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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

46 rumen fermentability and that their CT decreased CH_4 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.

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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; CH_4, methane; DM, dry matter; HP, hazelnut pericarps; IVDMD, *in*

57 *vitro* DM degradability; mDP, mean degree of polymerisation; N, nitrogen; NH_3, ammonia;

58 OM, organic matter; PC, procyanidins; PD, prodelphinidins; PEG, polyethylene glycol 4000;

59 PS, pellets of sainfoin; VFA, volatile fatty acids.

60

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 (N_2O), methane (CH_4) and

65 carbon dioxide (CO_2), 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

3

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 CH_4 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 flavonol 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 CH_4 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

4

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,

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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.

6

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 (CH_4, CO_2).

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 $3,400 \times g$ for 10

150   min at 4 °C. After sampling the supernatant for VFA and NH_3-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 $3,400 \times g$ 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 (CH_4 and CO_2) 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 NH_3 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 PROCMIXED 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 without 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

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

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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

4.2 Methane reduction

240   In the early and late phases of the fermentation, the CH_4 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-

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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.

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4.3 Protein degradability

270

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.

12

295

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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