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

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## SUMMARY

Two experiments studied the effects of dietary chicory against gastrointestinal nematodes in cattle. In Experiment (Exp.) 1, stabled calves were fed chicory silage (CHI<sub>1</sub>; *n* = 9) or ryegrass/clover hay (CTL<sub>1</sub>; *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 (CHI<sub>2</sub>; *n* = 10) or ryegrass/clover (CTL<sub>2</sub>; *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, CHI<sub>2</sub> 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 CHI<sub>1</sub> by 60% (geometric mean; *P* < 0.01), whereas *C. oncophora* burdens were unaffected (*P* = 0.12). In Exp. 2, FEC were markedly lowered in CHI<sub>2</sub> from D22 p.i. onwards (*P* < 0.01). *Ostertagia ostertagi* adult burdens were significantly reduced in CHI<sub>2</sub> 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

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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 vivo* studies with sheep suggest that higher levels of chicory (possibly  $\geq 50\%$  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 baermanized 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 baermannization 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 ( $\pm$ S.D.) 92  $\pm$  26 days; body weight (BW): 92  $\pm$  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<sup>®</sup>Vet, MSD Animal Health, 5 mg kg BW<sup>-1</sup>) 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 (CHI<sub>1</sub>,  $n=9$ ; three subgroups of three calves) or control (CTL<sub>1</sub>,  $n=6$ ; two subgroups of three calves) feeding groups. The calves were fed twice daily *ad libitum* with chicory silage (CHI<sub>1</sub>) or ryegrass/clover hay (CTL<sub>1</sub>) 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

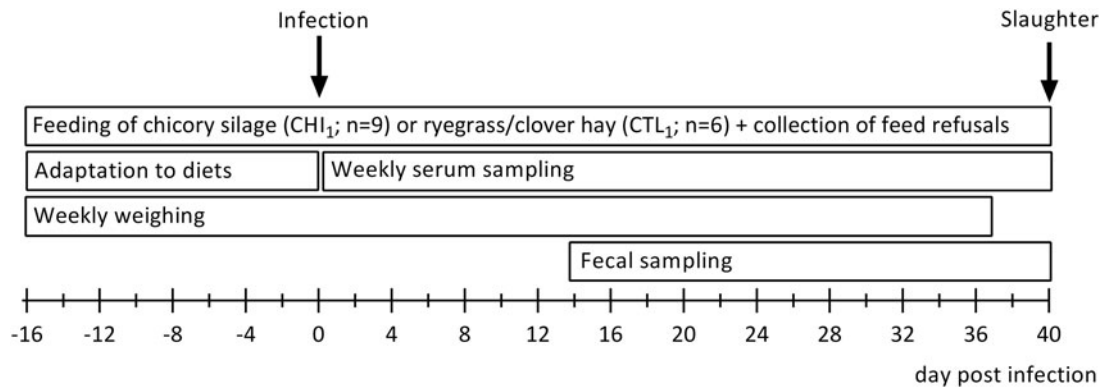


Fig. 1. Study design of Experiment 1 investigating the course of experimental infections with *Ostertagia ostertagi* and *Cooperia oncophora* in stabled calves fed with chicory silage and concentrate (CHI<sub>1</sub>) or ryegrass/clover hay and concentrate (CTL<sub>1</sub>) during 56 days.

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 (CHI<sub>2</sub>) or a control (CTL<sub>2</sub>) group of ten animals each. The CHI<sub>2</sub> and CTL<sub>2</sub> 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 CHI<sub>2</sub> and CTL<sub>2</sub> 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 CHI<sub>2</sub> 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 CHI<sub>2</sub> 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 CTL<sub>2</sub> 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<sup>-1</sup>)] on a 0.9 ha field at the experimental farm of the University of Copenhagen (55°67'48"N, 12°29'75"E). The soil

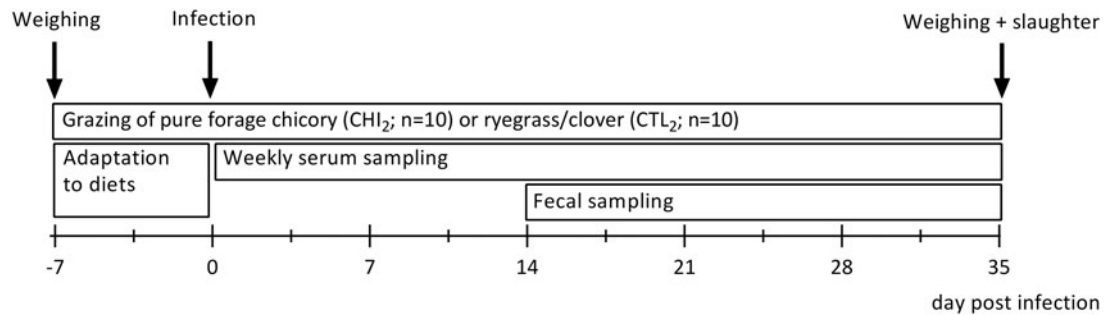


Fig. 2. Study design of Experiment 2 investigating the course of experimental infections with *Ostertagia ostertagi* in calves grazing pure forage chicory (CHI<sub>2</sub>) or ryegrass/clover (CTL<sub>2</sub>) during 42 days.

(moraine clay loam) was fertilized with 50 kg N ha<sup>-1</sup> 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<sup>®</sup> AS, 200 g inoculant 75 L<sup>-1</sup> 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<sup>®</sup>, 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<sup>-1</sup>. On May 2014, when the chicory plants were at the vegetative stage and chicory leaves constituted >90% of the total DM in the field, the sward was cut for ensiling. This chicory silage was produced to provide a potential supplement for calves allocated to grazing on chicory during Exp. 2. By early July 2014, the chicory plants were progressing into their reproductive stage and a cut 40 cm above the soil level was performed in order to remove long stems and flowers and prepare the field for the grazing trial. As a control pasture, a 0.8 ha ryegrass/clover sward located at the same farm and left ungrazed in 2013 was used. The control field was cut in mid-June 2014 and allowed to regrow for 2 months. At the start of Exp. 2, chicory leaves, stems and flowers constituted 90% of the total DM in the sward (chicory leaves = ~60%, chicory stem and flowers = ~40%) while the remaining 10% were a mix of unsown clover and shepherd's purse. Ryegrass

constituted 80% of the total DM in the control field, with the rest comprised mainly of clover.

**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 CTL<sub>1</sub> group consumed hay uniformly, whereas CHI<sub>1</sub> 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 cm<sup>2</sup>, 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 CHI<sub>2</sub> and CTL<sub>2</sub> 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 CHI<sub>2</sub> 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 (AAT), *in vitro* organic matter digestibility (OMD), ash and neutral detergent fibre (NDF). *In vivo* 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<sup>-1</sup> DM. The content of CT and SL were investigated 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 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 Vercruyse (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 (FEC<sub>DM</sub>) using the formula:

$$\text{FEC}_{\text{DM}} = \text{FEC} \times (100/\text{fecal DM\%})$$

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<sub>1</sub> and two CTL<sub>1</sub> 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 FEC<sub>DM</sub> at slaughter by the total number of females recovered from the same animal and was expressed as the number of eggs female<sup>-1</sup> per g of fecal DM.

#### Statistical analyses

All statistical analyses were performed in R version 3.2.2 (R Core Team, 2015). Untransformed FEC<sub>DM</sub>/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 FEC<sub>DM</sub> between feeding groups at each sampling point were analysed by generalized linear-mixed effect (*glmer*, R package 'lme4') models assuming a negative binomial (*nb*) distribution and testing the effect of feeding group (CHI<sub>1</sub> or CTL<sub>1</sub>) 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 FEC<sub>DM</sub> of each animal at the end of the trial was calculated according to Vercruyse *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

Table 1. Chemical composition of feeds offered to stabled calves infected with *Ostertagia ostertagi* and *Cooperia oncophora* in Experiment 1

	Experimental feeds		
	Chicory silage	Ryegrass/clover hay	Concentrate <sup>a</sup>
DM (%)	35.7	86.5	89.6
CP (g kg <sup>-1</sup> DM)	93	84	204
ME (MJ kg <sup>-1</sup> DM)	6.5	7.2	10.5
AAT (g kg <sup>-1</sup> DM)	54	63	114
OMD (% of DM)	62	59	83
Crude ash (g kg <sup>-1</sup> DM)	207	49	70
Sand (g kg <sup>-1</sup> DM)	72	0.0	0.0
NDF (g kg <sup>-1</sup> DM)	326	577	286
CT <sup>b</sup> (% DM)	n.d.	n.d.	n.d.
SL <sup>c</sup> (g kg <sup>-1</sup> DM)	12.3	0.0	0.0
DI-LAC	2.1	0.0	0.0
LAC	1.8	0.0	0.0
8-DOL	1.2	0.0	0.0
DI-8-DOL	3.8	0.0	0.0
DI-LCP	2.6	0.0	0.0
LCP	0.8	0.0	0.0

DM, dry matter; CP, crude protein; ME, metabolizable energy; AAT, aminoacid absorbed in the intestine; OMD = *in vivo* organic matter digestibility; NDF, neutral detergent fibre; CT, condensed tannins; SL, sesquiterpene lactones; LAC, lactucin; DI-LAC, 11, 13-dihydro-lactucin; 8-DOL, 8-deoxylactucin; DI-8-DOL, 11, 13-dihydro-8-deoxylactucin; LCP, lactucopicrin; DI-LCP, 11, 13-dihydro-lactucopicrin; n.d., not detected.

<sup>a</sup> Grønmix<sup>®</sup> (Danish Agro).

<sup>b</sup> According to Grabber *et al.* (2013; estimated detection limit = 0.2% of CT in DM).

<sup>c</sup> According to Peña-Espinoza *et al.* (2015).

(*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 ( $\pm$ S.D.). Percentage of *O. ostertagi* and *C. oncophora* L3 in larval cultures pooled by subgroup are reported as arithmetic means ( $\pm$ 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 (CHI<sub>2</sub> or CTL<sub>2</sub>) 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, AAT and OMD% were similar in chicory silage and ryegrass/clover hay. Chicory silage had a markedly higher content of crude ash (207 g kg<sup>-1</sup> DM) compared with ryegrass/clover hay (49 g kg<sup>-1</sup> DM) and concentrate (70 g kg<sup>-1</sup> DM). Sand, which was only detected in chicory silage, resulted in corrected ash content (crude ash minus sand) of 135 g kg<sup>-1</sup> DM chicory silage. Ryegrass/clover hay had the highest NDF content (577 g kg<sup>-1</sup> DM), followed by chicory silage (326 g kg<sup>-1</sup> DM) and concentrate (286 g kg<sup>-1</sup> 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<sup>-1</sup> DM), with 11,13-dihydro-8-deoxylactucin (DI-8-DOL) and 11,13-dihydro-lactucopicrin (DI-LCP) as the main SL (Table 1).

**Feed intake.** Mean daily intakes of DM, CP and ME over the experiment are presented in Table 2.

Table 2. Estimated dry matter, crude protein and metabolizable energy intakes in stabled calves fed with chicory silage and concentrate (Chicory) or ryegrass/clover hay and concentrate (Control) in Experiment 1

Group	Day post infection							
	0	7	14	21	28	35	40	
Chicory (n = 9)	Daily mean intake 100 kg BW <sup>-1</sup>	192.7 (±26.5)	192.7 (±26.5)	192.7 (±26.5)	192.7 (±26.5)	192.7 (±26.5)	192.7 (±26.5)	
	DM (kg)	1.7 (±0.1)	2.3 (±0.2)	2.4 (±0.1)	3.0 (±0.2)	3.1 (±0.1)	3.1 (±0.2)	3.0 (±0.2)
	CP (g)	12.9 (±1.4)	17.8 (±0.9)	18.9 (±0.5)	23.1 (±0.2)	23.0 (±0.2)	23.4 (±1.1)	22.9 (±1.1)
Control (n = 6)	Daily mean intake 100 kg BW <sup>-1</sup>	236.9 (±5.2)	236.9 (±5.2)	236.9 (±5.2)	236.9 (±5.2)	236.9 (±5.2)	236.9 (±5.2)	236.9 (±5.2)
	DM (kg)	2.0 (±0.2)	2.5 (±0.2)	2.6 (±0.5)	2.9 (±0.4)	3.1 (±0.5)	3.2 (±0.6)	3.1 (±0.6)
	CP (g)	16.8 (±0.9)	20.0 (±0.9)	21.3 (±3.2)	24.3 (±2.3)	26.2 (±2.9)	26.5 (±3.1)	25.9 (±2.9)

Data is based on daily measurements from subgroups of three calves and presented as daily mean intakes (±S.D.) of the preceding 5/7 days for a standard calf of 100 kg body weight (BW).

DM, dry matter; CP, crude protein; ME, metabolizable energy.

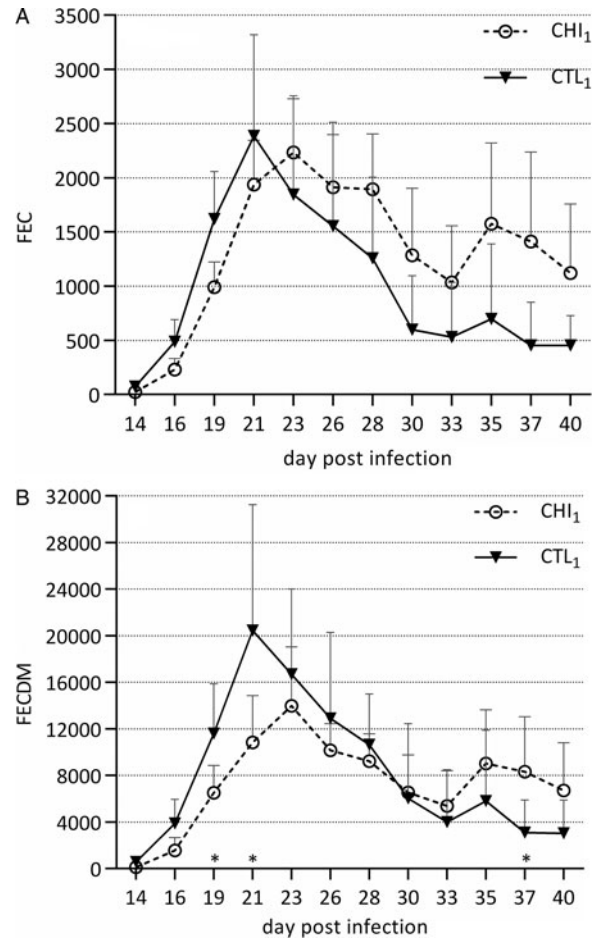


Fig. 3. Arithmetic mean fecal egg counts (FEC; A) and FEC per g of fecal dry matter (FECDM; B) in stabled calves experimentally infected with *Ostertagia ostertagi* and *Cooperia oncophora* and fed with chicory silage and concentrate (CHI<sub>1</sub>) or ryegrass/clover hay and concentrate (CTL<sub>1</sub>) in Experiment 1. Error bars indicate S.D. \*P < 0.05.

A comparable increase in DM intake was recorded in CHI<sub>1</sub> and CTL<sub>1</sub> animals during the trial. At D0 p.i., the DM intake in the CHI<sub>1</sub> group was composed of 76% chicory silage, whereas the CTL<sub>1</sub> 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 CHI<sub>1</sub> and CTL<sub>1</sub> 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 CTL<sub>1</sub> 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,



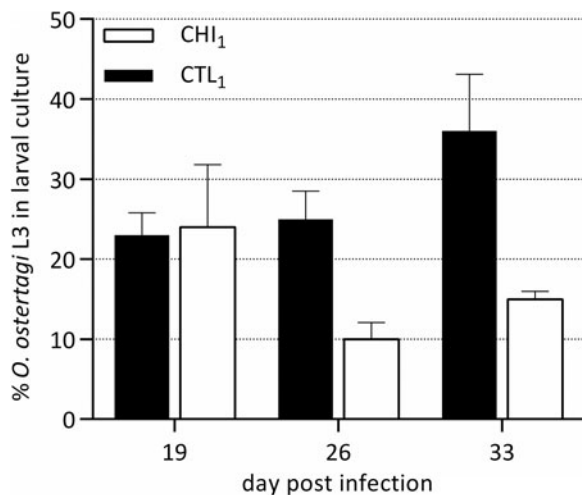


Fig. 4. Arithmetic mean percentage of *Ostertagia ostertagi* third-stage larvae (L3) identified in pooled fecal larval cultures of stabled calves experimentally infected with *O. ostertagi* and *Cooperia oncophora* and fed with chicory silage and concentrate (CHI<sub>1</sub>) or ryegrass/clover hay and concentrate (CTL<sub>1</sub>) in Experiment 1. Error bars indicate s.d.

D21 and D37 p.i. Higher FECDM was observed initially in CTL<sub>1</sub> (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 CHI<sub>1</sub> throughout the study period. However, the cumulative FECDM during the entire experiment was not significantly different between groups [mean (± s.d.) CHI<sub>1</sub> = 204 280 (±65 264) vs CTL<sub>1</sub> = 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 CHI<sub>1</sub> and CTL<sub>1</sub> 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 CHI<sub>1</sub> calves.

Post-mortem worm recovery in CHI<sub>1</sub> and CTL<sub>1</sub> 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 CTL<sub>1</sub> animals (*P* < 0·01). In contrast, adult counts of *C. oncophora* were not statistically different between groups (*P* = 0·12). In CTL<sub>1</sub> 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 CHI<sub>1</sub> and CTL<sub>1</sub> calves throughout the trial (Fig. 5). Mean (±s.d.)

Table 3. Worm counts of adult *Ostertagia ostertagi* and *Cooperia oncophora* detected post-mortem in stabled calves fed with chicory silage and concentrate (Chicory) or ryegrass/clover hay and concentrate (Control) in Experiment 1

Group	<i>O. ostertagi</i> adult worm counts			<i>C. oncophora</i> adult worm counts		
	Males	Females	Total	Males	Females	Total
Chicory ( <i>n</i> = 9)	878* (175–1915)	984** (250–1800)	1862** (425–3715)	17 537 (6750–25 450)	19 267 (11 200–25 750)	36 804 (17 950–51 200)
Control ( <i>n</i> = 6)	1747 (1205–2235)	2152 (1555–2960)	3899 (2760–5195)	10 392 (800–29 200)	12 750 (800–27 450)	23 142 (1600–56 650)

Results are presented as arithmetic means (range). \**P* < 0·05; \*\**P* < 0·01.

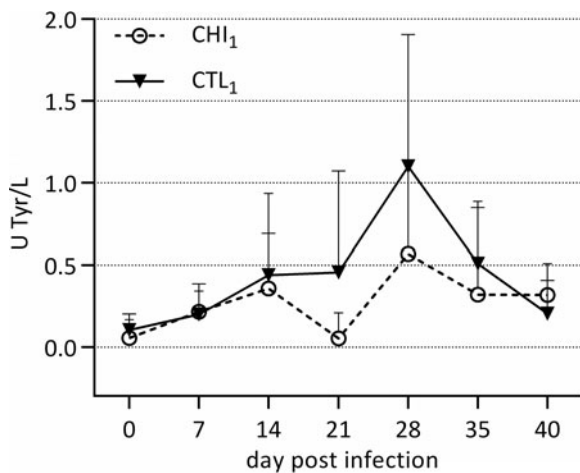


Fig. 5. Arithmetic mean serum pepsinogen levels (in units tyrosine; U Tyr/L) in stabled calves experimentally infected with *Ostertagia ostertagi* and *Cooperia oncophora* and fed with chicory silage and concentrate (CHI<sub>1</sub>) or ryegrass/clover hay and concentrate (CTL<sub>1</sub>) in Experiment 1. Error bars indicate s.d.

serum pepsinogen levels from D7 until D40 p.i. in CHI<sub>1</sub> and CTL<sub>1</sub> were 0.31 ( $\pm$  0.2) U Tyr/L and 0.48 ( $\pm$  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  $\times$  time interaction affected pepsinogen values ( $P > 0.18$ ). Serum TP and ALB levels were not affected by group or group  $\times$  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 influenced by time ( $P < 0.001$ ), but additionally group  $\times$  time interaction had a significant effect ( $P = 0.006$ ), with decreasing IP values during the trial in both groups but with consistently lower IP levels in the CTL<sub>1</sub> group (data not shown).

**Animal weights.** Bodyweight gains were significantly affected by time and group  $\times$  time interaction ( $P < 0.001$  and  $P = 0.004$ , respectively), with increasing BW in both groups but higher growth rates in CHI<sub>1</sub> calves. Similar mean ( $\pm$  s.d.) daily growth rates were observed in both groups until D19 p.i. [CHI<sub>1</sub> = 383 ( $\pm$  116) *vs* CTL<sub>1</sub> = 325 ( $\pm$  236) g day<sup>-1</sup>]. In contrast, from D19 until D37 p.i., CHI<sub>1</sub> and CTL<sub>1</sub> groups had mean growth rates of 811 ( $\pm$  59) and 391 ( $\pm$  132) g day<sup>-1</sup>, 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<sup>-1</sup> 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<sup>-1</sup> DM) and chicory silage (16.8 g total SL kg<sup>-1</sup> 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, CHI<sub>2</sub> calves had an estimated mean daily intake of 5.7 kg DM calf<sup>-1</sup> 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 CTL<sub>2</sub> animals had an estimated daily intake of 6.9 kg DM ryegrass/clover calf<sup>-1</sup> 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 CTL<sub>2</sub> animals from D24 p.i. onwards, with a mean ( $\pm$  s.d.) fecal DM% at slaughter of 18.72 ( $\pm$  1.9) and 12.40 ( $\pm$  2.2)% in CHI<sub>2</sub> and CTL<sub>2</sub> 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 CHI<sub>2</sub> and CTL<sub>2</sub> were 1589 and 1474 epg, respectively ( $P = 0.7$ ). From this point, mean FECDM in the CHI<sub>2</sub> group was rapidly and significantly reduced until the end of the trial: at D35 p.i., CHI<sub>2</sub> animals had a 65% lower mean FECDM compared with CTL<sub>2</sub> calves ( $P < 0.01$ ). Cumulative FECDM during the entire experiment was significantly reduced in the CHI<sub>2</sub> group [mean ( $\pm$  s.d.) CHI<sub>2</sub> = 12 870 ( $\pm$  8136) *vs* CTL<sub>2</sub> = 22 260 ( $\pm$  5671) epg of fecal DM;  $P < 0.01$ ].

Post-mortem worm recovery of CHI<sub>2</sub> calves revealed significantly lower numbers of *O. ostertagi* males [mean (range) CHI<sub>2</sub> = 2106 (283–5425) *vs* CTL<sub>2</sub> = 4692 (2775–6800) male worms;  $P < 0.01$ ] and females [CHI<sub>2</sub> = 2256 (617–4100) *vs* CTL<sub>2</sub> = 5386 (3700–6700) female worms;  $P < 0.001$ ], compared with CTL<sub>2</sub> 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) CHI<sub>2</sub> = 4362 (900–9525) *vs* CTL<sub>2</sub> = 10 078 (6475–13 325) total

Table 4. Chemical composition of feeds consumed by calves in Experiment 2

	Experimental feeds		
	Fresh forage chicory	Chicory silage	Ryegrass/clover
DM (%)	11.5 (±4.3)	23.5	22.0 (±2.1)
CP (g kg <sup>-1</sup> DM)	98.2 (±32.1)	117.2	159.8 (±4.1)
ME (MJ kg <sup>-1</sup> DM)	7.2 (±0.9)	8.6	8.3 (±0.5)
AAT (g kg <sup>-1</sup> DM)	67.5 (±6.4)	66.0	80.1 (±4.2)
OMD (% of DM)	63.1 (±9.5)	74.5	67.1 (±4.7)
Crude ash (g kg <sup>-1</sup> DM)	150.2 (±30.7)	147.2	108.6 (±5.2)
Sand (g kg <sup>-1</sup> DM)	17.0 (±1.4)	5.0	22.0 (±11.3)
NDF (g kg <sup>-1</sup> DM)	260.5 (±54.5)	326.4	430.2 (±24.3)
CT <sup>a</sup> (% DM)	n.d.	n.d.	n.d.
SL <sup>b</sup> (g kg <sup>-1</sup> DM)	22.5	16.8	0.0
DI-LAC	5.9	3.4	0.0
LAC	6.1	2.7	0.0
8-DOL	3.1	1.5	0.0
DI-8-DOL	3.5	4.1	0.0
DI-LCP	3.5	4.6	0.0
LCP	0.4	0.5	0.0

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.

DM, dry matter; CP, crude protein; ME, metabolizable energy; AAT, aminoacid absorbed in the intestine; OMD, *in vivo* organic matter digestibility; NDF, neutral detergent fibre; CT, condensed tannins; SL, sesquiterpene lactones; LAC, lactucin; DI-LAC, 11, 13-dihydro-lactucin; 8-DOL, 8-deoxylactucin; DI-8-DOL, 11, 13-dihydro-8-deoxylactucin; LCP, lactucopicrin; DI-LCP, 11, 13-dihydro-lactucopicrin; n.d., not detected.

<sup>a</sup> According to Grabber *et al.* (2013); estimated detection limit = 0.2% of CT in DM).

<sup>b</sup> According to Peña-Espinoza *et al.* (2015).

worms;  $P < 0.001$ ]. Per capita fecundity of *O. ostertagi* females was not statistically different between groups [mean (range) CHI<sub>2</sub> = 0.19 (0.05–0.39) vs CTL<sub>2</sub> = 0.25 (0.12–0.39) eggs female<sup>-1</sup> per g fecal DM;  $P = 0.24$ ].

**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.) CHI<sub>2</sub> = 1.02 ± 0.4 U Tyr/L; CTL<sub>2</sub> = 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 significantly affected by time ( $P < 0.001$ ), with equivalent and declining serum concentrations in both groups over the trial, and a significant effect of covariate ( $P < 0.05$ ) (data not shown). Serum IP concentrations were significantly influenced by covariate ( $P = 0.005$ ), group ( $P = 0.008$ ) and time ( $P < 0.001$ ), illustrated by constantly lower IP values in the CTL<sub>2</sub> group, but with decreasing IP levels in both groups until D21 p.i. and comparable values between groups at slaughter (data not shown).

**Animal weights.** Bodyweight gains were significantly influenced by group, time and group × time interaction ( $P < 0.001$ ), with mean (±s.d.) growth rates of 374 (±160) g day<sup>-1</sup> and 783 (±229) g day<sup>-1</sup> in CHI<sub>2</sub> and CTL<sub>2</sub> calves, respectively.

## DISCUSSION

In the present study, we demonstrated that cattle experimentally 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 experimental 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 CHI<sub>1</sub> and CTL<sub>1</sub> calves is explained by the unaffected *C. oncophora* population, which represented 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

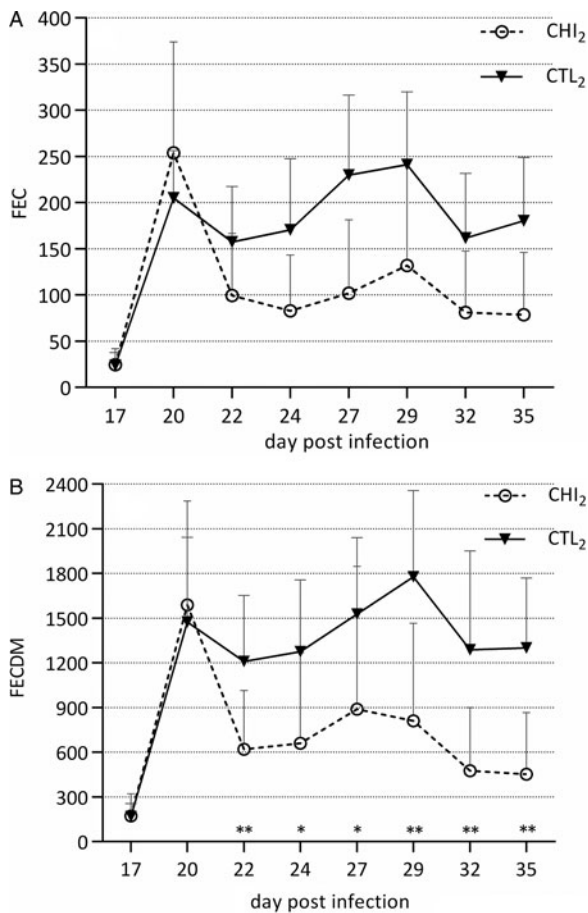


Fig. 6. Arithmetic mean fecal egg counts (FEC; A) and FEC per g of fecal dry matter (FECDM; B) in calves experimentally infected with *Ostertagia ostertagi* and grazing pure forage chicory (CHI<sub>2</sub>) or ryegrass/clover (CTL<sub>2</sub>) in Experiment 2. Error bars indicate S.D. \**P* < 0.05; \*\**P* < 0.01.

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, CTL<sub>1</sub> calves had a lower mean FECDM compared with CHI<sub>1</sub> animals (only significant at D37 p.i.). Post-mortem analyses demonstrated lower mean *C. oncophora* counts in CTL<sub>1</sub> animals compared with CHI<sub>1</sub> 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 CHI<sub>2</sub> 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

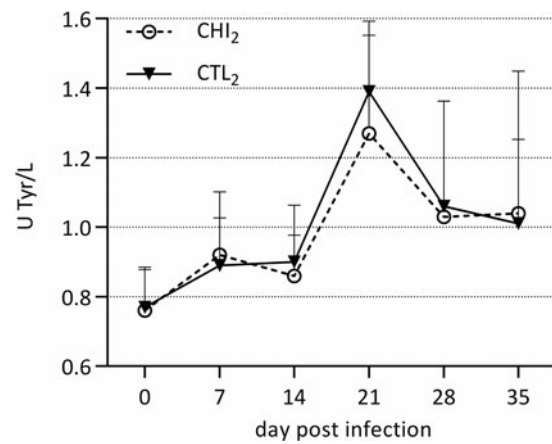


Fig. 7. Arithmetic mean serum pepsinogen levels (in units tyrosine; U Tyr/L) in calves experimentally infected with *Ostertagia ostertagi* and grazing pure forage chicory (CHI<sub>2</sub>) or ryegrass/clover (CTL<sub>2</sub>) in Experiment 2. Error bars indicate S.D.

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 (Vercruyse 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-naïve 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; Heckendorn *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 ( $\geq 70\%$  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  $\text{kg}^{-1}$  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 forage chicory. Several phytochemicals other than SL have been reported in chicory, e.g. derivatives of hydroxycinnamic acids (e.g. chicoric, chlorogenic and caffeic acid), coumarins (e.g. chicoriin) and flavonoids (Rees and Harborne, 1985; Heimler *et al.* 2009; Sinkovič *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 vivo* 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 CHI<sub>1</sub> and CTL<sub>1</sub> 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



'stomach tonic' effects in humans (Ahmed *et al.* 2003; Hitova and Melzig, 2014), which are believed to stimulate the appetite by increasing gastric secretions in the stomach. However, currently no studies have explored these effects in ruminants.

Experiments 1 and 2 had different study designs, which resulted in distinct voluntary intakes of chicory and animal growth between trials. In Exp. 1, we examined the effects of a chicory-rich diet against GIN while excluding nutritional-related effects by matching protein/energy intakes between groups with a commercial concentrate. As a result, chicory constituted ~70% of the daily DM intake in Exp. 1. In comparison, in Exp. 2 we investigated the anthelmintic effects of a pure chicory diet (>90% chicory DM intake) against *O. ostertagi* infections, without balancing nutritional levels between groups, but aiming for calves to exercise their voluntary feeding intake in the respective swards. In both experiments, chicory was readily eaten by the animals. In Exp. 1, significantly higher growth rates were detected in calves fed chicory silage, particularly from D19 p.i. onwards, and despite the comparable protein/energy intakes in both groups and the short duration of the experiment. Whether the markedly higher weight gain in chicory calves from D19 p.i. was related to the lower *O. ostertagi* worm burden in these animals, and/or to a higher nutritional value (e.g. in macro/micronutrients) of chicory silage compared with ryegrass/clover hay, was not further investigated. In contrast, weight gains in Exp. 2 were significantly lower in chicory-fed animals compared with controls. This was most likely due to the poor nutritional quality and low amounts of chicory on the pasture and thus lower DM intake (i.e. 5.7 vs 6.9 kg DM calf<sup>-1</sup> for CHI<sub>2</sub> and CTL<sub>2</sub>, respectively). In Exp. 2, our main problems were the rapid progression of the chicory plants into the reproductive stage from June 2014 onwards (2nd year sward), the very limited regrowth following the cut of the chicory sward in early July 2014 and the lower protein content of fresh chicory, as compared with the ryegrass/clover pasture. By the second half of Exp. 2 (late August 2014), the pure chicory sward was in the reproductive stage with few areas still containing chicory leaves, and therefore the calves needed to be supplemented with chicory silage. Despite this supplementary feeding, which also had lower protein content than the control pasture, the poor growth of the CHI<sub>2</sub> group was not reversed during the study period. Thus, close monitoring of the DM available in pure chicory swards grazed by parasitized calves, particularly during periods of rapid reproductive stem growth, seems to be critical to secure that animal production goals are met.

Finally, our findings suggest that the inclusion of a high percentage of fresh or conserved (ensiled) chicory in the diet of infected cattle could represent

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.

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