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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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21

22

23 **Abstract**

24 Information is lacking on the specific biological activity of feeds containing different types of
25 condensed tannin (CT) on rumen fermentation characteristics. The aim of this study was to
26 evaluate the *in vitro* rumen fermentation parameters of diets including sainfoin (*Onobrychis*
27 *viciifolia* Scop.) pellets (PS) and/or hazelnut (*Corylus avellana* L.) pericarps (HP) using a
28 batch culture system for 24 h. The treatments were a basal diet consisting of (dry matter (DM)
29 basis) 800 g/kg hay from permanent grassland and 200 g/kg concentrate mix (control), the
30 basal diet + 30.4% PS, the basal diet + 8.2% freeze-dried HP, and the basal diet + 15.2% PS +
31 4.1% HP. The diets were adjusted to be isotannic (20 g/kg DM, except for the control) and
32 isoproteic (132 g/kg DM). Total gas and methane (CH₄) productions were measured after 3.5
33 h and 24 h of incubation in buffered rumen fluid from sheep. At the end of incubation, pH, *in*
34 *vitro* DM degradability (IVDMD) and the concentration of fermentation end-products in the
35 medium were also measured. The CT structures in PS and HP, determined by the thiolysis
36 method, were very different: PS had mostly prodelphinidins and HP mostly procyanidins.
37 After 24 h of incubation, the total gas and CH₄ productions and IVDMD were greater for the
38 basal diet than for the CT-containing diets ($P<0.001$). The CH₄ production increased
39 significantly with the diet + HP in the presence of polyethylene glycol (PEG, 4000 Da
40 molecular weight), a CT-inactivating compound ($P<0.001$), and tended to increase for the diet
41 + PS ($P=0.062$). The volatile fatty acid (VFA: acetate, propionate, butyrate, minor and iso-
42 VFA) net productions were similar among treatments except for valerate (the lowest for PS-
43 containing diets, $P=0.003$), while the NH₃ concentration was lower for the diet + PS (with a
44 significant PEG effect) than for the diets including HP, and was highest for the basal diet. It
45 was concluded that the inclusion of PS and HP in a basal diet for ruminants results in lower

46 rumen fermentability and that their CT decreased CH₄ production and protein degradability.
47 The PS were more effective than HP for reducing rumen protein degradability with a potential
48 increase of duodenal nitrogen (N) flow.

49

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

52

53 *Abbreviations*

54 ADF, acid detergent fibre expressed inclusive of residual ash; aNDF, neutral detergent fibre
55 assayed with a heat-stable amylase and expressed inclusive of residual ash; CP, crude protein;
56 CT, condensed tannins; CH₄, methane; DM, dry matter; HP, hazelnut pericarps; IVDMD, *in*
57 *vitro* DM degradability; mDP, mean degree of polymerisation; N, nitrogen; NH₃, ammonia;
58 OM, organic matter; PC, procyanidins; PD, prodelphinidins; PEG, polyethylene glycol 4000;
59 PS, pellets of sainfoin; VFA, volatile fatty acids.

60

61 **1. Introduction**

62 The livestock sector plays an important role in climate change as it accounts for 14.5 % of
63 human-induced greenhouse gases emissions. Ruminant production contributes to about two-
64 thirds of the sector's emissions under the form of nitrous oxide (N₂O), methane (CH₄) and
65 carbon dioxide (CO₂), which are also losses of nitrogen (N), energy and organic matter that
66 undermine efficiency and productivity. Feed production and enteric fermentation from
67 ruminants are the two main sources of emissions, representing 45 and 39 % of sector
68 emissions, respectively (Gerber et al., 2013).

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

71 close system loops, reduce inputs and pollution, and improve animal health, (Dumont et al.,
72 2013). Improved feeding strategies offer a relevant level of action, especially when non-food
73 resources such as forage species or by-products from the human food industry are used.

74 Some natural resources such as forage legumes or by-products from the agroindustry
75 contain condensed tannins (CT; *syn.* proanthocyanidins) consisting in polymers of flavan-3-ol
76 units which have the potential to reduce pollution by decreasing CH₄ emissions and urinary N
77 losses through their ability to bind proteins (Mueller-Harvey et al., 2019). In addition, CT can
78 also help at controlling gastrointestinal nematodes, including small ruminant strains that are
79 multi-resistant against synthetic anthelmintic drugs (Gaudin et al., 2016). However,
80 considerable variability in the chemistry and the biological activity of CT has been observed
81 among natural resources. Although it had been widely assumed that CT act from a dose-
82 dependent manner, there is some evidence that the characteristics of CT play also an
83 important role on their activity in the rumen (Huyen et al., 2016a). Two structural features of
84 CT appear to merit particular attention: the structural characteristics of flavanols and the size
85 of polymers (Hatew et al., 2016). The structural differences consist in whether the flavanol is
86 (epi)gallocatechin giving rise to prodelphinidin (PD) or (epi)catechin giving rise to
87 procyanidin (PC), and in the mean degree of polymerisation (mDP). As the structural
88 composition of CT is uncommonly determined, very little is known on which CT traits are
89 best to decrease CH₄ production and excessive protein degradation in the rumen with
90 contradictory results in the literature (Hatew et al., 2015; Naumann et al., 2018). Moreover, to
91 date, there is no information on how different CT traits could be potentially complementary
92 when they are incubated together.

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

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

98

99 **2. Materials and methods**

100 *2.1 Treatments and plant materials*

101 Four diets were tested: i) a control basal diet consisting of, on a dry matter (DM) basis, 800
102 g/kg hay from permanent grassland (INRA Theix, 810 m above sea level, first cut, harvested
103 on 5 June 2015) containing approximately 70% grasses (mainly *Dactylis glomerata*, *Lolium*
104 *perenne*, *Festuca*), 25% dicotyledonous species including 5% *Trifolium repens*, and 200 g/kg
105 concentrate mix containing 320 g/kg barley, 180 g/kg rapeseed meal, 150 g/kg wheat, 110 g/kg
106 beet pulp, 180 g/kg other grains and 60 g/kg molasses and minerals, ii) the basal diet + PS
107 provided by the company MG2MIX (Châteaubourg, France, cv. Multifolia Perly, 3rd cut from
108 swards established around Viâpres-le-Petit, France, in 2015, high PD/PC ratio), iii) the basal
109 diet + freeze-dried HP provided by the company Inovfruit (Mussidan, France, low PD/PC ratio),
110 and iv) the basal diet + PS + HP. A treatment in which the basal diet was supplemented with
111 both HP and PS was added to determine whether mixing these CT-containing resources could
112 produce associative effects on rumen fermentation characteristics. For the basal diet, the
113 characteristics of hay was: DM = 950 g/kg fresh weight, organic matter (OM) = 902 g/kg DM,
114 crude protein (CP) = 86 g/kg DM, (neutral detergent fibre) aNDF = 528 g/kg DM, acid detergent
115 fibre (ADF) = 290 g/kg DM, *in vitro* digestibility = 615 g/kg DM. The chemical composition
116 of concentrate mix was: DM = 913 g/kg fresh weight, OM = 935 g/kg DM, CP = 170 g/kg DM,
117 cellulose = 87 g/kg DM and starch = 300 g/kg DM. The characteristics of PS and HP are
118 presented in Table 1. The treatments were prepared to be isotannic (20 g/kg of CT, except for
119 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 N₂ 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 (NH₃) present in the inoculum. Each treatment was
144 repeated three times over two weeks.

145 After 3.5 h and 24 h of incubation, the gas production was recorded using the pressure
146 transducer technique, as described by Theodorou et al. (1994) and gas samples were taken
147 from the headspace of the serum bottles for determination of gas composition (CH₄, CO₂).
148 After 24 h, the fermentation was stopped, the entire content of each serum bottle was
149 transferred into a pre-weighed Falcon tube, and tubes were centrifuged at 3,400 × g for 10
150 min at 4 °C. After sampling the supernatant for VFA and NH₃-N determination (for details,
151 see Niderkorn et al. (2012), the serum bottle was washed twice with distilled water to recover
152 all the nondegraded particles that were transferred into the Falcon tube. Tubes were again
153 centrifuged at 3,400 × g for 10 min at 4 °C, the supernatant was removed, and the DM of the
154 residue was determined to calculate *in vitro* DM degradability (IVDMD).

155

156 *2.3 Analytical methods*

157 Plant substrates and residues were analysed for DM by oven-drying at 103 ± 0.5 °C for
158 48h, and OM by ashing at 550 °C for 6 h in a muffle furnace. The aNDF and ADF contents
159 were determined according to the method described by Van Soest et al. (1991), using a Fibre
160 Analyser (Ankom Technology Corporation, Fairport, NY, USA). The CP content was
161 determined by the Dumas combustion method (AOAC, 1995) using a rapid N-cube protein/N
162 apparatus (Elementar Americas Inc., Mt Laurel, NJ, USA). The *in vitro* digestibility of hay
163 was evaluated according to the pepsin-cellulase method described by Aufrère and Michalet-
164 Doreau (1988). The gas composition (CH₄ and CO₂) was determined by gas chromatography
165 using a MicroGC 3000A (Agilent Technologies, France). The individual VFA (acetate,
166 propionate, butyrate, valerate, caproate, isobutyrate, isovalerate) in the supernatant fraction
167 was measured by gas chromatography and NH₃ was measured by Berthelot reaction
168 (Weatherburn, 1967). The IVDMD was determined by difference between DM of plant
169 material before the fermentation and DM of residue after 24 h of fermentation. The CT were

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

177

178 *2.4 Statistical analysis*

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

$$183 Y_{ijk} = \mu + D_i + P_j + (D \times P)_{ij} + e_{ij}$$

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

187

188 **3. Results**

189 *3.1 CT characteristics of resources*

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

195

196 *3.2 In vitro gas production and composition*

197 In the early phase of the fermentation (until 3.5 h), the total gas and CH₄ productions per g
198 of incubated DM were greater for the basal diet than for the diets including HP, themselves
199 being greater than for the diet + PS ($P=0.005$, data not shown). After 24 h of incubation, the
200 total gas production per g of incubated DM was greater for the basal diet than for the diet + HP
201 and the diet + PS, while the diet + HP + PS was intermediate ($P=0.002$, Figure 1A). For the diet
202 + HP, the presence of PEG significantly increased the gas production ($P=0.028$). The CH₄
203 production was higher for the basal diet than for the diet + HP + PS, itself being higher than for
204 the two other treatments ($P<0.001$, Figure 1B). The presence of PEG significantly increased
205 the CH₄ production for the diet + HP ($P=0.007$) and tended to increase it for the diet + PS
206 ($P=0.062$).

207

208 *3.3 Other in vitro rumen fermentation characteristics*

209 The pH of the medium was lower for the basal diet than for the diet + PS ($P=0.036$, Table
210 2). The IVDMD was greater for the basal diet than for the diet + HP and the diet + HP + PS,
211 while the diet + PS had the lowest value ($P<0.001$). The VFA net productions and profiles in
212 the medium were similar among treatments except for valerate, which was higher for the basal
213 diet than for the diet + PS and the diet + HP + PS ($P=0.003$, Table 2). The NH₃ concentration
214 was lower for the diet + PS than for the diets including HP, and was the highest for the basal
215 diet. Opposite PEG effects on NH₃ concentration were also detected for the basal diet
216 (incubation without PEG > with PEG, $P=0.041$) and the diet + PS (incubation without PEG <
217 with PEG, $P=0.016$) (Figure 1C).

218

219 **4. Discussion**

220 4.1 Fermentability of the diets including CT-containing resources

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

238

239 4.2 Methane reduction

240 In the early and late phases of the fermentation, the CH₄ production was consistently
241 higher for the basal diet compared to the CT-diets. The loss of fermentability due to CT when
242 PS or HP were included in the basal diet is certainly a part of the explanation. Besides, the
243 PEG effect observed after 24 h of incubation indicates clearly that CT in PS and HP also had
244 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 CH₄ 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 CH₄/apparently digestible OM also declined. In
248 our study, after 3.5 h and 24 h of incubation, the values for the CO₂:CH₄ 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 CH₄ 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 CH₄ formation
254 through a reduction of H₂ 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 CH₄ production can enhance fermentation
260 pathways that require a net incorporation of H₂ 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 H₂ 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 CH₄
267 production higher than for the CT-containing resources taken individually, likely due to the
268 higher total gas production.

269

270 4.3 Protein degradability

271 Regarding the fate of nitrogenous compounds, our results showed clearly that the inclusion
272 of PS and HP in the diet decreased the NH_3 concentration in the fermentation medium. As the
273 diets were isoproteic, it indicates that HP and PS resulted in a lower protein degradation or in
274 a greater incorporation of N in microbial biomass through microbial protein synthesis. As we
275 found that IVDMD, which can be partly linked to the microbial biomass, was lower in the
276 presence of HP and PS, it is more probable that less protein was degraded to NH_3 . The ability
277 of CT to form complexes with protein leading to their protection from ruminal degradation
278 has been recognised for a long time (Min et al., 2003). This role of CT was confirmed in our
279 study at least for the diet + PS for which the presence of PEG increased the NH_3
280 concentration. On the other hand, we found the lowest NH_3 concentration for the diet + PS,
281 suggesting that CT from PS were more active at reducing protein degradation than the CT
282 from HP as both diets were isotannic. It was reported that the size of CT is the key parameter
283 controlling protein binding activity as demonstrated using bovine serum albumin, for which
284 the activity clearly increased when the mDP values increased from 3 to 8 (Ropiak et al.,
285 2017). However, according to these authors there were only small differences in the efficacies
286 of larger CTs with mDP > 9 to aggregate the proteins. In our study, the mDP of CT of PS and
287 HP were similar (11 vs 13.5), suggesting that the size of these CT was not the main driver for
288 the activity differences. More importantly, the PD/PC ratio was the main difference and our
289 results suggest that a high PD/PC ratio as in PS gave rise to more active CT in terms of
290 protein binding activity than a low PD/PC ratio as in HP. This hypothesis is consistent with
291 the findings from Huyen et al. (2016a) who showed *in vitro* using CT extracts from different
292 plants that the proportion of PD in CT had the largest effect on rumen fermentation
293 characteristics. These authors argued that PD have more hydroxyl groups than PC, and thus
294 they are more able to bind fibre and protein, and impact on their degradability.

295

296 **5. Conclusions**

297 Under *in vitro* conditions, the inclusion of PS and HP in a basal diet for ruminants led to lowered
298 rumen fermentability and also decreased CH₄ production and protein degradability due to CT:
299 the PD-rich CT in PS were more effective than the PC-rich CT in HP with a possible increase
300 of duodenal N flow. The real impact on ruminant performances, the reduction in CH₄ emissions
301 and N urinary losses, and a potential increase in N use efficiency have to be evaluated *in vivo*.

302

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307 (Châteaubourg, France) and Inovfruit (Mussidan, France) which supplied PS and HP,
308 respectively.

309

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