Anthelmintic effects of forage chicory (Cichorium intybus) against gastrointestinal nematode parasites in experimentally infected cattle

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(Received 29 February 2016; revised 23 March 2016; accepted 29 March 2016; first published online 13 May 2016)

SUMMARY

Two experiments studied the effects of dietary chicory against gastrointestinal nematodes in cattle. In Experiment (Exp.) 1, stabled calves were fed chicory silage (CHI₁; n = 9) or ryegrass/clover hay (CTL₁; n = 6) with balanced protein/energy intakes between groups. After 16 days, all calves received 10,000 Ostertagia ostertagi and 66,000 Cooperia oncophora third-stage larvae (L₃) [day (D) 0 post-infection (p.i.)]. In Exp. 2, calves were assigned to pure chicory (CHI₂; n = 10) or ryegrass/ clover (CTL₂; n = 10) pastures. After 7 days, animals received 20,000 O. ostertagi L₃/calf (D₀ p.i.) and were moved regularly preventing pasture-borne infections. Due to poor regrowth of the chicory pasture, CHI₂ was supplemented with chicory silage. At D₄₀ p.i. (Exp. 1) and D₃₅ p.i. (Exp. 2) calves were slaughtered for worm recovery. In Exp. 1, fecal egg counts (FEC) were similar between groups. However, O. ostertagi counts were significantly reduced in CHI₁ by 60% (geometric mean; P < 0.01), whereas C. oncophora burdens were unaffected (P = 0.12). In Exp. 2, FEC were markedly lowered in CHI₂ from D₂₂ p.i. onwards (P < 0.01). Ostertagia ostertagi adult burdens were significantly reduced in CHI₂ by 66% (P < 0.001). Sesquiterpene lactones were identified only in chicory (fresh/silage). Chicory shows promise as an anti-Ostertagia feed for cattle and further studies should investigate its on-farm use.

Key words: Cichorium intybus, anthelmintic effects, cattle, Ostertagia ostertagi, Cooperia oncophora, sesquiterpene lactones, silage, ruminant.

INTRODUCTION

Parasitism with gastrointestinal nematodes (GIN) is ubiquitous in grazing cattle, and despite advances in vaccine development (Rinaldi and Geldhof, 2012; Vlaminck et al. 2015) on-farm control strategies largely rely on the regular use of anthelmintic drugs. As a result, reduced drug efficacy due to anthelmintic resistance (AR) is increasing among GIN of cattle in several countries (Sutherland and Leathwick, 2011; Gasbarre, 2014; Cotter et al. 2015; Geurden et al. 2015) threatening efforts to sustain animal health and productivity. The challenge posed by drug resistance urgently requires the development of effective alternatives, aiming at reducing the reliance on anthelmintics and lowering the selection pressure for AR on the still-effective drugs (Waller and Thamsborg, 2004; Sykes, 2010). Among other methods, the use of bioactive plants with direct in vivo anthelmintic activity has shown promise as a complementary parasite control strategy in small ruminants (reviewed by Hoste et al. 2006, 2015), but comparable evidence in large ruminants is scarce (Sandoval-Castro et al. 2012; Shepley et al. 2015).

One bioactive plant investigated as a potential anthelmintic crop in small ruminants, particularly in temperate regions, is chicory (Cichorium intybus L., Asteraceae). Chicory is a perennial herb that has been traditionally used for human consumption in Europe and other regions of the world, and since the 1980s forage cultivars for livestock have been developed (Rumball, 1986; Barry, 1998; Li and Kemp, 2005). Studies with forage chicory offered at levels ≥80% of the diet [based on dry matter (DM) intake] to parasitized lambs have consistently reported anthelmintic effects on abomasal worms (Teladorsagia circumcincta and Haemonchus contortus), while no apparent activity was observed against small intestinal nematodes (Scales et al. 1995; Marley et al. 2003; Athanasiadou et al. 2005; Tzamaloukas et al. 2005; Heckendorn et al. 2007). Direct anthelmintic effects of chicory are believed to derive from its content of plant secondary
metabolites (PSM), namely condensed tannins (CT) and sesquiterpene lactones (SL), but previous research has detected only marginal CT levels in chicory, although in vivo anti-parasitic activity was still observed (Tzamaloukas et al. 2005; Heckendorn et al. 2007). Until now, no studies have investigated the SL content of forage chicory used in anthelmintic trials in ruminants, but in vitro effects of SL-containing extracts against GIN have been demonstrated (Foster et al. 2011a; Peña-Espinoza et al. 2015). Additionally, it is known that high-protein/energy diets can indirectly affect GIN infections in both large and small ruminants by increasing the animals’ capacity to cope with, and eventually eliminate, helminth infections (Mansour et al. 1992; Van Houtert and Sykes, 1996; Coop and Kyriazakis, 2001; Tzamaloukas et al. 2006). Therefore, it is important to discern if the potential anti-parasitic effects of certain feeds are caused by direct anthelmintic activity or by indirect beneficial effects through improved nutrition (Hoste et al. 2015).

To date, only a single investigation has examined in vivo anthelmintic effects of chicory in cattle; the study reported that steers, naturally infected with GIN and grazing a mixed chicory-ryegrass pasture (24% chicory DM in the field), had no differences in fecal egg counts (FEC) or blood markers for GIN compared with infected controls grazing ryegrass (Marley et al. 2014). However, previous in vitro studies with sheep suggest that higher levels of chicory (possibly ≥50% chicory DM in the diet) may be necessary to affect GIN in the host, but this has yet to be demonstrated in cattle.

The main objective of our study was to investigate the anthelmintic effects of forage chicory-rich diets against experimental infections with GIN in cattle. First, we studied the effect of ensiled chicory in stabled calves infected with *Ostertagia ostertagi* and *Cooperia oncophora*, the most common GIN of cattle in Northern Europe, in comparison with infected animals fed a control and protein/energy-balanced diet [Experiment (Exp.) 1]. Next, we compared the course of mono infections with *O. ostertagi* in calves grazing either pure forage chicory or ryegrass/clover paddocks, while carefully avoiding any re-infections from pasture (Exp. 2). In addition, the chemical profiles of the different feeds were examined, with focus on their content of CT and SL.

**MATERIALS AND METHODS**

**Parasites**

For Exp. 1, infective third-stage larvae (L3) were isolated from four nematode-naïve donor calves, orally inoculated with anthelmintic-susceptible strains of *O. ostertagi* and *C. oncophora* (kindly provided by Prof. Dr. Janina Demeler, Freie Universität Berlin, Germany). Feces from the donor calves were collected rectally and used to prepare larval cultures (Roepstorff and Nansen, 1998). After 14 days, the cultures were baerminated and isolated L3 were pooled and maintained at 12 °C for 3 months until experimental infection. Two days prior to inoculation, motile L3 were selected by a further baermination and 100 larvae were morphologically identified according to van Wyk and Mayhew (2013); the final inoculum consisted of 13% *O. ostertagi* and 87% *C. oncophora* L3. For Exp. 2, *O. ostertagi* L3 were isolated from two nematode-naïve donor calves mono-infected with an ivermectin susceptible *O. ostertagi* strain (Batch number: OOSG10, Ridgeway Research, UK). *Ostertagia ostertagi* L3 recovered from larval cultures were maintained for 3 months at 12 °C prior to inoculation and infective doses were prepared as described for Exp. 1.

**Study designs and animals**

**Experiment 1.** This experiment was performed between October–December 2013 as a controlled trial to study the course of experimental infections with *O. ostertagi* and *C. oncophora* in stabled calves fed ensiled forage chicory or a control roughage diet with balanced protein and energy levels. The study design is summarized in Fig. 1. Fifteen Danish Jersey bull-calves [mean (±S.D.) 92 ± 26 days; body weight (BW): 92 ± 21 kg], reared indoor on a commercial farm, were included in the trial. Upon arrival at the experimental farm, all calves were orally drenched with fenbendazole (Panacur®Vet, MSD Animal Health, 5 mg kg BW⁻¹) in order to ensure nematode-free conditions (later confirmed with zero FEC in all animals 1 week post treatment). After drenching, calves were stratified by BW and blocked in five subgroups of three calves with similar BW. Calves were housed in straw-bedded pens with concrete floor, daily removal of feces, external feeders and free access to water. Subgroups of three animals were randomly assigned to chicory (CHI₁, n = 9; three subgroups of three calves) or control (CTL₁, n = 6; two subgroups of three calves) feeding groups. The calves were fed twice daily *ad libitum* with chicory silage (CHI₁) or ryegrass/clover hay (CTL₁) throughout the 56 days of experiment (see Feeds – Exp. 1). Additionally, both groups were offered a commercial concentrate to balance crude protein (CP) and metabolizable energy (ME) intakes between groups throughout the trial. The DM intake per pen was calculated on a daily basis by subtracting the residual feed unconsumed by each subgroup from the feed that was offered the previous day. The estimated daily DM, CP and ME intakes during the preceding 2/3 days were adjusted for the mean BW of each subgroup and used to define exact
daily amounts of concentrate for the next 2/3 days, in order to balance the expected CP (and ME) intakes between all subgroups. Feeding was introduced with a relatively high level of concentrate at the start of the trial and gradually replaced with increasing amounts of chicory silage or ryegrass/clover hay. At the day of infection, the concentrate represented ∼25% of the DM intake in all subgroups. After 16 days of feed adaptation [day (D) 0 post-infection (p. i.)] all calves were orally inoculated using a plastic syringe with a total of 76 000 \( L3 \) (10 000 \( O. ostertagi \) L3 and 66 000 \( C. oncophora \) L3) of the inoculum described above, administered over three consecutive days. At D40 p.i., all calves were euthanized by captive bolt pistol and exsanguinated for worm recovery.

**Experiment 2.** This study was designed as a grazing trial to investigate the course of experimental infections with \( O. ostertagi \) in calves grazing either pure forage chicory or ryegrass/clover (control) pastures, without supplementary concentrate feeding. The trial was conducted during 42 days in August–September 2014 and the study design is outlined in Fig. 2. Twenty Danish Holstein bull-calves (136 ± 16 days; 153 ± 24 kg BW), raised in a commercial farm without previous access to pasture, were used. At arrival, all animals were orally drenched with fenbendazole as described for Exp. 1 to remove any potential nematode infection from the farm of origin (confirmed with zero FEC in all calves 1 week post treatment). After treatment, calves were stratified by BW and randomly assigned to a chicory (CHI2) or a control (CTL2) group of ten animals each. The CHI2 and CTL2 groups were allocated to a pure forage chicory or ryegrass/clover pasture, respectively (see *Feeds – Exp. 2*). Animals had *ad libitum* access to water throughout the trial. After 7 days of diet adaptation (D0 p.i.), all calves were orally infected using a plastic syringe with a total of 20 000 \( O. ostertagi \) L3 of the inoculum described above, administered over two consecutive days. Infective doses were designed according to published guidelines for anthelmintic evaluation (Wood *et al.* 1995). Calves in CHI2 and CTL2 groups were strip grazed within their respective swards throughout the experiment. From D14 p.i. until the end of the trial, calves were moved to a new area with 5 days intervals, thus avoiding any potential reinfection on pasture. By the second half of Exp. 2, the pure chicory pasture was at the reproductive (flowering) stage. Calves in the CHI2 group quickly consumed the chicory leaves and the upper parts of stems and flowers in the new grazing strip areas, which, in addition to poor leaf regrowth, resulted in a low amount of chicory on the pasture by D16 p.i. Therefore, the CHI2 group was supplemented *ad libitum* with chicory silage (prepared on May 2014, see *Feeds – Exp. 2*) during the last 19 days of the trial (D16–D35 p.i.). The CTL2 group was not supplemented during the experiment. At D35 p.i., all animals were euthanized by captive bolt pistol and exsanguinated for worm recovery.

**Ethics**

Animal trials were approved by the Animal Experiments Inspectorate of the Danish Ministry of Environment and Food (License j. No. 2013-15-2934-00763) and were conducted in the experimental farm of the University of Copenhagen (Tåstrup, Denmark). Calves in both experiments were inspected at least twice daily during the entire study periods to detect any unusual behaviour or signs of clinical disease.

**Feeds**

**Experiment 1.** Forage chicory (cv. Spadona) was sown on 7 May 2013 as a pure sward [7·8 kg chicory seeds per hectare (ha\(^{-1}\)] on a 0·9 ha field at the experimental farm of the University of Copenhagen (35°67′48″N, 12°29′73″E). The soil

![Fig. 1. Study design of Experiment 1 investigating the course of experimental infections with *Ostertagia ostertagi* and *Cooperia oncophora* in stabled calves fed with chicory silage and concentrate (CHI1) or ryegrass/clover hay and concentrate (CTL1) during 56 days.](https://www.cambridge.org/core/asset/50031182016000706/3753D7F77E24369D9F6C9C6D04F6D33A/435333F205C4B94FB016A3F19855D581/fig1.jpg)
Experiment 2

Chicory leaves were cut on 23 September 2013 and let to wilt for 24 h on the field to prepare chicory silage. No further cuts were performed in the chicory sward during 2013. At harvest, chicory leaves represented 55% of the total DM in the field. Unsown plant species in the sward were mainly chamomile (Matricaria recutita) with some mugwort (Artemisia vulgaris) and shepherd’s purse (Capsella bursa-pastoris). After wilting, a silage inoculant (Silosolve®AS, 200 g inoculant 75 L−1 water, CHR Hansen, Denmark) was manually sprayed on the harvested field and the plant material was wrapped in bales of 300 kg. Ensiled chicory underwent anaerobic fermentation for an average of 8.5 weeks until use in the trial. Ryegrass/clover (Lolium perenne/Trifolium repens) hay was used as control feed. Throughout Exp. 1, all animals were supplemented with a concentrate for calves (Grønmix®, Danish Agro, Denmark) in order to balance CP and ME intakes between groups.

Experiment 2. In early March 2014, the same forage chicory sward used for Exp. 1 was fertilized with 80 kg N ha−1 in early May 2013. No herbicides were used. Chicory leaves were cut on 23 September 2013 and let to wilt for 24 h on the field to prepare chicory silage. No further cuts were performed in the chicory sward during 2013. At harvest, chicory leaves represented 55% of the total DM in the field. Unsown plant species in the sward were mainly chamomile (Matricaria recutita) with some mugwort (Artemisia vulgaris) and shepherd’s purse (Capsella bursa-pastoris). After wilting, a silage inoculant (Silosolve®AS, 200 g inoculant 75 L−1 water, CHR Hansen, Denmark) was manually sprayed on the harvested field and the plant material was wrapped in bales of 300 kg. Ensiled chicory underwent anaerobic fermentation for an average of 8.5 weeks until use in the trial. Ryegrass/clover (Lolium perenne/Trifolium repens) hay was used as control feed. Throughout Exp. 1, all animals were supplemented with a concentrate for calves (Grønmix®, Danish Agro, Denmark) in order to balance CP and ME intakes between groups.

Feed sampling and analyses. In Exp. 1, samples from chicory silage, ryegrass/clover hay and concentrate were collected for chemical analyses on D − 9 p.i. Calves in the CTL1 group consumed hay uniformly, whereas CH1 animals selectively consumed the soft chicory leaves in the silage while refusing thicker stems from unsown plant species (see Feeds – Exp. 1). Therefore, samples of the offered chicory silage and of the residual (unconsumed) feed were dried for 24 h at 90 °C for correction of DM chicory intake. In Exp. 2, fresh forage samples from the chicory and ryegrass/clover swards were collected at the start of the trial (D − 7 p.i.) and 1 week before the end of the trial (D28 p.i.) by harvesting 20 samples (50 × 50 cm2, cut 5–8 cm above soil level) at similar intervals following a diagonal line across each sward and pooled by field. The supplementary chicory silage prepared in 2014 was sampled at the start of Exp. 2. A rough estimate of the daily DM intake of CH1 and CTL2 groups from pasture was calculated by comparing the available DM in the fields at the first and last sampling. While the intake of chicory silage by CH1 calves was inferred from the number of opened silage bales and the estimated silage not consumed by the animals from D16 until D34 p.i. (corrected for DM).

All experimental feeds were submitted to an accredited laboratory (Eurofins Steins Laboratorium Ltd., Holstebro, Denmark) for analyses of DM, CP, net energy lactation (NEL), amino acids absorbed in the intestine (AAI), in vitro organic matter digestibility (OMD), ash and neutral detergent fibre (NDF). In vitro OMD% of ryegrass/clover (hay and pasture) was estimated by near-infrared spectrometry, while in vitro OMD% of chicory (silage and fresh) and concentrate were estimated according to Tilley and Terry (1963) or by the in vitro enzyme digestible organic matter (EFOS) method (Volden, 2011), respectively. Feeds were adjusted to in vivo OMD% following the regression formulas recommended by the Nordic Feed Evaluation System (NorFor; Volden, 2011) as.
follows: (a) chicory silage in vivo OMD% = 6.73 + 0.950 \times \text{in vitro OMD%}, (b) fresh chicory in vivo OMD% = 4.1 + 0.959 \times \text{in vitro OMD%}, (c) concentrate in vivo OMD% = 5.38 + 0.867 \times \text{in vitro OMD%}. ME from all feeds was calculated from NEL values divided by 0.65 (Nielsen et al. 2003) and expressed as mega joule (MJ) kg\(^{-1}\) DM. The content of CT and SL were analysed in samples of all experimental feeds collected at the start of Exp. 1 and 2; CT were analysed using the acetone–butanol–HCl method (Grabber et al. 2013), while total and individual SL were characterized as described by Peña-Espinoza et al. (2015).

**Animal measurements**

In Exp. 1, calves were individually weighed weekly from arrival until D37 p.i., while animals in Exp. 2 were weighed at arrival and on the day of slaughter, using an electronic scale. Blood samples from each calf were collected by jugular venipuncture at weekly intervals from D0 p.i. until slaughter, using an electronic scale. Blood samples were collected from each animal by jugular venipuncture at weekly intervals from D0 p.i. until slaughter in both trials. Recovered serum was analysed for total protein (TP), albumin (ALB), inorganic phosphate (IP) and serum pepsinogen. Serum pepsinogen was analysed according to Dorny and Vercruysse (1998) and expressed as units of tyrosine (U Tyr/L).

**Parasitological analyses**

**FEC and larval cultures.** In Exp. 1 and 2, fecal samples were collected rectally from each animal every 2–3 days from D14 p.i. until slaughter and analysed for strongyle FEC using an accredited, modified McMaster technique with a sensitivity of 5 eggs per g (epg) of feces (Henriksen and Aagard, 1975). The DM percentage of each fecal sample (fecal DM\%) was determined by drying 3 g of fresh feces for 24 h at 90°C. Subsequently, fecal DM\% was used to correct individual FEC per g of fecal DM (FECDM) using the formula:

\[
\text{FECDM} = \text{FEC} \times \left( \frac{100}{\text{fecal DM}\%} \right)
\]

In Exp. 1, larval cultures were prepared for each subgroup at D19, D26 and D33 p.i. by mixing 10 g of fresh feces from each animal into one pool which was cultured for 14 days as described by Roepstorff and Nansen (1998). After baermannization, 100 L3 from each subgroup were identified as *O. ostertagi* or *C. oncophora* according to van Wyk and Mayhew (2013).

**Worm counts.** Immediately after slaughter the abomasum and small intestine (Exp. 1) or only abomasum (Exp. 2) from each animal were removed and opened into individual buckets for worm recovery. The organs were thoroughly washed with warm (38°C) saline solution (0.9% NaCl) until total volumes of 5 L (abomasum) or 10 L (small intestine). Subsamples of 2×10% and 2×1% of stirred abomasal and small intestinal washed content, respectively (Exp. 1), and subsamples of 2×2% of stirred abomasal washed content (Exp. 2), were collected using a 25-μm-pore sieve. All worms collected in the two abomasal subsamples (Exp. 1 and 2) and on the two small intestinal subsamples (Exp. 1) per animal were counted. Male worms (*n = 20 per animal*) from abomasal and small intestinal samples were further transferred to microscope slides, added a drop of lactic acid (Sigma L1250, 10% v/v in distilled water) and observed by light microscopy to verify the nematode species (Barth and Visser, 1991). In Exp. 1, chemical digestion of abomasal mucosa for detection of inhibited *O. ostertagi* L4 was performed as described by Wood et al. (1995) in one animal from each subgroup (three CHI and two CTL1 calves).

**Ostertagia ostertagi female fecundity in mono-infected calves.** In Exp. 2, the per capita fecundity of *O. ostertagi* females was calculated by dividing the FECDM at slaughter by the total number of females recovered from the same animal and was expressed as the number of eggs female\(^{-1}\) per g of fecal DM.

**Statistical analyses**

All statistical analyses were performed in R version 3.2.2 (R Core Team, 2015). Untransformed FECDM/worm count data were analysed using generalized linear models, which have been advocated to increase statistical power and reduce risks of type I and II errors when analysing overdispersed (non-normally distributed) data, compared with the modelling of (log-)transformed counts (Wilson and Grenfell, 1997; Paterson and Lello, 2003). In Exp. 1, differences in FECDM between feeding groups at each sampling point were analysed by generalized linear-mixed effect (glmer, R package “lme4”) models assuming a negative binomial (\(\text{nb}\)) distribution and testing the effect of feeding group (CHI or CTL1) as fixed (main) factor and subgroup of three calves as random factor; the random factor (or random effect) included the variation of data between subgroups within the same feeding group (fixed factors) in the models. In addition, the cumulative FECDM of each animal at the end of the trial was calculated according to Vercruysse et al. (1993) and compared between feeding groups using the Wilcoxon rank sum test. Worm counts were analysed by fitting glmer.nb models, testing the effect of feeding group as fixed factor and subgroup as random factor. Fecal DM\%, BW gains and serum pepsinogen, TP, ALB and IP levels were analysed as repeated measures by fitting linear mixed-effects...
(lme, R package ‘nlme’) models, testing the effect of feeding group, time and their interaction as fixed factors, initial values as covariate and animal within subgroup as random factor (to account for the correlation of repeated measures from the same animal and the variation of data between subgroups of the same feeding group). Data acquired on a subgroup-basis were not subjected to statistical analysis. Total DM, CP and ME intakes recorded daily from each subgroup were adjusted to 100 kg BW and are presented as arithmetic means (±S.D.). Percentage of Ostertagia ostertagi and Cooperia oncophora L3 in larval cultures pooled by subgroup are reported as arithmetic means (±S.D.). In Exp. 2, differences in FECDM between feeding groups were analysed similarly as described for Exp.1, with the exception that all the models were fitted using generalized linear models (glm, package ‘MASS’), assuming a nb distribution. Worm counts and per capita female fecundity were analysed by glm.nb models with feeding group (CHI2 or CTL2) as fixed factor. Fecal DM%, BW gains and serum pepsinogen, TP, ALB and IP levels were analysed as repeated measures by fitting lme models, including group, time and their interaction as fixed factors, initial values as covariates and animal as random factor (to include correlation of data from the same calf). Repeated measures (not count) data were power-transformed if required to fit normality assumptions using the boxcox command (package ‘MASS’). All models were validated by analysis of residual plots. A level of $P < 0.05$ was considered significant.

RESULTS

Experiment 1

Feed analyses. Chemical compositions of the experimental feeds are presented in Table 1. Chicory silage had the lowest DM% of all feeds. Crude protein, ME and OMD% were similar in chicory silage and ryegrass/clover hay. Chicory silage had a markedly higher content of crude ash (207 g kg$^{-1}$ DM) compared with ryegrass/clover hay (49 g kg$^{-1}$ DM) and concentrate (70 g kg$^{-1}$ DM). Sand, which was only detected in chicory silage, resulted in corrected ash content (crude ash minus sand) of 135 g kg$^{-1}$ DM chicory silage. Ryegrass/clover hay had the highest NDF content (577 g kg$^{-1}$ DM), followed by chicory silage (326 g kg$^{-1}$ DM) and concentrate (286 g kg$^{-1}$ DM). Condensed tannins were not detected in any of the experimental feeds. Sesquiterpene lactones were identified only in chicory silage (12.3 g total SL kg$^{-1}$ DM), with 11,13-dihydro-lactucin (DI-LAC, 2.1 g kg$^{-1}$ DM), 11,13-dihydro-lactucopicrin (DI-LCP, 2.6 g kg$^{-1}$ DM) and 11,13-dihydro-8-deoxylactucin (8-DOL, 1.2 g kg$^{-1}$ DM) as the main SL (Table 1).

Feed intake. Mean daily intakes of DM, CP and ME over the experiment are presented in Table 2.
A comparable increase in DM intake was recorded in CHI1 and CTL1 animals during the trial. At D0 p.i., the DM intake in the CHI1 group was composed of 76% chicory silage, whereas the CTL1 consumed 80% ryegrass/clover hay. At D39 p.i. (day before slaughter), the DM intake constituted 74% chicory silage and 67% ryegrass/clover hay in the CHI1 and CTL1 groups, respectively. The remaining component of the diets was the concentrate, which was readily eaten by all subgroups. Balancing of protein and energy intakes resulted in marginally higher CP and ME intakes in CTL1 calves, but with comparable levels between groups throughout the experiment.

Parasitology. Fecal DM% was not significantly affected by feeding group, time or their interaction (P > 0.10), but a significant effect of initial values (covariate) was confirmed (P < 0.001). Arithmetic mean FEC and FECDM are presented in Fig. 3. Fecal strongyle eggs were detected from D14 p.i. onwards in calves from both groups, with significant differences in FECDM between groups only at D19.
D21 and D37 p.i. Higher FECDM was observed initially in CTL1 (from D14 to D28 p.i.), but following a marked reduction by D33 p.i. this group had the lowest FECDM during the last days of the trial. In comparison, a more stable egg excretion was observed in CHI1 throughout the study period. However, the cumulative FECDM during the entire experiment was not significantly different between groups [mean (± S.D.) CHI1 = 204 280 (±65 264) vs CTL1 = 225 128 (±94 501) epg of fecal DM; \(P=0·6\)]. Mean percentages of *O. ostertagi* L3 in pooled larval cultures from each group are presented in Fig. 4. At D19 p.i., similar proportions of *O. ostertagi* L3 were detected in CHI1 and CTL1 animals. On D26 and D33 p.i., in contrast, a markedly lower number of *O. ostertagi* L3 was observed in pooled larval cultures from the CHI1 calves.

Post-mortem worm recovery in CHI1 and CTL1 groups is summarized in Table 3. Calves fed with chicory silage had a significant arithmetic mean reduction of 52% (geometric mean reduction 60%) in the number of adult *O. ostertagi*, in comparison with CTL1 animals (\(P<0·01\)). In contrast, adult counts of *C. oncophora* were not statistically different between groups (\(P=0·12\)). In CTL1 calves, the number of adult *O. ostertagi* corresponded to 14% of the total worm counts in this group, which agrees with the proportion of *O. ostertagi* L3 in the original infective dose (13%). No inhibited *O. ostertagi* L4 were observed in the digested abomasal mucosa of the investigated calves.

**Biochemical parameters.** Very low serum pepsinogen values were detected in CHI1 and CTL1 calves throughout the trial (Fig. 5). Mean (\(\pm\) s.d.)
serum pepsinogen levels from D7 until D40 p.i. in CHI1 and CTL1 were 0.31 (± 0.2) U Tyr/L and 0.48 (± 0.2) U Tyr/L, respectively. Time had a significant influence on pepsinogen levels over the study period (P < 0.001), with a peak in both groups on D28 p.i., but neither group nor group × time interaction (P > 0.12), but were significantly influenced by time (P < 0.001), reflected in decreasing TP and ALB values in both groups from D0 p.i. until slaughter (data not shown). Similarly, serum IP levels were significantly reduced in the CHI2 group and fed with chicory silage and concentrate (CHI2) or ryegrass/clover hay and concentrate (CTL1) in Experiment 1. Error bars indicate s.d.

Animal weights. Bodyweight gains were significantly affected by time and group × time interaction (P < 0.001 and P = 0.004, respectively), with increasing BW in both groups but higher growth rates in CHI1 calves. Similar mean (±S.D.) daily growth rates were observed in both groups until D19 p.i. [CHI1 = 383 (±116) vs CTL1 = 325 (±236) g day⁻¹]. In contrast, from D19 until D37 p.i., CHI1 and CTL1 groups had mean growth rates of 811 (±59) and 391 (±132) g day⁻¹, respectively. In addition, initial BW (covariate) had a significant effect on growth rates, with a lower weight gain in lighter calves, irrespective of group (P < 0.001).

Experiment 2

Feed analyses and feed intake. Chemical profiles of the experimental feeds used in Exp. 2 are presented in Table 4. The chicory sward had markedly lower DM%, CP and NDF levels compared with the ryegrass/clover pasture. Chicory silage had a slightly higher CP content compared with fresh chicory, which reflects the high protein content of the chicory sward on May 2014 (142.6 g CP kg⁻¹ DM) when it was cut for ensiling. Comparable ME levels were identified in all feeds, while fresh chicory and chicory silage had higher ash content in comparison with fresh ryegrass/clover. No CT were detected in any of the experimental feeds. Sesquiterpene lactones were identified in fresh chicory (22.5 g total SL kg⁻¹ DM) and chicory silage (16.8 g total SL kg⁻¹ DM). In fresh chicory, the main SL detected were lactucin (LAC) and 11,13-dihydro-lactucin (DI-LAC), while in chicory silage the predominant SL were DI-LCP and DI-8-DOL. Based on sward measurements, CHI2 calves had an estimated mean daily intake of 5.7 kg DM calf⁻¹ during the trial, with increasing consumption of chicory silage from D16 p.i. onwards and ~70% of the DM intake being chicory silage at D34 p.i., whereas CTL2 animals had an estimated daily intake of 6.9 kg DM ryegrass/clover calf⁻¹ during the entire experiment.

Parasitology. Fecal DM% was significantly affected by group, time and their interaction and covariate (P < 0.05). Similar fecal DM content was observed between groups until D22 p.i., followed by markedly lower fecal DM% in CTL2 animals from D24 p.i. onwards, with a mean (±S.D.) fecal DM% at slaughter of 18.72 (±1.9) and 12.40 (±2.2)% in CHI2 and CTL2 groups, respectively (P < 0.001). Arithmetic mean FEC and FECDM are presented in Fig. 6. Fecal strongyle eggs were detected from D17 p.i. onwards in animals from both groups. At D20 p.i., mean FECDM of CHI2 and CTL2 were 1589 and 1474 epg, respectively (P = 0.7). From this point, mean FECDM in the CHI2 group was rapidly and significantly reduced until the end of the trial: at D35 p.i., CHI2 animals had a 65% lower mean FECDM compared with CTL2 calves (P < 0.01). Cumulative FECDM during the entire experiment was significantly reduced in the CHI2 group [mean (± S.D.) CHI2 = 12 870 (±8136) vs CTL2 = 22 60 (±5671) epg of fecal DM; P < 0.01].

Post-mortem worm recovery of CHI2 calves revealed significantly lower numbers of O. ostertagi males [mean (range) CHI2 = 2106 (283–5425) vs CTL2 = 4692 (2775–6800) male worms; P < 0.01] and females [CHI2 = 2256 (617–4100) vs CTL2 = 3386 (3700–6700) female worms; P < 0.001], compared with CTL2 animals. Calves fed pure forage Chicory had a highly significant arithmetic mean reduction of 57% (geometric mean reduction 66%) in the total worm burden of O. ostertagi, compared with control animals [mean (range) CHI2 = 4362 (900–9525) vs CTL2 = 10 078 (6475–13 325) total

Fig. 5. Arithmetic mean serum pepsinogen levels (in units tyrosine; U Tyr/L) in stabled calves experimentally infected with Ostertagia ostertagi and Cooperia oncophora and fed with chicory silage and concentrate (CHI1) or ryegrass/clover hay and concentrate (CTL1) in Experiment 1. Error bars indicate s.d.
Similarly, serum TP and ALB levels were only sign-
ificant in the CTL2 group, but with decreasing IP levels in
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cificant effect on pepsinogen levels (P < 0.001), with mean (±S.D.) growth
rates of 374 (±160) g day⁻¹ and 783 (±229) g day⁻¹ in CHI2 and CTL2 calves, respectively.

Results for fresh forage chicory and ryegrass/clover are presented as means (±S.D.), summarizing the samples collected from the swards at D = 7 and D28 p.i.

<table>
<thead>
<tr>
<th>Experimental feeds</th>
<th>Fresh forage chicory</th>
<th>Chicory silage</th>
<th>Ryegrass/clover</th>
</tr>
</thead>
<tbody>
<tr>
<td>DM (%)</td>
<td>11·5 (±4·3)</td>
<td>23·5</td>
<td>22·0 (±2·1)</td>
</tr>
<tr>
<td>CP (g kg⁻¹ DM)</td>
<td>98·2 (±32·1)</td>
<td>17·2</td>
<td>159·8 (±4·1)</td>
</tr>
<tr>
<td>ME (MJ kg⁻¹ DM)</td>
<td>7·2 (±6·9)</td>
<td>6·6</td>
<td>8·3 (±5·5)</td>
</tr>
<tr>
<td>AAT (g kg⁻¹ DM)</td>
<td>67·5 (±6·4)</td>
<td>66·0</td>
<td>80·1 (±4·2)</td>
</tr>
<tr>
<td>OMD (% of DM)</td>
<td>63·1 (±9·5)</td>
<td>74·5</td>
<td>67·1 (±4·7)</td>
</tr>
<tr>
<td>Crude ash (g kg⁻¹ DM)</td>
<td>150·2 (±30·7)</td>
<td>147·2</td>
<td>108·6 (±5·2)</td>
</tr>
<tr>
<td>Sand (g kg⁻¹ DM)</td>
<td>17·0 (±1·4)</td>
<td>5·0</td>
<td>22·0 (±11·3)</td>
</tr>
<tr>
<td>NDF (g kg⁻¹ DM)</td>
<td>260·5 (±5·4)</td>
<td>326·4</td>
<td>430·2 (±2·3)</td>
</tr>
<tr>
<td>CT (%) (DM)</td>
<td>n.d.</td>
<td>n.d.</td>
<td>n.d.</td>
</tr>
<tr>
<td>SL (g kg⁻¹ DM)</td>
<td>22·5</td>
<td>16·8</td>
<td>0·0</td>
</tr>
<tr>
<td>DI-LAC</td>
<td>5·9</td>
<td>3·4</td>
<td>0·0</td>
</tr>
<tr>
<td>LAC</td>
<td>6·1</td>
<td>2·7</td>
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<tr>
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</tr>
<tr>
<td>DI-LCP</td>
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Biochemical parameters. Mean serum pepsinogen levels are illustrated in Fig. 7, which shows almost identical profiles in both groups with a common peak at D21 p.i. and comparable levels at slaughter [mean (±S.D.) CHI2 = 1·02 ± 0·4 U Tyr/L; CTL2 = 1·01 ± 0·2 U Tyr/L]. Only time had a significant influence on pepsinogen levels (P < 0·001), but not group or group × time interaction (P > 0·8). Similarly, serum TP and ALB levels were only signif-
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DISCUSSION

In the present study, we demonstrated that cattle ex-
perimentally infected with GIN and fed forage chicory-rich diets had significant reductions in worm burdens of *O. ostertagi* (Exp. 1 and 2), but not of *C. oncophora* (Exp. 1), compared with infected animals fed control diets. In Exp. 1, calves fed with chicory silage had largely similar fecal egg excretions compared with control animals, but markedly lower numbers of *O. ostertagi* L3 in larval cultures from D26 p.i. These findings were confirmed in Exp. 2, where calves mono-infected with *O. ostertagi* and fed pure forage chicory (fresh and silage) had a significant reduction of nematode eggs in feces from D22 p.i. until slaughter, compared with control animals, without significant differences in the fecundity of female worms. In addition, SL were detected in both experiments only in forage chicory (fresh and silage), while CT were not identified in any of the experi-
mental feeds.

Differences in the FECDM of chicory-fed animals between experiments can be interpreted based on the distinct worm populations in the two trials. In Exp. 1 (animals co-infected with *O. ostertagi* and *C. oncophora*), the similar cumulative FECDM in CHI1 and CTL1 calves is explained by the unaffected *C. oncophora* population, which repre-
sented 87% of the infective dose and was responsible for approximately 65–90% of the egg excretion (based on larval cultures). In contrast, the lower number of *O. ostertagi* L3 in larval cultures of
chicory-fed calves from D26 p.i. onwards probably derived from a decreased fecal egg excretion of this species, as a consequence of the reduced number of *O. ostertagi* females in these animals. Towards the end of Exp. 1, CTL1 calves had a lower mean FECDM compared with CHI1 animals (only significant at D37 p.i.). Post-mortem analyses demonstrated lower mean *C. oncophora* counts in CTL1 animals compared with CHI1 calves, although with large variations in both groups and no statistical difference in *C. oncophora* burdens between groups. While in Exp. 2 (animals mono-infected with *O. ostertagi*), the significantly lower cumulative FECDM of the CHI2 group was likely a result of the reduced number of female *O. ostertagi* in these calves, later confirmed post-mortem. Nevertheless, FECDM (or FEC) alone as an indicator of egg excretion should be evaluated carefully while it is affected by the fecal output, which in turn is influenced by the DM% and digestibility of the feed and by the DM intake of the animal (Heckendorn et al. 2007). In our case, the accurate DM intake and fecal output of the calves in Exp. 2 was not monitored, and therefore, further studies in which the total daily fecal egg production is examined are required to confirm the effect of chicory feeding on *O. ostertagi* egg excretion and pasture contamination.

Mean serum pepsinogen levels of chicory and controls animals in both trials were <1·5 U Tyr/L, below the threshold of 2·0 U Tyr/L related with subclinical *O. ostertagi* infections (Vercruysse and Claerebout, 2001), reflecting the low infective doses with this species in our study. Consequently, our results are insufficient to determine possible consequences of chicory feeding in the pathophysiology of *O. ostertagi* in the infected animals, although no differences were detected in serum pepsinogen concentrations between feeding groups, despite the significantly lower burden of adult *O. ostertagi* in chicory-fed animals in both experiments.

Considering the comparable protein/energy intakes between groups in Exp. 1 and the low nutritional plane of chicory-fed calves in Exp. 2 (further discussed below), in addition to the slow development of immunity against *O. ostertagi* in parasite-naive calves (Gasbarre, 1997) and the minimal loss of worms before 50 days p.i. observed in similar infections (Michel, 1969), the present study demonstrates that forage chicory can selectively reduce *O. ostertagi* infections in cattle. Our results are consistent with earlier studies in sheep, which reported that chicory-rich diets induced selective anthelmintic effects only against abomasal nematodes (without affecting per capita female fecundity), but not towards small intestinal species (Scales et al. 1995; Marley et al. 2003; Athanasiadou et al. 2005; Tzamaloukas et al. 2005; Heckendorf et al. 2007). Similar to our findings in Exp. 1, Marley et al. (2014) reported no differences in FECDM and serum pepsinogen levels in cattle naturally infected with mixed species of GIN and grazing either
chicory/ryegrass or pure ryegrass for 126 days. But in contrast to our study, the cited authors did not observe any effect of chicory/ryegrass feeding on the number of *O. ostertagi* L3 in larval cultures. Post-mortem worm counts were not conducted. In the study by Marley *et al.* (2014), the animals grazed a sward with only 24% of chicory DM, much lower than the chicory levels offered in our study (≥70% of the daily DM intake), which may explain the lack of anthelmintic effect in that experiment. Previous studies have also reported a lower development and recovery of infective L3 from forage chicory swards that could reduce the larval challenge of animals grazing chicory (Moss and Vlassoff, 1993; Marley *et al.* 2006). However, this is unlikely to have contributed to the reduction in worm burdens in our Exp. 2 due to the artificial inoculation of the animals and the frequent relocation of the calves to clean areas, thus preventing any reinfection on pasture.

In relation to the PSM analysed in the experimental feeds, SL were only detected in chicory (ensiled and fresh) in both experiments. To our knowledge, this is the first report of the SL profile in forage chicory cv. Spadona. Previous studies have reported SL levels in other forage chicory cultivars, with levels ranging between 1·55 and 15·24 g total SL kg⁻¹ DM (Foster *et al.* 2006, 2011b). However, our findings are not directly comparable with these results because of differences between the studies regarding chicory cultivars, cultivation methods, locations, season, stage of plant growth and screening methods used, all of which can affect the detection and concentration of SL in chicory (Rees and Harborne, 1985; Foster *et al.* 2011b; Ferioli and D’Antuono, 2012). Whereas CT were not identified in any of the experimental feeds; however, we cannot conclude if CT were totally absent in chicory or if CT were present below the estimated detection limit of the method (0·2% CT in DM). Nevertheless, the undetectable levels of CT in all the experimental feeds indicate that CT were not required for the observed anthelmintic effects and suggest that SL and/or other PSM could be responsible for the anti-parasitic activity of chicory. Several phytochemicals other than SL have been reported in chicory, e.g. derivatives of hydroxycinnamic acids (e.g. chloric, chlorogenic and caffeic acid), coumarins (e.g. chicorion) and flavonoids (Rees and Harborne, 1985; Heimler *et al.* 2009; Sinković *et al.* 2015), and their potential significance in the anthelmintic effects of chicory needs further investigation.

Although our study was not designed to identify the specific nematode stages affected by chicory feeding, the similar FECDM in chicory and control groups in Exp. 2 up until D20 p.i. suggests that dietary chicory may not interfere with the exsheathment, establishment and development of the larval stages of *O. ostertagi* into egg-laying adults; rather, the marked drop in FECDM observed between D20 and D22 p.i. indicates that chicory may selectively (and rapidly) affect the survival of adult worms. Recently, we showed that SL-containing chicory extracts do not interfere with the artificial exsheathment of *O. ostertagi* L3 *in vitro*, but can induce a rapid paralysis of adult *O. ostertagi* in a dose-dependent manner (Peña-Espinoza *et al.* 2015). Previously, the *in vitro* activity of forage chicory against adult stages but not towards incoming L3 has been reported in sheep infected with *T. circumcincta* (Tzamaloukas *et al.* 2005). In Exp. 1, the absence of inhibited *O. ostertagi* L4 in the investigated CHI1 and CTL1 calves does not necessarily indicate a lack of arrested larvae in the other (unscreened) animals; however, it suggests that feeding with chicory silage did not interfere with the larval development into adult worms, although this remains to be demonstrated.

The mechanisms behind the selective activity of dietary chicory against abomasal worms but not towards small intestinal nematodes are unknown, but comparable findings in sheep by authors in different countries (Scales *et al.* 1995; Marley *et al.* 2003; Tzamaloukas *et al.* 2005) suggest that the mechanisms are preserved across ruminant species, abomasal nematodes, and perhaps, chicory cultivars and cultivation conditions. In our study, the lack of activity towards *C. oncophora* infections may indicate that there is none in chicory; interestingly, preliminary experiments from our group have demonstrated direct and dose-dependent *in vitro* activity of purified chicory extracts against adult *C. oncophora* (Peña-Espinoza *et al.* unpublished results). Hence, one explanation for the lack of *in vivo* activity against intestinal worms could be that PSM, such as SL, do not reach the small intestine in concentrations sufficient to exert their activity. Differences in the stability of PSM in different gut compartments have been documented for CT (Hoste *et al.* 2006), and studies suggest that SL may be more stable at low pH (Ferreira and Gonzalez, 2008; Saroglou *et al.* 2008). Currently, the understanding of the pharmacokinetics of SL in livestock is rudimentary, but previous studies have indicated that these phytochemicals can be metabolized by ruminants (cited by Barry, 1998; Ferreira and Gonzalez, 2008). Undoubtedly, more research is required to fully elucidate the fate of dietary SL from chicory in the digestive tract of ruminants and to establish how factors such as changing pH and host metabolism can affect the concentrations, and eventually the anthelmintic activity, of SL *in vivo*. Nonetheless, the selective effect of chicory against abomasal nematodes may also be explained by local changes induced only in the abomasum that result in worm expulsion, rather than or in combination with a direct effect on the worms. In traditional medicine, chicory has been described to have...
Effects of dietary chicory against cattle nematodes in vivo

A complementary and selective anti-Ostertagia strategy that may reduce infection levels and the need of drug treatments. Ostertagia ostertagi is considered the most important GIN infecting grazing cattle in temperate regions and cases of AR in this species have been reported (Edmonds et al. 2010; Gasbarre, 2014; Geurden et al. 2015). Moreover, forage chicory can be cultivated in a wide-range of climates (Li and Kemp, 2005), including areas where the conditions for other bioactive forages like tanniferous plants may be limited, as Northern Europe (Høgh-Jensen et al. 2006; Kidane et al. 2014). Our results also demonstrated that the conservation of chicory leaves as silage did not obstruct their anthelmintic effects, and therefore, this may be an acceptable method to preserve and use chicory in farms, independent of seasonal availability. Moreover, and in comparison with fresh forage, chicory silage prepared for Exp. 1 and 2 (in two consecutive years) contained increased concentrations of DI-LCP and DI-8-DOL; the latter SL has been recently linked with higher anthelmintic potency against O. ostertagi in vitro (Peña-Espinoza et al. 2015). Further studies are needed to clarify how ensiling affects the SL profile and the anthelmintic activity of chicory silage in comparison with fresh leaves and to evaluate the most appropriate on-farm use of forage chicory.

In conclusion, feeding forage chicory (≥70% of chicory DM in the diet) led to a significant reduction in worm burdens and FEC of O. ostertagi in experimentally infected calves in two independent experiments, while no apparent activity was observed against C. oncophora. Sesquiterpene lactones were identified and individually characterized in ensiled and fresh forage chicory, suggesting that these PSM may contribute to the observed anthelmintic effects of dietary chicory in vivo. However, further research is needed to fully elucidate the exact compound(s) and mechanism(s) behind the in vivo anti-parasitic effects of chicory in ruminants. Forage chicory (fresh and ensiled) was readily consumed by calves and we demonstrated that anti-parasitic effects were preserved despite ensiling, which may facilitate the use of chicory in integrated parasite control strategies on farms to reduce the reliance on anthelmintic drugs.

ACKNOWLEDGEMENTS

The authors gratefully acknowledge Preben Lund, Annett Christoffersen and the staff at Department of Experimental Medicine (Tåstrup) and Department of Veterinary Disease Biology, University of Copenhagen and the National Veterinary Institute, Technical University of Denmark for their valuable help and technical guidance. Matt Denwood is gratefully acknowledged for statistical advice. Johan Höglund is thanked for conducting serum pepsinogen analyses and Ida Hindrichsen (CHR Hansen) for providing the silage inoculant.


