In vitro rumen fermentation of diets with different types of condensed tannins derived from sainfoin (Onobrychis viciifolia Scop.) pellets and hazelnut (Corylus avellana L.) pericarps

Article

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In vitro rumen fermentation of diets with different types of condensed tannins derived from sainfoin (*Onobrychis viciifolia* Scop.) pellets and hazelnut (*Corylus avellana* L.) pericarps

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Abstract

Information is lacking on the specific biological activity of feeds containing different types of condensed tannin (CT) on rumen fermentation characteristics. The aim of this study was to evaluate the *in vitro* rumen fermentation parameters of diets including sainfoin (*Onobrychis viciifolia* Scop.) pellets (PS) and/or hazelnut (*Corylus avellana* L.) pericarps (HP) using a batch culture system for 24 h. The treatments were a basal diet consisting of (dry matter (DM) basis) 800 g/kg hay from permanent grassland and 200 g/kg concentrate mix (control), the basal diet + 30.4% PS, the basal diet + 8.2% freeze-dried HP, and the basal diet + 15.2% PS + 4.1% HP. The diets were adjusted to be isotannic (20 g/kg DM, except for the control) and isoproteic (132 g/kg DM). Total gas and methane (CH_4) productions were measured after 3.5 h and 24 h of incubation in buffered rumen fluid from sheep. At the end of incubation, pH, *in vitro* DM degradability (IVDMD) and the concentration of fermentation end-products in the medium were also measured. The CT structures in PS and HP, determined by the thiolysis method, were very different: PS had mostly prodelphinidins and HP mostly procyanidins. After 24 h of incubation, the total gas and CH_4 productions and IVDMD were greater for the basal diet than for the CT-containing diets ($P<0.001$). The CH_4 production increased significantly with the diet + HP in the presence of polyethylene glycol (PEG, 4000 Da molecular weight), a CT-inactivating compound ($P<0.001$), and tended to increase for the diet + PS ($P=0.062$). The volatile fatty acid (VFA: acetate, propionate, butyrate, minor and iso-VFA) net productions were similar among treatments except for valerate (the lowest for PS-containing diets, $P=0.003$), while the NH_3 concentration was lower for the diet + PS (with a significant PEG effect) than for the diets including HP, and was highest for the basal diet. It was concluded that the inclusion of PS and HP in a basal diet for ruminants results in lower

rumen fermentability and that their CT decreased CH_4 production and protein degradability. The PS were more effective than HP for reducing rumen protein degradability with a potential increase of duodenal nitrogen (N) flow.

Keywords: rumen fermentability, protein degradability, methane, tannin-containing feeds, prodelphinidins, procyanidins

Abbreviations

ADF, acid detergent fibre expressed inclusive of residual ash; aNDF, neutral detergent fibre assayed with a heat-stable amylase and expressed inclusive of residual ash; CP, crude protein; CT, condensed tannins; CH_4, methane; DM, dry matter; HP, hazelnut pericarps; IVDMD, *in vitro* DM degradability; mDP, mean degree of polymerisation; N, nitrogen; NH_3, ammonia; OM, organic matter; PC, procyanidins; PD, prodelphinidins; PEG, polyethylene glycol 4000; PS, pellets of sainfoin; VFA, volatile fatty acids.

1. Introduction

The livestock sector plays an important role in climate change as it accounts for 14.5 % of human-induced greenhouse gases emissions. Ruminant production contributes to about two-thirds of the sector's emissions under the form of nitrous oxide (N_2O), methane (CH_4) and carbon dioxide (CO_2), which are also losses of nitrogen (N), energy and organic matter that undermine efficiency and productivity. Feed production and enteric fermentation from ruminants are the two main sources of emissions, representing 45 and 39 % of sector emissions, respectively (Gerber et al., 2013).

Among the principles proposed for the design of sustainable ruminant production systems in the context of agroecology, practices are put forward that stimulate natural processes to

close system loops, reduce inputs and pollution, and improve animal health, (Dumont et al., 2013). Improved feeding strategies offer a relevant level of action, especially when non-food resources such as forage species or by-products from the human food industry are used.

Some natural resources such as forage legumes or by-products from the agroindustry contain condensed tannins (CT; *syn.* proanthocyanidins) consisting in polymers of flavan-3-ol units which have the potential to reduce pollution by decreasing CH_4 emissions and urinary N losses through their ability to bind proteins (Mueller-Harvey et al., 2019). In addition, CT can also help at controlling gastrointestinal nematodes, including small ruminant strains that are multi-resistant against synthetic anthelmintic drugs (Gaudin et al., 2016). However, considerable variability in the chemistry and the biological activity of CT has been observed among natural resources. Although it had been widely assumed that CT act from a dose-dependent manner, there is some evidence that the characteristics of CT play also an important role on their activity in the rumen (Huyen et al., 2016a). Two structural features of CT appear to merit particular attention: the structural characteristics of flavanols and the size of polymers (Hatew et al., 2016). The structural differences consist in whether the flavonol is (epi)gallocatechin giving rise to prodelphinidin (PD) or (epi)catechin giving rise to procyanidin (PC), and in the mean degree of polymerisation (mDP). As the structural composition of CT is uncommonly determined, very little is known on which CT traits are best to decrease CH_4 production and excessive protein degradation in the rumen with contradictory results in the literature (Hatew et al., 2015; Naumann et al., 2018). Moreover, to date, there is no information on how different CT traits could be potentially complementary when they are incubated together.

The aim of this study was to evaluate the effects on *in vitro* rumen fermentation parameters of including in a basal diet two resources contrasting by their CT characteristics, namely hazelnut pericarps (HP, *Corylus avellana* L.), which contain PC-type CT or pellets of sainfoin

(PS, *Onobrychis viciifolia* Scop.), which contain PD-type CT. In addition, these two resources were tested when mixed in a same diet to detect possible associative effects between them.

2. Materials and methods

2.1 Treatments and plant materials

Four diets were tested: i) a control basal diet consisting of, on a dry matter (DM) basis, 800 g/kg hay from permanent grassland (INRA Theix, 810 m above sea level, first cut, harvested on 5 June 2015) containing approximately 70% grasses (mainly *Dactylis glomerata*, *Lolium perenne*, *Festuca*), 25% dicotyledonous species including 5% *Trifolium repens*, and 200 g/kg concentrate mix containing 320 g/kg barley, 180 g/kg rapeseed meal, 150 g/kg wheat, 110 g/kg beet pulp, 180 g/kg other grains and 60 g/kg molasses and minerals, ii) the basal diet + PS provided by the company MG2MIX (Châteaubourg, France, cv. Multifolia Perly, 3rd cut from swards established around Viâpres-le-Petit, France, in 2015, high PD/PC ratio), iii) the basal diet + freeze-dried HP provided by the company Inovfruit (Mussidan, France, low PD/PC ratio), and iv) the basal diet + PS + HP. A treatment in which the basal diet was supplemented with both HP and PS was added to determine whether mixing these CT-containing resources could produce associative effects on rumen fermentation characteristics. For the basal diet, the characteristics of hay was: DM = 950 g/kg fresh weight, organic matter (OM) = 902 g/kg DM, crude protein (CP) = 86 g/kg DM, (neutral detergent fibre) aNDF = 528 g/kg DM, acid detergent fibre (ADF) = 290 g/kg DM, *in vitro* digestibility = 615 g/kg DM. The chemical composition of concentrate mix was: DM = 913 g/kg fresh weight, OM = 935 g/kg DM, CP = 170 g/kg DM, cellulose = 87 g/kg DM and starch = 300 g/kg DM. The characteristics of PS and HP are presented in Table 1. The treatments were prepared to be isotannic (20 g/kg of CT, except for the basal diet) and isoproteic (132 g/kg, adjusted with casein, Sigma-Aldrich, Saint Louis, MO,

120 USA). It represented 30.4% PS in the diet + PS, 8.2% HP in the diet + HP, and 15.2% PS +
121 4.1% HP in the diet + PS + HP.
122
123 *2.2 In vitro rumen fermentation assay*
124 All experimental procedures were conducted in accordance with the European Union
125 Directive 2010/63/EU, reviewed by the local ethics committee (C2E2A, "Comité d'Ethique
126 pour l'Expérimentation Animale en Auvergne") and authorised by the French Ministry for
127 Research (no. 7138-2016092709177605-V5).
128 Plant substrate (600 ± 0.5 mg in total) was placed in 120 ml serum bottles, pre-warmed at
129 39 ± 0.5 °C and flushed with N_2 to eliminate the oxygen present inside. Rumen fluid was
130 collected before morning feeding from three cannulated sheep (62 ± 7 kg on average) fed
131 daily 1200 g of a diet composed of, per kg (as fed), permanent grassland hay, first cut (800 g)
132 and the same concentrate mix used for the basal diet (400 g). Withdrawing and handling of
133 rumen fluid were as described previously (Macheboeuf et al., 2008). Forty ml of buffered
134 rumen fluid (strained rumen fluid diluted 1:2 (v/v) in an anaerobic phosphate:carbonate buffer
135 solution, initial pH 6.89 ± 0.02) was added in the serum bottle, which were subsequently
136 sealed hermetically with butyl rubber stopper and aluminium crimp seals. The buffer solution
137 was prepared as described by Goering and Van Soest (1970) and modified by Niderkorn et al.
138 (2011). The effects of CT were assessed by testing the treatments with and without
139 polyethylene glycol (PEG, 4000 Da molecular weight, 2.3 g/l) in the incubation medium, a
140 compound that can bind and inactivate CT. Blanks without any plant substrate (only buffered
141 rumen fluid) were incubated during the different runs. All bottles were incubated in a shaking
142 water bath at 39 °C. Samples of buffered rumen fluid were also taken at time 0 to determine
143 volatile fatty acids (VFA) and ammonia (NH_3) present in the inoculum. Each treatment was
144 repeated three times over two weeks.

After 3.5 h and 24 h of incubation, the gas production was recorded using the pressure transducer technique, as described by Theodorou et al. (1994) and gas samples were taken from the headspace of the serum bottles for determination of gas composition (CH_4, CO_2). After 24 h, the fermentation was stopped, the entire content of each serum bottle was transferred into a pre-weighed Falcon tube, and tubes were centrifuged at 3,400 × *g* for 10 min at 4 °C. After sampling the supernatant for VFA and NH_3-N determination (for details, see Niderkorn et al. (2012), the serum bottle was washed twice with distilled water to recover all the nondegraded particles that were transferred into the Falcon tube. Tubes were again centrifuged at 3,400 × *g* for 10 min at 4 °C, the supernatant was removed, and the DM of the residue was determined to calculate *in vitro* DM degradability (IVDMD).

2.3 Analytical methods

Plant substrates and residues were analysed for DM by oven-drying at 103 ± 0.5 °C for 48h, and OM by ashing at 550 °C for 6 h in a muffle furnace. The aNDF and ADF contents were determined according to the method described by Van Soest et al. (1991), using a Fibre Analyser (Ankom Technology Corporation, Fairport, NY, USA). The CP content was determined by the Dumas combustion method (AOAC, 1995) using a rapid N-cube protein/N apparatus (Elementar Americas Inc., Mt Laurel, NJ, USA). The *in vitro* digestibility of hay was evaluated according to the pepsin-cellulase method described by Aufrère and Michalet-Doreau (1988). The gas composition (CH_4 and CO_2) was determined by gas chromatography using a MicroGC 3000A (Agilent Technologies, France). The individual VFA (acetate, propionate, butyrate, valerate, caproate, isobutyrate, isovalerate) in the supernatant fraction was measured by gas chromatography and NH_3 was measured by Berthelot reaction (Weatherburn, 1967). The IVDMD was determined by difference between DM of plant material before the fermentation and DM of residue after 24 h of fermentation. The CT were

analysed by direct thiolysis of freeze-dried samples with benzyl mercaptan at 40 °C for 1 h to provide quantitative and qualitative information (Gea et al., 2011). In brief, the thiolysis reaction releases the terminal units as the catechin, epicatechin, gallocatechin, epigallocatechin and the extension units as their benzylmercaptan derivatives. Thiolysis provides quantitative data the content of extractable and unextractable CT (g CT/kg DM), and information on CT structures in terms of PD/PC and *cis/trans*-flavan-3-ol ratios (mol %) and mDP values.

2.4 Statistical analysis

Each vessel was considered as an experimental unit. All variables were analyzed using the PROCMIXED procedure by SAS (Mixed procedure, version 9.2; SAS Institute Inc., Cary, NC, USA). The type of diet, PEG and their interaction were used as fixed effects and incubation day as random factor using the following model:

$$Y_{ijk}=\mu+D_i+P_j+(D\times P)_{ij}+e_{ij}$$

where Y_{ijk} is the dependent variable, μ the overall mean, D_i the type of diet (j=4), P_j the effect of PEG (j=2, without and without the presence of PEG), $(D\times P)_{ij}$ the interaction of type of diet and PEG effect, and e_{ij} the residual error term. Significance was declared at $P<0.05$.

3. Results

3.1 CT characteristics of resources

The two CT-containing resources differed in terms of CP (PS > HP) and CT (HP > PS) contents (Table 1), but the treatments have been adjusted to be isoproteic and isotannic. The CT structures also differed between PS and HP (Table 1): PS had mostly PD with a PD/PC ratio of 75:25 and HP had mostly PC with a PD/PC ratio of 28:72. The mDP was 11.5 for PS and 13.3 for HP and the *cis/trans*-flavan-3-ol ratio was 85:15 for PS and 58:42 for HP.

3.2 In vitro gas production and composition

In the early phase of the fermentation (until 3.5 h), the total gas and CH_4 productions per g of incubated DM were greater for the basal diet than for the diets including HP, themselves being greater than for the diet + PS (*P*=0.005, data not shown). After 24 h of incubation, the total gas production per g of incubated DM was greater for the basal diet than for the diet + HP and the diet + PS, while the diet + HP + PS was intermediate (*P*=0.002, Figure 1A). For the diet + HP, the presence of PEG significantly increased the gas production (*P*=0.028). The CH_4 production was higher for the basal diet than for the diet + HP + PS, itself being higher than for the two other treatments (*P*<0.001, Figure 1B). The presence of PEG significantly increased the CH_4 production for the diet + HP (*P*=0.007) and tended to increase it for the diet + PS (*P*=0.062).

3.3 Other in vitro rumen fermentation characteristics

The pH of the medium was lower for the basal diet than for the diet + PS (*P*=0.036, Table 2). The IVDMD was greater for the basal diet than for the diet + HP and the diet + HP + PS, while the diet + PS had the lowest value (*P*<0.001). The VFA net productions and profiles in the medium were similar among treatments except for valerate, which was higher for the basal diet than for the diet + PS and the diet + HP + PS (*P*=0.003, Table 2). The NH_3 concentration was lower for the diet + PS than for the diets including HP, and was the highest for the basal diet. Opposite PEG effects on NH_3 concentration were also detected for the basal diet (incubation without PEG > with PEG, P=0.041) and the diet + PS (incubation without PEG < with PEG, *P*=0.016) (Figure 1C).

4. Discussion

4.1 Fermentability of the diets including CT-containing resources

In this study, the isotannic (except for the basal diet) and isoproteic substrates with different CT types in HP and PS allowed us to investigate the relationship between CT structure and their effects on rumen fermentation characteristics. Compared to the basal diet, the fermentability of the diets including PS and/or HP was negatively affected early in the incubation period as shown by the lower total gas productions after 3.5h of incubation. After 24 h of incubation, the fermentability was still reduced as evidenced by the lower values observed on total gas production and IVDMD. These results can be explained by the substitution of a part of the basal diet by less fermentable resources, but likely through different mechanisms. In the case of the inclusion of PS, it could be due to a low fibre degradability rather than a negative CT effect as this lack of fibre degradability/digestibility has been shown in *in vitro* and *in vivo* studies (Niderkorn et al., 2011; Huyen et al., 2016b) and because no significant difference in this study was observed between the diet + PS incubated with and without PEG. On the contrary, the increased total gas production when the diet + HP was incubated with PEG (CT inactivated) compared to the incubation without PEG indicates that the CT in HP decreased fibre fermentability. Interestingly, the total gas production for diet + PS + HP was not different compared from the basal diet, although the IVDMD was lower. This may indicate that the negative effect of PS on the fermentability was partly removed by a dilution effect from HP.

4.2 Methane reduction

In the early and late phases of the fermentation, the CH_4 production was consistently higher for the basal diet compared to the CT-diets. The loss of fermentability due to CT when PS or HP were included in the basal diet is certainly a part of the explanation. Besides, the PEG effect observed after 24 h of incubation indicates clearly that CT in PS and HP also had an anti-methanogenic effect. These results are in line with the conclusions of the meta-

245 analysis carried out by Jayanegara et al. (2012) that demonstrated that CH_4 reduction in the
246 presence of CT is mainly associated with a reduced apparent digestion of OM, and especially
247 fibre. However, these authors mentioned that CH_4/apparently digestible OM also declined. In
248 our study, after 3.5 h and 24 h of incubation, the values for the CO_2:CH_4 ratio were
249 consistently higher for the CT-containing diets than for the basal diet (P<0.001, data not
250 shown). These results indicate that the presence of PS or HP modifies the rumen metabolism
251 towards a lower proportion of CH_4 in the fermentation gas. Two hypotheses based on the
252 literature can be proposed: a direct effect of CT on the archaea-methanogen populations and
253 their activity (Saminathan et al., 2016) or a decrease of substrate for the CH_4 formation
254 through a reduction of H_2 availability (Tavendale et al., 2005). In our study, the results for
255 VFA net productions and profiles showed only a significant difference between treatments for
256 valerate and a trend for caproate. In particular, acetate and propionate productions were
257 similar and no PEG effect was detected on VFA (data not shown), indicating that the main
258 fermentation pathways were not strongly affected by the different CT from PS and HP. Some
259 authors have suggested that an inhibition of CH_4 production can enhance fermentation
260 pathways that require a net incorporation of H_2 into the VFA produced from glucose, leading
261 notably to increased valerate and caproate productions (Guyader et al., 2017). In our study,
262 we observed indeed a trend towards an increase of caproate concentration with the diet + HP,
263 but the diet + PS led to the lowest valerate concentration. These results suggest that H_2 may
264 have been redirected differently according to the type of CT-containing resource, possibly due
265 to the different structure of CT which has been shown to affect *in vitro* rumen fermentation
266 characteristics (Huyen et al., 2016a). It has to noted that mixing PS and HP led to CH_4
267 production higher than for the CT-containing resources taken individually, likely due to the
268 higher total gas production.
269

4.3 Protein degradability

Regarding the fate of nitrogenous compounds, our results showed clearly that the inclusion of PS and HP in the diet decreased the NH_3 concentration in the fermentation medium. As the diets were isoproteic, it indicates that HP and PS resulted in a lower protein degradation or in a greater incorporation of N in microbial biomass through microbial protein synthesis. As we found that IVDMD, which can be partly linked to the microbial biomass, was lower in the presence of HP and PS, it is more probable that less protein was degraded to NH_3. The ability of CT to form complexes with protein leading to their protection from ruminal degradation has been recognised for a long time (Min et al., 2003). This role of CT was confirmed in our study at least for the diet + PS for which the presence of PEG increased the NH_3 concentration. On the other hand, we found the lowest NH_3 concentration for the diet + PS, suggesting that CT from PS were more active at reducing protein degradation than the CT from HP as both diets were isotannic. It was reported that the size of CT is the key parameter controlling protein binding activity as demonstrated using bovine serum albumin, for which the activity clearly increased when the mDP values increased from 3 to 8 (Ropiak et al., 2017). However, according to these authors there were only small differences in the efficacies of larger CTs with mDP > 9 to aggregate the proteins. In our study, the mDP of CT of PS and HP were similar (11 vs 13.5), suggesting that the size of these CT was not the main driver for the activity differences. More importantly, the PD/PC ratio was the main difference and our results suggest that a high PD/PC ratio as in PS gave rise to more active CT in terms of protein binding activity than a low PD/PC ratio as in HP. This hypothesis is consistent with the findings from Huyen et al. (2016a) who showed *in vitro* using CT extracts from different plants that the proportion of PD in CT had the largest effect on rumen fermentation characteristics. These authors argued that PD have more hydroxyl groups than PC, and thus they are more able to bind fibre and protein, and impact on their degradability.

5. Conclusions

Under *in vitro* conditions, the inclusion of PS and HP in a basal diet for ruminants led to lowered rumen fermentability and also decreased CH_4 production and protein degradability due to CT: the PD-rich CT in PS were more effective than the PC-rich CT in HP with a possible increase of duodenal N flow. The real impact on ruminant performances, the reduction in CH_4 emissions and N urinary losses, and a potential increase in N use efficiency have to be evaluated *in vivo*.

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