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Abstract

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

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

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

1. Introduction

Infections by gastrointestinal nematodes (GIN) affect both health and welfare of grazing ruminants, causing anorexia, impaired digestion and nutrient absorption with related production losses, diarrhoea, anaemia and even death (Perry and Randolph, 1999; Stear et al., 2007; Hoste et al., 2016). Currently, the control of these parasites relies on repeated dosing with commercial anthelmintic drugs. However, the development of anthelmintic resistance in worm populations against one or multiple classes of anthelmintic has become a serious problem in several regions of the world, making it increasingly difficult to control parasitic infections (Kaplan, 2020). At the same time the increasing concerns of consumers about the presence of drug residues in foods and in the environment (McKellar, 1997) have stimulated the search for alternative or complementary solutions (Hoste and Torres-Acosta, 2011) within the context of organic farming and sustainable agriculture (Charlier et al., 2018).

Such alternatives include the use of bioactive plants with anthelmintic properties. Many results indicate that such resources, because of the presence of plant secondary metabolites (PSMs), might help to interfere with the biology of key-stages during nematode cycle and to reduce the consequences of GIN infections in grazing ruminants. Particular attention has been given to plants containing condensed tannins (CT) and some related polyphenols (see reviews by Terrill et al., 2012; Hoste et al., 2015, 2016). Based on previous studies, the need to explore new plant resources to develop non-drug-based strategies for the integrated control of nematode parasites in grazing ruminants has recently become a research priority in livestock production, especially in ruminant breeding as also reviewed by Morgan et al, 2020.

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

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

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

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

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

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

2. Materials and Methods

2.1. Stabling and animals

The experiments were carried out at the Asomaton Research Station of HAO Demeter on the island of Crete, Greece. The animal barn was of an open-sided shed type, with straw bedding. During the whole study the animals were kept indoors with each group in a separate pen of approximately 10 m^2 and 10 m^2 open yard. The study included three trials with lambs belonging to the local "Sfakion" breed. In order to achieve uniformity of the experimental animals, all lambs included were female, 6-month-old with a comparable body weight (BW), which was within the normal BW range of the breed (22-30 kg) at the specific age. The lambs were raised indoors under helminth-free conditions. Fourteen days before the start of each trial, they were drenched with albendazole at the higher commercially recommended dose (ALBENDAZOLE Drench, PROVET, 7.5 mg/kg) and they tested negative by faecal egg counts at the start of each trial. No anthelmintic resistance was previously recorded for this specific flock.

2.3. Infective larvae

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

2.4. Tannin-containing plant resources

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

2.5. Tannin concentration and composition

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

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

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

2.6. Experimental design

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

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

2.6.1. *Trial 1*

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

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

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

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

2.6.2. *Trial 2*

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

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

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

2.6.3. *Trial 3*

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

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

2.7. Pathophysiological parameters

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

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

2.8. Parasitological parameters

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

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

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

2.9. Statistical analyses

The data of FEC and adult worm counts (AWC) were log10(x+1) transformed prior to analysis. For the FEC values, comparison of all groups was first performed using an analysis of variance (ANOVA) with time as repeated measurement. Then, the comparison of results to the control values were carried out date by date, using one-way ANOVA completed by *the post-hoc* Bonferroni test for pairwise comparisons. Group means of AWC were compared by one-way ANOVA (Trial 1) or two-way ANOVA (Trial 2: CaBP +/- and PEG +/-; Trial 3: CaBP +/- and sainfoin +/-). Regarding the fecundity of female worms, the Shapiro-Wilk Test of normality, which is more appropriate for small sample sizes, was used. In cases where the data deviated significantly ($P<0.05$) from a normal distribution (Trial 1 and 3 for both parasite species and Trial 2 for *T. colubriformis*) the appropriate test to check the difference of fecundity between the groups, which is the non-parametric test of Kruskal-Wallis, was used. Where the dependent variable was normally distributed ($P>0.05$) the parametric test of one-way ANOVA (*H. contortus* of Trial 2) was used. Additionally, for the Trial 1, the model of linear regression was used, in order to be investigated if there was a negative correlation between the variables "percentages of carob" and "fecundity of female worms" for both parasite species (*H. contortus* and *T. colubriformis*). Finally, the Tukey HSD test was used for data of trial 3, in order to investigate statistically significant differences between groups.

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

2.10. Ethical considerations

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

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

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

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

No GIN larvae were recovered after pepsin digestion.

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

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

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

3.2.Trial 2

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

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

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

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

No GIN larvae were recovered after pepsin digestion.

The average daily gain (ADG) of lambs as calculated for the whole trial duration for (C), (C+PEG), (CaBP) and (CaBP+PEG) groups was 51.8(±30.1) (±s.d.), 69.8(±19.9), 60.8(±29.3) and 40.5(±25.6) g, respectively, which resulted in no significant differences between the groups.

3.3.Trial 3

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

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

No GIN larvae were recovered after pepsin digestion.

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

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

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

4. Discussion

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

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

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

Results of Trial 1 showed decreases in the mean values of FEC and AWC only in the group fed with the highest concentration of CaBP in the concentrate feed, although not significant. However, fecundity values showed a negative correlation to CaBP concentration in the feed indicating a dose-dependent fecundity suppression effect. 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.

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

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

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

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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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Abstract

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

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

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

1. Introduction

Infections by gastrointestinal nematodes (GIN) affect both health and welfare of grazing ruminants, causing anorexia, impaired digestion and nutrient absorption with related production losses, diarrhoea, anaemia and even death (Perry and Randolph, 1999; Stear et al., 2007; Hoste et al., 2016). Currently, the control of these parasites relies on repeated dosing with commercial anthelmintic drugs. However, the development of anthelmintic resistance in worm populations against one or multiple classes of anthelmintic has become a serious problem in several regions of the world, making it increasingly difficult to control parasitic infections (Kaplan, 2020). At the same time the increasing concerns of consumers about the presence of drug residues in foods and in the environment (McKellar, 1997) have stimulated the search for alternative or complementary solutions (Hoste and Torres-Acosta, 2011) within the context of organic farming and sustainable agriculture (Charlier et al., 2018).

Such alternatives include the use of bioactive plants with anthelmintic properties. Many results indicate that such resources, because of the presence of plant secondary metabolites (PSMs), might help to interfere with the biology of key-stages during nematode cycle and to reduce the consequences of GIN infections in grazing ruminants. Particular attention has been given to plants containing condensed tannins (CT) and some related polyphenols (see reviews by Terrill et al., 2012; Hoste et al., 2015, 2016). Based on previous studies, the need to explore new plant resources to develop non-drug-based strategies for the integrated control of nematode parasites in grazing ruminants has recently become a research priority in livestock production, especially in ruminant breeding as also reviewed by Morgan et al, 2020.

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

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

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

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

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

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

2. Materials and Methods

2.1. Stabling and animals

The experiments were carried out at the Asomaton Research Station of HAO Demeter on the island of Crete, Greece. The animal barn was of an open-sided shed type, with straw bedding. During the whole study the animals were kept indoors with each group in a separate pen of approximately 10 m^2 and 10 m^2 open yard. The study included three trials with lambs belonging to the local "Sfakion" breed. In order to achieve uniformity of the experimental animals, all lambs included were female, 6-month-old with a comparable body weight (BW), which was within the normal BW range of the breed (22-30 kg) at the specific age. The lambs were raised indoors under helminth-free conditions. Fourteen days before the start of each trial, they were drenched with albendazole at the higher commercially recommended dose (ALBENDAZOLE Drench, PROVET, 7.5 mg/kg) and they tested negative by faecal egg counts at the start of each trial. No anthelmintic resistance was previously recorded for this specific flock.

2.3. Infective larvae

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

2.4. Tannin-containing plant resources

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

2.5. Tannin concentration and composition

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

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

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

2.6. Experimental design

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

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

2.6.1. *Trial 1*

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

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

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

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

2.6.2. *Trial 2*

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

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

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

2.6.3. *Trial 3*

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

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

2.7. Pathophysiological parameters

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

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

2.8. Parasitological parameters

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

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

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

2.9. Statistical analyses

The data of FEC and adult worm counts (AWC) were log10(x+1) transformed prior to analysis. For the FEC values, comparison of all groups was first performed using an analysis of variance (ANOVA) with time as repeated measurement. Then, the comparison of results to the control values were carried out date by date, using one-way ANOVA completed by *the post-hoc* Bonferroni test for pairwise comparisons. Group means of AWC were compared by one-way ANOVA (Trial 1) or two-way ANOVA (Trial 2: CaBP +/- and PEG +/-; Trial 3: CaBP +/- and sainfoin +/-). Regarding the fecundity of female worms, the Shapiro-Wilk Test of normality, which is more appropriate for small sample sizes, was used. In cases where the data deviated significantly ($P<0.05$) from a normal distribution (Trial 1 and 3 for both parasite species and Trial 2 for *T. colubriformis*) the appropriate test to check the difference of fecundity between the groups, which is the non-parametric test of Kruskal-Wallis, was used. Where the dependent variable was normally distributed ($P>0.05$) the parametric test of one-way ANOVA (*H. contortus* of Trial 2) was used. Additionally, for the Trial 1, the model of linear regression was used, in order to be investigated if there was a negative correlation between the variables "percentages of carob" and "fecundity of female worms" for both parasite species (*H. contortus* and *T. colubriformis*). Finally, the Tukey HSD test was used for data of trial 3, in order to investigate statistically significant differences between groups.

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

2.10. Ethical considerations

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

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

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

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

No GIN larvae were recovered after pepsin digestion.

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

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

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

3.2.Trial 2

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

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

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

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

No GIN larvae were recovered after pepsin digestion.

The average daily gain (ADG) of lambs as calculated for the whole trial duration for (C), (C+PEG), (CaBP) and (CaBP+PEG) groups was 51.8(±30.1) (±s.d.), 69.8(±19.9), 60.8(±29.3) and 40.5(±25.6) g, respectively, which resulted in no significant differences between the groups.

3.3.Trial 3

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

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

No GIN larvae were recovered after pepsin digestion.

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

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

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

4. Discussion

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

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

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

Results of Trial 1 showed decreases in the mean values of FEC and AWC only in the group fed with the highest concentration of CaBP in the concentrate feed, although not significant. However, fecundity values showed a negative correlation to CaBP concentration in the feed indicating a dose-dependent fecundity suppression effect. 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.

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

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

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

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Table

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

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**	***cis/trans*-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)

[a]The 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	***H. contortus***			***T. colubriformis***		
	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	*H. contortus*			*T. colubriformis*		
	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.

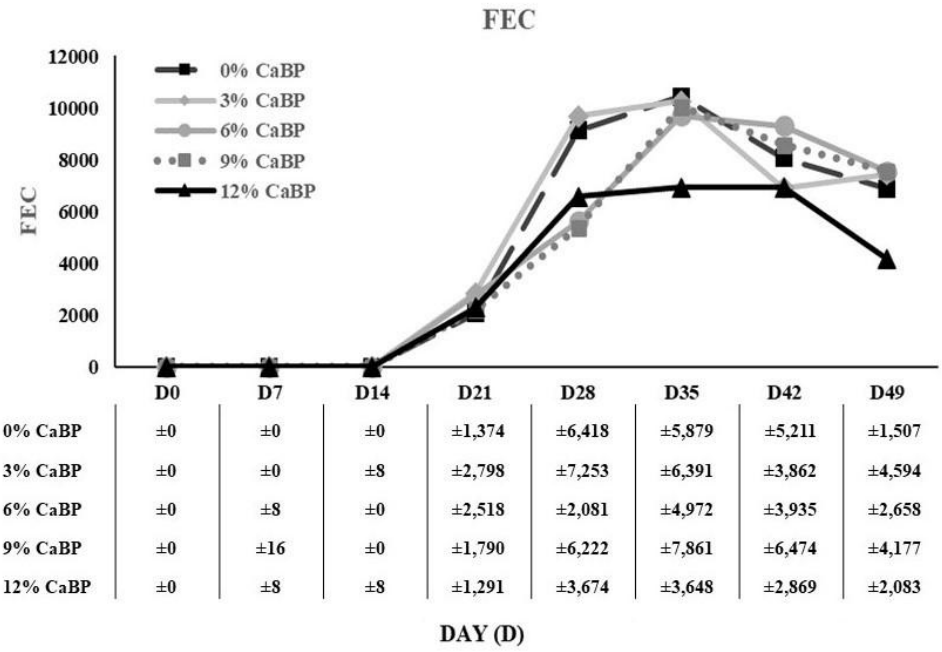
Trial 3: Effect of diet regimes containing different condensed tannin diets on adult worms recovered at necropsy in the different experimental lamb groups C (Control), CaBP (Carob), S (Sainfoin pellets), CaBP+S (Carob+Sainfoin pellets). Adult worm counts (AWC) shown as arithmetic mean of adult worms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

Treatment Group	*H. contortus*			*T. colubriformis*		
	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

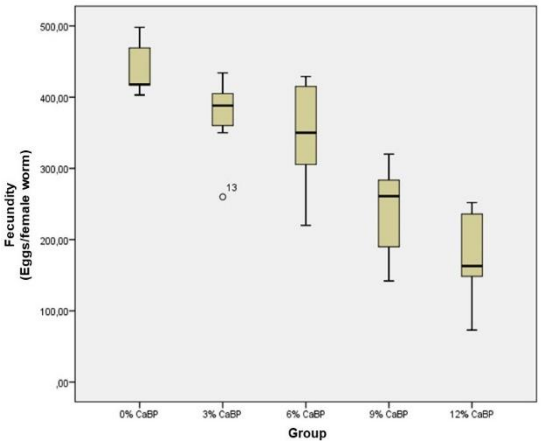
Figure 1. Trial 1: Effect of diet regimes containing different amounts of Carob pod meal (CaBP) on A) faecal egg counts (FEC) on Day 0 to 49) (SD in table below) and B) box-plots for female worm fecundity (95% confidence interval) in the different experimental lambs for *Haemonchus contortus* and *Trichostrongylus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

A.

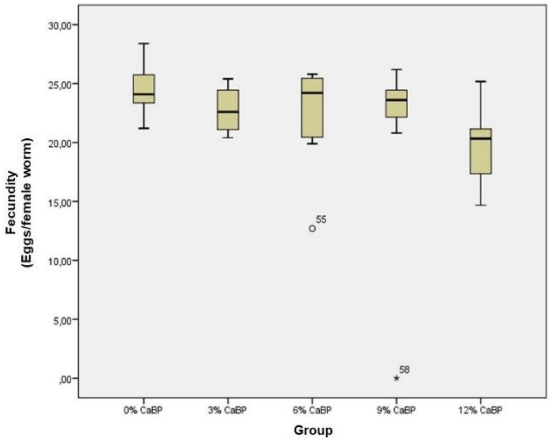


	D0	D7	D14	D21	D28	D35	D42	D49
0% CaBP	±0	±0	±0	±1,374	±6,418	±5,879	±5,211	±1,507
3% CaBP	±0	±0	±8	±2,798	±7,253	±6,391	±3,862	±4,594
6% CaBP	±0	±8	±0	±2,518	±2,081	±4,972	±3,935	±2,658
9% CaBP	±0	±16	±0	±1,790	±6,222	±7,861	±6,474	±4,177
12% CaBP	±0	±8	±8	±1,291	±3,674	±3,648	±2,869	±2,083

B.

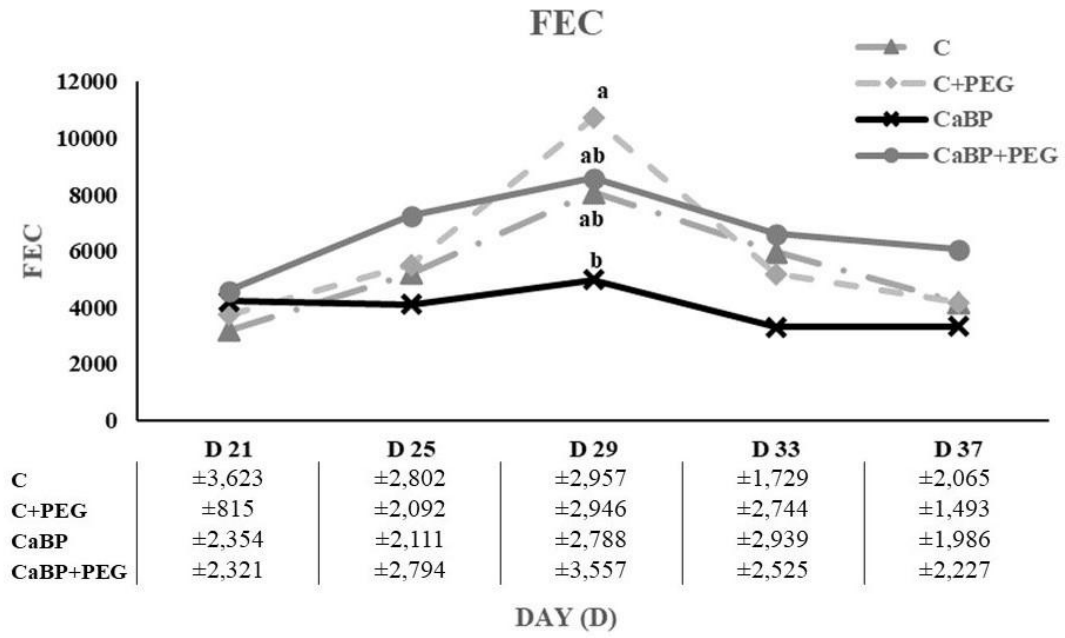


i. H. contortus



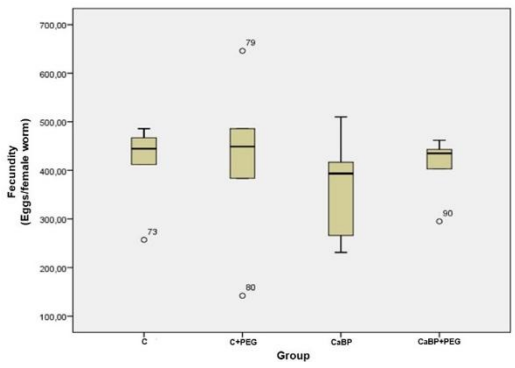
ii. T. colubriformis

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).

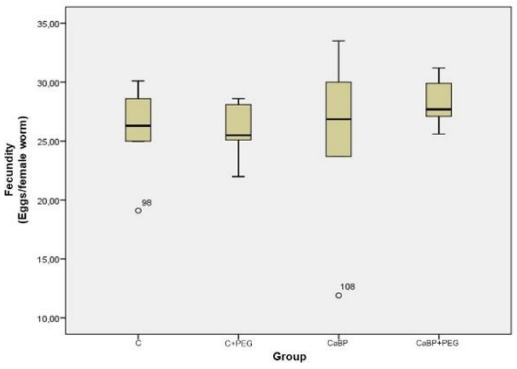


	D 21	D 25	D 29	D 33	D 37
C	±3,623	±2,802	±2,957	±1,729	±2,065
C+PEG	±815	±2,092	±2,946	±2,744	±1,493
CaBP	±2,354	±2,111	±2,788	±2,939	±1,986
CaBP+PEG	±2,321	±2,794	±3,557	±2,525	±2,227

B.



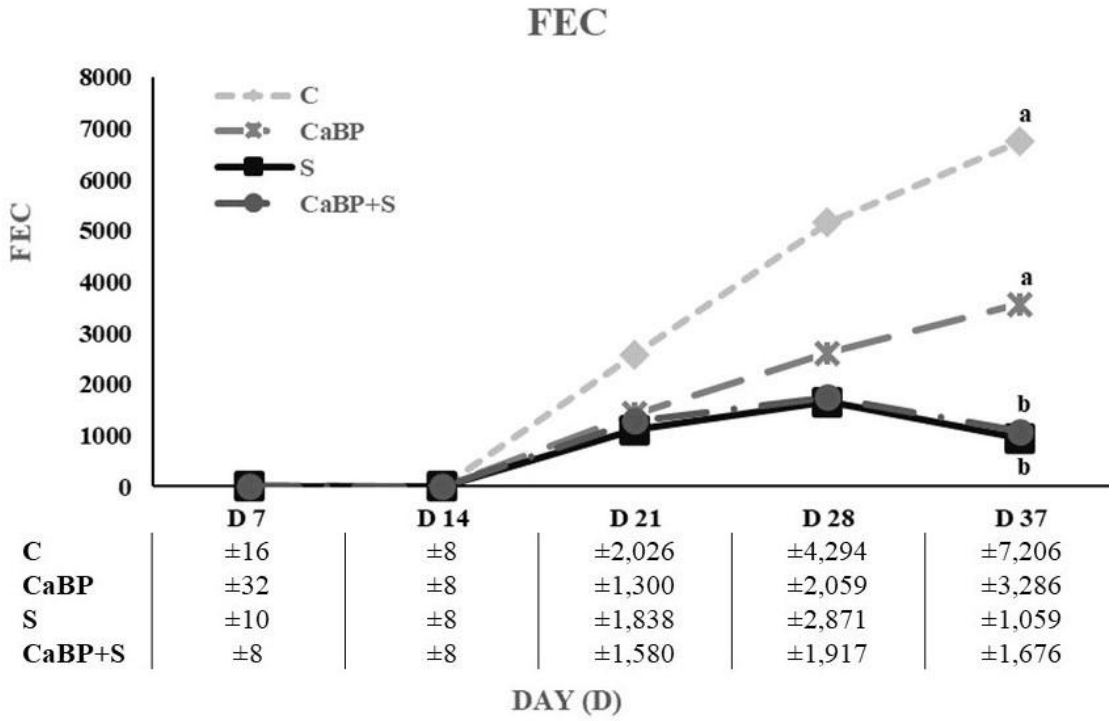
i. H. contortus



ii. T. colubriformis

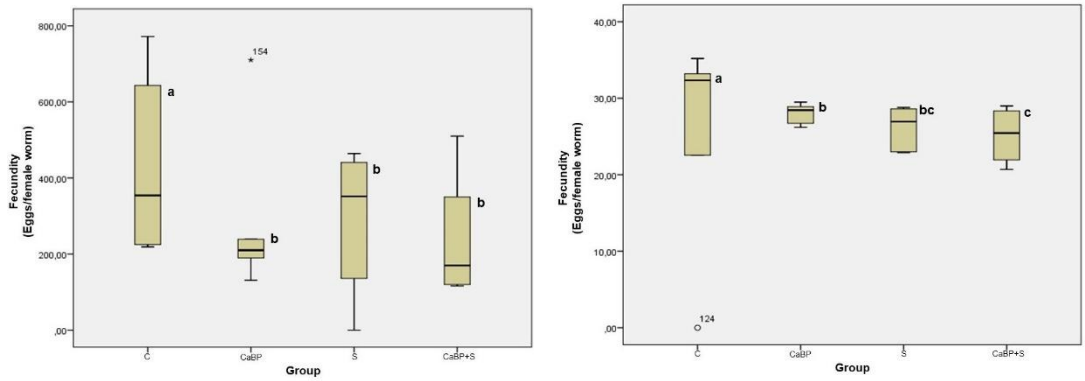
Figure 3. Trial 3: Effect of diet regimes containing different condensed tannin diets on A) faecal egg counts (FEC) on Day 7 to 37 (SD in table below) and B) box-plots for female worm fecundity (95% confidence interval), in the different experimental lambs groups C (Control), CaBP (Carob), S (Sainfoin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

A.



	D 7	D 14	D 21	D 28	D 37
C	±16	±8	±2,026	±4,294	±7,206
CaBP	±32	±8	±1,300	±2,059	±3,286
S	±10	±8	±1,838	±2,871	±1,059
CaBP+S	±8	±8	±1,580	±1,917	±1,676

B.



i. H. contortus *ii. T. colubriformis*

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***Conflict of Interest**

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