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Saratsi, K., Hoste, H., Voutzourakis, N., Tzanidakis, N., Stefanakis, A., Thamsborg, S. M., Mueller-Harvey, I., Hadjigeorgiou, I. and Sotiraki, S. (2020) Feeding of carob (*Ceratoniasiliqua*) to sheep infected with gastrointestinal nematodes reduces faecal egg counts and worm fecundity. *Veterinary Parasitology*, 284. 109200. ISSN 0304-4017 doi: <https://doi.org/10.1016/j.vetpar.2020.109200> Available at <http://centaur.reading.ac.uk/92265/>

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To link to this article DOI: <http://dx.doi.org/10.1016/j.vetpar.2020.109200>

Publisher: Elsevier

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Feeding of carob (*Ceratonia siliqua*) to sheep infected with gastrointestinal nematodes reduces faecal egg counts and worm fecundity

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26 **Abstract**

27 The present study explored the **anthelmintic effects** of condensed tannins (CT) in
28 carob (*Ceratonia siliqua*) pods fed to sheep against gastrointestinal nematodes. Three
29 independent *in vivo* trials tested whether i) carob pod (CaBP)-containing feed had an
30 anthelmintic effect and if yes, which was the optimal concentration in the diet; ii)
31 whether this effect could be attributed to tannins through the polyethylene glycol
32 (PEG) test and iii) whether there were any synergistic effects when combined with
33 another tannin-containing feed (e.g. sainfoin). In all trials 6-month old nematode-
34 naive lambs, experimentally infected with both *Haemonchus contortus* and
35 *Trichostrongylus colubriformis*, were used. Faecal egg counts (FEC) were performed
36 regularly and at the end of each trial adult worm counts (AWC) and female worm
37 fecundity were recorded. In trial 1, 35 lambs (five groups of seven lambs) were fed
38 different CaBP concentrations ranging from 0% to 12% w/w. FEC declined up to
39 39.2% only in the group fed with 12%CaBP, while a declining trend ($P<0.06$) was
40 demonstrated for the AWC of *T. colubriformis*, which was associated with the
41 increasing concentration of CaBP in feed. Female worm fecundity was reduced in
42 groups fed CaBP for both parasites, however this was only significant for *H.*
43 *contortus* ($P<0.001$), in a dose dependent manner. In trial 2, four groups of six
44 infected lambs each were used, which received the carob diets CaBP or CaBP+PEG,
45 and the tannin-free diets with or without PEG (C or C+PEG). Results showed that
46 FEC of Groups C, C+PEG, and CaBP+PEG were comparable throughout the trial,
47 while the group receiving only CaBP showed lower FEC from DAY 25 onwards.
48 AWC showed a reduction (67.7%) only for *H. contortus* ($P<0.03$). Reversal of the
49 anthelmintic effect of CaBP after PEG administration suggested that CT contributed
50 to the anthelmintic action. However, no effect of CaBP was observed on *T.*

51 *colubriformis* AWC and on female worm fecundity for both species. Finally, for trial
52 3 four groups of six lambs each received a diet based on CaBP, sainfoin (S) or a
53 combination (CaBP+S) and were compared to a control (C) diet of lucerne. On DAY
54 37 FEC values in groups CaBP+S and S tended to be lower compared to the two other
55 groups (C, CaBP), while for AWCs no significant differences were observed for both
56 parasites. The fecundity of *H. contortus* and *T. colubriformis* demonstrated significant
57 differences between the treated and control groups, with lower values in the animals
58 receiving CaBP+S. Overall, the results supported the hypothesis that carob had an
59 anthelmintic effect due to its CT, but there was no clear indication of a synergistic
60 effect with sainfoin.

61

62 **Keywords:** Carob, Sainfoin, *Haemonchus contortus*, *Trichostrongylus colubriformis*,
63 gastrointestinal nematodes, sheep, feed additives

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78 **1. Introduction**

79 Infections by gastrointestinal nematodes (GIN) affect both health and welfare of
80 grazing ruminants, causing anorexia, impaired digestion and nutrient absorption with
81 related production losses, diarrhoea, anaemia and even death (Perry and Randolph,
82 1999; Stear et al., 2007; Hoste et al., 2016). Currently, the control of these parasites
83 relies on repeated dosing with commercial anthelmintic drugs. However, the
84 development of anthelmintic resistance in worm populations against one or multiple
85 classes of anthelmintic has become a serious problem in several regions of the world,
86 making it increasingly difficult to control parasitic infections (Kaplan, 2020). At the
87 same time the increasing concerns of consumers about the presence of drug residues
88 in foods and in the environment (McKellar, 1997) have stimulated the search for
89 alternative or complementary solutions (Hoste and Torres-Acosta, 2011) within the
90 context of organic farming and sustainable agriculture (Charlier et al., 2018).
91 Such alternatives include the use of bioactive plants with anthelmintic properties.
92 Many results indicate that such resources, because of the presence of plant secondary
93 metabolites (PSMs), might help to interfere with the biology of key-stages during
94 nematode cycle and to reduce the consequences of GIN infections in grazing
95 ruminants. Particular attention has been given to plants containing condensed tannins
96 (CT) and some related polyphenols (see reviews by Terrill et al., 2012; Hoste et al.,
97 2015, 2016). Based on previous studies, the need to explore new plant resources to
98 develop non-drug-based strategies for the integrated control of nematode parasites in
99 grazing ruminants has recently become a research priority in livestock production,
100 especially in ruminant breeding as also reviewed by Morgan et al, 2020.

101 Small ruminants (sheep and goats) are a major component of the dairy sector in the
102 Mediterranean basin (Hadjigeorgiou et al., 2005). Sheep and goat production often
103 occupy marginal lands that are unsuitable for crop production but are rich in local
104 plants, such as rangeland vegetation, which can be exploited by animals as a feed
105 resource (Frutos et al., 2008; Méndez-Ortiz et al., 2018). Many rangeland plants also
106 contain PSMs, such as tannins (Papachristou et al., 2005) and several *in vitro* and *in*
107 *vivo* studies have evaluated their anthelmintic effects against GINs of small ruminants
108 (Manolaraki et al., 2010; Moreno-Gonzalo et al., 2012, 2013a,b, 2014; Arroyo-Lopez
109 et al., 2014; Silva Soares et al., 2018). Overall, CTs have been shown to directly or
110 indirectly interfere with the life cycle of several GINs and, therefore, CT-containing
111 plants, which also include many legumes, are proving to be beneficial nutritional
112 resources. However, a high degree of variability with respect to their anthelmintic
113 activity has also been recorded. Besides the total tannin concentration in ruminant
114 diets, recent studies have demonstrated that CT molecular composition or structural
115 characteristics can also affect anthelmintic activity (Mueller-Harvey et al., 2019).

116 Carob (*Ceratonia siliqua*) and sainfoin (*Onobrychis viciifolia*) are both resources of
117 the Fabaceae family and contain CTs. Carob is a leguminous tree that is widely
118 cultivated in the Mediterranean area. It is an important species both for economic and
119 environmental reasons (Batlle and Tous, 1997). Carob pods (fruits) are mostly used in
120 the food industry; pulp accounts for 90% by pod weight and seeds for 10%. They
121 contain high sugar (48–56%), but low protein (3–4%) and lipid concentrations (0.4–
122 0.8%) (Marakis, 1996; Batlle and Tous, 1997). Moreover, ripe carob pods contain
123 high concentrations of CTs (16–20% w/w DM) (Bravo et al., 1994; Batlle and Tous,
124 1997). This has been debated by Priolo et al. (2000, 2002) who claimed that the pods
125 have low content of CTs, but with exceptionally high biological activity. Silanikove et

126 al. (2006) have demonstrated that the yield of CTs is considerably affected by the
127 extraction method applied (from 5.0% with acidic methanol to 17.2% with urea-buffer
128 solution), suggesting that carob pods are a rich source of CTs. The high CT
129 concentration in by-products from carob pod processing justifies researching its value
130 as a feed additive with possible effect against GIN species.

131 Sainfoin, which can be found especially in southern parts of Europe, has been the
132 subject of renewed interest because of its beneficial effects in the context of
133 agroecology (Hayot Carbonero et al., 2011), its beneficial impact on ruminant
134 production and the environment and its potential antiparasitic effects on small
135 ruminants (Manolaraki et al., 2010; Hoste et al., 2015; Saratsis et al., 2016; Mueller-
136 Harvey et. al., 2019). *In vitro* studies have shown that sainfoin extracts have a dose-
137 dependent effect against different GIN species (Brunet et al., 2007; Manolaraki et al.,
138 2010; Novobilsky et al., 2013). Moreover, *in vivo* anthelmintic effects have also been
139 described in sheep and/or goats fed with sainfoin; i.e. 42-68% reduction in parasitic
140 egg excretion, which was associated with a 17.6% decrease in female worm fecundity
141 and a 45% decrease in worm numbers for *Haemonchus contortus* (Arroyo-Lopez et
142 al., 2014).

143 The present study, therefore, sought to explore the anthelmintic effects of feeding
144 regimes employing two CT-containing plant resources that may be relevant for
145 Mediterranean conditions. These were offered to lambs either alone or in combination
146 to evaluate their efficacies against two GIN species (*H. contortus* and
147 *Trichostrongylus colubriformis*). The specific objectives were to explore whether a)
148 the anthelmintic effect of carob in the feed is dose dependent (Trial 1), b) this
149 anthelmintic effect is associated with tannins by using polyethylene glycol (PEG) as a

150 tannin-inhibitor (Trial 2), and c) there are any synergistic effects between carob and
151 sainfoin feeds (Trial 3).

152 **2. Materials and Methods**

153 **2.1. Stabling and animals**

154 The experiments were carried out at the Asomaton Research Station of HAO Demeter
155 on the island of Crete, Greece. The animal barn was of an open-sided shed type, with
156 straw bedding. During the whole study the animals were kept indoors with each group
157 in a separate pen of approximately 10 m² and 10 m² open yard. The study included
158 three trials with lambs belonging to the local “Sfakion” breed. In order to achieve
159 uniformity of the experimental animals, all lambs included were female, 6-month-old
160 with a comparable body weight (BW), which was within the normal BW range of the
161 breed (22-30 kg) at the specific age. The lambs were raised indoors under helminth-
162 free conditions. Fourteen days before the start of each trial, they were drenched with
163 albendazole at the higher commercially recommended dose (ALBENDAZOLE
164 Drench, PROVET, 7.5 mg/kg) and they tested negative by faecal egg counts at the
165 start of each trial. No anthelmintic resistance was previously recorded for this specific
166 flock.

167 **2.3. Infective larvae**

168 Third-stage infective larvae of *H. contortus* and *T. colubriformis* strains, susceptible
169 to all classes of anthelmintic drugs, had been cultured from faeces of mono-
170 specifically infected donor sheep. Larvae were recovered using the Baermann
171 technique and then stored for 1-2 months at 4°C until use.

172

173 **2.4. Tannin-containing plant resources**

174 Carob pods (after removal of the seeds) were locally purchased and offered as crushed
175 flour meal incorporated in the concentrate feed supplement. Sainfoin pellets (Perly
176 cultivar, 3rd cut) were provided by Multifolia (Viapres le Petit 10380, France) as part
177 of the Research project CARES.

178 **2.5. Tannin concentration and composition**

179 Tannin concentrations and compositions were determined in triplicate using two
180 different assays, i.e. the acetone-butanol-HCl assay and the thiolytic degradation with
181 benzyl mercaptan. Both techniques were applied in order to ensure a comprehensive
182 analysis since it was previously demonstrated that, depending on the types of CTs, the
183 acetone-HCl-butanol assay can give higher CT concentrations than the thiolysis assay.

184 The acetone-HCl-butanol assay was carried out as previously described by Grabber et
185 al. (2013) and Desrues et al. (2017).

186 The thiolysis reaction was carried out with benzyl mercaptan (Gea et al., 2011;
187 Ropiak et al., 2016), the reaction products were identified by HPLC-MS analysis
188 (Williams et al., 2014; Desrues et al., 2017) and quantified based on peak areas at 280
189 nm (Gea et al., 2011; Ropiak et al., 2016). This provided information on CT
190 concentration (g CT/100 g DW), CT size (in terms of mean degree of polymerisation,
191 mDP), molar percentages of prodelphinidins (PD) and procyanidins (PC) within CTs,
192 and molar percentages of *cis*- vs *trans*- flavan-3-ol subunits (Ropiak et al., 2016).

193 **2.6. Experimental design**

194 All diets offered to the animals during the experimental period (with or without the
195 tannin sources) were formulated to meet the nutrient requirements of the animals
196 (NRC, 2007) and the total rations were always iso-nitrogenous and iso-energetic as

197 well as balanced for crude fibre, Ca, P and Ca/P ratio (Suppl Table 1). Animals had
198 access to clean water at all times. The animals' appetite was assessed and feed
199 consumption (as feed offered minus refusals) was recorded on a daily basis by the
200 farm manager.

201 **2.6.1. Trial 1**

202 To determine the anthelmintic effect of carob pod meal and to define the optimal
203 concentration in a sheep ration, a subset of 35 lambs were randomly allocated to 5
204 groups (n=7 lambs/diet) (Table 1).

205 Carob meal (CaBP) was offered as feed supplement, at increasing rates of 0%, 3%,
206 6%, 9% and 12% (g CaBP/100g DM) of the total ration. The highest proportion, of
207 carob meal contributed to concentrate feed was set to 12% (due to its poor energy and
208 protein contents) in order to enable formulating a ration, which could cover the
209 nutritional requirements of lambs.

210 Feeding the experimental diets started 2 weeks prior (D14) to experimental infection
211 with nematode larvae (D0) in order for the animals to adapt to the feed.

212 On DAY 0, all lambs in groups (i) to (v) were infected with a single dose of 12.000
213 3rd stage larvae (L3) of *H. contortus* and 12.000 L3 of *T. colubriformis*. At the end of
214 the experimental period (D49), all lambs were euthanised by injection of a massive
215 dose of pentobarbital (Dolethal®).

216 **2.6.2. Trial 2**

217 Four groups of 6 lambs were included in a two-factorial trial (diet and PEG-addition).
218 Two groups were offered CaBP as feed supplement at the rate of 12% in the total
219 ration, and two groups remained on standard diet (Table 1). Half of the lambs in each
220 diet group were offered PEG (Polyethylene Glycol 4000, Fisher Scientific USA)

221 orally (60 g/lamb diluted in 200 ml water) on a daily basis after being allocated into
222 groups.

223 On D0, all lambs were experimentally infected with 8.000 L3 of *H. contortus* and
224 16.000 L3 of *T. colubriformis*. On D21, after parasite infection was confirmed by
225 positive faecal examination, the animals were allocated into 4 groups of 6 lambs each,
226 according to the experimental diets. On D37 they were euthanised as described above.

227 **2.6.3. Trial 3**

228 To determine the possible synergistic anthelmintic effects between 2 CT-containing
229 resources namely carob (*C. siliqua*) and sainfoin (*O. viciifolia*), 4 groups of 6 lambs
230 were included in a two-factorial design (Table 1).

231 On D-14 each group of lambs received the allocated diet, containing **i**) carob meal
232 (CaBP) alone **ii**) sainfoin (S) pellets; **iii**) a combination of carob meal and sainfoin
233 pellets (CaBP+S) while **iv**) a control group (C), received an isoproteic diet based on
234 lucerne. Carob was offered as a feed supplement at the rate of 12% in the total ration.
235 Sainfoin was offered as pellets representing 35% of the total ration. On D0 all lambs
236 were infected with a single dose of 12.000 L3 of *H. contortus* and 12.000 L3 of *T.*
237 *colubriformis*. At the end of the experimental period (D37), all lambs were euthanised
238 as previously described.

239 **2.7. Pathophysiological parameters**

240 Individual blood samples were collected once weekly (from D0 to D49) during Trial 1
241 and once every two weeks (from D0 to D28) during Trial 3, by jugular venipuncture
242 into heparinized tubes (BD Vacutainer®, UK) to determine the packed cell volume
243 (PCV), as an indicator of anaemia, according to the micro-haematocrit method. In

244 Trial 2 due to its short duration, the recording of PCV values was not included in the
245 design.

246 **2.8. Parasitological parameters**

247 Individual faecal samples were collected weekly directly from the rectum, during the
248 1st and 3rd trial, and twice weekly during the 2nd trial in order to determine faecal egg
249 counts (FEC) using a modified McMaster technique (Roepstorff and Nansen, 1998).
250 FEC data were expressed as eggs per gram of faeces (EPG).

251 At necropsy, the abomasa and the first 12 meters of small intestine were separated,
252 ligated, rapidly removed and immediately processed to collect the adult worms from
253 the luminal contents. For the intra-mucosal larvae, pepsin digestion was applied both
254 on the abomasum and intestinal mucosa (MAFF, 1986). After 4h incubation at 37°C
255 the larvae were collected. After storage in 10% alcohol, worm counts were performed
256 according to a 10% aliquot technique (MAFF, 1986). Morphological identification of
257 worm stages, sex and species were conducted using standard procedures (MAFF,
258 1986).

259 The fecundity of female worms was measured on 10 worms per lamb. For *T.*
260 *colubriformis*, eggs were counted directly *in utero* after clearing in 85% lactic acid
261 solution. All egg counts were performed under a microscope set at 10 times
262 magnification (total 100 ×). For *H. contortus*, the fecundity was determined using the
263 method described by Kloostermann et al. (1978). Briefly, the worms were soaked for
264 5 min in a large volume of distilled water, before being placed individually in
265 microtubes with 1000 µl of 0.125% hypochlorite concentration solution and kept at
266 room temperature for 20 minutes. Treatment resulted in female worms disintegrating
267 thus enabling the direct counting of eggs under a stereo-microscope using an aliquot
268 (10%) of the total volume.

269 **2.9. Statistical analyses**

270 The data of FEC and adult worm counts (AWC) were $\log_{10}(x+1)$ transformed prior to
271 analysis. For the FEC values, comparison of all groups was first performed using an
272 analysis of variance (ANOVA) with time as repeated measurement. Then, the
273 comparison of results to the control values were carried out date by date, using one-
274 way ANOVA completed by *the post-hoc* Bonferroni test for pairwise comparisons.
275 Group means of AWC were compared by one-way ANOVA (Trial 1) or two-way
276 ANOVA (Trial 2: CaBP +/- and PEG +/-; Trial 3: CaBP +/- and sainfoin +/-).
277 Regarding the fecundity of female worms, the Shapiro-Wilk Test of normality, which
278 is more appropriate for small sample sizes, was used. In cases where the data deviated
279 significantly ($P < 0.05$) from a normal distribution (Trial 1 and 3 for both parasite
280 species and Trial 2 for *T. colubriformis*) the appropriate test to check the difference of
281 fecundity between the groups, which is the non-parametric test of Kruskal-Wallis,
282 was used. Where the dependent variable was normally distributed ($P > 0.05$) the
283 parametric test of one-way ANOVA (*H. contortus* of Trial 2) was used. Additionally,
284 for the Trial 1, the model of linear regression was used, in order to be investigated if
285 there was a negative correlation between the variables “percentages of carob” and
286 “fecundity of female worms” for both parasite species (*H. contortus* and *T.*
287 *colubriformis*). Finally, the Tukey HSD test was used for data of trial 3, in order to
288 investigate statistically significant differences between groups.

289 All statistical analyses were performed using the SyStat SPSS 9.0 Software.

290

291 **2.10. Ethical considerations**

292 The study was carried out in compliance with the national animal welfare regulations.

293 All trials took place in a Research Station of the Veterinary Research Institute. The

294 experimental protocol was approved by the responsible institutional committee (VRI
295 Committee for Approval of Experimental protocols as appointed at 26/5/2014,
296 Decision nr 972) . Euthanasia was performed in a humane manner according to EU
297 regulations.

298

299 **3. Results**

300 The CT concentrations and compositions are presented in Table 2. The HBA assay
301 yielded similar CT concentrations for both plant materials, whereas the thiolysis assay
302 generated lower CT concentrations for the sainfoin pellets. The thiolysis assay
303 revealed that: i) both carob and sainfoin CTs consisted mainly of prodelphinidins,
304 96.7 and 74.7 mole percentages, respectively; ii) carob CTs were highly galloylated
305 (i.e. 41.1% of flavan-3-ol subunits are galloylated), but sainfoin CTs did not contain
306 any esterified galloyl groups; iii) carob CTs were characterised by a relatively high
307 average molecular weight (mDP = 31.1), whereas sainfoin CTs had an mDP value of
308 11.5.

309

310 **3.1. Trial 1**

311 The results of Trial 1 are shown in Table 3 and Figure 1

312 The analyses of FEC, based on the ANOVA on Repeated Measures from D21 to D49,
313 showed an overall non-significant difference between groups, but significant
314 difference over time (between days of sampling). Meanwhile, the date-by-date
315 ANOVA of FEC showed no significant differences between groups, whatever the
316 date, as well as no dose effect. Reduction in FEC, up to 39.2% on DAY 49 as
317 compared to controls, was observed only for the group fed with the highest
318 concentration of carob meal.

319 For *H. contortus*, the AWC declined in the groups receiving the highest concentration
320 of carob meal but this effect was not statistically significant ($P= 0.964$). In contrast,
321 there was a declining trend ($P<0.06$) for the numbers of *T. colubriformis* with
322 increasing carob concentration.

323 The fecundity values showed significant differences (15.6%-59.3% lower than
324 0%CaBP respectively from the lowest to the highest CaBP concentration) between
325 groups for *H. contortus* demonstrating a dose dependent effect ($P<0.05$).

326 The Box plot (Figure 1b) for *H. contortus* fecundity suggests that worms from the
327 0%CaBP group tended to be more fecund than other CaBP groups and there may be
328 some degree of fecundity discrepancy between CaBP groups. This trend was
329 confirmed with the non-parametric test of Kruskal-Wallis, which showed that there
330 were statistically significant differences in fecundity between the groups ($P<0.001$).
331 More specifically, fecundity was statistically significantly greater for 0%CaBP group
332 than the other CaBP groups. On the other hand, regarding *T. colubriformis* fecundity,
333 there was no statistically significant difference between the groups ($P=0.128$).
334 However, the model of linear regression, which was implemented and was
335 statistically significant ($P<0.05$), showed a negative correlation between the variables
336 “group” and “fecundity” for both parasite species.

337 No GIN larvae were recovered after pepsin digestion.

338 Mean PCV values (\pm SD) for groups 0%CaBP, 3%CaBP, 6%CaBP, 9%CaBP, and
339 12%CaBP on the last day of the trial were 25.29 (\pm 5.96), 23.00 (\pm 5.72), 21.00
340 (\pm 6.32), 23.00 (\pm 5.89) and 24.00 (\pm 5.00) respectively. No significant differences were
341 found between the groups in PCV.

342 Average daily gain (ADG) as calculated for the whole trial duration for 0%CaBP,
343 3%CaBP, 6%CaBP, 9%CaBP and 12%CaBP groups was (mean \pm s.d.) 69.2 g (\pm 31.0),

344 61.5(\pm 36.1), 68.7(\pm 33.0), 74.8(\pm 37.5) and 64.4(\pm 32.9) g respectively, which yielded
345 no significant differences between the groups.

346 **3.2.Trial 2**

347 The results of Trial 2 are presented in Table 4 and Figure 2.

348 The Repeated Measurements Analyses of FEC showed an overall statistical difference
349 ($P < 0.001$) between the 4 groups. The date-by-date ANOVA of FEC indicated that
350 differences were most prominent on DAY 29 (significant statistical differences,
351 $P < 0.02$) and then on DAY 33 (trend, $P < 0.07$). Specifically, the values of the C,
352 C+PEG, CaBP+PEG groups were comparable throughout the trial, while the group
353 receiving only carob (CaBP) showed consistently lower FEC starting from DAY 25
354 until the last day of the experiment. It was evident that the effect of carob on FEC was
355 nullified by PEG.

356 Results on AWC, showed reduction only for *H. contortus* ($P < 0.03$) resulting in an
357 overall statistical difference between the 4 groups, since the lowest worm counts were
358 found for the CaBP group. Especially, for *H. contortus*, a reduction of approximately
359 65% was observed in the carob group compared to the control. The AWC in the
360 CaBP+PEG group were similar to the other 2 control groups showing no reduction in
361 worm population. On the other hand, no effect of carob was observed on *T.*
362 *colubriformis* worm counts.

363 No effect of carob on female fecundity was also observed, irrespective of the parasite
364 species. Both control and carob groups showed comparable levels of female fecundity
365 for the two parasite species. The Box plot in Figure 2b showed that the range of
366 fecundity of *H. contortus* for CaBP group was greater than for C, C+PEG and
367 CaBP+PEG groups and the interquartile range (middle 50% of the records) was lower
368 on the fecundity scale in the CaBP group than in the other groups.

369 No GIN larvae were recovered after pepsin digestion.
370 The average daily gain (ADG) of lambs as calculated for the whole trial duration for
371 (C), (C+PEG), (CaBP) and (CaBP+PEG) groups was 51.8(\pm 30.1) (\pm s.d.), 69.8(\pm 19.9),
372 60.8(\pm 29.3) and 40.5(\pm 25.6) g, respectively, which resulted in no significant
373 differences between the groups.

374 **3.3.Trial 3**

375 The results of Trial 3 are shown in Table 5 and Figure 3.

376 The FEC values of all experimental groups remained at very low levels up to DAY
377 21. The overall repeated analyses based on 3 dates of the patent phase (DAY 21,
378 DAY 28, DAY 37) showed a trend for differences ($P < 0.07$) between groups. The
379 results of the date-by-date ANOVA test did not show difference on DAY 21 and on
380 DAY 28, while on DAY 37, the values of FEC in groups CaBP+S and S tended to be
381 reduced ($P < 0.06$) compared to the two other groups. When compared to the control
382 values of FEC, the reductions in the 3 treated groups ranged from 44.6% to
383 approximately 86 %. These differences were mainly found for the sainfoin group (S)
384 and carob+sainfoin (CaBP+S) groups. As regards the AWCs, no significant
385 differences were observed neither in the number of *H. contortus* and *T. colubriformis*.
386 No GIN larvae were recovered after pepsin digestion.

387 The non-parametric test of Kruskal-Wallis showed that there were statistically
388 significant differences in fecundity between the groups ($P < 0.001$). Specifically, the C
389 group presented the highest fecundity values, while the CaBP+S group presented the
390 lowest ones for both parasite species. Tukey HSD test for *H. contortus* showed that
391 the C group differed significantly from CaBP, S and CaBP+S, while for *T.*
392 *colubriformis* fecundity for CaBP group was also statistically different from CaBP+S
393 (Figure 3b).

394 When exploring the pathophysiological parameters (i.e. PCV), the analysis of
395 variance on repeated measures and also the date by date ANOVA did not show
396 significant differences between the groups. Specific values for mean PCV (\pm SD) on
397 DAY 28 of the respective groups C, CaBP, S and CaBP+S were 31.67 (\pm 3.39), 33.00
398 (\pm 4.86), 31.33 (\pm 3.61) and 30.50 (\pm 4.37).

399 The average daily gain (ADG) as calculated for the whole trial duration for (C),
400 (CaBP), (S) and (CaBP+S) groups was (mean \pm s.d.) 122.5(\pm 38.1), 88.2(\pm 39.2),
401 104.6(\pm 11.9) and 124.8(\pm 39.7) g, respectively and there were no significant
402 differences between the groups.

403 **4. Discussion**

404 The literature contains several *in vitro* and *in vivo* studies, conducted on small
405 ruminants, which evaluated the anthelmintic effect of tannin-containing plants. Such
406 studies first examined temperate forage legumes fed through grazing, as hay, silage or
407 pellets. Examples are sainfoin (Hoste et al., 2016; Legendre et al., 2018; Mueller-
408 Harvey et al., 2019), sericea lespedeza (*Lespedeza cuneata*) (Burke et al., 2012a,b;
409 Mechineni et al., 2014; Kommuru et al., 2014, 2015), and sulla (*Hedysarum*
410 *coronarium*) (Niezen et al., 1995, 2002). More recently, there has been also a growing
411 interest in tannin-containing by-products from the food industry as illustrated by
412 studies with hazelnut peels (*Corylus avellana* fruits) (Desrues et al., 2012; Girard et
413 al., 2013), carob pods (Manolaraki et al., 2010; Arroyo-Lopez et al., 2014) and
414 browse plants such as *Pistacia lentiscus* (Landau et al., 2010; Manolaraki et al.,
415 2010), *Quercus coccifera* (Manolaraki et al., 2010) and *Salix* spp (Mupeyo et al.,
416 2011).

417 In the current study, we further explored the *in vivo* anthelmintic effects of carob pod
418 meal since it represents a common feed resource in the Mediterranean region and
419 there was some previous evidence of its anthelmintic (Arroyo-Lopez et al., 2014) and
420 anticoccidial (Saratsis et al., 2016; Legendre et al., 2018) properties. In order to
421 develop a practical implementation tool for carob as dietary intervention, we wanted
422 to identify a) the optimal carob concentration in the feed for bioactivity, b) whether
423 CTs contributed to such an activity and c) whether there were any synergistic effects
424 with other plant sources with different types of CTs (i.e. sainfoin). For all 3 trials a
425 balanced and palatable ration was specifically designed for all animals. This aimed to
426 achieve similar production indexes in all groups and ensured that any observed
427 differences in the effects of parasitism would not stem from quantitative differences in
428 the dietary composition but rather from differences in the bioactive CTs (Coop and
429 Kyriazakis, 1999; Athanasiadou et al., 2008; Hoste et al., 2015).

430 The parasites that served as models for this study (*H. contortus* and *T. colubriformis*)
431 are the most pathogenic and/or prevalent GIN species in European sheep and goats
432 (Charlier et al., 2018). These experiments allowed us to investigate carob-pods
433 efficacy against nematodes in the different anatomical location within the gut, as
434 location can affect the exposure of worms to different CT concentrations (Desrues et
435 al., 2017; Quijada et al., 2018).

436 Results of Trial 1 showed decreases in the mean values of FEC and AWC only in the
437 group fed with the highest concentration of CaBP in the concentrate feed, although
438 not significant. However, fecundity values showed a negative correlation to CaBP
439 concentration in the feed indicating a dose-dependent fecundity suppression effect.
440 The results suggest that carob used in feed at 12% has a potential anthelmintic effect

441 and this effect is due mainly to the reduction of female worm fecundity
442 (predominantly in *H. contortus*) and to a lesser extent to the reduction of
443 establishment and development of the worms. Since *H. contortus* produce a
444 remarkably high daily egg output compared to *T. colubriformis* (Besier et al., 2016),
445 we suggest that the reduction in FEC seen in this trial can be attributed to the effect
446 the carob diet had against *H. contortus*. Overall, the results of this trial suggest that
447 the higher the concentration of carob in the ration the higher the anthelmintic activity;
448 this effect that was more evident for *H. contortus*. Unfortunately, there are limitations
449 to the quantity of carob pod meal that can be included in a well balanced ration since
450 carob pods contain high sugar but low protein and lipid concentrations (Priolo et al.,
451 1998; Karabulut et al., 2006).

452 During Trial 2, the main results **i)** confirmed that CaBP reduced FEC in lambs, as
453 these reductions compared to control values ranged from 20% to 45%, **ii)** that these
454 reductions in FEC seemed to be mainly due to the lower numbers from the highly
455 prolific *H. contortus* species and not from *T. colubriformis*, and that there were no
456 effects on female fecundity of both species and **iii)** that the anthelmintic effect of
457 CaBP may be attributed to CTs, because a restoration to control values for FEC and
458 *Haemonchus* worm numbers was observed in the CaBP + PEG group. PEG is a non-
459 nutritive synthetic polymer that is capable of binding and deactivating CTs; it has
460 been used in many animal nutrition studies to increase the intake of CT-containing
461 feeds and to improve protein absorption (Silanikove et al., 1996; Bermingham et al.,
462 2001; Theodoridou et al., 2012). This ability has also been used to test (Brunet et al.,
463 2007, 2008; Debela et al., 2012; Brito et al., 2018) whether any observed *in vivo*
464 anthelmintic activity was linked to the presence of CTs.

465 Finally, the aim of Trial 3 was to investigate two hypotheses: firstly, that carob CTs
466 generate a stronger anthelmintic effect than sainfoin CTs and secondly, that
467 synergistic effects could be achieved by combining carob with sainfoin. The rationale
468 for these hypotheses is based on the fact that carob and sainfoin contain different
469 types of CTs and that these could target different stages of the GIN life cycle. Carob
470 CTs are highly galloylated prodelphinidins, whereas sainfoin CTs are non-galloylated
471 prodelphinidins. Previous studies found two structural features in CTs that enhance
472 anthelmintic activity *in vitro*: i) prodelphinidin CTs are more potent than procyanidin
473 CTs and ii) galloylation increases the anthelmintic effect of CTs (Hoste et al., 2016;
474 Kommuru et al., 2014, 2015). Therefore, carob CTs, which have a high
475 prodelphinidin/procyanidin ratio (96.7% prodelphinidins/3.3% procyanidins) and are
476 also highly galloylated (i.e. 41.1% of the flavan-3-ol subunits are galloylated) should
477 produce a stronger anthelmintic effect than sainfoin, as sainfoin CTs have less
478 prodelphinidins (74.8%) and no galloyl groups (N.B. % stands for mole percent
479 within CT molecules; Table 2).

480 There are several important reasons that could explain why the results from Trial 3
481 did not support either of these hypotheses. Firstly, sainfoin - but not carob - was fed in
482 a pelleted form, while it has been demonstrated previously that the pelleting process
483 has a marked effect on CTs in terms of their analysis (Mueller-Harvey et al., 2019).
484 Table 2 shows that the CT concentrations in sainfoin pellets differed considerably
485 between the two assays (6.5 and 1.7 g CT/100 g DW) in contrast to the carob meal
486 data (5.8 and 7.2 g CT/100g DW). However, we currently do not know whether the
487 pelleting process enhances the anthelmintic activity of CTs or not. Secondly, up to
488 now most attempts to unravel links between CT structural features and anthelmintic
489 effects have employed *in vitro* assays. Therefore, *in vivo* feeding trials such as the

490 present ones are vital to test the laboratory data. It may turn out that the esterified
491 galloyl groups are not stable in the digestive tract and that the prodelphinidins in
492 carob and sainfoin were the active CTs.

493 Therefore, preliminary conclusions from the Trial 3 data could be that galloylation is
494 unlikely to enhance anthelmintic activity *in vivo* in terms of *H. contortus* fecundity or
495 total worm counts and that pelleting of CT-plants might lead to lower FEC. These
496 indications will, however, need rigorous testing in the future.

497 The nutritional and/or anthelmintic properties of sainfoin fed as direct grazing, silage,
498 hay or pellets have been evaluated in both sheep and goats, with promising
499 anthelmintic results when used either alone (Paolini et al., 2005; Heckendorn et al.,
500 2006; Ríos-de Alvarez et al., 2008; Gaudin et al., 2016) or in combination with other
501 CT sources (Girard et al., 2013). Previous results have demonstrated that sainfoin
502 consumption under different forms of preservation can reduce FEC and also reduce
503 female worm fecundity of *H. contortus* (Manolaraki et al., 2010; Arroyo-Lopez et al.,
504 2014) or *T. colubriformis* (Manolaraki et al., 2010); however, in other studies a lack
505 of effect has been observed (Heckendorn et al., 2006). The issue of the variable
506 results has also been addressed in several reviews (Hoste et al., 2015; Hoste and
507 Niderkorn, 2019).

508 To summarise, the main results of trial 3 for FECs were i) a confirmation of
509 significant reductions of FEC due to the consumption of both CaBP and sainfoin
510 pellets; ii) a temporal increase in the anthelmintic effects of sainfoin but not for CaBP,
511 and iii) no synergistic effects of the combination CaBP + sainfoin. In addition, it
512 would appear that these results can largely be explained by significant effects on
513 female fecundity of both species, but there were only limited effects on the worm
514 populations. No significant effects on AWC were observed for any of the species. On

515 the other hand, although the differences were not significant, the percentage of
516 reduction compared to the controls (Group C) for *H. contortus* worm numbers were
517 respectively, for Groups CaBP 35.5%, S 62.1% and CaBP+S 53.5%.

518 In conclusion, the results of these three trials, which focussed on carob pod meal
519 alone or in combination, raised future research questions regarding what causes the
520 differences in results when different CT-containing resources are used and what is
521 required for a more rational use of CT-containing resources as nutraceutical feeds
522 under farm conditions and in different production systems (Hoste et al., 2015).

523 Our results confirmed that **i)** the consumption of CT containing resources can
524 modulate the biology of GINs; **ii)** that CT were involved in the anthelmintic effects of
525 carob and **iii)** the concentration in the diet influenced the anthelmintic effects as
526 previously shown in other *in vivo* studies with sericea lespedeza (Shaik et al., 2004,
527 2006) or sainfoin (Brunet et al., 2007) and **iv)** different mechanisms appeared to affect
528 the worm population and could explain the reduction of FECs: either a reduced
529 fecundity of female adult worms (see Trial 1 and 3) and /or a reduction of the number
530 of worms (see Trial 2).

531 The data of these 3 studies also illustrated that results depended on the type of
532 nematode species (abomasal or intestinal species) and/or on the nature of CT
533 resources (in our case carob vs sainfoin) and on the CTs. As stated by Quijada (2015)
534 and Desrues et al. (2016) the quantitative and qualitative differences in CTs appear to
535 influence the anthelmintic activity on the different species of parasitic nematodes.

536

537 Our results suggest that, when worm populations are exposed to CTs in the
538 gastrointestinal tract, upon their ingestion by the host the most evident effect recorded
539 is the reduction of female fecundity. Particularly for *H. contortus*, it appears that

540 fecundity is only affected when the worms are exposed to CTs during maturation
541 (Trial 1 and 3) and not when they are already mature adults (Trial 2). On the other
542 hand, when CaBP was consumed for two weeks by animals in which adult worm
543 populations were already established and patent, the main finding was a significant
544 decrease in *H. contortus* worm counts. Moreover, the current study adds further
545 support to the observation that most of the CT effect is related to abomasal parasite –
546 and not so much to the small intestinal parasite. This is possibly due to the higher CT
547 concentration in the abomasum compared with the rumen and intestines, along with
548 higher prodelphinidin percentage as already shown in studies on the cattle abomasal
549 parasite *Ostertagia ostertagi* (Desrues et al., 2017).

550 **Acknowledgements**

551 This article is based upon work from EMIDA ERA-Net project CARES and COST
552 Action COMBAR CA16230 (European Cooperation in Science and Technology). The
553 authors would like to thank Chris Drake for the chemical analyses.

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Feeding of carob (*Ceratonia siliqua*) to sheep infected with gastrointestinal nematodes reduces faecal egg counts and worm fecundity

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26 **Abstract**

27 The present study explored the anthelmintic effects of condensed tannins (CT) in
28 carob (*Ceratonia siliqua*) pods fed to sheep against gastrointestinal nematodes. Three
29 independent *in vivo* trials tested whether i) carob pod (CaBP)-containing feed had an
30 anthelmintic effect and if yes, which was the optimal concentration in the diet; ii)
31 whether this effect could be attributed to tannins through the polyethylene glycol
32 (PEG) test and iii) whether there were any synergistic effects when combined with
33 another tannin-containing feed (e.g. sainfoin). In all trials 6-month old nematode-
34 naive lambs, experimentally infected with both *Haemonchus contortus* and
35 *Trichostrongylus colubriformis*, were used. Faecal egg counts (FEC) were performed
36 regularly and at the end of each trial adult worm counts (AWC) and female worm
37 fecundity were recorded. In trial 1, 35 lambs (five groups of seven lambs) were fed
38 different CaBP concentrations ranging from 0% to 12% w/w. FEC declined up to
39 39.2% only in the group fed with 12%CaBP, while a declining trend ($P<0.06$) was
40 demonstrated for the AWC of *T. colubriformis*, which was associated with the
41 increasing concentration of CaBP in feed. Female worm fecundity was reduced in
42 groups fed CaBP for both parasites, however this was only significant for *H.*
43 *contortus* ($P<0.001$), in a dose dependent manner. In trial 2, four groups of six
44 infected lambs each were used, which received the carob diets CaBP or CaBP+PEG,
45 and the tannin-free diets with or without PEG (C or C+PEG). Results showed that
46 FEC of Groups C, C+PEG, and CaBP+PEG were comparable throughout the trial,
47 while the group receiving only CaBP showed lower FEC from DAY 25 onwards.
48 AWC showed a reduction (67.7%) only for *H. contortus* ($P<0.03$). Reversal of the
49 anthelmintic effect of CaBP after PEG administration suggested that CT contributed
50 to the anthelmintic action. However, no effect of CaBP was observed on *T.*

51 *colubriformis* AWC and on female worm fecundity for both species. Finally, for trial
52 3 four groups of six lambs each received a diet based on CaBP, sainfoin (S) or a
53 combination (CaBP+S) and were compared to a control (C) diet of lucerne. On DAY
54 37 FEC values in groups CaBP+S and S tended to be lower compared to the two other
55 groups (C, CaBP), while for AWCs no significant differences were observed for both
56 parasites. The fecundity of *H. contortus* and *T. colubriformis* demonstrated significant
57 differences between the treated and control groups, with lower values in the animals
58 receiving CaBP+S. Overall, the results supported the hypothesis that carob had an
59 anthelmintic effect due to its CT, but there was no clear indication of a synergistic
60 effect with sainfoin.

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62 **Keywords:** Carob, Sainfoin, *Haemonchus contortus*, *Trichostrongylus colubriformis*,
63 gastrointestinal nematodes, sheep, feed additives

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78 **1. Introduction**

79 Infections by gastrointestinal nematodes (GIN) affect both health and welfare of
80 grazing ruminants, causing anorexia, impaired digestion and nutrient absorption with
81 related production losses, diarrhoea, anaemia and even death (Perry and Randolph,
82 1999; Stear et al., 2007; Hoste et al., 2016). Currently, the control of these parasites
83 relies on repeated dosing with commercial anthelmintic drugs. However, the
84 development of anthelmintic resistance in worm populations against one or multiple
85 classes of anthelmintic has become a serious problem in several regions of the world,
86 making it increasingly difficult to control parasitic infections (Kaplan, 2020). At the
87 same time the increasing concerns of consumers about the presence of drug residues
88 in foods and in the environment (McKellar, 1997) have stimulated the search for
89 alternative or complementary solutions (Hoste and Torres-Acosta, 2011) within the
90 context of organic farming and sustainable agriculture (Charlier et al., 2018).
91 Such alternatives include the use of bioactive plants with anthelmintic properties.
92 Many results indicate that such resources, because of the presence of plant secondary
93 metabolites (PSMs), might help to interfere with the biology of key-stages during
94 nematode cycle and to reduce the consequences of GIN infections in grazing
95 ruminants. Particular attention has been given to plants containing condensed tannins
96 (CT) and some related polyphenols (see reviews by Terrill et al., 2012; Hoste et al.,
97 2015, 2016). Based on previous studies, the need to explore new plant resources to
98 develop non-drug-based strategies for the integrated control of nematode parasites in
99 grazing ruminants has recently become a research priority in livestock production,
100 especially in ruminant breeding as also reviewed by Morgan et al, 2020.

101 Small ruminants (sheep and goats) are a major component of the dairy sector in the
102 Mediterranean basin (Hadjigeorgiou et al., 2005). Sheep and goat production often
103 occupy marginal lands that are unsuitable for crop production but are rich in local
104 plants, such as rangeland vegetation, which can be exploited by animals as a feed
105 resource (Frutos et al., 2008; Méndez-Ortiz et al., 2018). Many rangeland plants also
106 contain PSMs, such as tannins (Papachristou et al., 2005) and several *in vitro* and *in*
107 *vivo* studies have evaluated their anthelmintic effects against GINs of small ruminants
108 (Manolaraki et al., 2010; Moreno-Gonzalo et al., 2012, 2013a,b, 2014; Arroyo-Lopez
109 et al., 2014; Silva Soares et al., 2018). Overall, CTs have been shown to directly or
110 indirectly interfere with the life cycle of several GINs and, therefore, CT-containing
111 plants, which also include many legumes, are proving to be beneficial nutritional
112 resources. However, a high degree of variability with respect to their anthelmintic
113 activity has also been recorded. Besides the total tannin concentration in ruminant
114 diets, recent studies have demonstrated that CT molecular composition or structural
115 characteristics can also affect anthelmintic activity (Mueller-Harvey et al., 2019).

116 Carob (*Ceratonia siliqua*) and sainfoin (*Onobrychis viciifolia*) are both resources of
117 the Fabaceae family and contain CTs. Carob is a leguminous tree that is widely
118 cultivated in the Mediterranean area. It is an important species both for economic and
119 environmental reasons (Batlle and Tous, 1997). Carob pods (fruits) are mostly used in
120 the food industry; pulp accounts for 90% by pod weight and seeds for 10%. They
121 contain high sugar (48–56%), but low protein (3–4%) and lipid concentrations (0.4–
122 0.8%) (Marakis, 1996; Batlle and Tous, 1997). Moreover, ripe carob pods contain
123 high concentrations of CTs (16–20% w/w DM) (Bravo et al., 1994; Batlle and Tous,
124 1997). This has been debated by Priolo et al. (2000, 2002) who claimed that the pods
125 have low content of CTs, but with exceptionally high biological activity. Silanikove et

126 al. (2006) have demonstrated that the yield of CTs is considerably affected by the
127 extraction method applied (from 5.0% with acidic methanol to 17.2% with urea-buffer
128 solution), suggesting that carob pods are a rich source of CTs. The high CT
129 concentration in by-products from carob pod processing justifies researching its value
130 as a feed additive with possible effect against GIN species.

131 Sainfoin, which can be found especially in southern parts of Europe, has been the
132 subject of renewed interest because of its beneficial effects in the context of
133 agroecology (Hayot Carbonero et al., 2011), its beneficial impact on ruminant
134 production and the environment and its potential antiparasitic effects on small
135 ruminants (Manolaraki et al., 2010; Hoste et al., 2015; Saratsis et al., 2016; Mueller-
136 Harvey et. al., 2019). *In vitro* studies have shown that sainfoin extracts have a dose-
137 dependent effect against different GIN species (Brunet et al., 2007; Manolaraki et al.,
138 2010; Novobilsky et al., 2013). Moreover, *in vivo* anthelmintic effects have also been
139 described in sheep and/or goats fed with sainfoin; i.e. 42-68% reduction in parasitic
140 egg excretion, which was associated with a 17.6% decrease in female worm fecundity
141 and a 45% decrease in worm numbers for *Haemonchus contortus* (Arroyo-Lopez et
142 al., 2014).

143 The present study, therefore, sought to explore the anthelmintic effects of feeding
144 regimes employing two CT-containing plant resources that may be relevant for
145 Mediterranean conditions. These were offered to lambs either alone or in combination
146 to evaluate their efficacies against two GIN species (*H. contortus* and
147 *Trichostrongylus colubriformis*). The specific objectives were to explore whether a)
148 the anthelmintic effect of carob in the feed is dose dependent (Trial 1), b) this
149 anthelmintic effect is associated with tannins by using polyethylene glycol (PEG) as a

150 tannin-inhibitor (Trial 2), and c) there are any synergistic effects between carob and
151 sainfoin feeds (Trial 3).

152 **2. Materials and Methods**

153 **2.1. Stabling and animals**

154 The experiments were carried out at the Asomaton Research Station of HAO Demeter
155 on the island of Crete, Greece. The animal barn was of an open-sided shed type, with
156 straw bedding. During the whole study the animals were kept indoors with each group
157 in a separate pen of approximately 10 m² and 10 m² open yard. The study included
158 three trials with lambs belonging to the local “Sfakion” breed. In order to achieve
159 uniformity of the experimental animals, all lambs included were female, 6-month-old
160 with a comparable body weight (BW), which was within the normal BW range of the
161 breed (22-30 kg) at the specific age. The lambs were raised indoors under helminth-
162 free conditions. Fourteen days before the start of each trial, they were drenched with
163 albendazole at the higher commercially recommended dose (ALBENDAZOLE
164 Drench, PROVET, 7.5 mg/kg) and they tested negative by faecal egg counts at the
165 start of each trial. No anthelmintic resistance was previously recorded for this specific
166 flock.

167 **2.3. Infective larvae**

168 Third-stage infective larvae of *H. contortus* and *T. colubriformis* strains, susceptible
169 to all classes of anthelmintic drugs, had been cultured from faeces of mono-
170 specifically infected donor sheep. Larvae were recovered using the Baermann
171 technique and then stored for 1-2 months at 4°C until use.

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173 **2.4. Tannin-containing plant resources**

174 Carob pods (after removal of the seeds) were locally purchased and offered as crushed
175 flour meal incorporated in the concentrate feed supplement. Sainfoin pellets (Perly
176 cultivar, 3rd cut) were provided by Multifolia (Viapres le Petit 10380, France) as part
177 of the Research project CARES.

178 **2.5. Tannin concentration and composition**

179 Tannin concentrations and compositions were determined in triplicate using two
180 different assays, i.e. the acetone-butanol-HCl assay and the thiolytic degradation with
181 benzyl mercaptan. Both techniques were applied in order to ensure a comprehensive
182 analysis since it was previously demonstrated that, depending on the types of CTs, the
183 acetone-HCl-butanol assay can give higher CT concentrations than the thiolysis assay.

184 The acetone-HCl-butanol assay was carried out as previously described by Grabber et
185 al. (2013) and Desrues et al. (2017).

186 The thiolysis reaction was carried out with benzyl mercaptan (Gea et al., 2011;
187 Ropiak et al., 2016), the reaction products were identified by HPLC-MS analysis
188 (Williams et al., 2014; Desrues et al., 2017) and quantified based on peak areas at 280
189 nm (Gea et al., 2011; Ropiak et al., 2016). This provided information on CT
190 concentration (g CT/100 g DW), CT size (in terms of mean degree of polymerisation,
191 mDP), molar percentages of prodelphinidins (PD) and procyanidins (PC) within CTs,
192 and molar percentages of *cis*- vs *trans*- flavan-3-ol subunits (Ropiak et al., 2016).

193 **2.6. Experimental design**

194 All diets offered to the animals during the experimental period (with or without the
195 tannin sources) were formulated to meet the nutrient requirements of the animals
196 (NRC, 2007) and the total rations were always iso-nitrogenous and iso-energetic as

197 well as balanced for crude fibre, Ca, P and Ca/P ratio (Suppl Table 1). Animals had
198 access to clean water at all times. The animals' appetite was assessed and feed
199 consumption (as feed offered minus refusals) was recorded on a daily basis by the
200 farm manager.

201 **2.6.1. Trial 1**

202 To determine the anthelmintic effect of carob pod meal and to define the optimal
203 concentration in a sheep ration, a subset of 35 lambs were randomly allocated to 5
204 groups (n=7 lambs/diet) (Table 1).

205 Carob meal (CaBP) was offered as feed supplement, at increasing rates of 0%, 3%,
206 6%, 9% and 12% (g CaBP/100g DM) of the total ration. The highest proportion, of
207 carob meal contributed to concentrate feed was set to 12% (due to its poor energy and
208 protein contents) in order to enable formulating a ration, which could cover the
209 nutritional requirements of lambs.

210 Feeding the experimental diets started 2 weeks prior (D14) to experimental infection
211 with nematode larvae (D0) in order for the animals to adapt to the feed.

212 On DAY 0, all lambs in groups (i) to (v) were infected with a single dose of 12.000
213 3rd stage larvae (L3) of *H. contortus* and 12.000 L3 of *T. colubriformis*. At the end of
214 the experimental period (D49), all lambs were euthanised by injection of a massive
215 dose of pentobarbital (Dolethal®).

216 **2.6.2. Trial 2**

217 Four groups of 6 lambs were included in a two-factorial trial (diet and PEG-addition).

218 Two groups were offered CaBP as feed supplement at the rate of 12% in the total
219 ration, and two groups remained on standard diet (Table 1). Half of the lambs in each
220 diet group were offered PEG (Polyethylene Glycol 4000, Fisher Scientific USA)

221 orally (60 g/lamb diluted in 200 ml water) on a daily basis after being allocated into
222 groups.

223 On D0, all lambs were experimentally infected with 8.000 L3 of *H. contortus* and
224 16.000 L3 of *T. colubriformis*. On D21, after parasite infection was confirmed by
225 positive faecal examination, the animals were allocated into 4 groups of 6 lambs each,
226 according to the experimental diets. On D37 they were euthanised as described above.

227 **2.6.3. Trial 3**

228 To determine the possible synergistic anthelmintic effects between 2 CT-containing
229 resources namely carob (*C. siliqua*) and sainfoin (*O. viciifolia*), 4 groups of 6 lambs
230 were included in a two-factorial design (Table 1).

231 On D-14 each group of lambs received the allocated diet, containing **i**) carob meal
232 (CaBP) alone **ii**) sainfoin (S) pellets; **iii**) a combination of carob meal and sainfoin
233 pellets (CaBP+S) while **iv**) a control group (C), received an isoproteic diet based on
234 lucerne. Carob was offered as a feed supplement at the rate of 12% in the total ration.
235 Sainfoin was offered as pellets representing 35% of the total ration. On D0 all lambs
236 were infected with a single dose of 12.000 L3 of *H. contortus* and 12.000 L3 of *T.*
237 *colubriformis*. At the end of the experimental period (D37), all lambs were euthanised
238 as previously described.

239 **2.7. Pathophysiological parameters**

240 Individual blood samples were collected once weekly (from D0 to D49) during Trial 1
241 and once every two weeks (from D0 to D28) during Trial 3, by jugular venipuncture
242 into heparinized tubes (BD Vacutainer®, UK) to determine the packed cell volume
243 (PCV), as an indicator of anaemia, according to the micro-haematocrit method. In

244 Trial 2 due to its short duration, the recording of PCV values was not included in the
245 design.

246 **2.8. Parasitological parameters**

247 Individual faecal samples were collected weekly directly from the rectum, during the
248 1st and 3rd trial, and twice weekly during the 2nd trial in order to determine faecal egg
249 counts (FEC) using a modified McMaster technique (Roepstorff and Nansen, 1998).
250 FEC data were expressed as eggs per gram of faeces (EPG).

251 At necropsy, the abomasa and the first 12 meters of small intestine were separated,
252 ligated, rapidly removed and immediately processed to collect the adult worms from
253 the luminal contents. For the intra-mucosal larvae, pepsin digestion was applied both
254 on the abomasum and intestinal mucosa (MAFF, 1986). After 4h incubation at 37°C
255 the larvae were collected. After storage in 10% alcohol, worm counts were performed
256 according to a 10% aliquot technique (MAFF, 1986). Morphological identification of
257 worm stages, sex and species were conducted using standard procedures (MAFF,
258 1986).

259 The fecundity of female worms was measured on 10 worms per lamb. For *T.*
260 *colubriformis*, eggs were counted directly *in utero* after clearing in 85% lactic acid
261 solution. All egg counts were performed under a microscope set at 10 times
262 magnification (total 100 ×). For *H. contortus*, the fecundity was determined using the
263 method described by Kloostermann et al. (1978). Briefly, the worms were soaked for
264 5 min in a large volume of distilled water, before being placed individually in
265 microtubes with 1000 µl of 0.125% hypochlorite concentration solution and kept at
266 room temperature for 20 minutes. Treatment resulted in female worms disintegrating
267 thus enabling the direct counting of eggs under a stereo-microscope using an aliquot
268 (10%) of the total volume.

269 **2.9. Statistical analyses**

270 The data of FEC and adult worm counts (AWC) were $\log_{10}(x+1)$ transformed prior to
271 analysis. For the FEC values, comparison of all groups was first performed using an
272 analysis of variance (ANOVA) with time as repeated measurement. Then, the
273 comparison of results to the control values were carried out date by date, using one-
274 way ANOVA completed by *the post-hoc* Bonferroni test for pairwise comparisons.
275 Group means of AWC were compared by one-way ANOVA (Trial 1) or two-way
276 ANOVA (Trial 2: CaBP +/- and PEG +/-; Trial 3: CaBP +/- and sainfoin +/-).
277 Regarding the fecundity of female worms, the Shapiro-Wilk Test of normality, which
278 is more appropriate for small sample sizes, was used. In cases where the data deviated
279 significantly ($P < 0.05$) from a normal distribution (Trial 1 and 3 for both parasite
280 species and Trial 2 for *T. colubriformis*) the appropriate test to check the difference of
281 fecundity between the groups, which is the non-parametric test of Kruskal-Wallis,
282 was used. Where the dependent variable was normally distributed ($P > 0.05$) the
283 parametric test of one-way ANOVA (*H. contortus* of Trial 2) was used. Additionally,
284 for the Trial 1, the model of linear regression was used, in order to be investigated if
285 there was a negative correlation between the variables “percentages of carob” and
286 “fecundity of female worms” for both parasite species (*H. contortus* and *T.*
287 *colubriformis*). Finally, the Tukey HSD test was used for data of trial 3, in order to
288 investigate statistically significant differences between groups.

289 All statistical analyses were performed using the SyStat SPSS 9.0 Software.

290

291 **2.10. Ethical considerations**

292 The study was carried out in compliance with the national animal welfare regulations.

293 All trials took place in a Research Station of the Veterinary Research Institute. The

294 experimental protocol was approved by the responsible institutional committee (VRI
295 Committee for Approval of Experimental protocols as appointed at 26/5/2014,
296 Decision nr 972) . Euthanasia was performed in a humane manner according to EU
297 regulations.

298

299 **3. Results**

300 The CT concentrations and compositions are presented in Table 2. The HBA assay
301 yielded similar CT concentrations for both plant materials, whereas the thiolysis assay
302 generated lower CT concentrations for the sainfoin pellets. The thiolysis assay
303 revealed that: i) both carob and sainfoin CTs consisted mainly of prodelphinidins,
304 96.7 and 74.7 mole percentages, respectively; ii) carob CTs were highly galloylated
305 (i.e. 41.1% of flavan-3-ol subunits are galloylated), but sainfoin CTs did not contain
306 any esterified galloyl groups; iii) carob CTs were characterised by a relatively high
307 average molecular weight (mDP = 31.1), whereas sainfoin CTs had an mDP value of
308 11.5.

309

310 **3.1. Trial 1**

311 The results of Trial 1 are shown in Table 3 and Figure 1

312 The analyses of FEC, based on the ANOVA on Repeated Measures from D21 to D49,
313 showed an overall non-significant difference between groups, but significant
314 difference over time (between days of sampling). Meanwhile, the date-by-date
315 ANOVA of FEC showed no significant differences between groups, whatever the
316 date, as well as no dose effect. Reduction in FEC, up to 39.2% on DAY 49 as
317 compared to controls, was observed only for the group fed with the highest
318 concentration of carob meal.

319 For *H. contortus*, the AWC declined in the groups receiving the highest concentration
320 of carob meal but this effect was not statistically significant ($P= 0.964$). In contrast,
321 there was a declining trend ($P<0.06$) for the numbers of *T. colubriformis* with
322 increasing carob concentration.

323 The fecundity values showed significant differences (15.6%-59.3% lower than
324 0%CaBP respectively from the lowest to the highest CaBP concentration) between
325 groups for *H. contortus* demonstrating a dose dependent effect ($P<0.05$).

326 The Box plot (Figure 1b) for *H. contortus* fecundity suggests that worms from the
327 0%CaBP group tended to be more fecund than other CaBP groups and there may be
328 some degree of fecundity discrepancy between CaBP groups. This trend was
329 confirmed with the non-parametric test of Kruskal-Wallis, which showed that there
330 were statistically significant differences in fecundity between the groups ($P<0.001$).
331 More specifically, fecundity was statistically significantly greater for 0%CaBP group
332 than the other CaBP groups. On the other hand, regarding *T. colubriformis* fecundity,
333 there was no statistically significant difference between the groups ($P=0.128$).
334 However, the model of linear regression, which was implemented and was
335 statistically significant ($P<0.05$), showed a negative correlation between the variables
336 “group” and “fecundity” for both parasite species.

337 No GIN larvae were recovered after pepsin digestion.

338 Mean PCV values (\pm SD) for groups 0%CaBP, 3%CaBP, 6%CaBP, 9%CaBP, and
339 12%CaBP on the last day of the trial were 25.29 (\pm 5.96), 23.00 (\pm 5.72), 21.00
340 (\pm 6.32), 23.00 (\pm 5.89) and 24.00 (\pm 5.00) respectively. No significant differences were
341 found between the groups in PCV.

342 Average daily gain (ADG) as calculated for the whole trial duration for 0%CaBP,
343 3%CaBP, 6%CaBP, 9%CaBP and 12%CaBP groups was (mean \pm s.d.) 69.2 g (\pm 31.0),

344 61.5(\pm 36.1), 68.7(\pm 33.0), 74.8(\pm 37.5) and 64.4(\pm 32.9) g respectively, which yielded
345 no significant differences between the groups.

346 **3.2.Trial 2**

347 The results of Trial 2 are presented in Table 4 and Figure 2.

348 The Repeated Measurements Analyses of FEC showed an overall statistical difference
349 ($P < 0.001$) between the 4 groups. The date-by-date ANOVA of FEC indicated that
350 differences were most prominent on DAY 29 (significant statistical differences,
351 $P < 0.02$) and then on DAY 33 (trend, $P < 0.07$). Specifically, the values of the C,
352 C+PEG, CaBP+PEG groups were comparable throughout the trial, while the group
353 receiving only carob (CaBP) showed consistently lower FEC starting from DAY 25
354 until the last day of the experiment. It was evident that the effect of carob on FEC was
355 nullified by PEG.

356 Results on AWC, showed reduction only for *H. contortus* ($P < 0.03$) resulting in an
357 overall statistical difference between the 4 groups, since the lowest worm counts were
358 found for the CaBP group. Especially, for *H. contortus*, a reduction of approximately
359 65% was observed in the carob group compared to the control. The AWC in the
360 CaBP+PEG group were similar to the other 2 control groups showing no reduction in
361 worm population. On the other hand, no effect of carob was observed on *T.*
362 *colubriformis* worm counts.

363 No effect of carob on female fecundity was also observed, irrespective of the parasite
364 species. Both control and carob groups showed comparable levels of female fecundity
365 for the two parasite species. The Box plot in Figure 2b showed that the range of
366 fecundity of *H. contortus* for CaBP group was greater than for C, C+PEG and
367 CaBP+PEG groups and the interquartile range (middle 50% of the records) was lower
368 on the fecundity scale in the CaBP group than in the other groups.

369 No GIN larvae were recovered after pepsin digestion.
370 The average daily gain (ADG) of lambs as calculated for the whole trial duration for
371 (C), (C+PEG), (CaBP) and (CaBP+PEG) groups was 51.8(\pm 30.1) (\pm s.d.), 69.8(\pm 19.9),
372 60.8(\pm 29.3) and 40.5(\pm 25.6) g, respectively, which resulted in no significant
373 differences between the groups.

374 **3.3.Trial 3**

375 The results of Trial 3 are shown in Table 5 and Figure 3.

376 The FEC values of all experimental groups remained at very low levels up to DAY
377 21. The overall repeated analyses based on 3 dates of the patent phase (DAY 21,
378 DAY 28, DAY 37) showed a trend for differences ($P < 0.07$) between groups. The
379 results of the date-by-date ANOVA test did not show difference on DAY 21 and on
380 DAY 28, while on DAY 37, the values of FEC in groups CaBP+S and S tended to be
381 reduced ($P < 0.06$) compared to the two other groups. When compared to the control
382 values of FEC, the reductions in the 3 treated groups ranged from 44.6% to
383 approximately 86 %. These differences were mainly found for the sainfoin group (S)
384 and carob+sainfoin (CaBP+S) groups. As regards the AWCs, no significant
385 differences were observed neither in the number of *H. contortus* and *T. colubriformis*.
386 No GIN larvae were recovered after pepsin digestion.

387 The non-parametric test of Kruskal-Wallis showed that there were statistically
388 significant differences in fecundity between the groups ($P < 0.001$). Specifically, the C
389 group presented the highest fecundity values, while the CaBP+S group presented the
390 lowest ones for both parasite species. Tukey HSD test for *H. contortus* showed that
391 the C group differed significantly from CaBP, S and CaBP+S, while for *T.*
392 *colubriformis* fecundity for CaBP group was also statistically different from CaBP+S
393 (Figure 3b).

394 When exploring the pathophysiological parameters (i.e. PCV), the analysis of
395 variance on repeated measures and also the date by date ANOVA did not show
396 significant differences between the groups. Specific values for mean PCV (\pm SD) on
397 DAY 28 of the respective groups C, CaBP, S and CaBP+S were 31.67 (\pm 3.39), 33.00
398 (\pm 4.86), 31.33 (\pm 3.61) and 30.50 (\pm 4.37).

399 The average daily gain (ADG) as calculated for the whole trial duration for (C),
400 (CaBP), (S) and (CaBP+S) groups was (mean \pm s.d.) 122.5(\pm 38.1), 88.2(\pm 39.2),
401 104.6(\pm 11.9) and 124.8(\pm 39.7) g, respectively and there were no significant
402 differences between the groups.

403 **4. Discussion**

404 The literature contains several *in vitro* and *in vivo* studies, conducted on small
405 ruminants, which evaluated the anthelmintic effect of tannin-containing plants. Such
406 studies first examined temperate forage legumes fed through grazing, as hay, silage or
407 pellets. Examples are sainfoin (Hoste et al., 2016; Legendre et al., 2018; Mueller-
408 Harvey et al., 2019), sericea lespedeza (*Lespedeza cuneata*) (Burke et al., 2012a,b;
409 Mechineni et al., 2014; Kommuru et al., 2014, 2015), and sulla (*Hedysarum*
410 *coronarium*) (Niezen et al., 1995, 2002). More recently, there has been also a growing
411 interest in tannin-containing by-products from the food industry as illustrated by
412 studies with hazelnut peels (*Corylus avellana* fruits) (Desrues et al., 2012; Girard et
413 al., 2013), carob pods (Manolaraki et al., 2010; Arroyo-Lopez et al., 2014) and
414 browse plants such as *Pistacia lentiscus* (Landau et al., 2010; Manolaraki et al.,
415 2010), *Quercus coccifera* (Manolaraki et al., 2010) and *Salix* spp (Mupeyo et al.,
416 2011).

417 In the current study, we further explored the *in vivo* anthelmintic effects of carob pod
418 meal since it represents a common feed resource in the Mediterranean region and
419 there was some previous evidence of its anthelmintic (Arroyo-Lopez et al., 2014) and
420 anticoccidial (Saratsis et al., 2016; Legendre et al., 2018) properties. In order to
421 develop a practical implementation tool for carob as dietary intervention, we wanted
422 to identify a) the optimal carob concentration in the feed for bioactivity, b) whether
423 CTs contributed to such an activity and c) whether there were any synergistic effects
424 with other plant sources with different types of CTs (i.e. sainfoin). For all 3 trials a
425 balanced and palatable ration was specifically designed for all animals. This aimed to
426 achieve similar production indexes in all groups and ensured that any observed
427 differences in the effects of parasitism would not stem from quantitative differences in
428 the dietary composition but rather from differences in the bioactive CTs (Coop and
429 Kyriazakis, 1999; Athanasiadou et al., 2008; Hoste et al., 2015).

430 The parasites that served as models for this study (*H. contortus* and *T. colubriformis*)
431 are the most pathogenic and/or prevalent GIN species in European sheep and goats
432 (Charlier et al., 2018). These experiments allowed us to investigate carob-pods
433 efficacy against nematodes in the different anatomical location within the gut, as
434 location can affect the exposure of worms to different CT concentrations (Desrues et
435 al., 2017; Quijada et al., 2018).

436 Results of Trial 1 showed decreases in the mean values of FEC and AWC only in the
437 group fed with the highest concentration of CaBP in the concentrate feed, although
438 not significant. However, fecundity values showed a negative correlation to CaBP
439 concentration in the feed indicating a dose-dependent fecundity suppression effect.
440 The results suggest that carob used in feed at 12% has a potential anthelmintic effect

441 and this effect is due mainly to the reduction of female worm fecundity
442 (predominantly in *H. contortus*) and to a lesser extent to the reduction of
443 establishment and development of the worms. Since *H. contortus* produce a
444 remarkably high daily egg output compared to *T. colubriformis* (Besier et al., 2016),
445 we suggest that the reduction in FEC seen in this trial can be attributed to the effect
446 the carob diet had against *H. contortus*. Overall, the results of this trial suggest that
447 the higher the concentration of carob in the ration the higher the anthelmintic activity;
448 this effect that was more evident for *H. contortus*. Unfortunately, there are limitations
449 to the quantity of carob pod meal that can be included in a well balanced ration since
450 carob pods contain high sugar but low protein and lipid concentrations (Priolo et al.,
451 1998; Karabulut et al., 2006).

452 During Trial 2, the main results **i)** confirmed that CaBP reduced FEC in lambs, as
453 these reductions compared to control values ranged from 20% to 45%, **ii)** that these
454 reductions in FEC seemed to be mainly due to the lower numbers from the highly
455 prolific *H. contortus* species and not from *T. colubriformis*, and that there were no
456 effects on female fecundity of both species and **iii)** that the anthelmintic effect of
457 CaBP may be attributed to CTs, because a restoration to control values for FEC and
458 *Haemonchus* worm numbers was observed in the CaBP + PEG group. PEG is a non-
459 nutritive synthetic polymer that is capable of binding and deactivating CTs; it has
460 been used in many animal nutrition studies to increase the intake of CT-containing
461 feeds and to improve protein absorption (Silanikove et al., 1996; Bermingham et al.,
462 2001; Theodoridou et al., 2012). This ability has also been used to test (Brunet et al.,
463 2007, 2008; Debela et al., 2012; Brito et al., 2018) whether any observed *in vivo*
464 anthelmintic activity was linked to the presence of CTs.

465 Finally, the aim of Trial 3 was to investigate two hypotheses: firstly, that carob CTs
466 generate a stronger anthelmintic effect than sainfoin CTs and secondly, that
467 synergistic effects could be achieved by combining carob with sainfoin. The rationale
468 for these hypotheses is based on the fact that carob and sainfoin contain different
469 types of CTs and that these could target different stages of the GIN life cycle. Carob
470 CTs are highly galloylated prodelphinidins, whereas sainfoin CTs are non-galloylated
471 prodelphinidins. Previous studies found two structural features in CTs that enhance
472 anthelmintic activity *in vitro*: i) prodelphinidin CTs are more potent than procyanidin
473 CTs and ii) galloylation increases the anthelmintic effect of CTs (Hoste et al., 2016;
474 Kommuru et al., 2014, 2015). Therefore, carob CTs, which have a high
475 prodelphinidin/procyanidin ratio (96.7% prodelphinidins/3.3% procyanidins) and are
476 also highly galloylated (i.e. 41.1% of the flavan-3-ol subunits are galloylated) should
477 produce a stronger anthelmintic effect than sainfoin, as sainfoin CTs have less
478 prodelphinidins (74.8%) and no galloyl groups (N.B. % stands for mole percent
479 within CT molecules; Table 2).

480 There are several important reasons that could explain why the results from Trial 3
481 did not support either of these hypotheses. Firstly, sainfoin - but not carob - was fed in
482 a pelleted form, while it has been demonstrated previously that the pelleting process
483 has a marked effect on CTs in terms of their analysis (Mueller-Harvey et al., 2019).
484 Table 2 shows that the CT concentrations in sainfoin pellets differed considerably
485 between the two assays (6.5 and 1.7 g CT/100 g DW) in contrast to the carob meal
486 data (5.8 and 7.2 g CT/100g DW). However, we currently do not know whether the
487 pelleting process enhances the anthelmintic activity of CTs or not. Secondly, up to
488 now most attempts to unravel links between CT structural features and anthelmintic
489 effects have employed *in vitro* assays. Therefore, *in vivo* feeding trials such as the

490 present ones are vital to test the laboratory data. It may turn out that the esterified
491 galloyl groups are not stable in the digestive tract and that the prodelphinidins in
492 carob and sainfoin were the active CTs.

493 Therefore, preliminary conclusions from the Trial 3 data could be that galloylation is
494 unlikely to enhance anthelmintic activity *in vivo* in terms of *H. contortus* fecundity or
495 total worm counts and that pelleting of CT-plants might lead to lower FEC. These
496 indications will, however, need rigorous testing in the future.

497 The nutritional and/or anthelmintic properties of sainfoin fed as direct grazing, silage,
498 hay or pellets have been evaluated in both sheep and goats, with promising
499 anthelmintic results when used either alone (Paolini et al., 2005; Heckendorn et al.,
500 2006; Ríos-de Alvarez et al., 2008; Gaudin et al., 2016) or in combination with other
501 CT sources (Girard et al., 2013). Previous results have demonstrated that sainfoin
502 consumption under different forms of preservation can reduce FEC and also reduce
503 female worm fecundity of *H. contortus* (Manolaraki et al., 2010; Arroyo-Lopez et al.,
504 2014) or *T. colubriformis* (Manolaraki et al., 2010); however, in other studies a lack
505 of effect has been observed (Heckendorn et al., 2006). The issue of the variable
506 results has also been addressed in several reviews (Hoste et al., 2015; Hoste and
507 Niderkorn, 2019).

508 To summarise, the main results of trial 3 for FECs were i) a confirmation of
509 significant reductions of FEC due to the consumption of both CaBP and sainfoin
510 pellets; ii) a temporal increase in the anthelmintic effects of sainfoin but not for CaBP,
511 and iii) no synergistic effects of the combination CaBP + sainfoin. In addition, it
512 would appear that these results can largely be explained by significant effects on
513 female fecundity of both species, but there were only limited effects on the worm
514 populations. No significant effects on AWC were observed for any of the species. On

515 the other hand, although the differences were not significant, the percentage of
516 reduction compared to the controls (Group C) for *H. contortus* worm numbers were
517 respectively, for Groups CaBP 35.5%, S 62.1% and CaBP+S 53.5%.

518 In conclusion, the results of these three trials, which focussed on carob pod meal
519 alone or in combination, raised future research questions regarding what causes the
520 differences in results when different CT-containing resources are used and what is
521 required for a more rational use of CT-containing resources as nutraceutical feeds
522 under farm conditions and in different production systems (Hoste et al., 2015).

523 Our results confirmed that **i)** the consumption of CT containing resources can
524 modulate the biology of GINs; **ii)** that CT were involved in the anthelmintic effects of
525 carob and **iii)** the concentration in the diet influenced the anthelmintic effects as
526 previously shown in other *in vivo* studies with sericea lespedeza (Shaik et al., 2004,
527 2006) or sainfoin (Brunet et al., 2007) and **iv)** different mechanisms appeared to affect
528 the worm population and could explain the reduction of FECs: either a reduced
529 fecundity of female adult worms (see Trial 1 and 3) and /or a reduction of the number
530 of worms (see Trial 2).

531 The data of these 3 studies also illustrated that results depended on the type of
532 nematode species (abomasal or intestinal species) and/or on the nature of CT
533 resources (in our case carob vs sainfoin) and on the CTs. As stated by Quijada (2015)
534 and Desrues et al. (2016) the quantitative and qualitative differences in CTs appear to
535 influence the anthelmintic activity on the different species of parasitic nematodes.

536

537 Our results suggest that, when worm populations are exposed to CTs in the
538 gastrointestinal tract, upon their ingestion by the host the most evident effect recorded
539 is the reduction of female fecundity. Particularly for *H. contortus*, it appears that

540 fecundity is only affected when the worms are exposed to CTs during maturation
541 (Trial 1 and 3) and not when they are already mature adults (Trial 2). On the other
542 hand, when CaBP was consumed for two weeks by animals in which adult worm
543 populations were already established and patent, the main finding was a significant
544 decrease in *H. contortus* worm counts. Moreover, the current study adds further
545 support to the observation that most of the CT effect is related to abomasal parasite –
546 and not so much to the small intestinal parasite. This is possibly due to the higher CT
547 concentration in the abomasum compared with the rumen and intestines, along with
548 higher prodelphinidin percentage as already shown in studies on the cattle abomasal
549 parasite *Ostertagia ostertagi* (Desrues et al., 2017).

550 **Acknowledgements**

551 This article is based upon work from EMIDA ERA-Net project CARES and COST
552 Action COMBAR CA16230 (European Cooperation in Science and Technology). The
553 authors would like to thank Chris Drake for the chemical analyses.

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Table 1: Experimental design (CaBP=Carob; CaBP+PEG=Carob+PEG; C=Control; C+PEG=Control+PEG; CaBP+S=Carob+Sainfoin; S=Sainfoin)

Trial	Groups	Lambs/ group	Mean BW at start \pm s.d. (kg)	Day feeding started	Infection Day	Inoculation dose (L3)	Day trial ended
1	CaBP 0%	7	25.8 \pm 1.1	-14	0	12,000 <i>H. contortus</i> & 12,000 <i>T. colubriformis</i>	49
	CaBP 3%		26.2 \pm 3.7				
	CaBP 6%		25.2 \pm 2.6				
	CaBP 9%		27.1 \pm 3.5				
	CaBP 12%		26.4 \pm 2.8				
2	C	6	26.4 \pm 2.7	21	0	8,000 L3 <i>H. contortus</i> & 16,000 L3 <i>T. colubriformis</i>	37
	C+PEG		26.5 \pm 2.6				
	CaBP (12%)		26.3 \pm 2.4				
	CaBP+PEG		27.0 \pm 1.7				
3	C	6	27.0 \pm 3.1	-14	0	12,000 L3 <i>H. contortus</i> & 12,000 L3 <i>T. colubriformis</i>	37
	CaBP (12%)		27.1 \pm 2.5				
	CaBP+S		27.1 \pm 2.9				
	S		26.8 \pm 3.2				

Table 2. Condensed tannin concentrations (expressed as g CT/100 g DW) measured either with the acetone-HCl-butanol or the thiolysis assays as well as tannin compositions in the two different feeds [abbreviations: % refers to molar percentages of galloylation, prodelphinidins (PD), procyanidins (PC), *cis*- or *trans*- flavan-3-ol subunits; mean degree of polymerisation (mDP)].
 ND: non detected

	% galloylation	PD/PC	Tannins (acetone-HCl/butanol)	Tannins (thiolysis)	mDP	<i>cis/trans</i>-flavan-3-ols
Carob meal	41.1 (± 0.6)	96.7/3.3 (± 0.1)	5.84 (± 0.2)	7.20 (± 0.0)	31.2 (± 0.1)	45.9/54.1 (± 0.0)
Sainfoin pellets^a	ND	74.8/25.2 (± 0.5)	6.50 (± 0.3)	1.70 (± 0.1)	11.5 (± 0.3)	85.3/14.7 (± 0.1)

^aThe same sainfoin pellets were used in another study (Quijada et al., 2018) and the data are reported here for comparison purposes.

Table 3.

Trial 1: Effect of diet regimes containing different concentration of Carob (CaBP) on adult worms recovered at necropsy in the different experimental lamb groups. Adult worm counts (AWC) shown as arithmetic mean of adult worms (and SD in brackets) per group fed different amount of Carob pod meal (CaBP = Carob).

Treatment Group	<i>H. contortus</i>			<i>T. colubriformis</i>		
	Female	Male	Total	Female	Male	Total
0% CaBP	2,777 (±1,579)	2,331 (±1,382)	5,109 (±2,802)	2,063 (±534)	1,097 (±471)	3,160 (±944)
3% CaBP	2,789 (±1,606)	2,006 (±1,117)	4,794 (±2,709)	2,493 (±986)	1,163 (±568)	3,656 (±1,461)
6% CaBP	3,584 (±1,570)	2,570 (±1,178)	6,154 (±2,595)	2,514 (±497)	903 (±676)	3,417 (±1,073)
9% CaBP	3,029 (±1,385)	2,799 (±1,415)	5,827 (±2,692)	1,910 (±1,043)	633 (±427)	2,543 (±1,418)
12% CaBP	2,160 (±1,362)	2,039 (±1,212)	4,199 (±2,422)	1,550 (±801)	944 (±630)	2,494 (±1,416)

Table 4.

Trial 2: Effect of PEG intake on adult worms recovered at necropsy in the different groups of lambs fed with carob rich diet (Groups: CaBP (Carob) and CaBP+PEG (Carob+PEG)) or not (Groups: C (Control), C+PEG (Control+PEG)). Adult worm counts (AWC) shown as arithmetic mean of adult worms (female, male, total) (and SD in brackets) per group.

Treatment Group	<i>H. contortus</i>			<i>T. colubriformis</i>		
	Female	Male	Total	Female	Male	Total
C	897 (±736)	584 (±461)	1,480 ^a (±1,194)	5,783 (±2,104)	4,382 (±1,529)	10,166 (±3,599)
C+PEG	1,002 (±323)	710 (±279)	1,712 ^a (±593)	6,028 (±2,740)	4,713 (±2,067)	10,742 (±4,776)
CaBP	288 (±220)	243 (±199)	532 ^b (±399)	5,397 (±2,280)	4,882 (±2,171)	10,279 (±4,439)
CaBP+PEG	701 (±250)	617 (±178)	1,318 ^a (±424)	5,751 (±2,387)	5,303 (±1,743)	11,054 (±4,091)

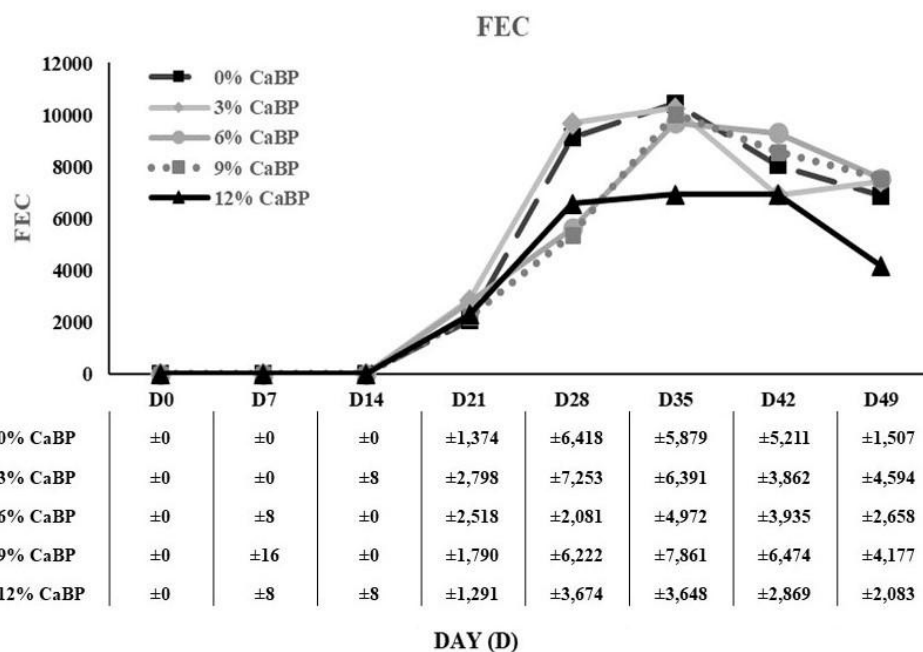
Table 5.

Trial 3: Effect of diet regimes containing different condensed tannin diets on adult worms recovered at necropsy in the different experimental lamb groups C (Control), CaBP (Carob), S (Sainfoin pellets), CaBP+S (Carob+Sainfoin pellets). Adult worm counts (AWC) shown as arithmetic mean of adult worms (female, male, total) (and SD in brackets) per group.

Treatment Group	<i>H. contortus</i>			<i>T. colubriformis</i>		
	Female	Male	Total	Female	Male	Total
C	3,482 (±3,170)	3,302 (±2,797)	6,783 (±5,924)	1,370 (±443)	1,082 (±438)	2,452 (±842)
CaBP	2,087 (±2,339)	2,288 (±2,562)	4,375 (±4,788)	1,140 (±373)	1,052 (±259)	2,192 (±617)
S	1,315 (±1,594)	1,255 (±1,707)	2,570 (±3,286)	1,735 (±1,414)	1,453 (±1,029)	3,188 (±2,406)
CaBP+S	1,470 (±1,257)	1,685 (±1,393)	3,155 (±2,607)	1,088 (±1,198)	863 (±961)	1,952 (±2,156)

Figure 1. Trial 1: Effect of diet regimes containing different amounts of Carob pod meal (CaBP) on A) faecal egg counts (FEC) on Day 0 to 49) (SD in table below) and B) box-plots for female worm fecundity (95% confidence interval) in the different experimental lambs for *Haemonchus contortus* and *Trichostrongylus colubriformis*.

A.



B.

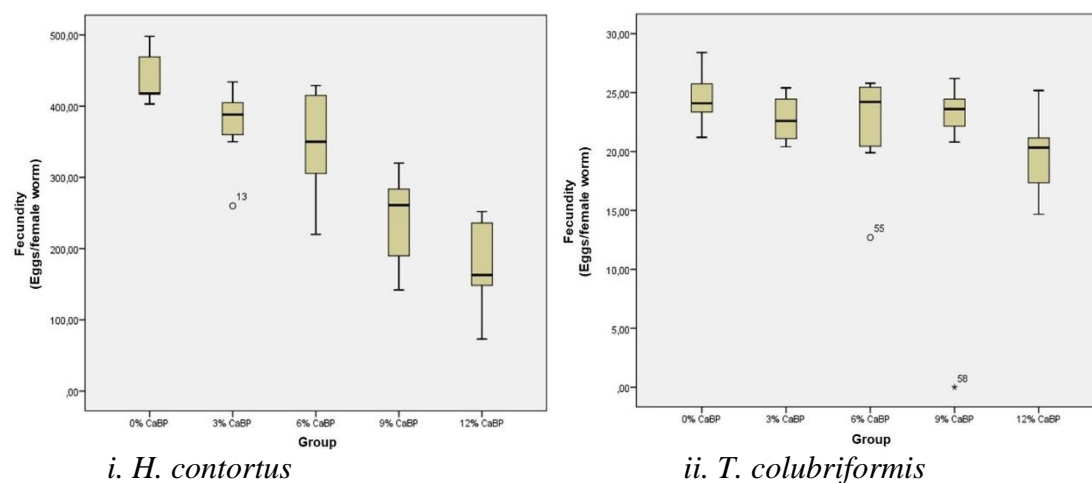
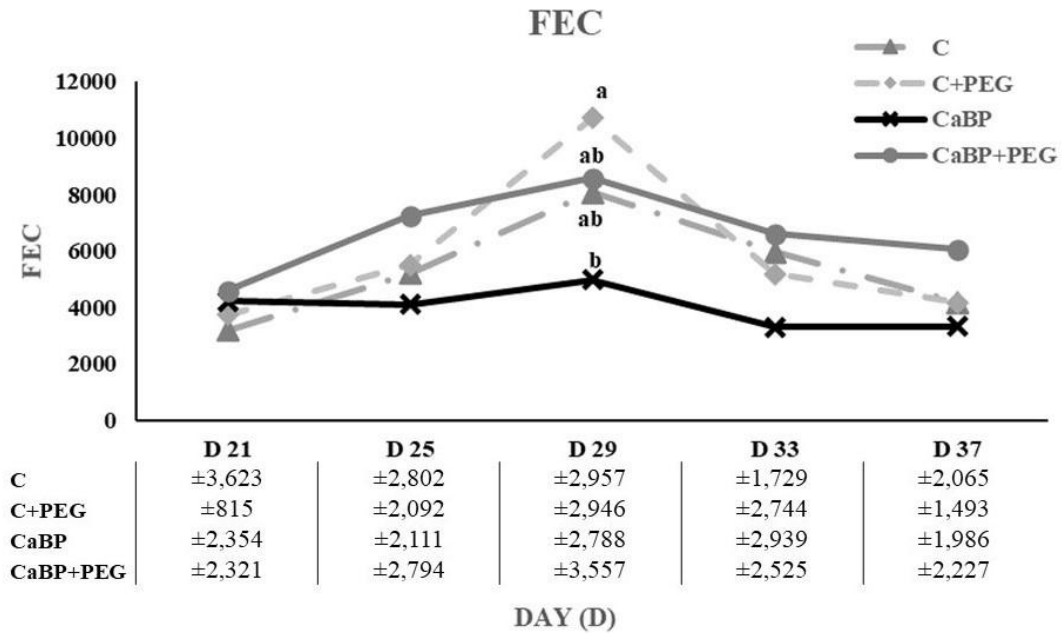
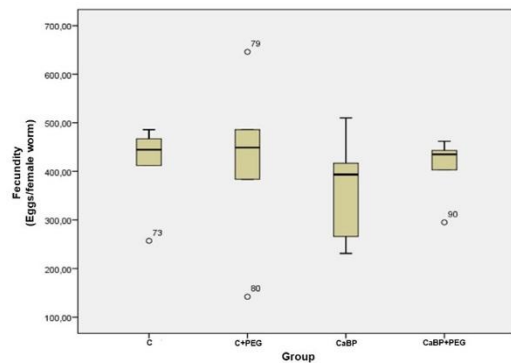


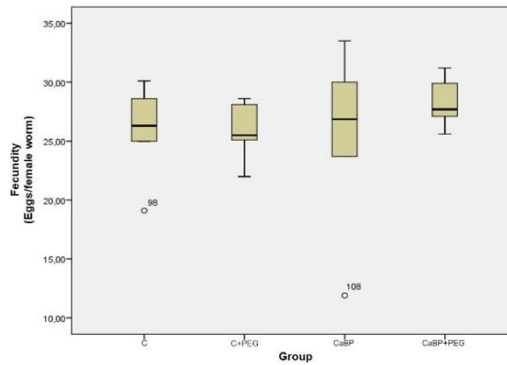
Figure 2. Trial 2: Effect of PEG intake on A) faecal egg counts (FEC) on Day 21 to 37 (SD in table below) and B) box-plots for female worm fecundity (95% confidence interval) for *Haemonchus contortus* and *Trichostrongylus colubriformis* in the different experimental lambs fed with carob pods meal at 12% (CaBP and CaBP+PEG) or served as Controls (C and C+PEG).



B.



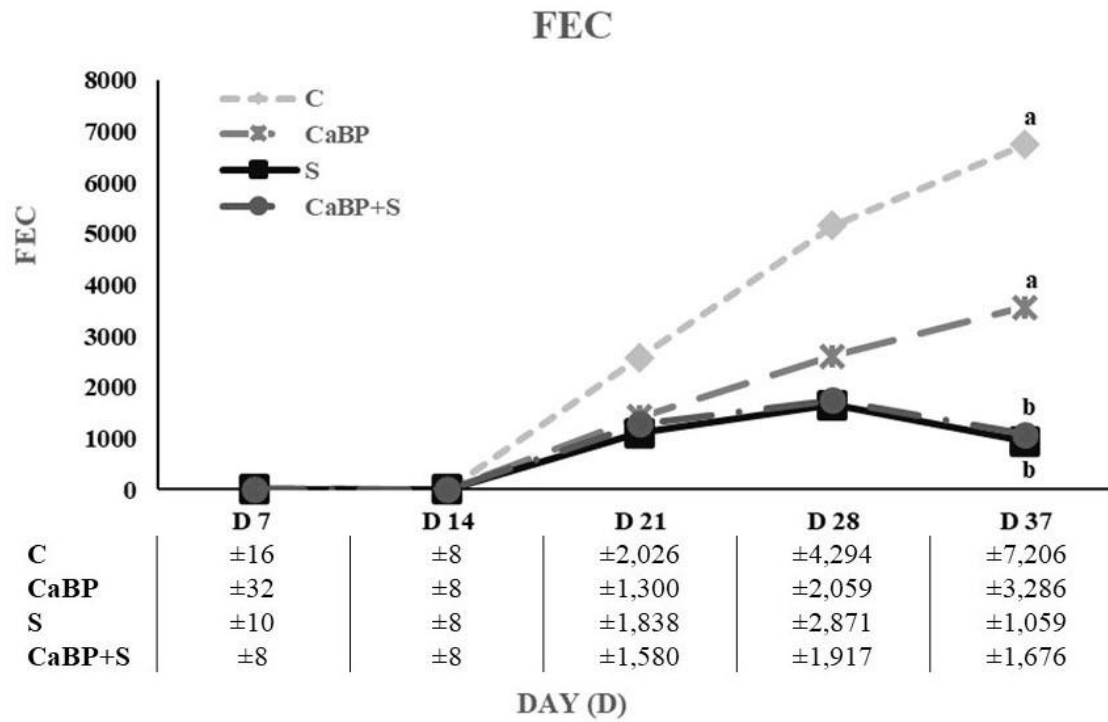
i. H. contortus



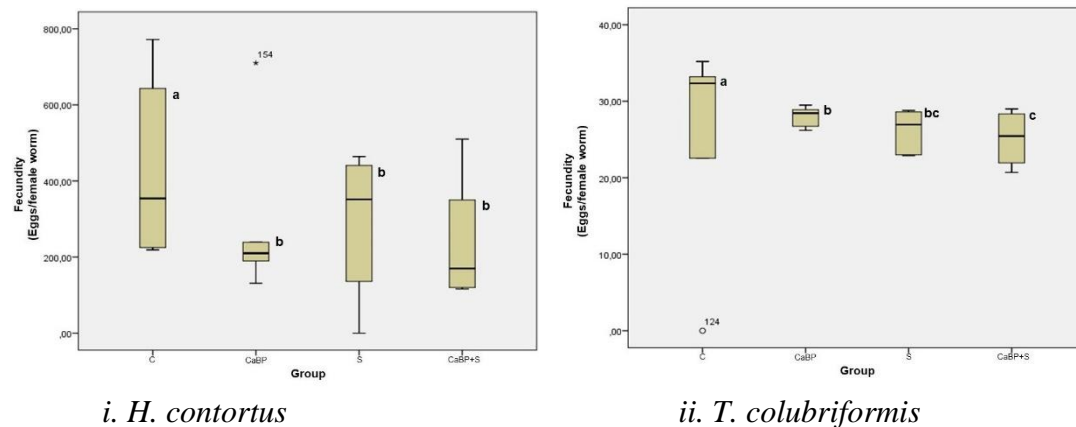
ii. T. colubriformis

Figure 3. Trial 3: Effect of diet regimes containing different condensed tannin diets on A) faecal egg counts (FEC) on Day 7 to 37 (SD in table below) and B) box-plots for female worm fecundity (95% confidence interval), in the different experimental lambs groups C (Control), CaBP (Carob), S (Sainfoin pellets), CaBP+S (Carob+Sainfoin pellets) for *Haemonchus contortus* and *Trichostrongylus colubriformis*.

A.



B.



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Declaration of interests

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests:

Author Contribution Statement

SARATSI K was a PhD student and the paper is part of her PhD Thesis, **SOTIRAKI S**, **HADJIGEORGIOU I**, and **HOSTE H** were the Supervisors of her Thesis including the current work. **VOUTZOURAKIS N** and **STEFANAKIS A** are specialist in animal nutrition and supporting the preparation of ration and acquiring the samples, data interpretation and writing the paper. **TZANIDAKIS N**, is a veterinarian supporting with laboratory techniques, **THAMSBORG SM**, is a senior scientists coordinator of CARES project who supervised the trials and supported data analyses, interpretation and writing the paper and **MUELLER-HARVEY I** is a senior researcher expert in tannin analyses who supported in chemical analysis of the feeds and data interpretation and writing the paper.

According to CRediT:

SARATSI K: Conceptualization, Visualization, Investigation, Resources, Formal Analyses, Writing- Original draft preparation, Reviewing and Editing, **SOTIRAKI S**, **HADJIGEORGIOU I**, **HOSTE H** and **THAMSBORG SM:** Conceptualization, Supervision, Writing, Reviewing and Editing. **VOUTZOURAKIS N**, **TZANIDAKIS N** and **STEFANAKIS A** Investigation, Resources, Reviewing and Editing, **MUELLER-HARVEY I:** Methodology, Investigation, Reviewing and Editing