c
c
b

Figure 2.

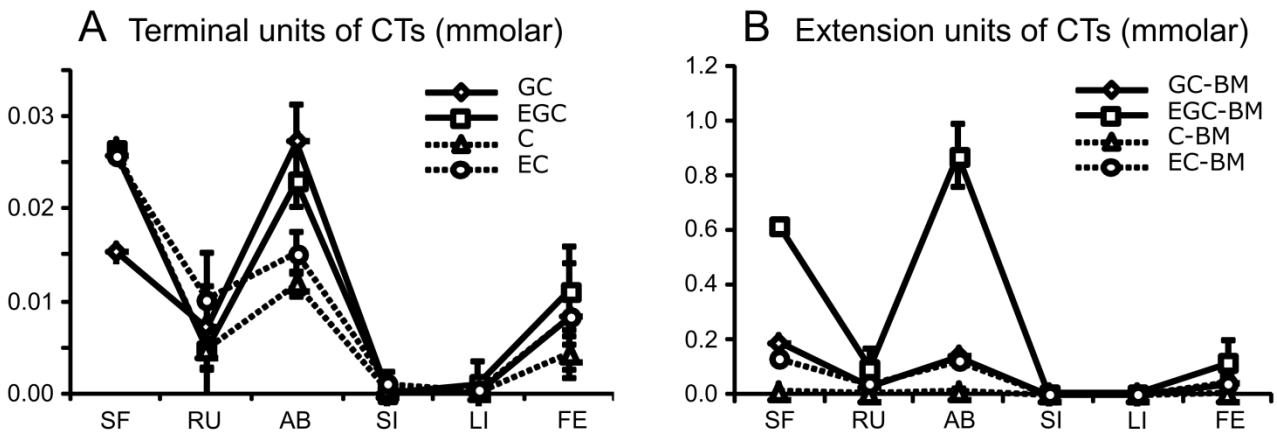

A Terminal units of CTs (mmolar)
GC
EGC
C
EC
0.03
0.02
0.01
0.00
SF
RU
AB
SI
LI
FE
B Extension units of CTs (mmolar)
GC-BM
EGC-BM
C-BM
EC-BM
1.2
1.0
0.8
0.6
0.4
0.2
0.0
SF
RU
AB
SI
LI
FE

Figure 3.

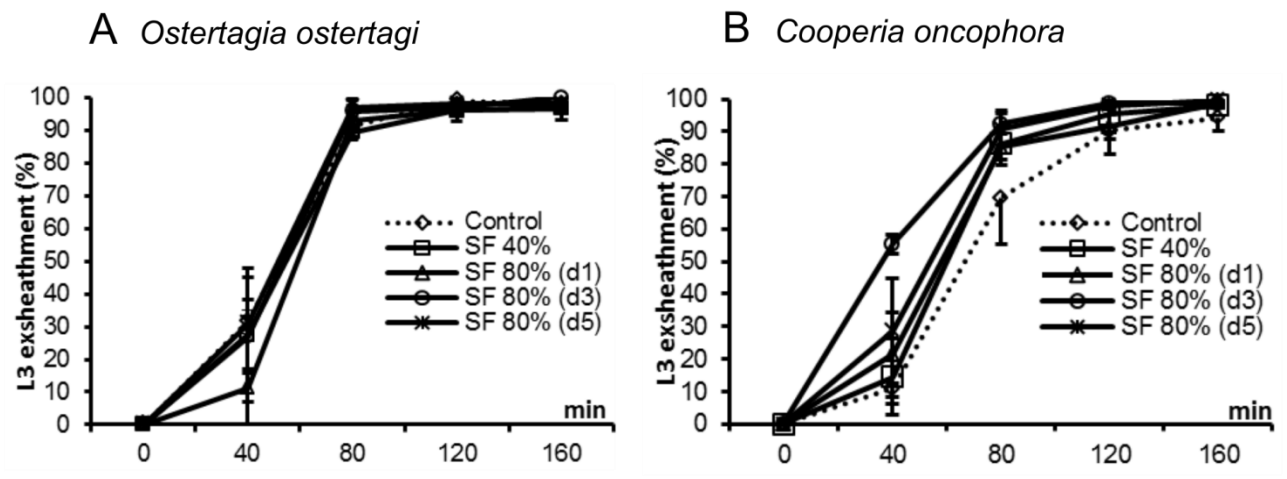
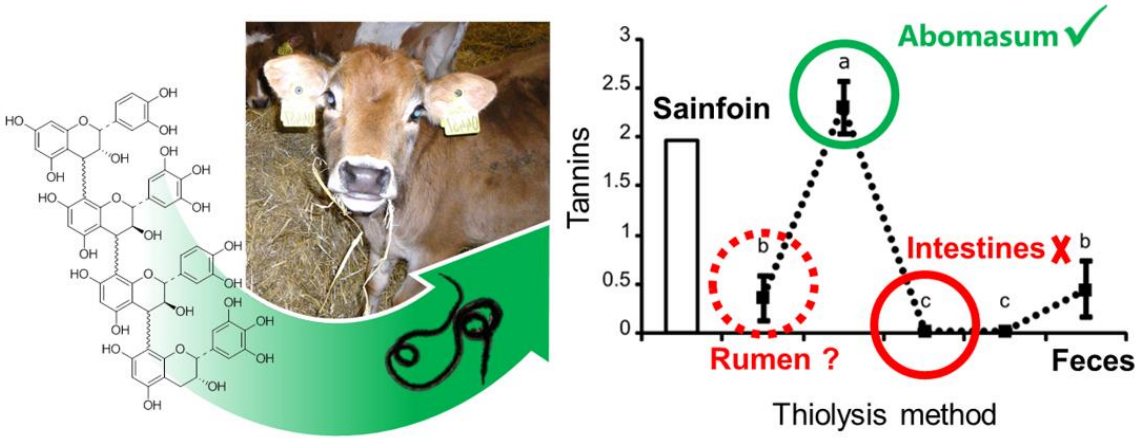

A Ostertagia ostertagi
B Cooperia oncophora
L3 exsheathment (%)
100
90
80
70
60
50
40
30
20
10
0
0
40
80
120
160
min
Control
SF 40%
SF 80% (d1)
SF 80% (d3)
SF 80% (d5)

Figure 4.



Graphic for table of contents