



## Research paper

## Heather (*Calluna vulgaris*) supplementation does not reduce trapping ability of *Duddingtonia flagrans* in faeces of *Haemonchus contortus* infected lambs

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## ABSTRACT

Infection with gastro-intestinal nematodes (GIN) seriously impairs productivity and health of grazing animals. Due to the considerable rise in anthelmintic resistance and the increasing popularity of organic farming, alternative control strategies will replace or complement traditional anthelmintics. The efficacy of two potential alternatives (i) feeding the tanniferous forage heather (*Calluna vulgaris*) and (ii) the nematophagous fungus *Duddingtonia flagrans* (isolate FiBL-DF-P14), was tested in a feeding experiment with lambs artificially infected with *Haemonchus contortus*. Animals received hay supplemented with heather or with a late cut hay (ecohay) as a control feed *ad libitum* for three weeks. Two doses ( $1 \times 10^5$  and  $5 \times 10^4$  chlsp/kg LW) of *D. flagrans* chlamydospores (chlsp) were administered to animals of each roughage treatment and *H. contortus* larval recovery from faecal cultures was compared with an untreated control (6 animals per *D. flagrans*-heather combination). Protein, crude fiber and energy contents of ecohay and heather were similar but heather contained approximately twice more fat, four times more lignin and ten times more of all condensed tannin fractions. Heather contained 17.3 mg Proanthocyanidin per g dry matter (DM) while contents of ecohay were 1.7 mg/g DM. Daily average feed intake across both treatments was 1.5 kg DM/animal/day, of which heather/ecohay intake accounted for 0.17/0.19 kg. Overall, there was no significant effect of heather on faecal egg counts (FEC). There was a tendency for a significant interaction between feed supplement and time and a significantly ( $p = 0.030$ ) lower FEC of nominally 1799 EPG in the heather treatment at the end of the heather feeding period compared with the ecohay treatment. Lambs in this study consumed less heather than grazing sheep in other studies, even though condensed tannin contents were comparably low. Heather supplementation did not affect larval recovery in faecal cultures and trapping ability of *D. flagrans*. As compared with the untreated control, both doses of *D. flagrans* reduced larval recovery by 96.2 % and 95.5 %, respectively ( $p < 0.001$ ), with no significant difference between the doses. The isolate FiBL-DF-P14 was at least as effective as isolates tested in other studies and achieved over 95 % reduction at a low dosage of  $5 \times 10^4$  chlsp/kg LW. In conclusion, our results confirm the potential of and indicate no negative interactions between both alternative GIN control methods.

### 1. Introduction

Infection with gastro-intestinal nematodes (GIN) seriously impairs productivity and health of grazing farm animals (Sargison, 2012). Among the GIN species, *Haemonchus contortus* is a major threat to sheep and goat health and profitability (Kearney et al., 2016). The livestock industry, in particular small ruminant farmers, therefore rely heavily on anthelmintic chemicals for GIN control (Kaplan, 2020). These

compounds, designed to eliminate the worm burden from the animal, have been used frequently for decades and have led to an increasing number of parasite populations which are resistant to chemical anthelmintics (Kaplan and Vidyashankar, 2012) with resistance occurring rapidly after introduction of new compounds (Scott et al., 2013).

Due to the considerable rise in anthelmintic resistance over the past 50 years (Sangster et al., 2018) and the increasing popularity of organic farming across Europe and beyond (Willer et al., 2021), alternative

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control strategies are being developed to replace or complement traditional anthelmintics (Ali et al., 2021; Knox et al., 2012). Although these strategies have largely been developed independently and their underlying mechanisms are usually considered in isolation, it is increasingly being recognized that non-chemical parasite control strategies may need to be combined to control gastrointestinal parasitism more effectively, with resultant resilient production systems and reduce reliance on anthelmintics (Houdijk et al., 2012).

There has been increasing interest in the use of bioactive plant feeding as a sustainable gastrointestinal parasite control method for small ruminants. Plants rich in secondary metabolites, such as condensed tannins (CT), have been shown to have anthelmintic properties *in vitro* and *in vivo* (Athanasiadou et al., 2007; Hoste et al., 2006). The perennial shrub, heather (*Calluna vulgaris*), is abundant across European countries and is rich in CT (Tolera et al., 1997). Previous studies have shown that heather extracts have anthelmintic effects *in vitro* on parasite functions including exsheathment and hatching, and *in vivo* effects including reduction in larvae establishment and a decrease in faecal egg counts (FEC) in goats (Moreno-Gonzalo et al., 2013b, a).

The nematophagous fungus *Duddingtonia flagrans* is a biocontrol tool to block the migration of infective GIN larvae from the faeces to the herbage and thus prevents grazing animals from reinfection. The oral administration of *D. flagrans* chlamydo-spores (chlsp) has repeatedly been shown to reduce the number of migrating GIN larvae from the faeces of infected grazing animals by 50–100 % (Jobim et al., 2008; Mendoza De Gives et al., 2006; Paraud, 2005), and the number of infective larvae on pasture (de Almeida et al., 2012). In grazing studies, *D. flagrans* reduced worm burdens of sheep (Healey et al., 2018), goats (Gomez-Rincon et al., 2007), and cattle (Assis et al., 2012). *D. flagrans* chlsp are highly resistant to environmental impact and survive intestinal passage in ruminants or horses (Ojeda-Robertos et al., 2009). When shed with faeces, oxygen availability triggers the growth of the fungal mycelium and GIN larvae having emerged from eggs within the faeces serve as nutrition for *D. flagrans* (Waller and Faedo, 1996). It has been reported that the nematophagous (biocontrol) activity of *D. flagrans* depends on the GIN species (Mendoza De Gives et al., 2006), the fungal strain (Gronvold et al., 1993), and the administered chlsp dose (Terrill et al., 2004). Fungal growth depends on the culture medium (Braga et al., 2013), a large proportion of ingested CT are excreted with the faeces (Quijada et al., 2018), and antifungal activity of a tannin-rich plant has been observed (Morey et al., 2016). Interactions of the tannin-rich heather with biocontrol activity of *D. flagrans* may therefore exist.

The aim of our study was to investigate possible antagonistic effects of heather feeding and *D. flagrans* administration to parasitised sheep on the development of GIN larvae from the faeces. Our hypothesis was that the combined administration of heather and *D. flagrans* spores to sheep infected with the abomasal nematode *H. contortus* might have a negative effect on *D. flagrans* growth and trapping ability, due to previously reported anti-fungal activity of CT. Therefore, we expected that a combined application of the two strategies might result in lower efficacy compared with applying the two approaches separately.

## 2. Animals, material and methods

### 2.1. Animals and parasitic infection

Thirty-six uncastrated male crossbred lambs from Lacaune dairy ewes and Texel sires were purchased from an organic farm. Lambs were born in winter (from the end of October to beginning of March) and had never been on pasture according to the breeder. Despite this, seven lambs excreted trichostrongylid eggs (< 100 eggs per gram faeces [EPG]) during the acclimatisation period. All animals were therefore treated with 2.5 mg Monepantel/kg LW (Zolvix®) three weeks before artificial infection. As two lambs still excreted trichostrongylid eggs two weeks before artificial infection (< 150 EPG), the whole herd was

treated again with 12.0 mg Triclabendazole and 7.5 mg Levamisolhydrochloride/kg LW (Endex®). All animals had zero EPG at a lower detection level of 25 EPG for trichostrongylid eggs one week prior to artificial infection.

On the day of artificial infection (day -22) each lamb was infected orally with 4000 third stage larvae of *H. contortus*. At this time, lambs were on average aged 153 days (95–230 days; SD 35.9) and weighed on average 42.9 kg (31.5–51.5 kg LW; SD 4.9). Until the onset of the feeding experiment (day 1), they had grown on average 0.170 kg/day (0.07 – 0.32 kg/day; SD 0.06).

### 2.2. Experimental setup

The experiment was set up as a nested design with six treatments, namely three levels of *D. flagrans* spore supplementation (*D. flagrans* treatments; n, l, c) nested within two roughage supplements (roughage treatments; H, C). Fig. 1 gives an overview of the timeline and samples taken.

Following the artificial infection (days -22 to 0), animals were kept indoors in one large group on straw and were offered hay *ad libitum* from hayracks. From day 1, they were housed indoors in 12 pens. Animals were first assigned to one of six groups based on their LW and FEC on day 1 in order to obtain six groups with similar average and range of LW and FEC to minimize variation between groups. Groups were then randomly assigned to six treatments (Hn, Hl, Hc, Cn, Cl, Cc). Six pens were assigned to each roughage treatment (H, C). Three animals (one of each *D. flagrans* treatment n, l, c) were then penned together in each of the 12 pens. Assignment of individual animals to pens was stratified based on LW of the animals within treatment so that each of the 12 pens consisted of 3 animals of similar LW. Consequently, feed consumption of animals was expected to be similar within pen.

From day 1 onwards and for 20 days in total, all animals received hay *ad libitum* (base diet); in addition, animals in six pens (H) received heather, whereas animals in the six remaining pens (C) received a late cut hay as roughage supplements. Starting on day 12, one animal in each pen received a normal dose of *D. flagrans* spores (n), one animal in each pen received a low dose (l) and one control animal in each pen received no *D. flagrans* spores (c) daily for eight days. This resulted in a total of six animals per treatment (Hn, Hl, Hc, Cn, Cl, Cc).

From day 21 onwards all animals were offered the base diet *ad libitum* for another period of 17 days to investigate whether heather consumption may have a residual impact on performance and individual FEC of the animals.

During the 37 trial days, live weight (LW) was determined 4 times with an accuracy of 0.5 kg and FEC were assessed 6 times during the trial. During *D. flagrans* supplementation, faeces were collected twice and two series of coprocultures were prepared to determine the percentage of infective larvae emerged from faeces of individual animals.

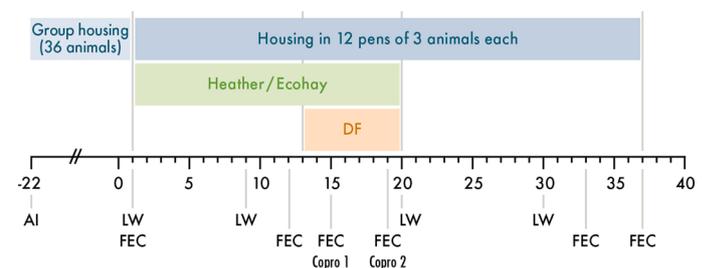


Fig. 1. Overview of experimental design, recorded parameters and samples taken. Heather/Ecohay: heather and ecohay feeding period; DF: period of *Duddingtonia flagrans* administration; AI: artificial infection; LW: weighing; FEC: Faecal egg counts; Copro: Coprocultures.

### 2.3. Housing and feeding

Each pen measured 5 m<sup>2</sup>; softwood granules were used for bedding (Tierwohl Super®) to ensure animals were not ingesting additional nutrients. The base diet consisted of hay (second cut of UFA 330® grass-clover standard mixture) and was provided in hayracks, whereas the roughage supplements were offered in hay nets. Animals had access to water from automatic drinkers and commercial mineral blocks *ad libitum* (MetaFit®). During the period of *D. flagrans* administration, each animal received 20 g of concentrate (UFA 272 Bio®; crude protein: 170 g/kg DM, net energy meat: 7.8 MJ/kg DM) per day.

Heather (*Calluna vulgaris*) was collected weekly (on days 0, 7, 14) from the pre-alpine site "Oberer Hummel" (municipality of Einsiedeln, Switzerland) at an altitude of 1400–1500 m above sea level. Green shoots from the heather plants were selected, to ensure palatability (Gilbert, 2008) and presence of active compounds (Zhao, 2011), and stored in large bags at 4°C until time of feeding.

The ecohay used as control roughage supplement was a late cut hay from an extensively farmed meadow with a species-rich plant composition (herbs, grasses, legumes) cut after the main grasses have flowered. Its crude protein (CP) and acid detergent fiber (ADF) content as determined by NIRS prior to the experiment (6.7 % CP, 41.7 % ADF) were similar to nutrient values of *C. vulgaris* reported in literature (6.1 % - 9.8 % CP, 43.6–44.6 % ADF; Celaya et al., 2010; Frutos et al., 2008; Tolera et al., 1997).

Feed intake (hay, heather, ecohay) was recorded on pen basis by weighing feed offered and refused daily during the heather feeding period. For feed intake and feed conversion ratio calculations, the first eight days of heather feeding were considered as an acclimatization period and excluded from analysis and from graphs.

To determine whether all individual animals consumed heather and ecohay, the eating behaviour (eating/not eating) of the lambs was observed by instantaneous scan sampling (Pullin et al., 2017) on seven occasions during days 13–20. On each occasion, four or five observations were made per animal, resulting in 32 observations per animal.

Samples of heather offered and refused in the hay nets were drawn from each batch of heather collected; samples of the batches of hay and ecohay were taken once for feed analysis.

### 2.4. *Duddingtonia flagrans* additive

*Duddingtonia flagrans* (isolate FiBL-DF-P14; CBS 138751) chlsp were administered as a granulated feed additive containing  $1 \times 10^7$  viable chlsp/g additive. The number of viable chlsp/g additive (NV) was calculated as  $NV = NT \cdot v$ , where NT is the total number of chlsp/g additive, as determined using a Neubauer counting chamber (Roth AG, Arlesheim), and v is their viability defined as the proportion of germinated spores on potato dextrose agar plates after 44 h incubation at room temperature (Oberhänsli, 2016).

Consequently, 0.01 g of the additive per kg LW were administered daily in sheep that received the normal dose n ( $1 \times 10^5$  chlsp/kg LW) and 0.005 g/kg LW were administered in those that received the low dose I ( $5 \times 10^4$  chlsp/kg LW). The normal dose was selected based on previous experiments with the same strain of *D. flagrans* (Maurer et al., 2021) whereas the low dose was similar to the dose used by Healey et al. (2018).

The appropriate amount of additive was prepared for each sheep individually, based on its LW on day 9. The additive was mixed with 20 g of concentrate feed and offered to sheep in individual buckets before the daily feeding from day 12 to day 19. Control animals received 20 g of concentrates without additive.

### 2.5. Nutrient composition and polyphenol content

Coarsely chopped hay and heather samples were dried at 40 °C until weight was constant after 24 h. Samples were then ground with a cutting

mill (Retsch SM100) at a grinding grade of 0.5 mm.

Chemical composition Weender analysis of hay, ecohay and heather was performed by LUFÄ, Speyer, Germany.

Heather and ecohay samples were analysed using UPLC-MS/MS to determine the proanthocyanidin (PA; syn. condensed tannins CT) contents including the amount, size and ratio of subtypes. For sample preparation 20 mg of finely ground heather powder was extracted with  $2 \times 1.4$  mL acetone/water (80:20, v/v) on a rotary shaker for  $2 \times 3$  h (280 rpm), followed by centrifugation for 10 min. The supernatant was transferred to a new micro centrifuge tube and evaporated to water phase in an Eppendorf concentrator (5301, Eppendorf AG). Aqueous samples were frozen at -20 °C and lyophilized. The freeze-dried phenolic extract was dissolved in 1 mL of Milli-Q purified water, vortexed for 5 min, and filtered with a 0.20 µm PTFE filter into UPLC vials. The sample was  $5 \times$  diluted with water and analysed according to Engström et al. (2014).

### 2.6. Faecal egg counts and coprocultures

Faecal egg counts were carried out with a modified McMaster technique with a minimum detection limit of 25 EPG (Schwarz et al., 2020).

Quantitative coprocultures were prepared on days 15 and 19 from faeces collected in fabric bags fixed to the sheep for 1.5–2 h. For each animal and sampling time, two independent coprocultures were prepared resulting in 12 coprocultures per treatment and day. For each coproculture, FEC were determined and 30 g of faeces were cultured in a wide-necked honey jar (ø65 mm; volume 212 mL) lined with two layers of medical gauze  $20 \times 20$  cm (IVF Hartmann AG). After incubating for 14 days at 25 °C and 80 % RH, cultures were placed in Baermann funnels. Migrated third stage larvae were collected from the funnels after 24 h and counted using a light microscope at 40x magnification. The percentage of larvae recovered from the culture (PR) was calculated as  $PR = LH/(FEC \cdot W) \cdot 100$  using the total number of larvae harvested from the culture (LH), the FEC of the native faeces (FEC) and the weight of faeces in the culture (W).

Faecal dry matter was determined after drying approximately 20 g of fresh faeces at 105 °C for 24 h.

### 2.7. Statistical analysis

To determine the effect of roughage treatment (H, C) on feed intake the pen was the experimental unit ( $n = 12$ ), whereas to determine the effects of treatment on FEC and LW the animal was the experimental unit ( $n = 36$ ). The roughage  $\times$  *D. flagrans* interactions on weight gain and parasitological parameters (Hn, Hl, Hc, Cn, Cl, Cc) were determined at individual animal level (6 replicates per treatment). One animal of the treatment Cl was excluded from all analyses due to health problems not associated with the experimental treatments (chronic diarrhea and poor growth); individual animal and corresponding pen level measurements (feed consumption and feed conversion) were therefore excluded from follow up analyses (5 replicates for treatment Cl).

All statistical analyses were executed using the software R (R Core Team, 2020). Effects of the treatments on outcome variables were modelled with linear mixed effect models (function "lmer"; package "lme4"; Bates et al., 2015). Models for outcome variables that had been assessed on pen level (feed intake) included roughage treatment, time and their interaction as fixed effect and time nested in pen as random effect. Models that had been assessed on animal level (weight gain, feed conversion ratio, faecal dry matter) included roughage treatment as fixed effect and animal nested in pen as random effect. The model for LW included time and the interaction between time and roughage treatment as fixed effect as it was measured several times. Since two FEC were carried out per animal and time, the model for FEC included the number of FEC (1, 2) within time within animal within pen as nested random effect and roughage treatment, time and their interaction as fixed effects. The model for recovery percentage of *H. contortus* larvae

comprised roughage treatment, *D. flagrans* treatment, their interaction, time and the interaction between time and *D. flagrans* treatment as fixed effects, while random effects were the same as for FEC. Similarly, feeding behaviour was modelled with roughage treatment as fixed and scan within time within animal within pen as nested random effect. Model residuals were visually assessed for deviances from normality or homogeneity of variance. To meet model assumptions, the outcome variables EPG and larval recovery percentages were square-root transformed. Factorial fixed effects were coded as dummy variables with sum contrasts which enabled to test for main effects even in presence of interactions.

P-values were computed by comparing the full model, including all fixed effects, with a model reduced by the effect or interaction of interest. P-values, model estimates and confidence intervals were calculated using parametric bootstrap simulations (functions “Pbmodcomp” and “bootMer”; packages “pbkrtest” and “lme4”; Bates et al., 2015; Halekoh and Hojsgaard, 2014).

### 3. Results

#### 3.1. Feed composition

Feed composition is summarized in Table 1.

With regard to crude protein, crude fiber and energy, ecohay had similar values to heather. However, heather contained approximately twice more fat, four times more lignin (ADL) and less crude ash (mainly Potassium). The average dry matter of all fresh heather samples was lower (60.4 %) than that of both hay types (90.9 and 91.5 %).

Nutritional values and polyphenol contents of heather offered vs heather refused were similar, indicating that sheep did not select a certain part of the heather shots that were offered to them.

From the polyphenol analysis of heather samples, it was confirmed that CTs were the dominating polyphenol and CT content are therefore shown in Table 1, which includes type, size and procyanidin/prodelphinidin (PC/PD) ratio. The contents of PC, PD and PA were 10 times

higher in heather compared with ecohay.

#### 3.2. Feed intake and daily weight gains

Initial LW at day 1 of animals in roughage treatments H and C did not differ (average  $\pm$  SD; min.-max:  $43.3 \pm 4.5$ ;  $37\text{--}55.5$  for treatment H and  $44.9 \pm 3.7$ ;  $38\text{--}51.5$  for treatment C).

Feeding behavior observations confirmed that all animals across pens consumed roughage (heather or ecohay) from the haynets. Feeding was recorded during 37 % and 30 % of the observations for the animals fed heather and ecohay, respectively, indicating that animals offered heather displayed similar feeding visits compared with those offered ecohay.

Total daily average feed intake across all treatments was 1.7 kg of fresh (1.5 kg dry) matter (Table 2). Average feed intake, daily weight gain and feed conversion ratio did not differ between roughage treatments H and C. There was an interaction between time and roughage treatment for LW ( $p = 0.04$ ) but no main effect of the roughage treatment ( $p = 0.90$ ). Average intake of PC, PD and PA was numerically ten times higher in the H than in the C roughage treatment.

Fig. 2A presents total feed intake per animal/day in sheep offered heather or ecohay during days 9–20 and intake of heather and ecohay is presented in Fig. 2B. Animals consumed significantly ( $p < 0.001$ ) more DM at the end than at the beginning of the feeding period. Over time, intake of the base diet ( $p = 0.07$ ) and of both, heather and ecohay ( $p = 0.001$ ), increased. There was no difference between the increase of heather and ecohay intake over time ( $p = 0.27$ ).

#### 3.3. Faecal egg counts

Initial *H. contortus* FEC (day 1) of animals in both roughage and in all *D. flagrans* treatments did not differ significantly (EPG; average  $\pm$  SD; min.-max.:  $1478 \pm 697$ ;  $550\text{--}2900$  for treatment H;  $1697 \pm 833$ ;  $500\text{--}3200$  for treatment C;  $1558 \pm 764$ ;  $500\text{--}2900$  for treatment c;  $1641 \pm 854$ ;  $550\text{--}3200$  for treatment I;  $1571 \pm 703$ ;  $550\text{--}2550$  for treatment

**Table 1**

Composition of feedstuffs used in the experiment: hay fed as a base diet, heather *Calluna vulgaris* offered and refused (average  $\pm$  standard deviation of three samples taken from each of three weekly batches), and ecohay used as a control roughage.

Parameter	Unit	Hay (base diet)	<i>Calluna vulgaris</i> ; offered			<i>Calluna vulgaris</i> ; refused			Ecohay (control)
			Average	$\pm$	SD	Average	$\pm$	SD	
<i>Nutrient composition</i>									
Dry matter	g/kgFM	909	526	$\pm$	51.1	681	$\pm$	9.3	915
Crude protein	g/kgDM	132	86	$\pm$	3.0	86	$\pm$	6.0	91
Crude fat	g/kg DM	21	36	$\pm$	0.7	37	$\pm$	4.5	19
Crude fiber	g/kg DM	250	305	$\pm$	28.2	313	$\pm$	54.7	299
Crude ash	g/kg DM	95	36	$\pm$	3.4	39	$\pm$	5.2	74
ADF(OM)	g/kg DM	317	431	$\pm$	25.9	444	$\pm$	46.3	378
ADL	g/kg DM	29	245	$\pm$	10.4	252	$\pm$	8.0	57
<i>Energy</i>									
ME Energy	MJ/kg DM	9.72	8	$\pm$	0.4	8	$\pm$	0.7	8.63
Net energy growth	MJ/kg DM	5.75	5	$\pm$	0.3	5	$\pm$	0.5	4.98
<i>Minerals and trace elements</i>									
Calcium	g/kg DM	5.7	4	$\pm$	0.3	5	$\pm$	0.4	8.0
Phosphorus	g/kg DM	2.1	1	$\pm$	0.0	1	$\pm$	0.1	1.3
Magnesium	g/kg DM	2.3	1	$\pm$	0.1	1	$\pm$	0.1	3.3
Sodium	g/kg DM	0.2	<0.1			<0.1			<0.1
Potassium	g/kg DM	30.9	5	$\pm$	0.2	6	$\pm$	0.5	18.4
<i>Polyphenols</i>									
Procyanidin total	mg/g	nd	15.6	$\pm$	2.69	15.0	$\pm$	1.58	1.5
Prodelphinidin total	mg/g	nd	1.8	$\pm$	0.08	1.7	$\pm$	0.08	0.2
Proanthocyanidin total	mg/g	nd	17.3	$\pm$	2.70	16.8	$\pm$	1.65	1.7
mDP_PA <sup>1</sup>	nd	nd	5.7	$\pm$	0.096	5.6	$\pm$	0.20	4.5
Procyanidin/: Prodelphinidin	Ratio	nd	8.7			8.8			7.5

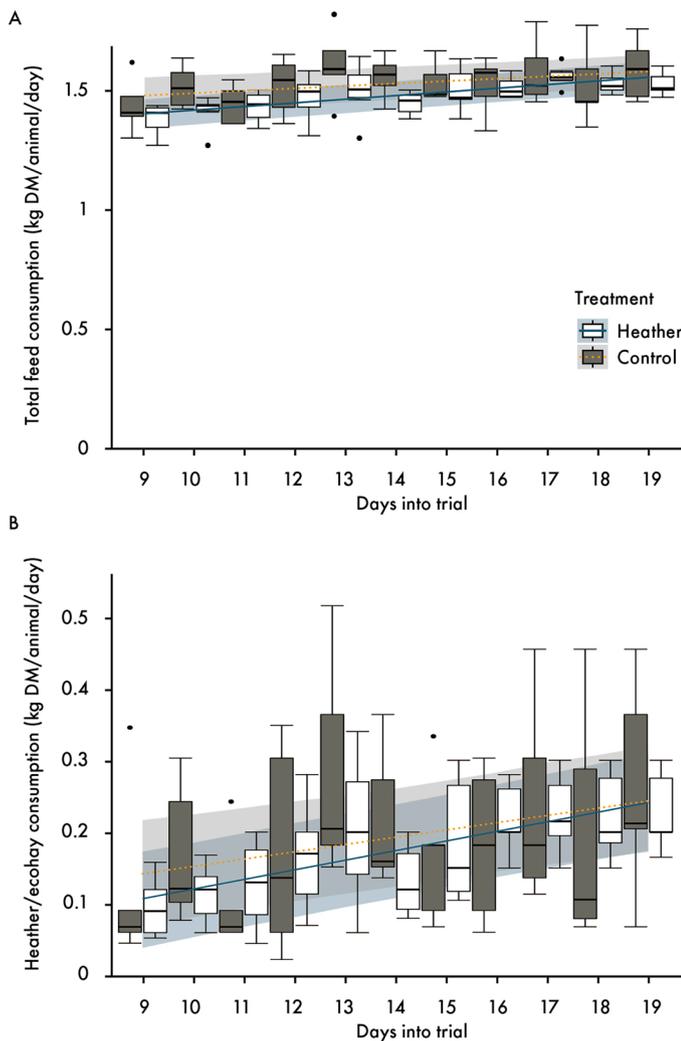
<sup>1</sup> Mean degree of polymerization of proanthocyanidins.

**Table 2**

Feed intake and production parameters during the heather feeding period. Average intake of hay (base diet), heather and ecohay was determined on pen level; daily weight gains were determined on individual animal level.

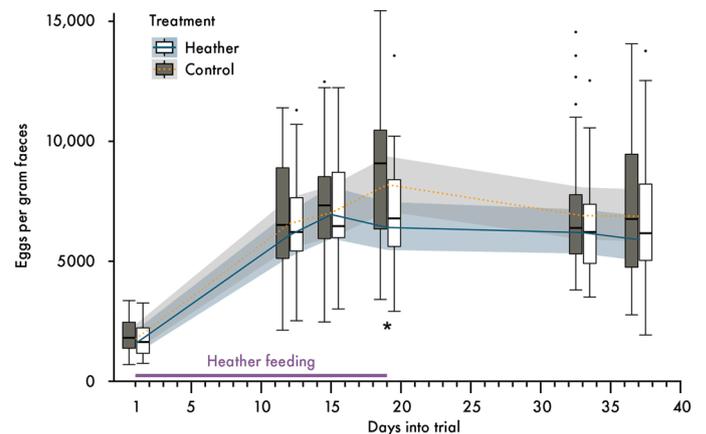
Parameter	Heather			N	Ecohay			N	p
	Average	±	SD		Average	±	SD		
Total feed intake [kg FM/animal/day]	1.73	±	0.13	6	1.68	±	0.13	5	0.30
Total feed intake [kg DM/animal/day]	1.48	±	0.09	6	1.53	±	0.12	5	0.12
Hay (base diet) intake [kg DM/animal/day]	1.31	±	0.04	6	1.34	±	0.05	5	0.13
Heather/ecohay intake [kg DM/animal/day]	0.17	±	0.08	6	0.19	±	0.13	5	0.48
Estimated average Procyanidin intake [mg/animal/day] <sup>1</sup>	2644				294				
Estimated average Prodelphinidin intake [mg/animal/day] <sup>1</sup>	302				30				
Estimated average Proanthocyanidin intake [mg/animal/day] <sup>1</sup>	2946				325				
Daily weight gain [kg]	0.21	±	0.07	18	0.26	±	0.08	17	0.12
Feed conversion ratio [kg feed/kg weight gain]	7.53	±	2.81	6	6.83	±	2.54	5	0.39

<sup>1</sup> Estimated based on heather/ecohay intake and contents of the CT fraction.



**Fig. 2.** Daily total feed intake (A), and intake of heather and ecohay (B) of lambs. Lines and shades display model estimates and confidence intervals of the two roughage treatments heather H and ecohay C. Days 1-8 of heather feeding were regarded as acclimatization period for heather consumption.

n). **Fig. 3** presents FEC data and estimates of the model for FEC during the heather feeding period and 17 days after. There was a tendency for a significant interaction between feed type and time ( $p = 0.08$ ) and the post hoc test revealed a significantly lower FEC of nominally 1799 EPG in the H treatment at the end of the heather feeding period compared with the C treatment (day 19). Overall, heather feeding as main effect,



**Fig. 3.** Box plots of faecal egg counts (EPG) before, during and after the heather feeding period. Lines and shades display model estimates and confidence intervals, respectively, of the two roughage treatments. \* denotes a significant ( $p = 0.030$ ) difference between FEC of roughage treatments at day 19.

independently of the interaction with time, did not affect FEC ( $p = 0.29$ ).

Model estimates of faecal dry matter at the end of the heather feeding period were 29.3 % for the C treatment and 30.2 % for the H treatment ( $p = 0.50$ ).

### 3.4. Larval recovery from faecal cultures

Faecal egg counts were on average 7137 (min.-max.: 2550–12325) after 3 and 7476 (min.-max.: 2750–14875) after 7 days of *D. flagrans* administration. There was no significant difference in FEC between the six groups.

In all *D. flagrans* treatments, larval recovery percentages were higher after three days (65.7 % in c, 2.7 % in l, 3.4 % in n) than after seven days (39.6 % in c, 1.4 % in l, 1.5 % in n) of *D. flagrans* spore administration ( $p = 0.001$ ). There was no interaction between roughage and *D. flagrans* treatments ( $p = 0.22$ ), and there was no significant difference between larval recovery from faecal cultures of animals in roughage treatments C and H ( $p = 0.82$ ). **Fig. 4** presents larval recovery percentages for each *D. flagrans* treatment (c, l, n) pooled for the H and C treatments and for the two dates.

Larval recovery for both low and normal doses of *D. flagrans* was reduced compared to the untreated control by 96.2 % and 95.5 %, respectively ( $p < 0.001$ ). Recovery percentages of the low and normal doses did not differ.

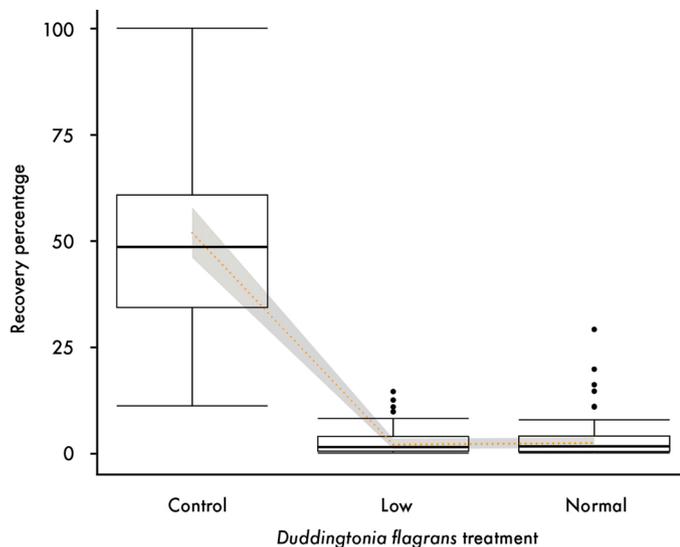


Fig. 4. Boxplot of *Haemonchus contortus* larvae recovery percentages pooled by *D. flagrans* treatment (control, low, normal) for both series of coprocultures (day 15 and 19) and for both roughage treatments (heather and ecohay). Figure includes model estimates (dotted line) and confidence intervals (grey area).

#### 4. Discussion

Our study showed that the combined administration of heather and *D. flagrans* spores to sheep infected with the abomasal nematode *H. contortus* did not have antagonistic effects that would result in higher larval recovery compared with applying the two approaches separately. Animals that consumed heather tended to have lower FEC compared with control animals, but only at the end of the heather feeding period. There was no negative impact on the performance of animals that consumed heather compared with control animals.

When offered heather and hay *ad libitum*, approximately 11 % of the daily intake of our growing Lacaune x Texel crossbred sheep was heather. To our knowledge, no data from a feeding trial using cut heather are available, but Hester et al. (1999) observed that grazing Scottish Blackface ewes spent about 50 % of their grazing time grazing on heather in mixed grass/heather mosaics, where heather was dominant and the proportion of grass was low and hence made grass less accessible to the animals. Spanish Gallega sheep consumed a proportion of 21 % of heather in heather dominated pastures (Osoro et al., 2013).

The lower proportion of heather in the diet of our sheep might be due to *ad libitum* availability of palatable hay, lack of prior exposure in consuming heather, or to genetic effects on consumption of tanniferous forages, as have been shown by Glasser et al. (2009) for goats. Ingested amounts of heather in this trial increased with time, leading to the assumption that lack of prior exposure was in fact important and that there were adjustment effects. It is unlikely that reduced heather intake is attributed to the nutritional qualities of heather in this study. Crude protein and fiber content of the *C. vulgaris* consumed in our study was similar to that of *C. vulgaris* from Spain (Celaya et al., 2010; Frutos et al., 2008) and Scotland (Tolera et al., 1997). Nutrient composition was also comparable to other *C. vulgaris* samples that were recently collected in Norway, Germany, Spain, and Scotland (SRUC, unpublished data).

Ecohay was found to have ten times lower CT values than heather samples, which confirms it was appropriately used as a control roughage. Previous studies have shown that total tannins in heather can range from 22 to 84 g tannic acid equivalents (TAE)/kg DM (Celaya et al., 2010; Frutos et al., 2008; Gonzalez-Hernandez et al., 2003; Moreno-Gonzalo et al., 2014b; Osoro et al., 2007). Tolera et al. (1997) demonstrated that *C. vulgaris* samples contained 97.3 g/kg DM total PA. Offered heather from our study contained 17.3 g/kg of total PA, the

majority of which was of the PC type. Previous studies have suggested plants rich in PD show better anthelmintic ability due to their larger size meaning more hydrogen bonds can be formed with parasitic proteins (Mueller-Harvey et al., 2019; Naumann et al., 2017; Quijada et al., 2015). The lower levels of CTs and, in particular, PDs in our study, along with the relatively low heather intake could be associated with the lack of a stronger heather effect on the FEC.

Faecal egg counts were numerically 1800 epg (22 %) lower in the heather than in the ecohay treatment after 19 days of heather feeding. This is less than the 40–80 % reduction reported for goats with mixed GIN species by Celaya et al. (2010); Frutos et al. (2008) and Osoro et al. (2007) after up to five months of heather feeding. Faecal egg counts dropped by 80 % after one week of feeding the tanniferous *Sericea lespedeza* hay (Shaik et al., 2006) to goats infected with *H. contortus*.

Larval development in faecal cultures was not affected by heather consumption compared with the control. Similarly, incubation of *H. contortus* eggs in *C. vulgaris* extracts also did not have an impact on egg hatching *in vitro* (Moreno-Gonzalo et al., 2013a) but to our knowledge, this effect has not been previously evaluated in the faeces of animals following the consumption of *C. vulgaris*. In contrast, this effect has been shown for other tanniferous forages when fed to animals infected with *H. contortus*, e.g. *S. lespedeza* (22.4 % total CTs; Shaik et al., 2006), *Acacia nilotica* and *Eucalyptus drepanophylla* (21.5 % and 13.6 % total CTs, respectively; Moreno et al., 2012) and *Acacia karroo* (2.1 % total CTs; Marume et al., 2012).

The Swiss isolate of *D. flagrans* used in the present study reduced larval recovery by over 95 % at both dosages and at both time points tested. World-wide, *D. flagrans* shows low genetic variation (Ahren et al., 2004) and previous similar studies used various isolates. Faedo et al. (1997) obtained similar reductions of larval recovery from faecal cultures with similar doses ( $5 \times 10^4$  –  $2.5 \times 10^5$ ) of *D. flagrans* chlsp of different wild strains.

Some previously published trials with *D. flagrans* used a higher dose ( $5 \times 10^5$  or  $1 \times 10^7$ /kg LW; Fontenot et al., 2003; Waghorn et al., 2003), than applied in our study. Our low dose ( $5 \times 10^4$ /kg LW) is similar to that used by Healey et al. (2018;  $3 \times 10^4$ /kg LW) and the normal dose ( $1 \times 10^5$ /kg LW) corresponds to the dose applied in other studies (Burke et al., 2005). Dose rate was observed to play a more important role in trapping efficacy for the slower moving L3 of *Ostertagia (Teladorsagia) circumcincta* than for the more mobile *H. contortus* (Waghorn et al., 2003). Trapping efficacy is also dependent on the GIN egg/chlsp ratio in the faeces (Sagües et al., 2021), because excreta of larvae stimulate trap formation and hence trapping efficacy of *D. flagrans* (Anan'ko and Teplyakova, 2011). In our experiment, the GIN species and high FEC were favouring the high trapping efficacy of *D. flagrans* as it was observed.

Larval recovery from coprocultures was lower at day 7 (40 % in control) compared with day 3 (66 % in control) post chlsp administration. However, lower recovery was consistent in all *D. flagrans* treatments and the results of days three and seven did not differ with regard to percentage of larval recovery reduction in the *D. flagrans* treatments as compared with the control treatment. Similarly, Chandrawathani et al. (2003) report development rates of 10 and 50 % in cultures produced at two subsequent days from faeces of untreated sheep without being able to give a conclusive explanation. Morbidity of *H. contortus* females is unlikely to play a role at time points only slightly higher than 30 days after infection, which is the minimum survival time of *H. contortus* females (Saccareau et al., 2017). We found no evidence of a lower hatchability of nematode eggs laid by older females and estimate that this effect would unlikely happen within four days. The reason for consistently lower recovery rates from cultures from day seven remains therefore unknown.

Although there was no additive, beneficial effect of the combined use of heather and *D. flagrans*, importantly there were no adverse effects of heather feeding on *D. flagrans* trapping ability. Polyphenols of another Ericaceae tree species have been shown to have antifungal effects

(Martins et al., 2021), which could have an impact on growth and the trapping ability of *D. flagrans*. *D. flagrans* is susceptible to several anthelmintics and fungicides when applied *in vitro* (Vieira et al., 2017; Wang et al., 2021) and *in vivo* and a wash-out period of four days (Paraud et al., 2004) to two weeks has been recommended before using *D. flagrans* after application of benzimidazoles (Sanyal et al., 2004). Not all fungicides have an impact on *D. flagrans* trapping ability though; in a study by Burke et al. (2005) there was no effect of copper wire particle administration to lambs on *D. flagrans* trapping ability in the faeces, even though copper is a potent fungicide widely used for plant protection (Thuerig et al., 2018).

## 5. Conclusions

Our study showed that short-term exposure to heather feeding might have a small effect on reducing FEC in parasitized sheep; this effect appeared to be short-lived, as it was only present during the heather feeding phase and was not sustained in the longer term. The combined heather and *D. flagrans* consumption did not result in an additive effect, as it would have been demonstrated by lower larval recovery in coprocultures of animals subjected to both approaches, but importantly heather did not have a negative impact on *D. flagrans* trapping ability. Under standard agricultural practices, where animals are grazing on parasite contaminated pastures, a reduction in FEC as a consequence of heather feeding and a further reduction in larval recovery as a consequence of *D. flagrans* administration have the potential to impact on parasite epidemiology.

## Animal welfare statement

The authors confirm that they have followed EU standards for the protection of animals used for scientific purposes (EU directive 2010/63/EU for animal experiments). The trial was approved by the cantonal veterinary office Aargau, Switzerland (permission number AG 75'728) and all animal-related procedures were conducted according to the Swiss legislation on animal welfare.

## CRedit authorship contribution statement

**Veronika Maurer:** Conceptualisation, Methodology, Investigation, Writing – original draft, Writing – review & editing, Project administration, Funding acquisition. **Mirjam Holinger:** Methodology, Formal analysis, Visualization, Writing – review & editing. **Thomas Oberhänkli:** Methodology. **Susann Thüer:** Investigation. **Steffen Werne:** Methodology, Writing – review & editing. **Francesca Shepherd:** Investigation, Writing – review and editing. **Spiridoula Athanasiadou:** Conceptualisation, Methodology, Writing – original draft, Writing – review & editing.

## Declaration of Competing Interest

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.

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