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24 ***An in vitro* evaluation of the effects of a *Yucca schidigera* extract and chestnut**
25 **tannins on composition and metabolic profiles of canine and feline faecal**
26 **microbiota**

27

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64

65 **Abstract**

66 The *in vitro* effect of a *Yucca schidigera* extract and tannins from chestnut wood on
67 composition and metabolic activity of canine and feline faecal microbiota was
68 evaluated. Four treatments were carried out: control diet (Group CTRL), chestnut
69 tannins (Group CT), *Y. schidigera* extract (Group YSE) and Group CT+YSE. The YSE
70 was added to canine and feline faecal cultures at 0.1 g/l, while CT were added at 0.3
71 g/l for a 24 h incubation. A total of 130 volatile compounds were detected by means
72 of HS-SPME-GC/MS analyses. Several changes in the metabolite profiles of
73 fermentation fluids were found including a decrease of alcohols (- 19%) and esters (-
74 42%) in feline and canine inoculum, respectively, due to the antibacterial properties of

75 tannins. In canine inoculum, after 6 h, Group YSE+CT resulted in lower cadaverine
76 concentrations (- 37%), while ammonia (- 4%) and quinolone (- 27%) were reduced
77 by Group CT. After 24 h, the presence of CT resulted in a decrease of sulphur
78 compounds, such as dimethyl sulphide (- 69%) and dimethyl disulphide (- 20%). In
79 feline faecal cultures, after 6 h, Group CT lowered the amount of indole (- 48%),
80 whereas Group YSE tended to decrease trimethylamine levels (- 16%). Both in canine
81 and feline inoculum, Group CT and, to a minor extent, Group YSE affected volatile
82 fatty acids patterns. In canine faecal cultures, tannins exerted an inhibitory effect on
83 *E. coli* population (- 0.45 log₁₀ numbers of DNA copies/ml), while enterococci were
84 increased (+ 2.06 log₁₀ numbers of DNA copies/ml) by Group YSE. The results from
85 the present study show that *Y. schidigera* extract and tannins from chestnut wood exert
86 different effects on the composition and metabolism of canine and feline faecal
87 microbiota. In particular, the supplementation of *Y. schidigera* and tannins to diets for
88 dogs and cats may be beneficial due to the reduction of some potentially toxic volatile
89 metabolites.

90

91 *Keywords:* dogs; cats; intestinal microorganisms; *Yucca schidigera*; tannins; volatile
92 compounds.

93

94 *Abbreviations:* ADF, acid detergent fibre; ADL, acid detergent lignin; CT, tannins
95 from chestnut wood; DM, dry matter; FOS, fructo-oligosaccharides; GC/MS, gas
96 chromatography–mass spectrometry; NDF, neutral detergent fibre; PCA, principal
97 components analysis; HS-SPME, headspace solid-phase microextraction; VFA,
98 volatile fatty acids; VOCs, volatile organic compounds; YSE, *Yucca schidigera*
99 extract.

100

101 **1. Introduction**

102 The canine and feline gastrointestinal tract is inhabited by a variety of complex
103 microbial communities that play a fundamental role in maintaining the nutritional and
104 health status of the host. Diet is recognized as one of the major factors driving the
105 composition and metabolism of the gut microbiota (Russell et al. 2013). While many
106 studies have highlighted the beneficial effects deriving from the dietary inclusion of
107 non-digestible carbohydrates and prebiotics in dogs and cats (Pinna and Biagi 2014;
108 Rochus et al. 2014), little is known about feeding functional food components to these
109 animals.

110 *Yucca schidigera* Roezl ex Ortgies and tannin extracts are naturally occurring
111 plant substances that have been widely investigated in swine and poultry production
112 as potential phytochemical compounds for reducing odour and ammonia emissions
113 (Windisch et al. 2008; Biagi et al. 2010).

114 *Y. schidigera* is known to have antimicrobial (Wang et al. 2000), antiprotozoal
115 (McAllister et al. 2001), and antifungal (Miyakoshi et al. 2000) activities. The
116 biological effects of *Y. schidigera* have been attributed to its high content of steroidal
117 saponin fraction (Patra and Saxena 2009); however, Duffy et al. (2001) reported that
118 the observed effects were also due to the non-saponin fraction.

119 Tannins are natural polyphenolic compounds that can be chemically divided
120 into condensed and hydrolyzable tannins, whose structural diversity influences their
121 metabolism and bioavailability (Aura 2008). Tannins extracted from chestnut
122 (*Castanea sativa* Miller) wood are characterized by the presence of hydrolyzable
123 tannins, which are known to act as effective microbiota modulatory agents in poultry
124 (Jamroz et al. 2009), swine (Biagi et al. 2010), and ruminants (Hassanat and Benchaar
125 2013). While the administration of *Y. schidigera* to dogs and cats resulted in lower
126 faecal odour (Lowe et al. 1997; Lowe and Kershaw 1997; Giffard et al. 2001), to the

127 best of our knowledge, there are no studies on the influence of tannins on the canine
128 and feline intestinal ecosystems.

129 Considering that very little is known about the effects of *Y. schidigera* and
130 tannin extracts in dogs and cats, the aim of the present study was to evaluate *in vitro*
131 the effect of these extracts on the composition and activity of canine and feline faecal
132 microbiota. We hypothesized that adding *Y. schidigera* and tannin to canine and feline
133 faecal inocula may reduce the presence of potential pathogenic bacteria and toxic
134 compounds.

135

136 **2. Material and methods**

137 The current study was carried out at the Laboratory of Animal Production of the
138 Department of Veterinary Medical Sciences, University of Bologna, Italy.

139

140 ***2.1 Canine experiment***

141 Five healthy adult dogs (mixed breed; average BW 18.0 kg; age 4 to 6 year) were fed
142 the same commercial dry diet for adult dogs (Effeffe Pet Food S.p.A., Pieve di Porto
143 Morone, Italy), for 4 week before collection of fresh faecal samples. The diet contained
144 the following ingredients: dried poultry meat, rice and other cereals, oils and fat, dried
145 eggs by-products, vitamins, and minerals. The macronutrient composition of the diet
146 (as fed) was the following: water 53 g/kg, crude protein 229 g/kg, ether extract 154
147 g/kg, crude ash 69 g/kg, starch 370 g/kg, crude fibre 16 g/kg, neutral detergent fibre
148 (NDF) 137 g/kg, acid detergent fibre (ADF) 90 g/kg, and acid detergent lignin (ADL)
149 43 g/kg. A sample of fresh faeces was collected from each dog immediately after
150 excretion, pooled, and suspended at 10 g/l in prerduced Wilkins Chalgren anaerobe
151 broth. The faecal suspension was used to inoculate (100 ml/l) a previously warmed (39
152 °C) and prerduced medium prepared according to Sunvold et al. (1995). Five 30-ml

153 bottles (each bottle containing 21 ml of faecal culture) were set up per treatment. The
154 same dry food that was fed to the dogs used as faecal donors, was digested in triplicate
155 using the 2-step procedure proposed by Biagi et al. (2016). After *in vitro* digestion, the
156 undigested fraction was dried at 70 °C until a constant dry weight was obtained (19.3
157 g of undigested residue were obtained from 100 g of food dry matter (DM)) and its
158 chemical composition was the following: crude protein 169 g/kg, ether extract 100
159 g/kg, starch 17 g/kg, crude ash 238 g/kg, crude fibre 76 g/kg, NDF 313 g/kg, ADF 162
160 g/kg, and ADL 59 g/kg.

161 Four treatments were carried out: 1) control diet (CTRL) with no addition of
162 substrates, 2) group *Yucca schidigera* extract (group YSE), Syntonise (saponins
163 content 10.5%, Sintofarm S.p.A., Guastalla, Italy), 3) group chestnut tannins (group
164 CT), Farmatan 75 (tannins from chestnut wood, tannic acids 75.6%, Sintofarm S.p.A.,
165 Guastalla, Italy), 4) group YSE+CT. The YSE was added at a final concentration of
166 0.1 g/l, while CT was added at a final concentration of 0.3 g/l. The bottles also
167 contained the *in vitro* digested commercial dry food for dogs at 20 g/l. These
168 concentrations should reflect the amount of *Y. schidigera* and tannins extracts that
169 reach the hindgut when they are included in a commercial extruded food for dogs (with
170 a digestibility of approximately 80%) at a concentration of 1.0 and 3.0 g/kg,
171 respectively. In each study, five bottles were prepared without any experimental
172 substrate and with no addition of the digested food as a negative control. The pH of
173 faecal cultures was adjusted to 6.7; bottles were sealed and incubated for 24 h at 39 °C
174 in an anaerobic cabinet (Anaerobic System; Forma Scientific Co., Marietta, OH; under
175 an 85% N₂, 10% CO₂, and 5% H₂ atmosphere). Samples of fermentation fluid were
176 collected from each bottle at 6 and 24 h for the determination of pH, ammonia,
177 biogenic amines, volatile fatty acids (VFA), volatile compounds (VOCs), and for
178 microbial analysis.

179 **2.2 Feline experiment**

180 Four healthy European shorthair female cats (average BW 4.0 kg; age 4 to 6 year) were
181 fed the same commercial dry diet for adult cats (COOP Italia, Bologna, Italy), for 4
182 week before collection of fresh faecal samples. The diet contained the following
183 ingredients: cereals, meat and meat by-products, vegetable by-products, fish by-
184 products, protein plant extract, oils and fat, minerals, and vegetables. The
185 macronutrient composition of the diet (as fed) was the following: water 63 g/kg, crude
186 protein 277 g/kg, ether extract 94 g/kg, crude ash 98 g/kg, starch 397 g/kg, crude fibre
187 33 g/kg, NDF 258 g/kg, ADF 50 g/kg, and ADL 16 g/kg. The same dry food that was
188 fed to the cats used as faecal donors was digested in triplicate (Biagi et al. 2016). After
189 in vitro digestion of the diet fed to the cats, the undigested fraction was dried at 70 °C
190 (23.6 g of undigested residue were obtained from 100 g of food DM) and its chemical
191 composition was the following: crude protein 135 g/kg, ether extract 23 g/kg, starch
192 56 g/kg, crude ash 281 g/kg, NDF 322 g/kg, ADF 133 g/kg, and ADL 53 g/kg.
193 The method used in this experiment reflects the one adopted for the canine experiment.

194

195 **2.3 Chemical analyses**

196 Analyses of commercial dry food and digested diets were performed according to
197 AOAC International standard methods (AOAC, 2000; method 950.46 for water,
198 method 954.01 for crude protein, method 920.39 for ether extract, method 920.40 for
199 starch, method 942.05 for crude ash, and method 962.09 for crude fibre). Fibre
200 fractions were determined according to the procedure described by Van Soest et al.
201 (1991), where NDF was assayed with a heat stable amylase and expressed inclusive of
202 residual ash, ADF was expressed inclusive of residual ash, and lignin was determined
203 by solubilization of cellulose with sulfuric acid. Ammonia was measured using a
204 commercial kit (Urea/BUN – Color; BioSystems S.A., Spain). Volatile fatty acids

205 were analysed by HPLC. For the determination of biogenic amines, samples were
206 diluted 1:5 with perchloric acid (0.3 M); biogenic amines were later separated by
207 HPLC and quantified through fluorimetry.

208

209 **2.4 Microbial analysis**

210 A 1-ml portion of fermentation fluid was collected from each vessel and centrifuged
211 at 4 °C for 5 min at 18000 g. The supernatant was removed and immediately frozen at
212 – 80°C for further analysis. Bacterial genomic DNA was extracted from remaining
213 pellet using the QIAamp Fast DNA Stool Mini-Kit (QIAGEN GmbH, Germany).
214 Isolated DNA concentration (ng/μl) and purity were measured using a NanoDrop 1000
215 spectrophotometer (Thermo Scientific, Wilmington, DE). Template DNA was diluted
216 to 50 ng/μl and stored at – 20 °C until further analysis. *Escherichia coli* (Malinen
217 2003), *Bifidobacterium* genus (Matsuki et al. 2002), *Lactobacillus* genus (Collier et al.
218 2003), and *Enterococcus* genus (Rinttilä et al. 2004) were quantified via qPCR using
219 specific primers.

220 The qPCR assay was performed using a CFX96 Touch thermal cycler (Bio-
221 Rad, USA). Amplification was performed in duplicate for each bacterial group within
222 each sample. Briefly, the PCR reaction contained 7.5 μl 2X SensiFAST No-ROX PCR
223 Master Mix (Bioline GmbH, Germany), 4.8 μL of nuclease-free water, 0.6 μl of each
224 10 pmol primer, and 1.5 μl of template DNA for a final reaction volume of 15 μl. The
225 amplification cycle was as follows: initial denaturation at 95 °C for 2 min, 95 °C for 5
226 s, primer annealing at 55–61 °C for 10 s and 72 °C for 8 s. The cycle was repeated 40
227 times. A negative control (without the DNA template) was also run for each primer
228 pair. Standard curves were constructed from eight 10-fold dilutions for *Escherichia*
229 *coli*, *Bifidobacterium* genus, *Lactobacillus* genus, and *Enterococcus* genus. Cycle
230 threshold (Ct) values were plotted against standard curves for quantification of the

231 target bacterial DNA from faecal inoculum. Melting curves were checked after
232 amplification to ensure single product amplification of consistent melting temperature.

233

234 ***2.5 Determination of total volatile compounds (VOCs)***

235 A 1-ml portion of fermentation fluid was collected from each vessel, placed in a 7-ml
236 vial with PTFE/red rubber septa (Supelco, Bellefonte, PA, USA) and capped. Volatile
237 compounds were extracted by headspace-solid phase microextraction (HS-SPME)
238 using a fused-silica fibre (10 mm length) coated with a 50/30 mm thickness of
239 divinylbenzene/carboxen/polydimethylsiloxane (DVB/CAR/PDMS) and determined
240 by GC/MS (QP-2010 Plus, Shimadzu, Japan), interfaced with a computerized system
241 for data acquisition (Software GC-MS Solution V. 2.5, Shimadzu, Japan). A RTX-
242 WAX column (30 m, 0.25 mm i.d., 0.25 µm film thickness, Restek, USA) coated with
243 a stationary phase of polyethylene glycol, was used. The SPME fibre was first
244 conditioned by heating it in a GC injection port at 270 °C for 60 min; then, it was
245 inserted into the sample vial through the septum and exposed to the headspace (10 min
246 at 60 °C) of the previously conditioned sample in a water bath at 60 °C for 60 min.
247 Thereafter, the fibre was withdrawn into the needle and transferred to the injection port
248 of the GC/MS system. The SPME fibre was desorbed and maintained in the injection
249 port at 240 °C for 10 min. The sample was injected in split mode at a 1:30 split ratio.
250 Helium was used as the carrier gas at a constant flow rate of 1 mL/min and a linear
251 velocity of 36.2 cm/s. The oven temperature was kept at 40 °C for 10 min, then raised
252 to 200 °C at 3 °C/min, and finally increased to 230 °C at 10 °C/min; final temperature
253 was held for 5 min. The total run time was 74.33 min. Both injector and interface
254 temperatures were set at 240 °C. The ion source temperature was set at 200 °C. The
255 filament emission current was 70 eV. A mass range from 40 to 250 m/z was scanned
256 at a rate of 0.25 scans/s. The acquisition and integration modes were Full Scan (TIC)

257 and Single Ion Monitoring (SIM), respectively. Compounds were identified by
258 comparing their mass spectra with those contained in the NIST08 (National Institute
259 of Standards and Technology, Gaithersburg) library and those reported in a previous
260 study (Francioso et al. 2010). In order to control and prevent eventual environmental
261 contamination, a blank injection of fibre and vials was carried out daily.

262

263 ***2.6 Statistical analyses***

264 Data were analysed by 2-way ANOVA, with group YSE and group CT as the main
265 effects; the Newman-Keuls test was used as the post hoc test. Five bottles ($n = 5$) were
266 set up per treatment; each bottle represented an independent replicate. Differences
267 were considered statistically significant at $p < 0.05$. The principal components analysis
268 (PCA) was used to better understand the data variability. Statistical analyses were
269 performed using Statistica 10.0 software (Stat Soft Italia, Italy).

270

271 **3. Results**

272 ***3.1 Chemical and microbial analyses***

273 ***3.1.1 Canine experiment***

274 pH values and concentration of ammonia and biogenic amines after 6 and 24 h of
275 incubation with canine faecal inoculum are shown in Table 1. The pH values at 6 h
276 were unaffected by treatments ($p > 0.05$), while, after 24 h of incubation, pH was
277 significantly higher ($p < 0.001$) in vessels containing group CT. After 6 h of
278 fermentation, ammonia was lowered ($p < 0.05$) by group CT. At 6 h, in the group
279 CT+YSE vessels, the concentration of cadaverine was reduced ($p < 0.001$). After 24 h
280 of incubation, group CT tended to lower the concentration of putrescine ($p = 0.088$)
281 and decreased spermidine ($p = 0.010$). Finally, after 24 h of incubation, group YSE
282 tended to reduce spermine concentrations ($p = 0.066$).

283 Volatile fatty acids concentrations at 6 and 24 h of fermentation with canine
284 faecal inoculum are shown in Table 2. Only traces of n-valeric acid were detected (data
285 not shown). Production of total VFA was decreased ($p < 0.004$) by group CT after 6
286 h, but not after 24 h of incubation. After 6 h of incubation, addition of group CT to the
287 inocula resulted ($p < 0.05$) in lower concentrations of acetate, propionate and
288 isobutyrate; the latter was decreased ($p = 0.009$) also by group YSE. After 24 h of
289 incubation, group CT tended to reduce ($p = 0.083$) the concentrations of propionic
290 acid.

291 The abundances of some bacterial populations in canine faecal inoculum are
292 presented in Table 3. After 6 h, group YSE resulted in an increase ($p = 0.035$) of the
293 presence of *Enterococcus* spp., whereas *E. coli* abundances were lowered by group CT
294 ($p = 0.009$). After 24 h of incubation, the abundances of the assessed microbial
295 populations were not affected by treatments ($p > 0.05$), but group YSE tended to reduce
296 the presence of lactobacilli ($p = 0.053$). Bifidobacteria were inconsistently detected in
297 canine faecal inoculum (data not shown). (Tables 1, 2 and 3 near here).

298

299 3.1.2 Feline experiment

300 The values of pH and concentration of ammonia and biogenic amines determined after
301 6 and 24 h of incubation with feline faecal inoculum, are shown in Table 4.

302 After 6 h of incubation, the pH level was reduced ($p = 0.021$) by group YSE,
303 while, at 24 h, pH was higher ($p < 0.001$) in vessels containing group CT. Ammonia
304 concentrations at 6 and 24 h were not affected by treatment ($p > 0.05$). Regarding the
305 concentration of biogenic amines, cadaverine was lowered by group YSE at both 6 (p
306 = 0.017) and 24 h (- 20%; $p < 0.001$), while an increase ($p = 0.040$) in spermine
307 concentration was observed at 24 h in the group YSE vessels.

308 Concentrations of volatile fatty acids at 6 and 24 h of fermentation with feline
309 faecal inoculum are shown in Table 5. After 6 h, concentration of total VFA tended to
310 be lower for both group CT ($p = 0.092$) and group YSE ($p = 0.052$) treatments, while,
311 at 24 h, total VFA concentration was significantly reduced ($p = 0.002$) by group CT.
312 At 6 h, the presence of group YSE resulted in lower concentrations of acetic ($p = 0.010$)
313 and valeric acids ($p = 0.006$). In bottles containing group CT, a reduction ($p = 0.010$)
314 of the concentration of propionic acid was observed at 6 h, while a decrement of acetate
315 ($p = 0.044$), propionate ($p < 0.0001$) and isovalerate ($p = 0.039$) was noted at 24 h.
316 Similarly, at 24 h, group CT tended to reduce ($p = 0.095$) the concentration of butyrate.

317 Abundances of investigated microbial populations in the feline faecal inoculum
318 are shown in Table 6. At 6 h, the presence of enterococci was reduced by group
319 CT+YSE ($p = 0.008$). (Tables 4, 5 and 6 near here).

320

321 ***3.2 Volatile compounds (VOCs) detected by HS-SPME-GC/MS analysis***

322 In all samples, a total of 130 different metabolites were identified, of which 36 were
323 cat-related and 20 were dog-related (Supplemental Table 1). Most of VOCs that have
324 been identified belong to the families of esters and alcohols; however, after 6 h of
325 incubation, one of the most represented metabolites in feline and canine faecal
326 inoculum was indole, which accounted for 47.1% and 39.7% of total VOCs area,
327 respectively. After 24 h of fermentation, indole proportions decreased in both dog and
328 cat inoculum (22.2% and 17.8% of total VOCs area, respectively). Other
329 representative metabolites detected in both dog and cat vessels after 6 h of incubation,
330 were ethanol, 1-propanol, 1-butanol, 3-methyl-1-butanol, and 1-hexanol (belonging to
331 the family of alcohols), while phenol and dimethyl trisulphide were more predominant
332 in dog faecal inoculum. After 24 h of fermentation, phenol was the most abundant
333 metabolite detected in the dog faecal inoculum (32.9% of total VOCs area), followed

334 by indole, dimethyl trisulphide and dimethyl disulphide (22.2%, 11.2% and 6.7% of
335 total VOCs area, respectively).

336

337 *3.2.1 Canine experiment*

338 Absolute area of VOCs obtained from the HS-SPME-GC/MS analysis of dog faecal
339 inoculum is shown in Supplemental Table 2. After 6 h of incubation, hydrogen
340 sulphide was higher ($p < 0.05$) in group CT vessels. Regarding aldehyde compounds,
341 the addition of group CT resulted in higher amounts of caproaldehyde ($p = 0.024$) and
342 nonaldehyde ($p = 0.017$) after 6 h of fermentation, and higher amounts of
343 isovaleraldehyde at both 6 ($p < 0.001$) and 24 h ($p < 0.001$). Similarly,
344 isovaleraldehyde amount was increased ($p < 0.05$) by group YSE at 24 h. On the
345 contrary, after 6 h of incubation, a significant decrease ($p = 0.025$) of caproaldehyde
346 was observed in YSE vessels. Quinoline was decreased by the presence of group CT
347 at both 6 and 24 h ($p < 0.05$), while phenol was increased at 6 h by group CT ($p =$
348 0.007).

349 After 24 h of incubation, a decrease of the amount of dimethyl sulphide ($p =$
350 0.058) and dimethyl disulphide ($p < 0.05$) was observed in group CT batches;
351 similarly, group CT decreased the amount of acetic acid butyl ester ($p < 0.001$), acetic
352 acid propyl ester ($p = 0.003$) and total esters ($p = 0.006$).

353

354 *3.2.2 Feline experiment*

355 Absolute areas of the significantly different VOCs detected by HS-SPME-GC/MS
356 analysis of feline faecal inoculum are shown in Supplemental Table 3. After 6 h of
357 incubation, lower amounts of indole ($p < 0.001$), 1-butanol ($p = 0.008$), 1-butanol, 3-
358 methyl ($p < 0.01$), 1-pentanol ($p = 0.019$) and total alcohols ($p = 0.035$), were observed
359 in vessels containing group CT. Conversely, group CT increased ($p < 0.05$) the amount

360 of hydrogen sulphide after 6 h of incubation. Addition of YSE resulted in a decreasing
361 trend in trimethylamine ($p = 0.069$) and ethanol ($p = 0.073$) amounts.

362 After 24 h, hydrogen sulphide amount was lower ($p < 0.05$) in batches where
363 the combination CT+YSE had been added, while a reduction tendency ($p = 0.092$) of
364 this metabolite was observed in group YSE vessels. Likewise, the addition of CT+YSE
365 to the inocula tended to reduce total aldehydes ($p = 0.095$), 2-propanone ($p = 0.071$)
366 and acetic acid ethyl ester ($p = 0.095$). After 24 h of incubation, a decrease ($p < 0.05$)
367 of propanethioate-S-methyl and acetic acid 3-methylbutyl ester was observed in group
368 CT vessels. On the contrary, the amount of *p*-cresol was increased significantly ($p =$
369 0.002) by group CT.

370

371 ***3.3 Principal Component Analysis (PCA)***

372 *3.3.1 PCA of canine experiment*

373 To evaluate the effect of CT and YSE addition on VOCs formation and to define which
374 compounds were the most representative of variance, the data were subjected to PCA.
375 When samples of dog faecal inoculum were tested, the first two principal components
376 reached 77.08% of variance (Supplemental Figure 1). The factor 1 explains 65.63% of
377 variance; in particular, it is possible to observe that sulphur compounds, as well as
378 acetic acid esters, alcohols, organic acids and quinolone, are inversely correlated to
379 indole and carbon disulphide. In addition, a third cluster consisting mainly of
380 aldehydes (isovaleraldehyde, caproaldehyde, nonanal, benzaldehyde and 2-
381 methylbutyraldehyde) was more correlated to Factor 2 (11.45% of variance) (Figure
382 2). Considering the Biplot (Supplemental Figure 2), results at 6-h were well separated
383 from the 24-h ones; in particular, the 6-h group CT assay was completely segregated
384 from the other treatments, while all 24-h assays were plotted in the same cluster and

385 were mainly characterized by the presence of sulphur compounds, alcohols and acetic
386 acid esters.

387 In order to better understand the variance of results, the PCA of all determined
388 parameters from the canine experiment was carried out. As reported in Biplot (Figure
389 1), a total of 86.26% of variance was comprised in the first two principal components,
390 of which PC1 explains 69.98% of total variance. In addition, it is possible to observe
391 the presence of several clusters; in fact, PC2 evidences how lactobacilli, enterococci,
392 total organic acids and 2-propanone were inversely correlated to the main aldehydes
393 (e.g. caproaldehyde, benzaldehyde, etc.) and the 6-h control sample. On the other hand,
394 the 24-h samples did not separate and were mainly characterized by the VOCs
395 presence as reported above, which thus demonstrate a non-significant effect of group
396 CT and group YSE after 24 h. (Figure 1 near here).

397

398 *3.3.2 PCA of feline experiment*

399 A PCA analysis of feline faecal VOCs was also carried out. Supplemental Figure 3
400 reports the component loading in the cat experiment; the first two principal
401 components explained 81.78% of the total variance. In general, aldehydes, alcohols,
402 organic acids and sulphur compounds were more correlated to Factor 1, which
403 explained 67.26% of variance, whereas acetic acid esters, phenol and indole were
404 correlated to Factor 2, which explained 14.52% of variance. In Supplemental Figure
405 4, a clear separation between 6-h and 24-h results is evidenced. After 6 h, group YSE
406 and group CT treatments were mainly characterized by the presence of aldehydes (3-
407 methylbenzaldehyde and isovaleraldehyde) and alcohols (3-methyl-1-butanol and
408 ethanol), while VOCs at 24 h are mainly depicted by acetic acid esters, phenol, sulphur
409 compounds, butyraldehyde and benzaldehyde. However, it might be pointed out that,

410 at 6 h, the combination YSE+CT was inversely correlated to the concentrations of
411 indole, acetic acid propyl- and ethyl-esters and methyl propane thioate.

412 In addition, the PCA of all determined parameters was carried out (Figure 2).
413 The 73.54% of total variance explained by the first two principal components, of which
414 PC2 was more correlated to *Escherichia coli*, ethyl acetate and ketones. As shown in
415 Figure 2, different clusters could be distinguished, in particular the 6-h samples were
416 all gathered in the same cluster, characterized by aldehydes and ethanol, and inversely
417 correlated to the presence of lactobacilli and organic acids. On the other hand, the 24-
418 h samples (except for CTRL 24h) were mainly depicted by the presence of ammonia,
419 short-chain alcohols and sulphur compounds. (Figure 2 near here).

420

421 **4. Discussion**

422 The results from the present *in vitro* study showed that *Y. schidigera* extract and
423 chestnut tannins were able to exert some influence on the metabolism of canine and
424 feline faecal microbiota.

425

426 ***4.1 pH values, SCFA production and bacterial protein catabolism***

427 It is known that a low intestinal pH helps to improve the health of the gut mucosa by
428 reducing unwanted bacteria and absorption of ammonia, while higher intestinal pH is
429 thought to promote the growth of detrimental microorganisms (Zentek et al. 2013).

430 When incubated with both canine and feline faecal inocula, tannins increased the pH
431 of the fermentation fluid at 24 h, presumably as a consequence of the reduction of total
432 VFA production. In fact, addition of CT to canine and feline slurries resulted in lower
433 concentrations of acetate and propionate. Bravo et al. (1994) similarly reported that
434 fermentation of tannic acid (1 g/l) with a rat caecal inoculum led to a decrease in acetic
435 and propionic acid concentrations, while tannic acid at 2.5 g/l resulted in the total

436 suppression of acetic acid production, suggesting that tannic acid could be a specific
437 inhibitor of acetate-producing bacteria. A decrease of SCFA concentrations in the
438 colonic lumen may lead to mucosal atrophy as a consequence of a lack of energy for
439 colonocytes (Machiels et al. 2014).

440 Compared with CTRL, group CT and group YSE significantly reduced the
441 concentration of isobutyric acid in canine inocula, while group CT lowered the amount
442 of isovalerate in feline inocula after 6 h. Isobutyric and isovaleric acid, as well as 2-
443 methylbutyrate, mainly derive from the degradation of valine, isoleucine and leucine
444 (Hughes et al. 2000), and are used as faecal markers for bacterial protein catabolism.
445 In the first experiment, ammonia concentration, as well as spermidine concentration,
446 were reduced by group CT, while the association CT+YSE decreased the amount of
447 cadaverine. In presence of feline faecal inocula, group CT failed to decrease the
448 amount of biogenic amines, even though group YSE reduced cadaverine but increased
449 spermine concentration. Ammonia and biogenic amines are also products which derive
450 from proteolytic reactions; in particular, biogenic amines are generally formed through
451 the decarboxylation of specific free amino acids by microbial decarboxylases (Ku et
452 al. 2013). The inhibitory effects of tannins on microbial proteolysis had already been
453 observed by Biagi et al. (2010) in piglets (both *in vitro* and *in vivo*), and by Hassanat
454 and Benchaar (2013) in presence of ruminal inoculum. *Yucca schidigera* is commonly
455 used as a dietary additive in livestock and companion animals (Lowe et al. 1997; Lowe
456 and Kershaw 1997) for the reduction of ammonia and faecal odour in animal excreta.
457 Several mechanisms have been proposed to explain the effect of *Y. schidigera* extracts
458 on ammonia concentrations in animal faeces, among which are the ability to inhibit
459 the enzyme urease (Balog et al. 1994), to bind directly ammonia (McCrorry and Hobbs
460 2001) and to inhibit specific bacterial strains (Patra and Saxena, 2009). However, in
461 the present study, YSE failed to reduce ammonia concentrations. Similarly, ammonia

462 content of caecal material did not decrease in rats receiving sarsaponin (steroidal
463 glycosides extracted from *Y. schidigera*) with the diet (120 mg/kg of diet; Preston et
464 al., 1987).

465

466 ***4.2 Faecal microbiota***

467 In this study, the addition of YSE to canine faecal inoculum resulted in an increase of
468 enterococci but the same effect was not observed when group YSE was incubated in
469 presence of the feline faecal inoculum. There is no evidence in the literature suggesting
470 that *Y. schidigera* may act as a prebiotic so that, at present, we do not know if the
471 increment of enterococci was the consequence of a direct effect of YSE on their growth
472 or the result of the inhibition of enterococci antagonists by the saponins contained in
473 the YSE (Killeen et al. 1998). Among the tested substrates, group CT resulted in a
474 reduction of *E. coli* in the first experiment, while, in presence of feline faecal inoculum,
475 *E. coli* abundances tended to be lower when CT was used in association with YSE.
476 These results are not in agreement with those of Biagi et al. (2010), who reported a
477 lack of antibacterial activity of chestnut tannins against coliforms, both *in vitro* and *in*
478 *vivo*. It has been observed that yucca saponins may exhibit antibacterial effects on *E.*
479 *coli* (Sen et al. 1998), but other authors did not report any bacteriostatic effect of *Y.*
480 *schidigera* on pure cultures of *E. coli* (Killeen et al. 1998). However, Wang et al.
481 (2000) showed that saponins from different sources, including *Y. schidigera*, are more
482 effective against gram-positive than gram-negative bacteria (e.g. *E. coli*). The
483 reduction of the presence of *E. coli* in the animals' intestine may be considered
484 beneficial. However, when chestnut tannins were added to canine faecal inocula, minor
485 effects were observed only after 6 h of incubation, which are probably without any
486 physiological importance.

487

488 ***4.4 Volatile organic compounds in faecal inocula***

489 To the best of our knowledge, our results provide the first characterization of VOCs
490 profile of canine and feline faecal inocula by HS-SPME-GC/MS. A great variety of
491 VOCs was found in the fermentation fluids. The addition of chestnut tannins to the
492 canine faecal inocula led to an increase of hydrogen sulphide and a decrease of
493 dimethyl sulphide and dimethyl disulphide, making the interpretation of these results
494 very difficult, as both metabolites are produced from the catabolism of S-containing
495 amino acids. Hydrogen sulphide is the main product of sulphate-reducing bacterial
496 catabolism of cysteine and, when present at high concentrations, may inhibit the
497 oxidation of butyrate by the colonic epithelium (Roediger et al. 1993). In general,
498 hydrogen sulphide is methylated by the colonic epithelium to less harmful products,
499 such as methanethiol and dimethyl sulphide (Weiseger et al. 1980). In the feline
500 experiment, an increase of carbon sulphide and hydrogen sulphide was observed at 6
501 h in the vessels containing CT, while hydrogen sulphide was decreased at 24 h by
502 group CT+YSE. In a previous *in vitro* study with dog faecal inoculum, the addition of
503 *Y. schidigera* (0.17 g/l) resulted in lower (- 38%) hydrogen sulphide but did not affect
504 total gas production (Giffard et al. 2001). In the study by Swanson et al. (2002), feeding
505 dogs with a *Lactobacillus acidophilus* supplemented diet tended to increase dimethyl
506 sulphide, methanethiol and hydrogen sulphide faecal concentrations. The ability of
507 some species of *Lactobacillus* spp. to produce hydrogen sulphide has been reported by
508 Arici et al. (2004). In the already cited study by Swanson et al. (2002), dogs fed fructo-
509 oligosaccharides (FOS) at 2 g/d had the lowest faecal concentrations of S-compounds.
510 Conversely, in the study by Hesta et al. (2005), the administration of FOS at 31 g/kg
511 of diet (on DM basis) failed to reduce the faecal concentration of sulphur compounds
512 in cats receiving a protein-restricted diet. Although in this study S-compounds
513 accounted only for a small part of identified chemicals (1.40% and 2.65% of total

514 VOCs area after 6 h of incubation in presence of feline and canine faecal inocula,
515 respectively), they are in general considered responsible for the distinctive bad odour
516 of faeces. In fact, as reported by Hesta et al. (2005), S-containing components have an
517 important impact on faecal odour, since six out of 10 compounds with the lowest odour
518 detection threshold (degree of sensitivity of humans) contain sulphur.

519 While the utilization of CT led to contradictory results with regard to the
520 concentrations of S-compounds, the experimental substrates used in this study were
521 more effective in limiting the presence of N-compounds. In fact, group CT lowered
522 the amount of quinoline (in presence of canine faecal inoculum) and indole (when
523 incubated with feline faecal inoculum), while group YSE tended to lower the amount
524 of trimethylamine in presence of cat faecal inoculum. Quinoline and indole are
525 aromatic heterocyclic organic compounds; indole derives from microbial degradation
526 of tryptophan in the colon (Shimada et al. 2013). In the past, indole was suspected to
527 contribute to colon carcinogenesis, but Shimada et al. (2013) recently attributed to
528 indole immunomodulatory and anti-inflammatory properties on intestinal epithelial
529 cells. However, in a study by Nowak and Libudzisz (2006), indole showed negative
530 effects on the *in vitro* viability of lactic acid bacteria. Trimethylamine is a tertiary
531 amine derived from the bacterial catabolism of choline and, to a less extent, carnitine
532 (Russell et al. 2013); trimethylamine can be further metabolized by gut microbiota to
533 trimethylamine-N-oxide, a catabolite recently considered to be involved in human
534 colorectal cancer pathogenesis (Xu et al. 2015). In the present study, trimethylamine
535 levels tended to be reduced by group YSE.

536 The utilization of CT resulted in higher concentrations of phenol and *p*-cresol
537 (4-methylphenol) in canine and feline faecal inocula, respectively. Phenol and *p*-cresol
538 are recognized as potentially harmful by-products deriving from the bacterial
539 catabolism of aromatic amino acids and are correlated with the development of cancer

540 in the human colon (Nowak and Libudzisz 2006). It has been shown that numerous
541 mammals host bacteria that are able to degrade hydrolyzable tannins (Kohl et al. 2015).
542 Hydrolyzable tannins are polyesters consisting of a carbohydrate core and phenolic
543 acids (mainly gallic and ellagic acids), which are characterized by a phenolic ring and
544 a carboxyl group. Based on our results, it may be supposed that the phenolic acids
545 released from the tannins may be further metabolized to phenol and *p*-cresol by the gut
546 microbiota. The slightly higher amount of phenol detected in vessels containing YSE
547 and canine faecal inoculum may be the consequence of microbial degradation of
548 polyphenolic compounds (resveratrol and yuccaols) that can be found in *Y. schidigera*.
549 However, the microbial metabolism of resveratrol in the large intestine is still
550 uncertain (Selma et al. 2009). Interestingly, the association CT+YSE resulted in a
551 strong reduction of phenol concentrations, suggesting a synergistic effect of CT and
552 YSE for which we do not have an explanation.

553 The observed reduction of the amounts of ketones and alcohols as a result of
554 CT addition, seems to suggest a decreased microbial activity induced by tannins. In
555 fact, ketones can derive from the microbial catabolism of fatty acids, while alcohols
556 can be produced during sugar fermentation or reduction of organic acids by bacteria
557 (de Lacy Costello et al. 2014).

558 Esters, the most numerous chemical class (36 identified compounds) in our
559 samples, derive from the esterification of alcohols and fatty acids by the gut microbiota
560 or by the esterase activity of the enterocytes (Garner et al. 2007). The lowering effect
561 of group CT on esters abundance in the canine and, to a lesser extent, feline faecal
562 inocula may be related, as observed by Daniel et al. (1991) in rats, to the inhibition of
563 esterase activity by tannins.

564 Changes in the composition and abundances of VOCs in the headspace of
565 vessels that were observed in this study can be directly related to the gut microbiota

566 metabolism and to the presence of the experimental substrates. Feeding *Y. schidigera*
567 to dogs and cats has been demonstrated to reduce faecal malodour by sensory testing
568 (Lowe and Kershaw 1997), while a concurrent study with dogs and cats showed
569 changes in the faecal concentrations of several volatile components after *Y. schidigera*
570 extract supplementation (Lowe et al. 1997). However, the authors did not find a direct
571 correlation between the changes in the amounts of VOCs and the aroma amelioration.
572 In fact, faecal odour is the resultant of absolute and relative concentrations of several
573 compounds, the possible interactions among different chemicals and the impact of both
574 texture and microstructure of faeces on release of odour compounds (Hesta et al. 2005;
575 de Lacy Costello et al. 2014).

576

577 **5. Conclusions**

578 The results from the present study show that *Y. schidigera* extract and tannins from
579 chestnut wood exert different effects on the composition and metabolism of canine and
580 feline faecal microbiota. Although a decrease in malodorous compounds was
581 observed, it is not possible to state whether or not the tested substrates lowered the
582 inoculum odour. However, the dietary supplementation of *Y. schidigera* and tannins to
583 dogs and cats may be beneficial due to the reduction of some potentially toxic volatile
584 metabolites.

585

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588

589 **Disclosure statement**

590 No potential conflict of interest was reported by the authors.

591

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595

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737

738

Table 1. pH values and mean concentrations (n = 5) of ammonia [mmol/l] and biogenic amines [$\mu\text{mol/l}$] after 6 and 24 h of an in vitro incubation of canine faecal inoculum with a control diet to which different substrates were added.

	Group CTRL	Group CT	Group YSE	Group CT + YSE	Pooled SEM	ANOVA <i>p</i> -value		
						Group CT	Group YSE	Group CT + YSE
At 6 h								
pH	6.37	6.37	6.36	6.36	0.01	0.955	0.436	0.822
NH ₃	38.5	37.1	38.7	36.8	0.48	0.015	0.979	0.641
Putrescine	928	1030	1036	854	42.3	0.634	0.684	0.104
Cadaverine	221	225	267	140	15.7	< 0.001	0.219	< 0.001
Spermidine	32.2	32.2	29.6	34.6	1.66	0.151	0.953	0.151
Spermine	18.0	17.0	15.6	19.4	1.48	0.357	0.990	0.124
At 24 h								
pH	6.12	6.19	6.09	6.19	0.001	< 0.001	0.186	0.253
NH ₃	38.2	40.8	41.3	43.8	2.77	0.366	0.287	0.980
Putrescine	1336	1326	1497	1318	52.1	0.088	0.162	0.123
Cadaverine	322	335	342	312	20.5	0.684	0.950	0.310
Spermidine	40.0	33.6	40.6	30.4	2.84	0.010	0.653	0.513
Spermine	24.6	21.0	20.0	16.2	2.38	0.140	0.066	0.967

Table 2. Mean concentrations (n = 5) of VFA [mmol/l] after 6 and 24 h of an *in vitro* incubation of canine faecal inoculum with a control diet to which different substrates were added.

	Group CTRL	Group CT	Group YSE	Group CT + YSE	Pooled SEM	ANOVA <i>p</i> -value		
						Group CT	Group YSE	Group CT + YSE
At 6 h								
Acetic acid	12.0	11.1	11.7	10.7	0.30	0.016	0.373	0.895
Propionic acid	9.86	8.62	9.40	8.95	0.26	0.019	0.837	0.232
Isobutyric acid	1.11	0.67	0.61	0.42	0.10	0.023	0.009	0.310
<i>n</i> -Butyric acid	4.88	4.41	4.70	4.47	0.18	0.138	0.795	0.612
Isovaleric acid	0.90	0.87	0.87	0.66	0.12	0.193	0.199	0.321
Total VFA	28.8	25.7	27.3	25.2	0.60	0.004	0.215	0.528
At 24 h								
Acetic acid	16.3	14.9	16.7	16.1	0.86	0.276	0.389	0.651
Propionic acid	14.1	11.8	15.5	13.8	0.82	0.083	0.172	0.343
Isobutyric acid	1.07	1.02	1.30	1.22	0.13	0.587	0.109	0.927
<i>n</i> -Butyric acid	5.84	5.78	6.09	6.26	0.41	0.893	0.399	0.771
Isovaleric acid	1.77	1.81	1.87	1.86	0.15	0.910	0.636	0.887
Total VFA	39.3	35.4	40.5	39.4	2.24	0.286	0.262	0.553

Table 3. Microbial populations [log 10 DNA copies/ml] detected by qPCR in canine faecal inoculum with a control diet to which different substrates were added.

Values are the means of 5 bottles per treatment.

	Group CTRL	Group CT	Group YSE	Group CT + YSE	Pooled SEM	ANOVA <i>p</i> -value		
						Group CT	Group YSE	Group CT + YSE
At 6 h								
<i>Lactobacillus</i> spp.	8.06	6.35	7.11	7.06	0.54	0.132	0.837	0.157
<i>Enterococcus</i> spp.	7.38	6.36	9.44	8.22	0.82	0.206	0.035	0.908
<i>E. coli</i>	5.19	4.74	5.95	4.68	0.28	0.009	0.246	0.175
At 24 h								
<i>Lactobacillus</i> spp.	8.41	7.74	7.31	7.54	0.30	0.500	0.053	0.170
<i>Enterococcus</i> spp.	8.10	8.77	7.12	7.86	1.01	0.509	0.381	0.974
<i>E. coli</i>	5.33	4.93	4.84	5.37	0.33	0.857	0.942	0.193

Table 4. pH values and mean concentrations (n = 5) of ammonia [mmol/l] and biogenic amines [$\mu\text{mol/l}$] after 6 and 24 h of an in vitro incubation of feline faecal inoculum with a control diet to which different substrates were added.

	Group CTRL	Group CT	Group YSE	Group CT + YSE	Pooled SEM	ANOVA <i>p</i> -value		
						Group CT	Group YSE	Group CT + YSE
At 6 h								
pH	6.28	6.28	6.24	6.26	0.01	0.377	0.021	0.372
NH ₃	33.4	34.9	34.0	33.5	0.38	0.571	0.664	0.233
Putrescine	187	200	190	179	7.74	0.860	0.188	0.074
Cadaverine	243	245	181	196	21.3	0.689	0.017	0.774
Spermidine	29.0	30.8	32.1	30.4	1.99	0.795	0.641	0.503
Spermine	5.60	5.50	5.00	4.04	0.52	0.355	0.083	0.451
At 24 h								
pH	6.10	6.14	6.05	6.17	0.01	< 0.0001	0.344	0.004
NH ₃	52.2	50.9	48.7	52.0	1.68	0.777	0.744	0.536
Putrescine	303	379	373	304	23.8	0.872	0.924	0.007
Cadaverine	265	235	211	212	9.77	0.142	<0.001	0.132
Spermidine	32.0	28.4	27.4	34.4	2.68	0.534	0.797	0.065
Spermine	6.38	7.46	9.40	8.88	0.99	0.781	0.040	0.432

Table 5. Mean concentrations (n = 5) of VFA [mmol/l] after 6 and 24 h of an *in vitro* incubation of feline faecal inoculum with a control diet to which different substrates were added.

	Group CTRL	Group CT	Group YSE	Group CT + YSE	Pooled SEM	ANOVA <i>p</i> -value		
						Group CT	Group YSE	Group CT + YSE
At 6 h								
Acetic acid	14.8	13.3	12.7	12.1	0.34	0.082	0.010	0.385
Propionic acid	7.09	6.51	7.16	5.99	0.18	0.010	0.446	0.335
Isobutyric acid	0.53	0.39	0.51	0.53	0.05	0.583	0.602	0.498
<i>n</i> -Butyric acid	7.54	7.96	7.58	7.49	0.16	0.666	0.558	0.489
Isovaleric acid	0.63	0.65	0.62	0.68	0.04	0.646	0.894	0.788
<i>n</i> -Valeric acid	0.38	0.32	0.25	0.24	0.02	0.348	0.006	0.455
Total VFA	31.0	29.1	28.8	27.1	0.55	0.092	0.052	0.942
At 24 h								
Acetic acid	24.2	22.4	23.6	21.7	0.44	0.044	0.451	0.961
Propionic acid	12.7	10.6	12.8	10.0	0.34	<0.0001	0.681	0.393
Isobutyric acid	1.21	1.21	1.11	1.10	0.05	0.974	0.383	0.987
<i>n</i> -Butyric acid	10.1	9.68	9.86	9.29	0.14	0.095	0.292	0.718
Isovaleric acid	2.01	1.95	2.26	1.80	0.07	0.039	0.686	0.108
<i>n</i> -Valeric acid	2.29	3.03	2.60	3.12	0.19	0.109	0.599	0.774
Total VFA	52.5	48.9	52.3	47.1	0.75	0.002	0.422	0.497

Table 6. Microbial populations [log₁₀ DNA copies/ml] detected by qPCR in feline faecal inoculum with a control diet to which different substrates were added.

Values are the means of 5 bottles per treatment.

	Group CTRL	Group CT	Group YSE	Group CT + YSE	Pooled SEM	ANOVA <i>p</i> -value		
						Group CT	Group YSE	Group CT + YSE
At 6 h								
<i>Lactobacillus</i> spp.	8.63	7.67	7.87	7.83	0.17	0.153	0.368	0.181
<i>Enterococcus</i> spp.	6.13	7.82	9.42	3.87	0.73	0.105	0.769	0.008
<i>Bifidobacterium</i> spp.	2.70	1.51	1.09	1.60	0.28	0.154	0.160	0.119
<i>E. coli</i>	8.26	7.21	7.00	7.29	0.21	0.311	0.132	0.092
At 24 h								
<i>Lactobacillus</i> spp.	7.18	6.46	6.65	6.27	0.17	0.122	0.294	0.611
<i>Enterococcus</i> spp.	7.53	7.84	5.66	7.64	0.48	0.245	0.290	0.388
<i>Bifidobacterium</i> spp.	2.39	1.39	1.46	1.16	0.26	0.228	0.278	0.512
<i>E. coli</i>	6.32	5.53	5.62	5.32	0.23	0.259	0.335	0.603

Figure 1. PCA score plot of pH, bacterial metabolites and microbial populations on canine faecal inoculum

Figure 2. PCA score plot of pH, bacterial metabolites and microbial populations on feline faecal inoculum