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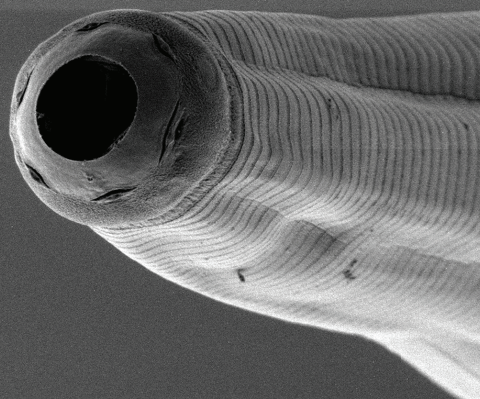
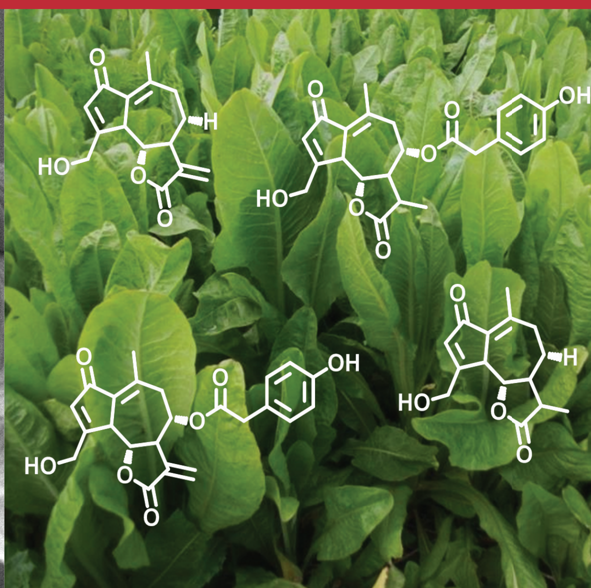
Anthelmintic activity of forage chicory (Cichorium intybus) and field efficacy of ivermectin against gastrointestinal nematodes in Danish cattle

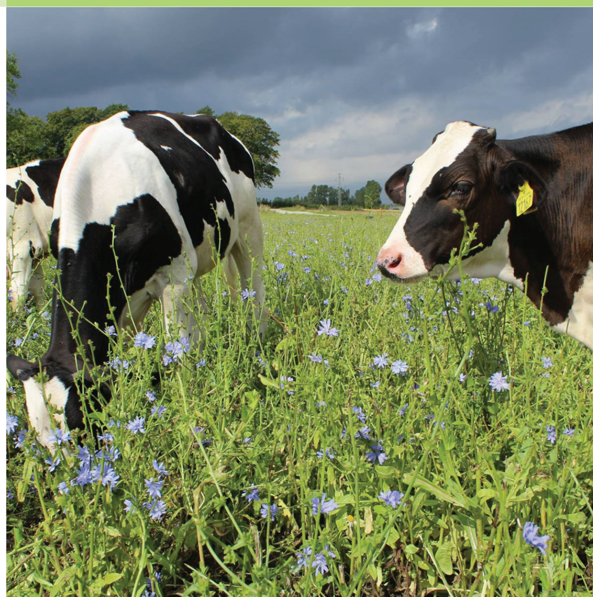
PhD thesis





Miguel Peña-Espinoza

2016



DTU Vet
National Veterinary Institute

Anthelmintic activity of forage chicory (*Cichorium intybus*) and field efficacy of ivermectin against gastrointestinal nematodes in Danish cattle

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PhD thesis

Section for Bacteriology, Pathology and Parasitology
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Preface

The research work presented in this PhD thesis was conducted between October 2012 and December 2015 in the Section for Bacteriology, Pathology and Parasitology and Section for Immunology and Vaccinology at the National Veterinary Institute, Technical University of Denmark, and in the Section for Parasitology and Aquatic Diseases, Department of Veterinary Disease Biology, University of Copenhagen. The PhD studies were funded by the EMIDA ERA-NET project “Coping with anthelmintic resistance in ruminants” (CARES; 3405-11-0430/32) and CONICYT Chile (Becas Chile, segunda convocatoria 2009).

The thesis is organized as a succinct review of gastrointestinal nematodes in cattle, anthelmintic resistance and the use of bioactive plants as an alternative helminth control approach, with focus on studies with forage chicory. Next, a brief description of the performed investigations is presented, including the experimental designs and methods, a summary of the major findings and conclusions, and final remarks on future research. The thesis culminates with the following scientific articles describing in detail the research work conducted during the PhD studies:

Manuscript I. Efficacy of ivermectin against gastrointestinal nematodes of cattle in Denmark evaluated by different methods for analysis of faecal egg count reduction. International Journal for Parasitology: Drugs and Drug Resistance (*in press*).

Manuscript II. Anthelmintic effects of forage chicory (*Cichorium intybus*) against gastrointestinal nematode parasites in experimentally infected cattle. Parasitology 2016, 143, 1279−1293.

Manuscript III. Sesquiterpene lactone containing extracts from two cultivars of forage chicory (*Cichorium intybus*) show distinctive chemical profiles and *in vitro* activity against *Ostertagia ostertagi.* International Journal for Parasitology: Drugs and Drug Resistance 2015, 5, 191−200.

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My years in Copenhagen have been an amazing experience of learning and discovery thanks to the company, inspiration and the big help of all my friends in Denmark, and now around the world. I feel really grateful to have met you all and to have shared so many brilliant moments in the dark winter days and the sunny summer nights. My wholehearted thanks to my sisters and brothers in the road, Paula, Giovanna, Miriam, Noelia, Teresa, Eva, Marcela, Alejandro, Olivier, Sundar, César, Leo, Laue, Sebastian, Kristian, Myriam, José and all the big CPH family. *Tusind tusind tak!!!*

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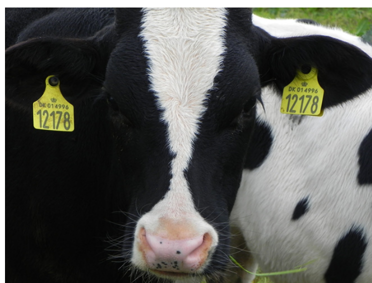
And finally, to each calf that was involved in our studies. Respectfully and gratefully, *moooooohchas gracias*!



Miguel Peña-Espinoza

Abstract

Parasitism with gastrointestinal (GI) nematodes is ubiquitous in grazing cattle and its control is critical to sustain satisfactory levels of animal health, welfare and productivity. Currently, conventional parasite control strategies relying exclusively on anthelmintic drugs are severely threatened by the development of anthelmintic resistance (AR) in bovine nematodes in several countries. In Denmark, cattle farmers and their veterinarians depend largely on macrocyclic lactones (ML), mainly ivermectin (IVM), for nematode control. However, no studies have evaluated the field efficacy of IVM against GI nematodes in Danish cattle. Alternative control approaches to minimize selection for AR and that can complement the use of the still effective anthelmintics in integrated control strategies are urgently needed. A promising complementary control strategy is to include bioactive forages with anti-parasitic effects in the animals' diets. Forage chicory (*Cichorium intybus*) has demonstrated anthelmintic effects against sheep nematodes, but comparable evidence in cattle is absent. The direct anthelmintic activity of forage chicory has been associated with its content of plant secondary metabolites (PSM), particularly sesquiterpene lactones (SL). Still, no studies have investigated the content of SL in chicory used as anthelmintic forage and no evidence exists of a direct effect of chicory SL against parasitic nematode stages. Thus, the main objective of the present investigations was to contribute towards the integrated control of bovine GI nematodes by evaluating the field efficacy of ivermectin in naturally-infected Danish cattle and by exploring the direct anthelmintic activity of forage chicory and its SL against bovine nematodes *in vivo* and *in vitro*.

Faecal egg count reduction tests (FECRT) were performed to evaluate the field efficacy of IVM against natural infections with GI nematodes (Manuscript I). Six cattle farms with a history of macrocyclic lactones use were included. At day of treatment (Day 0), 20 naturally infected grazing calves per farm were stratified by initial faecal egg counts (FEC) and randomly allocated to a treatment group (IVM; n=10) or an untreated control group (CTL; n=10). Calves in the IVM group were treated with 0.2 mg IVM kg^{-1} body weight (s.c.). Individual FEC were obtained at Day 0 and 14 days post-treatment and pooled larval cultures for species-specific detection of *Ostertagia ostertagi* and *Cooperia oncophora* by real-time PCR. Treatment efficacies were analysed as FEC reduction percentages (FECR%) using five different methods of calculations: the recommended WAAVP method (with and without CTL) and two Bayesian methods; Bayescount (with and without CTL) and EggCounts. A simulation study was performed to evaluate the performance of the three methods (without CTL) to correctly identify the FECR% of simulated populations with values as observed in three investigated farms. In the FECRT, reduced IVM efficacy was confirmed in three farms by all methods based on the FEC of treated animals only and in one farm according to the two methods including the FEC from treated and untreated cattle. Post-treatment, *C. oncophora* was detected in treated groups of all herds and *O.*

ostertagi was identified in one farm with confirmed reduced IVM efficacy. In the simulation study, high FEC aggregation markedly affected the performance of the methods of calculation. Furthermore, there is an urgent need for standardised international guidelines for the design and evaluation of FECRT in cattle. This is the first report of reduced efficacy of an anthelmintic in Danish cattle. However, due to the low number of enrolled farms in the FECRT, this study may not represent the general situation in Danish cattle. In addition, the presence of IVM-resistant nematode strains suggested by the FECRT in the investigated farms should preferably be confirmed by a controlled efficacy test. Yet, evidence of IVM inefficacy to reduce FEC in Danish cattle suggests that farmers and their advisors should closely monitor the efficacy of the anthelmintics treatments and include alternative control strategies to reduce their dependence on anthelmintics.

Two independent trials studied the direct anthelmintic effects of forage chicory-rich diets against GI nematodes in experimentally infected cattle (Manuscript II). In Experiment 1, 15 stabled, nematode-naïve Jersey-bull calves (body weight [BW]: 92±21 kg) were randomly allocated, after BW stratification, into chicory (CHI_1, n=9) or control (CTL_1, n=6) groups and fed *ad libitum* with forage chicory (cultivar [cv.] Spadona) silage or ryegrass/clover hay, respectively. Animals were supplemented with concentrate to balance protein/energy intakes between groups throughout the trial. After 16 days of adaptation to diets, all calves were infected with 10,000 *O. ostertagi* and 66,000 *C. oncophora* L3 (Day 0 post infection [p.i.]). In Experiment 2, 20 parasite-naïve Holstein bull-calves (153±24 kg) were stratified by BW and randomly assigned to pastures of either pure forage chicory (cv. Spadona; CHI_2, n=10) or ryegrass/clover (CTL_2, n=10). After 7 days, all calves were infected with 20,000 *O. ostertagi* L3 (Day 0 p.i.) and moved regularly to avoid re-infections from pasture. Due to poor regrowth of the chicory pasture, the CHI_2 group was supplemented with chicory silage. Faecal egg counts per g of faecal dry matter (FECDM) were performed in both trials. Condensed tannins (CT) and SL were analysed in all the experimental feeds. At Day 40 p.i. (Experiment 1) and Day 35 p.i. (Experiment 2) calves were slaughtered for worm counts. In Experiment 1, cumulative FECDM was not significantly different between groups, but lower numbers of *O. ostertagi* L3 were detected in pooled larval cultures of CHI_1 calves from Day 26 p.i. At slaughter, *O. ostertagi* counts were significantly reduced in CHI_1 by 60% (geometric mean reduction; $P<0.01$), whereas *C. oncophora* burdens were unaffected ($P=0.12$). In Experiment 2, FECDM were markedly lowered in CHI_2 from Day 22 p.i onwards ($P<0.01$) and *O. ostertagi* adult burdens were significantly reduced in CHI_2 by 66% ($P<0.001$). Condensed tannins were not detected in any of the experimental feeds and SL were only identified in forage chicory (fresh and silage) in both experiments, at levels ranging from 12.3 to 22.5 g SL kg^{-1} dry matter. Forage chicory-rich diets (≥ 70% of chicory dry matter in the diet) led to a significant reduction in adult worm burdens and FECDM of *O. ostertagi* in calves, while no activity was observed against *C. oncophora.* Chicory (fresh and silage) was readily consumed by calves and the anthelmintic effects were preserved despite ensiling.

To confirm the relationship between the observed *in vivo* anthelmintic effects of forage chicory and its content of SL, *in vitro* studies were performed to test the direct anti-parasitic activity of SL-containing chicory extracts against free-living and parasitic stages of *O. ostertagi* (Manuscript III). Total (free and unbound) SL were extracted from leaves of chicory cv. Spadona and cv. Puna II. *O. ostertagi* eggs isolated from mono-infected calves were hatched and L1 were used in a larval feeding inhibition assay (LFIA), while cultured L3 were used in a larval exsheathment inhibition assay (LEIA). Adult worms were immediately recovered after slaughter and used for motility inhibition assays (AMIA). Analyses by high-performance liquid chromatography of Spadona and Puna II extracts revealed the presence of lactucin (LAC), 8-deoxylactucin (8-DOL) and lactucopicrin (LCP). In addition, the 11, 13-dihydro (DI) derivatives of the mentioned SL were also detected. Between 60 – 65% of the tested extracts was composed of SL, while the remaining (unknown) compounds were not determined. Comparable levels of total and individual SL and unknown compounds were detected in both extracts; however, Spadona-extract contained significantly higher concentrations of DI-8-DOL (P=0.01), while Puna II-extract had increased levels of DI-LAC (P<0.0001). In the LFIA, both extracts reduced larval feeding at increasing concentrations, but Spadona extract showed higher potency confirmed by significantly lower EC_{50} (P<0.0001). In the LEIA, neither of the two extracts interfered with the exsheathment of L3 (P>0.05). In the AMIA, both extracts induced a dose-dependent worm paralysis effect but Spadona extract exerted a more rapid and lethal activity on adult worms than Puna II extract, with significantly lower EC_{50} (P<0.0001). Sesquiterpene lactone-containing extracts from forage chicory demonstrated a direct and potent anti-parasitic activity against *O. ostertagi* L1 and exerted a rapid and lethal paralysis of *O. ostertagi* adults, and these molecules may be the responsible active compounds for the anthelmintic activity of chicory. Differences in the anti-parasitic activity between extracts from two chicory cultivars seem related with their distinct content of SL and could help to further identify the most active compounds.

In conclusion, reduced IVM efficacy was confirmed in three of six Danish cattle farms and emphasizes the need to perform larger surveys, monitor the efficacy of prescribed anthelmintic treatments and to include alternative control methods to reduce the selection pressure for AR. Forage chicory-rich diets show promise as selective anti-*Ostertagia* feed for cattle and may help to reduce infection levels and the need for anthelmintic treatments in grazing cattle. However, further studies are needed to investigate the on-farm use of forage chicory as part of integrated parasite control strategies for cattle. Sesquiterpene lactone-containing chicory extracts demonstrated direct and dose-dependent anthelmintic effects against *O. ostertagi in vitro* and distinct potencies observed between chicory cultivars may be related to differences in their individual SL.

Sammendrag

Gastrointestinale nematoder (GIN), også kaldet løbetarmorm, er almindeligt forekommende hos græssende kvæg, og det er afgørende for opretholdelsen af sundhed, velfærd og produktivitet i kvægbruget at kontrollere disse parasitter. Flere steder i verden udgør udviklingen af ormemiddelresistens (OR) igennem de senere år imidlertid en alvorlig trussel mod den gængse parasitkontrol, der oftest udelukkende er baseret på anvendelse af ormemidler. I Danmark kontrolleres GIN hos kvæg overvejende ved anvendelse af ormemidler tilhørende gruppen af makrocykliske laktoner (ML), især ivermectin (IVM), men hidtil har ingen studier undersøgt effekten af disse medikamenter i danske kvægbesætninger. Alternative kontrolmetoder er nødvendige med henblik på at mindske selektionen for OR. Anvendelse af bioaktive planter med antiparasitær effekt i foderet har vist lovende resultater som et supplement til traditionelle kontrolforanstaltninger. Fodring med cikorie (*Cichorium intybus*) har således vist god effekt mod GIN hos får, men tilsvarende resultater overfor kvægets GIN findes ikke. Cikoriens anthelmintiske aktivitet er blevet sat i forbindelse med dens indhold af bioaktive plantemetabolitter, især stofferne sesquiterpene-laktoner (SL). Hovedformålet med de nærværende studier var derfor at bidrage mod en integreret kontrol af GIN hos kvæg i) ved at evaluere effekten af IVM i danske besætninger; og ii) ved at undersøge den anti-parasitære effekt af cikorieekstrakter og cikorie dels *in vitro* dels *in vivo* overfor kvægets GIN.

Fækale ægreduktionstests blev gennemført i 6 danske kvægbesætninger med henblik på at vurdere IVMs effekt overfor naturlige infektioner med GIN. Førsteårsgræssende kalve i hver besætning blev behandlet ved injektion med den anbefalde dosis IVM (CHI_1; n=10) eller de indgik i forsøget som ubehandlede kontroller (CTL_1; n=10). Den fækale ægudskillelse blev vurderet ved behandling samt 14 dage senere, og samtidig blev larvekulturer opsat for hver behandlingsgruppe med henblik på artsidentifikation af 3. stadielarver (L3) v.h.a. «real-time quantitative PCR». Reduktionsprocenten blev beregnet ud fra fem forskellige metoder, bl.a. gældende retningslinier fra World Association for the Advancement of Veterinary Parasitology (WAAVP, med eller uden CTL) og to Bayesiansk metoderne: EggCounts (uden CTL) and Bayescount (med eller uden CTL). Effekten af IVM var lavere end forventet i alle 6 besætninger, og nedsat effekt, defineret som en reduktionsprocent under 95% og en nedre grænse for 95% konfidensintervallet under 90%, blev bekræftet i 3 besætninger ved anvendelse af de tre metoder uden inklusion af ubehandlede kontroller. Efter behandling viste larvekulturerne, at tyndtarmsormen, *Cooperia oncophora* var til stede i alle de behandlede grupper, mens løbeormen, *Ostertagia ostertagi* blev fundet i en af besætningerne med bekræftet nedsat effekt of IVM.

Den direkte antiparasittære effekt af fodercikorie blev undersøgt i to uafhængige forsøg hhv. et opstaldnings- og et markforsøg. I Eksperiment 1 blev 15 nematode-naïve Jersey kalve (92±21 kg) tilfældigt fordelt (stratificeret efter vægt) mellem en cikorie- (CHI_1, n=9) eller kontrol- (CTL_1, n=6) gruppe, som blev fodret *ad libitum* med henholdsvis cikorie-ensilage af sorten Spadona eller med hø af kløvergræs. Med henblik på afbalancering af protein- og energiindtagelsen mellem grupperne blev foderet suppleret med kalvepiller (kraftfoder) igennem hele forsøgsperioden. Efter en adaptationsperiode på 16 dage blev kalvene inficeret med 10.000 *O. ostertagi* og 66.000 *C. onchophora* L3-larver (Dag 0 post infektion [p.i.]). I Eksperiment 2 blev 20 nematode-naïve kalve af racen Dansk Holstein (153±24 kg) stratificeret efter vægt og tilfældigt allokeret til marker med ren fodercikorie af sorten Spadona (CHI_2; n=10) eller kløvergræs (CTL_2; n=10). Alle kalvene blev eksperimentelt inficerede med 20.000 *O. ostertagi* L3-larver efter 7 dages adaptation (Dag 0 p.i.). Forsøgsgruppen (CHI_2) blev fodret supplerende med cikorie-ensilage p.g.a. ringe plantevækst i forsøgsperioden. Under begge eksperimenter blev der foretaget fækale ægtællinger pr. g tørstof (faecal egg counts per g of faecal dry matter [FECDM]), og koncentrationen af kondenserede tanniner (CT) samt SL i foderet blev analyseret. Ormebyrden i de eksperimentelt inficerede kalve blev optalt efter slagtning Dag 40 p.i. (Eksperiment 1) og Dag 35 p.i. (Eksperiment 2). Det kumulative antal æg pr. g tørstof var ikke signifikant forskellig mellem grupperne i Eksperiment 1, men fra Dag 26 p.i. blev der påvist lavere antal *O. ostertagi* i larvekulturerne fra CHI_1 kalvene. Ved slagtning var antallet *O. ostertagi* significant reduceret med 60% i CHI_1 gruppen (gennemsnitlig geometrisk reduktion; $P<0.01$), mens antallet af *C. oncophora* var uændret i forhold til kontrolgruppen ($P=0.12$). I Experiment 2, var FECDM markant lavere hos CHI_2 kalvene fra Dag 22 p.i ($P<0.01$) og antallet af *O. ostertagi* var signifikant reducet med 66% ($P<0.001$). Kondenserede tanniner kunne ikke påvises i foderet i hverken det ene eller det andet forsøg, og SL i koncentrationer mellem 12.3 og 22.5 g SL pr. kg fodertørstof blev udelukkende identificeret i cikorie (både frisk og ensileret). Fodring med cikorie i høje koncentrationer (≥70 % cikorie tørstof på diæt) medførte en significant reduktion i både ægudskillelsen og antallet af *O. ostertagi* hos kalvene, mens der ikke blev fundet nogen anthelmintisk effekt overfor *C. oncophora.* Kalvene optog villigt cikorien (frisk såvel som ensileret), og den anthelmintiske effect blev bevaret trods ensilering.

Med henblik på undersøgelse af sammenhængen mellem indholdet af SL i cikorien og de observerede anthelmintiske effekter *in vivo* blev der gennemført *in vitro* studier for at teste den direkte antiparasittære effect af SL-holdige ekstrakter overfor fritlevende og parasitære stadier of *O. ostertagi.* Sesquiterpene-laktoner blev ekstraheret fra cikoriesorterne Spadona og Puna II og testet i «larval feeding inhibition assay» (LFIA) vha. L1-larver isoleret fra kalve mono-inficerede med *O. ostertagi*. Tillige blev den anthelmintiske effekt overfor L3 testet ved «larval exsheathment inhibition assay» (LEIA) og adulte nematoder testet ved «motility inhibition assay» (AMIA) på *O. ostertagi* isoleret umiddelbart efter slagtning. Analyse af cikorieekstrakterne vha. "High-performance

væskekromatografi" (HPLC) afslørede forekomst af lactucin (LAC), 8-deoxylactucin (8-DOL) and lactucopicrin (LCP) samt disses 11, 13-dihydro (DI) derivater. Sesquiterpene-laktoner udgjorde mellem 60 og 65 % af de testede ekstrakter, mens de resterende, ukendte forbindelser ikke kunne bestemmes. Der blev fundet sammenlignelige koncentrationer af SL (total og individuelle) og "ukendte" forbindelser i ekstrakter fra begge cikoriesorter. Spadona-ekstrakt indeholdt signifikant højere koncentrationer af DI-8-DOL (P=0.01), mens Puna II havde højere koncentrationer af DI-LAC (P<0.0001). Der var en omvendt korrelation mellem stigende koncentrationer af ekstrakter fra begge cikoriesorter og fødeoptaget hos *O. ostertagi* L1-larver i LFIA-testen, men Spadona ekstrakt var mere potent end Puna II reflekteret i en signifikant lavere EC_{50}-værdi (P<0.0001). Ingen af ekstrakterne påvirkede L3-larverne i LEIA-testen (P>0.05), mens begge cikoriesorter inducerede en dosisafhængig paralyse af adulte orm i AMIA; Spadona ekstrakt udviste dog en hurtigere og mere dødelig aktivitet end Puna II, hvilket blev afspejlet i en signifikant lavere EC_{50}–værdi (P<0.0001). Ekstrakter af fodercikorie indeholdende SL havde således en direkte og potent antiparasitær effekt overfor *O. ostertagi* L1-larver og var i stand til at paralysere adulte *O. ostertagi* hurtigt og effektivt. Det er derfor sandsynligt, at disse molekyler er afgørende for cikoriens antiparasitære aktivitet. De markante forskelle i potens mellem de to cikoriesorter var tilsyneladende korrelleret med forskelle i SL profilerne.

Studierne gennemført i forbindelse med nærværende ph.d-afhandling viste nedsat effekt af IVM i 3 af 6 danske kvægbesætninger, hvilket understreger behovet for løbende overvågning af effekten af de ormemidler, som anvendes i danske besætninger samt for alternative kontrolmetoder, der kan reducere udviklingen af OR. Fodring med cikorie viste lovende resultater overfor *O. ostertagi*, og kan medvirke til at nedbringe infektionsniveauet samt behovet for behandling med ormemidler, men yderligere undersøgelser er nødvendige med henblik på implementering i praksis. Ekstrakter af cikorie indeholdende SL demonstrerede en direkte og dosisafhængig anthelmintisk effekt overfor *O. ostertagi in vitro*, og vores resultater tyder på, at forskellene i effekten mellem cikoriesorterne kan være relaterede til forskelle i deres indhold af SL.

Resumen

El parasitismo con nematodos gastrointestinales (GI) es frecuente en el ganado bovino a pastoreo y su control es crítico para sostener niveles satisfactorios de salud, bienestar y productividad animal. En la actualidad, las estrategias de control parasitario convencionales dependientes exclusivamente del uso de fármacos antihelmínticos están seriamente amenazadas por el desarrollo de resistencia antihelmíntica (AR) en nematodos bovinos en distintos países. En Dinamarca, los ganaderos y sus asesores veterinarios basan mayoritariamente el control parasitario del ganado bovino en el uso de lactonas macrocíclicas (ML), principalmente ivermectina (IVM). Sin embargo, ningún estudio a la fecha ha evaluado la eficacia en terreno de IVM contra nematodos GI en rebaños bovinos daneses. Más aún, enfoques alternativos de control que minimicen la selección de AR, y que se puedan complementar con el uso de los fármacos antihelmínticos aún efectivos en estrategias de control integrado, son urgentemente necesarios. Una potencial estrategia de control complementaria es la introducción de forrajes bioactivos con actividad antiparasitaria en la dieta de los animales. La achicoria forrajera (*Cichorium intybus*) ha demostrado efectos antihelmínticos contra nematodos del ovino, pero hasta el momento no existe evidencia comparada en bovinos. Los efectos antiparasitarios directos de la achicoria forrajera han sido asociados con la presencia de metabolitos secundarios en la planta, particularmente lactonas sesquiterpénicas (SL). No obstante, ningún estudio a la fecha ha investigado el contenido de SL en achicoria usada como forraje antihelmíntico y no existe evidencia de un efecto directo de SL de achicoria contra estadios parasitarios de nematodos. Por lo tanto, el principal objetivo de las presentes investigaciones fue contribuir al control integrado de nematodos GI del bovino a través del estudio de la eficacia en terreno de IVM contra infecciones parasitarias en ganado bovino danés y la investigación de la actividad antihelmíntica de achicoria forrajera y sus SL contra nematodos del bovino *in vivo* e *in vitro*.

Tests de la reducción de la oviposición (“Faecal egg count reduction tests”/FECRT) fueron realizadas para evaluar la eficacia en terreno de IVM contra infecciones naturales con nematodos GI (Manuscrito I). Seis predios bovinos con uso previo de ML fueron incluidos en los estudios. En el día del tratamiento (Día 0), 20 terneros a pastoreo naturalmente infectados en cada predio fueron estratificados en base al conteo de huevos de nematodos en las heces (“Faecal egg counts”/FEC) y asignados al azar a un grupo tratado (IVM; n=10) o a un grupo control no tratado (CTL; n=10) con niveles similares de FEC. Terneros en el grupo IVM fueron tratados con 0.2 mg IVM kg^{-1} peso vivo (s.c.). Muestras fecales fueron obtenidas el Día 0 y 14 días post-tratamiento para realizar FEC individuales y la preparación de cultivos de larvas por grupo para la detección de *Ostertagia ostertagi* y *Cooperia oncophora* por medio de PCR en tiempo real. La eficacia de los tratamientos fue analizada como el porcentaje de reducción de FEC (FECR%) usando cinco métodos de

cálculo distintos: el método sugerido por la WAAVP (con y sin grupo CTL) y dos métodos bayesianos; Bayescount (con y sin grupo CTL) y EggCounts. Además, se llevó a cabo un estudio de simulación para evaluar el rendimiento de los tres métodos (sin grupo CTL) para identificar correctamente FECR% de poblaciones de nematodos simuladas con valores observados en los rebaños investigados. En los FECRT, una eficacia reducida de IVM fue confirmada en tres predios por todos los métodos incluyendo el FEC de animales tratados solamente y en un predio por los dos métodos incluyendo el FEC de animales tratados y no tratados. Post-tratamiento, se detectó la presencia de *C. oncophora* en grupos tratados de todos los predios y *O. ostertagi* en un predio con una confirmada eficacia reducida de IVM. En el estudio de simulación, una alta agregación del FEC afectó el rendimiento de todos los métodos de cálculo. Guías armonizadas internacionales para el diseño y evaluación de FECRT en ganado bovino son urgentemente necesarias. Este es el primer estudio que reporta la eficacia reducida de un antihelmíntico en ganado bovino danés. Sin embargo, y debido al bajo número de predios investigados, este estudio no es representativo de la situación general en Dinamarca. Además, cepas de nematodos resistentes a IVM indicadas en el FECRT deben ser confirmadas por medio de un test de eficacia controlada, que incluye el tratamiento de animales y el conteo de nematodos post-mortem. Sin embargo, la evidencia de una eficacia reducida de IVM en ganado bovino danés sugiere que los productores y sus asesores técnicos deben monitorear atentamente la eficacia de los tratamientos antihelmínticos utilizados e incluir estrategias de control parasitario alternativas para reducir la dependencia a los antihelmínticos.

Dos ensayos independientes se llevaron a cabo para estudiar el efecto antiparasitario de dietas ricas en achicoria forrajera contra nematodos GI en bovinos infectados experimentalmente (Manuscrito II). En el Experimento 1, 15 terneros Jersey machos estabulados sin contacto previo con nematodos (peso vivo 92±21 kg) fueron asignados al azar, luego de randomización en base a peso vivo, a un grupo achicoria (CHI_1, n=9) o grupo control (CTL_1, n=6) de pesos similares y alimentados *ad libitum* con ensilaje de achicoria forrajera (cultivo [cv.] Spadona) o heno de ballica/trébol, respectivamente. Los animales fueron suplementados con concentrado para equilibrar los consumos de proteína/energía entre los grupos a lo largo del ensayo. Luego de 16 días de adaptación a las dietas, todos los terneros fueron infectados con 10,000 L3 de *O. ostertagi* y 66,000 L3 *C. oncophora* (Día 0 post-infección [p.i.]). En el Experimento 2, 20 terneros Holstein machos sin contacto previo con nematodos (153±24 kg) fueron estratificados en base a peso vivo y asignados al azar a praderas con achicoria forrajera pura (cv. Spadona; CHI_2, n=10) o con ballica/trébol (CTL_2, n=10). Luego de 7 días, todos los terneros fueron infectados con 20,000 L3 de *O. ostertagi* (Día 0 p.i.) y se rotaron regularmente para prevenir reinfecciones en pradera. Debido al pobre rebrote de la pradera de achicoria, el grupo CHI_2 fue suplementado con ensilaje de achicoria. Conteo de huevos en las heces por g de materia seca fecal (FECDM) fueron realizados en ambos ensayos. Taninos condensados (CT) y lactonas sesquiterpénicas (SL) fueron analizados en todos los forrajes y concentrados. Al Día 40 p.i. (Experimento 1) y Día

35 p.i. (Experimento 2) los terneros fueron eutanasiados para conteo de nematodos post-mortem. En el Experimento 1, no se detectaron diferencias significativas en el FECDM acumulativo entre grupos, pero se identificó un número reducido de L3 de *O. ostertagi* en los cultivos de larvas del grupo CHI_1 desde el Día 26 p.i. Post-mortem, la carga parasitaria de *O. ostertagi* fue significativamente reducida in CHI_1 en un 60% (reducción media geométrica; $P<0.01$), mientras que la carga de *C. oncophora* no fue afectada ($P=0.12$). En el Experimento 2, el FECDM fue marcadamente reducido en CHI_2 desde el Día 22 p.i. ($P<0.01$) y la carga de *O. ostertagi* adultos fue significativamente reducida en CHI_2 en un 66% ($P<0.001$). No se detectaron CT en ninguno de los alimentos experimentales y SL fueron identificadas sólo en achicoria forrajera (pradera y ensilaje) en ambos experimentos, a niveles entre 12.3 y 22.5 g SL kg^{-1} materia seca. Dietas ricas en achicoria forrajera (≥ 70% de achicoria materia seca en la dieta) indujeron una reducción significativa en la carga de nematodos adultos y FECDM de *O. ostertagi* en terneros, mientras que no se observó actividad contra *C. oncophora.* Achicoria (fresca y ensilaje) fue consumida sin problemas por los terneros y los efectos antihelmínticos se conservaron a pesar del ensilado.

Para confirmar la relación entre los efectos antihelmínticos *in vivo* de achicoria forrajera y su contenido de lactonas sesquiterpénicas (SL), se realizaron estudios *in vitro* para evaluar la actividad antiparasitaria directa de extractos de achicoria conteniendo SL contra estadios de vida libre y parasitarios de *O. ostertagi* (Manuscrito III). Se extrajeron SL totales de hojas de achicoria cv. Spadona y cv. Puna II. Huevos de *O. ostertagi* aislados de terneros infectados fueron eclosionados y las L1 usadas para el ensayo de inhibición de la alimentación larvaria ("larval feeding inhibition assay"/LFIA), mientras que L3 cultivadas se usaron para el ensayo de inhibición del desenvaine larvario ("larval exsheathment inhibition assay"/LEIA). Estadios adultos fueron aislados inmediatamente post-mortem y usados en el ensayo de inhibición de la motilidad ("adult motility inhibition assay"/AMIA). Análisis químicos con cromatografía líquida de alta eficacia de los extractos de Spadona y Puna II revelaron la presencia de las SL lactucin (LAC), 8-deoxylactucin (8-DOL) y lactucopicrin (LCP). Además, los 11, 13-dihydro (DI) derivados de las mencionadas SL fueron detectados. Entre el 60 – 65% del contenido de los extractos testeados correspondió a SL, mientras que los restantes compuestos (desconocidos) no fueron determinados. Niveles similares de SL totales e individuales y de compuestos desconocidos fueron detectados en ambos extractos; sin embargo, extractos de Spadona contenían concentraciones significativamente altas de DI-8-DOL ($P=0.01$), mientras que extractos de Puna II tenían altos niveles de DI-LAC ($P<0.0001$). En el LFIA, concentraciones crecientes de ambos extractos inhibieron la alimentación de las L1, pero el extracto de Spadona demostró una mayor potencia que fue confirmada por valores de EC_{50} significativamente menores ($P<0.0001$). En el LEIA, ninguno de los dos extractos impidió el desenvaine de las L3 ($P>0.05$). En el AMIA, ambos extractos causaron una parálisis dosis-dependiente en nematodos, pero el extracto de Spadona ejerció una actividad más rápida y letal en los nematodos adultos que el extracto de Puna II, con valores de EC_{50} significativamente

menores (*P*<0.0001). Extractos de achicoria forrajera conteniendo SL demostraron potentes y directos efectos antiparasitarios contra L1 de *O. ostertagi* y causaron una rápida y letal parálisis en estadios adultos de *O. ostertagi*. Las SL son probablemente los compuestos activos responsables por la actividad antihelmíntica de achicoria. Las diferencias en los efectos antiparasitarios entre extractos de dos cultivos de achicoria parecen estar relacionadas con su contenido particular de SL y podría ayudar a identificar los compuestos más activos.

En conclusión, se confirmó una eficacia reducida de IVM en tres de seis predios bovinos en Dinamarca, lo que recalca la necesidad de realizar estudios con un mayor número de rebaños, el monitoreo de la eficacia de los tratamientos antihelmínticos prescritos y el incluir métodos alternativos de control parasitario para reducir la selección de AR. Dietas ricas en achicoria forrajera tienen el potencial de ser usadas como forraje anti-*Ostertagia* en ganado bovino, lo que podría ayudar a reducir los niveles de infección parasitaria y la necesidad de efectuar tratamientos antihelmínticos. Sin embargo, se necesitan de estudios adicionales que investiguen el uso de achicoria forrajera en un contexto productivo en predios bovinos como parte de estrategias de control integrado de nematodos GI. Extractos de achicoria forrajera conteniendo SL demostraron efectos antihelmínticos directos y dosis-dependientes contra *O. ostertagi in vitro*, y las diferencias en potencias antiparasitarias entre cultivos de achicoria puede estar relacionada con su contenido particular de SL individuales.

Abbreviations

AMIA	adult motility inhibition assay
AR	anthelmintic resistance
BW	body weight
CP	crude protein
CT	condensed tannins
CET	controlled efficacy test
CI	confidence interval
cv.	cultivar
DI-8-DOL	11,13-dihydro-8-deoxylactucin
DM	dry matter
DMSO	dimethyl sulfoxide
EM	electron microscopy
EPG	nematode eggs per g of faeces
FEC	faecal egg counts
FECDM	faecal egg counts adjusted for faecal dry matter
FECR	faecal egg count reduction
FECRT	faecal egg count reduction test
FSG	first-season grazing
GI	gastrointestinal
GIN	gastrointestinal nematodes
ha	hectare
HPLC-MS	high-performance liquid chromatography with mass spectrometry
IVM	ivermectin
ITS-2	second internal transcribed spacer
L1/L2/L3	first/second/third-stage nematode larvae
LEIA	larval exsheathment inhibition assay
LFIA	larval feeding inhibition assay
MCMC	Markov chain Monte Carlo
ML	macrocyclic lactones
MOX	moxidectin
NDF	neutral detergent fibre
PBS	phosphate-buffer solution
p.i.	post-infection
PSM	plant secondary metabolites
s.c.	subcutaneous
SEM	scanning electron microscopy
SL	sesquiterpene lactones
TEM	transmission electron microscopy
TST	targeted selective treatment
VetStat	Danish system for surveillance of the veterinary use of drugs for production animals
WAAVP	World Association for the Advancement of Veterinary Parasitology

Introduction

Cattle farming is a human activity of vast socio-economic significance, contributing to societies in terms of food production and as source of income, agricultural diversification and asset savings for rural communities (FAO, 2002; Herrero et al., 2009; Swanepoel et al., 2010). However, the sustainability of cattle farming is facing multiple and remarkable challenges in the first half of the 21st century. The foreseen growing demand for animal products by 2050, mostly in low and middle-income economies, is projected to be met by higher yields from an increasingly constrained array of natural resources (Thornton, 2010). Concurrently, the widely recognized environmental impact of cattle farming, mainly caused by greenhouse gas emissions, land and water use, and the food-feed competition resulting from the use of human-edible crops for livestock feeding, are expected to augment following the increasing pace of food production (Herrero et al., 2011; Makkar, 2016).

Generally speaking, the main strategies suggested to address these concomitant challenges are: to increase the efficiency of livestock systems aiming to maximize productivity, in order to reduce the environmental impacts per unit of product (Thornton, 2010; FAO, 2014), and to reduce the consumption of animal products, hence leading to shrinking of livestock numbers and their environmental impacts (McMichael et al., 2007; Foley et al., 2011). A complementary approach is to increase the feeding of ruminants with feed components that do not directly compete with human consumption ("not food-competing feedstuffs"), like grasslands, crop residues or by-products from food production (Eisler et al., 2014). Although the production from roughage-fed cattle systems is expected to be lower than from concentrate-fed cattle, thus resulting in a lower carbon footprint for the latter, the lower production efficiency from the first approach is offset by the benefit of reducing the production of human-edible crops for livestock in arable lands (Schader et al., 2015). As a result, it has been suggested that a global food system with pasture-based ruminant products can provide sufficient food for the expected increase in global demand and to reduce livestock environmental impacts (Schader et al., 2015), although with reductions in the consumption of animal products particularly from monogastric animals (usually fed with food-competing feeds). Furthermore, well-managed grasslands can also act as "carbon sinks" (FAO, 2010; Smith, 2014) and access to pasture has been related with improved animal health and welfare parameters (Burow et al., 2011; 2013).

Certainly, the success of current and future pasture-based cattle systems will depend on permanent advancements in animal breeding, nutrition and health. This PhD thesis tries to contribute to this wider goal through the study of novel strategies for the control of gastrointestinal nematodes in cattle, which remain the most prevalent infections in grazing livestock in Denmark and worldwide.

Chapter 1: Background

1.1 Gastrointestinal (GI) nematodes of cattle: *Ostertagia ostertagi* and *Cooperia oncophora*

Parasitic infections with GI nematodes are ubiquitous in grazing cattle of all ages, inducing clinical and subclinical disease with negative consequences for animal health, welfare and productivity. Worldwide, the most common GI nematodes infecting cattle are from the genus *Haemonchus*, *Ostertagia*, *Trichostrongylus*, *Cooperia* and *Nematodirus* (Armour, 1989; Taylor *et al.*, 2016). In Denmark, as in other temperate regions, the most prevalent and economically important GI nematodes are *Ostertagia ostertagi* and *Cooperia oncophora* (Nansen et al., 1993; Monrad, 2010), both belonging to the order *Strongylida*, family *Trichostrongylidae* (Anderson, 2000).

1.1.1 Life cycle

Ostertagia ostertagi (Stiles, 1982) infects the abomasum of cattle while *C. oncophora* (Railliet, 1898) parasitise the small intestine, with both species having a direct life cycle (Fig. 1.1). In *O. ostertagi*, eggs at the morula stage are passed with the faeces to the pasture. Inside the dung pat, eggs develop into L1, which then moult to L2 until reaching the infective L3 stage that retains the cuticle (sheath) of the L2. The development from egg to L3 varies according to the temperature in the dung pat, and under optimal conditions (25°C) can occurs within 7 days (Pandey, 1972). Ensheathed L3 actively migrate outside the dung pat or are translocated by rain splash into the surrounding pasture (Grønvold and Høgh-Schmidt, 1989). When cattle consume the infective larvae with the contaminated herbage, the L3 undergo exsheathment in the rumen and migrates to the abomasum, infecting the abomasal gastric glands. Inside the glands, *O. ostertagi* develop into the L4 and L5 stages, and approximately 18 days post-infection, the L5 emerge from the gastric glands and the worms moult into the adult stage, which can be found on the abomasal mucosa. After mating, female worms start laying eggs around 21 days post-infection (pre-patent period), which are passed to the pasture with the faeces. Some infective L3 ingested by cattle during the end of the grazing season (autumn) do not develop into adult worms in three weeks as described above; instead they remain "arrested" as early L4 (hypobiotic larvae) in the gastric glands (Michel, 1974). Arrested L4 usually resume their development in late winter or early spring, and the synchronized emergence of a large number of L5 into the abomasal lumen can lead the clinical condition known as "Ostertagiosis Type II" (Entrocasso et al., 1986).

The life cycle of *C. oncophora* is essentially similar to that described for *O. ostertagi*, apart from the fact that infective L3, which undergo exsheathment in the abomasum, migrate to the small intestine where the nematodes develop into L4, L5 and adult worms, with the first females laying eggs as soon as 15 days post-infection (Frankena, 1987). Arrested development has also been described in *C. oncophora* (Michel et al., 1978)

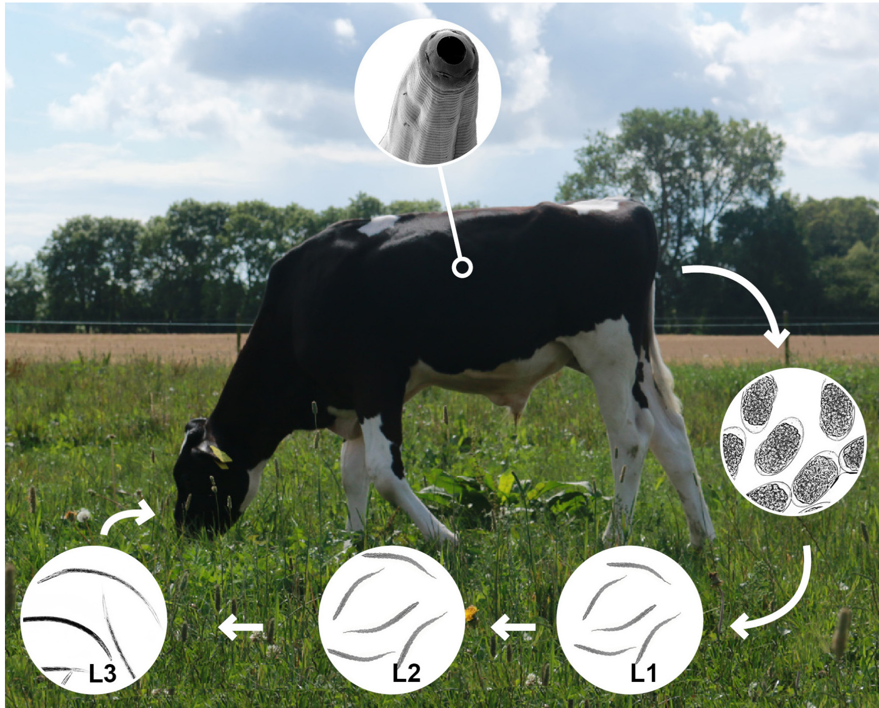


Fig. 1.1. Life cycle of *Ostertagia ostertagi*. Development from egg to L3 occurs inside the dung pat and the L3 must migrate onto the herbage to be ingested by the host and continue its development until adult stage.

1.1.2 Pathophysiology

1.1.2.1 *Ostertagia ostertagi*

Ostertagia ostertagi is commonly regarded as the most pathogenic cattle nematode in temperate areas due to the severe damage that can induce in the abomasum. The pathogenicity of *O. ostertagi* is mainly attributed to the emergence of L5 from the gastric glands and the presence of adult worms in the abomasal lumen, which can induce severe epithelial damage (resulting in protein loss to the lumen), loss of parietal cells and hyperplasia of mucous-secreting cells, inhibition of acid secretion (thus increasing abomasal pH and allowing survival of anaerobic bacteria) and increased serum gastrin and pepsinogen levels (McKellar et al., 1986; Taylor et al., 1989; Fox, 1997; Simpson, 2000). In chronic infections, macroscopic lesions in the abomasal mucosa can be observed, such as oedema, haemorrhages and formation of small nodules. Fox et al. (1989) reported that calves trickle infected with 10,000 *O. ostertagi* L3 daily during 46 days had significantly lower nitrogen digestibility and rate of passage of digesta and a marked reduction in voluntary feed intake. In subsequent studies, reduced feeding intake has been further confirmed as the main factor responsible for the reduced weight gain in infected calves (Fox, 1993; Fox, 1997; Forbes et al., 2000)

1.1.2.2 *Cooperia oncophora*

Cooperia oncophora has been considered a mild pathogen in cattle and a small number of studies have investigated its pathological effects. Coop et al. (1979) observed that calves daily infected with up to 20,000 *C. oncophora* L3 during 20 weeks had only a slight hypoalbuminemia and minor pathological damage in the small intestine, with a limited compression of the intestinal villi only in the areas with presence of worms, without penetration of the mucosa. In the same study, only a small reduction in weight gains were observed with no differences in the DM intake between infected or non-infected groups. In contrast, Armour et al. (1987) reported that calves daily infected with 10,000 *C. oncophora* L3 over six weeks had atrophied and thickened intestinal villi with excessive mucus production, loss of plasma protein into the intestinal lumen, reduced feed intake and weight loss; however, 12 weeks post-infection the authors observed a significant regeneration of the intestinal mucosa, associated with the expulsion of most of the worms, although loss of plasma protein to the intestine continued.

1.1.2.3 Interactions of *O. ostertagi* and *C. oncophora*

Parkins et al. (1990) reported that mixed infections with *O. ostertagi* and *C. oncophora*, which are the norm in the field, can induce larger pathological effects than mono-infections, leading to diarrhoea, weight loss, anorexia and hypoalbuminemia. However, the authors only studied the course of mixed infections, without comparing with mono-infections under the same conditions. Satrija and Nansen (1993) described watery diarrhoea in calves co-infected with *O. ostertagi* and *C. oncophora*, but did not observe clinical signs in calves mono-infected with *O. ostertagi* and no differences were detected in serum pepsinogen, albumin or weight gains between groups. In the same study, *C. oncophora* did not affect the establishment or the course of *O. ostertagi* infections.

1.1.3 Impact of GI nematodes on animal health and productivity

Clinical parasitism with GI nematodes, particularly in susceptible cattle, is usually manifested by poor body condition, anorexia, lower weight gain or weight loss, dull hair coat, (watery) diarrhoea, FEC>200 EPG and serum pepsinogen >5 unit of tyrosine (Eysker and Ploeger, 2000; Vercruysse and Claerebout, 2001). Although clinical disease can still be observed, it is widely recognized that the major impact of GI nematodes under a context of widespread use of (effective) anthelmintic drugs is at the subclinical level, inducing production loses without obvious clinical symptoms (Corwin, 1997; Charlier et al., 2014). Subclinical parasitism with GI nematodes has been reported to induce reduced growth rate (calves), loss of carcass yield and quality (heifers), decreased pregnancy rates (cows and heifers), reduce milk yield (cows) and longer calving to conception interval (primiparous and multiparous cows) (Gibbs, 1992; Hawkins, 1993; Shaw et al., 1998; Vercruysse and Claerebout, 2001; Charlier et al., 2009). Growth rates of FSG calves treated chemo-prophylactically with anthelmintics have been reported to increase by 150/315 g day compared with untreated calves suffering subclinical/clinical infections (Shaw et al., 1998), while in treated cows an average increase in milk yield of ~1 kg/day has been observed (Charlier et al., 2009). Also, it has been reported that reduced growth rates as a result of GI parasitism during the first grazing season can persist in heifers during their second grazing season, thus suggesting a lasting impact of helminth infections on animal production (Larsson et al., 2011).

1.1.4 Immune response

Cattle is able to develop acquired immunity against GI nematode infections, but the rate and extent of this response varies largely towards different worm species (Armour, 1989; Claerebout and Vercruysse, 2000). Under field conditions, a high level of immunity to *C. oncophora* is observed in FSG calves after around 4 months of exposure, illustrated by a marked reduction in FEC and adult worms at winter housing (Armour, 1989). Under experimental conditions, challenge with 100,000 *C. oncophora* L3 resulted in the expulsion of adult worms from most animals after 35-42 days post-infection (Kanobana et al., 2001). Yearling and adult cattle exposed to *C. oncophora* during previous grazing seasons have none or very low infection levels of this species (Armour, 1989), although *C. oncophora* adult stages can still be detected (yet very rarely) in cows (Borgsteede et al., 2000). In contrast, immunity towards *O. ostertagi* develops slower (Gasbarre, 1997) and calves at housing after their first grazing season can still have a high worm burden (Armour, 1989). Yearling cattle are still susceptible to new infections in their second grazing season, and a marked decrease in *O. ostertagi* burdens and/or reduce establishment of newly acquired L3 do not occur before the animals are >18-24 months-old (Williams et al., 1993; Gasbarre, 1997). However, a reduced egg output related with development of acquired immunity in young cattle exposed to *O. ostertagi* in the previous grazing season is clear (Grønvold et al., 1992; Gasbarre et al., 2001). Interestingly, it is known that the development of immunity to *O. ostertagi* in yearling cattle is inversely correlated to the efficacy (and intensity) of anthelmintic treatments performed during the first grazing season, which limit the exposure to infections and the resulting build-up of immunity (Vercruysse et al., 1994). Adult cattle can still harbour small numbers of *O. ostertagi* adults (Agneessens et al., 2000; Claerebout and Vercruysse, 2000) and have a very low faecal egg excretion, although enough to preserve the contamination of the pastures with this species (Armour, 1989).

1.1.5 Epidemiology

Similarly as in other North European countries, the epidemiology of GI nematodes of cattle in Denmark is determined by the presence of overwintered L3 on pastures as a source of infection for cattle turned out (~early May) on that fields (Nansen et al., 1978; 1987). Infective L3 ingested by FSG (naïve) calves develop into adult worms that generally reach a peak of faecal egg excretion around 2 months post-turn out, resulting in the accumulation of newly developed L3 on pasture from mid-summer onwards (Fig. 1.2). Under Danish conditions, development of L3 in the field is slow during the first half of the grazing season (<15°C) but rapidly increases as the temperature rises, leading to a "synchronized" larval development of infective larvae from mid-summer (Monrad, 2010). By late summer/early autumn, a high concentration of L3 is expected on the pastures which can lead to severe clinical disease of grazing animals, condition known as "Ostertagiosis Type I" (Henriksen et al., 1976; Nansen, 1987). Higher stocking rates exacerbates the level

of infections as animals are forced to graze more close to the faecal pats, where most of the infective L3 are present (Nansen et al., 1988). Susceptible calves are infected mainly with *C. oncophora* during the first-half of the grazing season, while contamination of pastures with *O. ostertagi* L3 increases and peaks from mid-summer until early autumn (Henriksen et al., 1976). However, unusual climatic conditions can significantly change the described pattern of pasture infectivity (Nansen et al., 1989). During very dry Danish summers in the mid-1970's, faecal pats were not disintegrated and infective L3 developed inside the dung did not migrate onto the grass (thus pasture infectivity remained low in late summer). The following spring, the same dung pats acted as reservoirs of a high number of overwintered larvae that were translocated onto the herbage, leading to clinical disease already on early spring/summer, termed "early-season Ostertagiosis Type I" (Nansen et al., 1989).

The infection dynamics described above imply that susceptible cattle are kept on the same permanent pasture the entire grazing season. Certainly, farming practices that reduce the contact with a high number of infective larvae may effectively prevent significant production losses and clinical parasitism. Differences in parasite exposure are greatly influenced by production systems and climatic factors, as recently described for *O. ostertagi* in dairy cows from different European countries (Bennema et al., 2010). Moreover, the influence of climate change on both the worm biology and livestock management is likely to further alter the epidemiology of pasture-borne parasitism in cattle (Morgan and Wall, 2009), to which the control strategies will need to adapt.

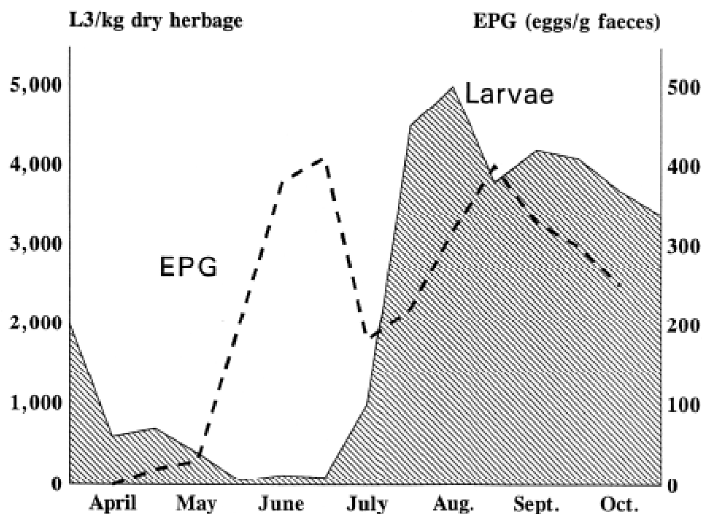


Fig. 1.2. Number of infective L3 on pasture (L3/kg dry herbage; hatched area) on a permanent pasture grazed by FSG calves in Denmark with turn-out mid-April. Faecal egg counts (EPG; dotted line) are presented as group means for each time point (adapted from Monrad, 2010).

1.2. Control of GI nematodes of cattle with anthelmintics

Since the release of the first broad-spectrum benzimidazoles in the 1960's and the advent of ivermectin and the macrocyclic lactones (ML) in the 1980's (Egerton et al., 1981), the use of synthetic anthelmintics has become the basis for the control of GI nematodes in intensively grazed cattle (Geurden et al., 2015; Sutherland and Bullen, 2015). Apart from the low toxicity of these drugs in treated animals and their initial high anti-parasitic efficacy when released, farmers also rapidly adopted anthelmintics because they could be used in different production systems without requiring major changes in husbandry practices (Nansen, 1993). In Denmark, drugs within three broad-spectrum anthelmintic classes with distinct modes of action are currently registered for use in cattle: *i)* benzimidazoles (e.g. fenbendazole, albendazole), *ii)* imidazothiazoloes/tetrahydro-pyrimidines (e.g. levamisole) and *iii)* ML (e.g. ivermectin, moxidectin, eprinomectin, etc.). Additionally, two novel broad-spectrum anthelmintic groups have been described: the amino-acetonitrile derivatives (ADD; Kaminsky et al. (2008) and the spiroindoles (Little et al., 2011). The ADD have a high efficacy against GI nematodes of ruminants and a commercial product from this group, monepantel, has been registered in the EU for use in sheep (EMA, 2009). In Denmark, as in other European countries, the ML are the most widely used anti-parasitics in cattle, mainly due to their high efficacy against adult and larval stages of parasitic nematodes and their persistent efficacy against re-infections, but also because of their effect against ectoparasites and availability as topical (pour-ons) formulations (Vercruysse and Rew, 2002).

1.2.1 Ivermectin: mechanisms of action and expected *in vivo* efficacy in cattle

Ivermectin (IVM) and other ML selectively increase the permeability of excitable membranes in the nervous system of parasitic nematodes (Gill and Lacey, 1998) by binding irreversibly to glutamate-gated chloride ion channels (and in higher concentrations also to γ-aminobutyric acid [GABA] and glycine receptors), interfering with the muscle contractions that regulate worm feeding, reproduction and movement (Cully et al., 1994; Kotze et al., 2014). However, not all cattle nematodes are equally susceptible to IVM. When IVM was released, clinical trials in cattle revealed that *C. oncophora* was the "dose limiting" species for this drug, requiring a minimum dosage of 0.2 mg IVM kg BW^{-1} (s.c. injection) to eliminate adults and L4 stages by ≥97% (Egerton et al., 1981). In contrast, *O. ostertagi* adults and L4 were eliminated by >99% with a dose of 0.05 mg IVM kg^{-1} BW s.c. (Egerton et al., 1981). Later research demonstrated that IVM can be detected at higher active concentrations in the abomasal mucosa, in comparison with the small intestine (Lifschitz et al., 2000). As a result, the recommended dose for injectable IVM was established to 0.2 mg kg BW^{-1}, but even at this dosage, small percentages of exposed worms are able to survive each treatment, thereby contributing to the selection of resistant nematode populations.

1.2.2 Anthelmintic resistance in GI nematodes of cattle

Anthelmintic resistance (AR) has been defined as the capacity of a worm population (or individual worms within the population) to tolerate doses of an anthelmintic that would have otherwise kill a normal population from the same species, and to transmit this "resistant" fitness to their progeny (Prichard et al., 1980). The accumulation of resistant genes in a nematode population is therefore an evolutionary process which depends on: *i)* the genetic diversity of the parasite populations under selection for AR, *ii)* the selection pressure (i.e. applications of anthelmintics) and *iii)* time (Prichard, 2002). Therefore, it can be expected that parasite populations exposed to anthelmintic drugs will evolve gradually from fully susceptible to fully resistant, and at different speeds under different circumstances (Kaplan et al., 2007).

Anthelmintic resistance is nowadays a widespread problem for the control of GI nematodes of small ruminants (Kaplan and Vidyashankar, 2012). In bovine nematodes, AR was originally sporadically identified (Jackson et al., 1987; Vermuntt et al., 1996; Stafford and Coles, 1999). However, since the 2000's, cases of AR in GI nematodes of cattle have been continuously reported in surveys worldwide, sometimes even involving multi-drug resistant populations (e.g.: Mejía et al., 2003; Waghorn et al., 2006; Soutello et al., 2007; Sutherland and Leathwick, 2011; Gasbarre, 2014; Geurden et al., 2015). In the majority of these studies, AR has been declared to ML, and in temperate regions mainly involving *C. oncophora*, although cases of AR in *O. ostertagi* developed in the field have also been confirmed (Edmonds *et al.* 2010; Gasbarre, 2014). In Scandinavia, the presence of AR in cattle nematodes has been only investigated in Sweden and clear indications were detected of reduced field efficacies of injectable and pour-on ML (Demeler et al., 2009; Areskog et al., 2013).

It is evident that, at the moment, the development and impact of AR in cattle has not reached the alarming levels observed in sheep and goat farming (Kaplan and Vidyashankar, 2012). In addition, the impact of an inefficacious treatment is likely to be more obvious in small ruminants (particularly if infected with *Haemonchus contortus*) than in cattle, where a reduced drug efficacy may only translates in subclinical production losses. Nevertheless, it has been suggested that some differences in the husbandry of cattle have limited the progression of AR in bovine helminths. One explanation is the higher level of the cattle nematode population *in refugia*, i.e. the proportion of the worm population not exposed to anthelmintics which is outside the host as free-living stages or in untreated animals (Coles, 2002). In cattle nematodes, worms *in refugia* are expected to be present mainly as free-living stages in the dung and on pasture, which may constitute > 99% of the entire parasite population in a farm (Nansen, 1993). In addition, adult cattle are not frequently treated with anthelmintics, in contrast with adult sheep, and this may have mitigated a rapid selection pressure (Prichard, 2002). Nevertheless, an intensification of treatments in adult

cattle, due to ease of administration and development of drug formulations without milk withdrawal to maximise production, may increase the selection for AR by decreasing the *in refugia* population (Stafford and Coles, 1999; Coles, 2002; Sutherland and Leathwick, 2011). Furthermore, topical (pour-ons) anthelmintics are widely used in cattle, and whether the varying drug concentrations observed in animals treated with these formulations can increase the selection for AR needs to be evaluated (Sargison et al., 2009; Sutherland and Bullen, 2015). Considering the current situation of AR in cattle nematodes, it seems opportune to reduce the selection pressure on the still effective anthelmintics before drug-resistance becomes a more extensive problem (Prichard, 2002; Taylor et al., 2010). A first step towards this goal is the precise detection of the drug efficacy on-farm.

1.2.3 Field detection of anthelmintic resistance in GI nematodes of cattle

In the absence of quantitative molecular techniques for detection of AR to ML and the high cost of the controlled efficacy test (the gold standard method to diagnose AR), at present the only readily available technique to diagnose AR on farms is the faecal egg count reduction test (FECRT). Various *in vitro* assays have also been described (Demeler et al., 2010; 2012), although they are currently not widely available.

The FECRT evaluates the efficacy of an anthelmintic by measuring the reduction in FEC in a treated group before and after treatment or between a treated and untreated group (Taylor et al., 2002). Current guidelines for the design and analysis of the FECRT in cattle are derived from recommendations developed for the detection of AR in sheep nematodes (WAAVP; Coles et al., 1992). Since then, several possible biases have been highlighted concerning the use of FECRT in cattle, mainly the high degree of density-dependence of cattle nematodes (thus reducing the correlation between FEC and actual worm burden), the lower faecal egg excretion commonly detected in cattle as compared with sheep, and the highly aggregated (over-dispersed) distribution of FEC between animals (Coles, 2002; Coles et al., 2006; Demeler et al., 2010; El-Abdellati et al., 2010; Sutherland and Leathwick, 2011). In addition, the inclusion of untreated control animals in the FECRT has been subject of much scientific debate (Dobson et al., 2012; Calvete and Uriarte, 2013; Lyndal-Murphy et al., 2014), and although untreated controls have been included in FECRT in cattle (Waghorn et al., 2006; Lyndal-Murphy et al., 2010), no field studies have compared the validity of such designs. Another problem is unreliable detection of AR when the drug efficacy is still high (80 – 95%), which prevents implementation of measures at an early stage to avoid the progression of AR into treatment failure. To reduce the uncertainty of the FECRT, much information on the true drug efficacy can be gained when studying the 95% confidence interval of a treatment (Levecke et al., 2012). Robust statistical analyses by Bayesian modelling using Markov chain Monte Carlo (MCMC) methods have been advocated to cope with low FEC data and to increase the accuracy in detecting uncertainty intervals (Denwood et al., 2010; Torgerson et al., 2014). These methods are being

increasingly used for monitoring AR in cattle nematodes (Neves et al., 2014; O'Shaughnessy et al., 2014; Geurden et al., 2015; Ramos et al., 2016). However, the influence of varying levels of aggregation observed in cattle FEC data on the performance of such Bayesian methods has not been evaluated. In addition, sensitive and species-specific tests to detect the nematode species surviving treatment are critical for a specific diagnosis of AR and are urgently required for cattle (Coles, 2002; Sutherland and Leathwick, 2011).

1.3 Alternative parasite control strategies

In parallel to the development of AR in worm populations, there has been an increasing public concern on the prophylactic use of veterinary drugs in food animals and the potential residues in the environment, as well as a wish to reduce the input of synthetic chemicals in organic and sustainable farming systems (Waller and Thamsborg, 2004; Hoste et al., 2014). All these factors have motivated the search for alternative and sustainable control methods that can complement or substitute the sole use of anthelmintics in farms (Thamsborg et al., 1999; Waller, 2006). Various alternative parasite control approaches to anthelmintics have been explored in ruminants (Hoste and Torres-Acosta, 2011). A common objective of these strategies is to reduce the parasite burden in the animals below a threshold of clinical or production impact, instead of achieve a high efficacy like anthelmintics, and to combine different methods as part of integrated parasite control programs (Thamsborg et al., 1999; Waller and Thamsborg, 2004; Ketzis et al., 2006). In general, an integrated parasite control program combines: *a)* strategies that reduced or eliminate the parasite burden in the host, *b)* strategies that increase the immunity and/or the resilience of the host to a parasite infection and *c)* strategies that lower the infection rate of the host (Hoste and Torres-Acosta, 2011). In cattle, some alternative or complementary approaches to synthetic anthelmintics for the control of GI nematodes have been investigated, like: pasture management, selection of resistant breeds, biological control and development of vaccines (Vercruysse and Dorny, 1999; Waller, 1999; Rinaldi and Geldhof, 2012; Vlaminck et al., 2015). However, one of the most promising novel approaches for nematode control, and not yet widely explored in cattle, is the use of bioactive plants to modulate the parasite biology.

1.4 Bioactive plants

In the Nordic countries, as in many other areas of the world, there is a long tradition of using plants with anthelmintic properties to treat parasitised animals (Waller et al., 2001). In less favoured regions of the world plants or plant extracts are normally used as dewormers, particularly where the access to synthetic anthelmintics is limited or costly. However, the believed anthelmintic activity of these plants is generally based on anecdotal evidence without scientific validation (Githiori et al., 2006). Yet, during the last decades, and largely driven by the development of AR in parasitic nematodes, extensive research has been conducted to confirm and validate the anthelmintic activity of several bioactive plants (Athanasiadou et al., 2007; Hoste et al., 2015). Most of these studies have explored the potential use of forages with anthelmintic activity as part of the animal's diets, as a nutraceutical approach (Waller and Thamsborg, 2004; Hoste et al., 2014). A nutraceutical, in the context of veterinary science, has been defined as any livestock feed which combines nutritional value with beneficial effects on animal health, and is usually offered to animals during several days or weeks (Hoste et al., 2015). Under this approach, the available scientific evidence indicates that bioactive plants may control parasitic infections by exerting direct anthelmintic effects in the host and/or indirectly by enhancing the immune response of the host to nematode infections through an improved nutrition (Hoste et al., 2012). The direct anthelmintic effects of plant nutraceuticals have been attributed to the activity of certain phytochemicals or plant secondary metabolites (PSM) (Barry et al., 2001; Athanasiadou and Kyriazakis, 2007). Condensed tannins (CT) have been the most widely investigated PSM for anthelmintic effects and promising results have been obtained using tannin-rich plants of the family *Fabaceae* (legumes), mainly against GI nematodes in small ruminants (reviewed by Hoste et al., 2006; 2012). Plants with PSM other than CT have also been examined for anthelmintic effects, like papaya, fig and pineapple containing cysteine proteinases (Stepek et al., 2004) and forage chicory containing sesquiterpene lactones (further discussed in Section 1.5).

To date, very few *in vivo* studies have examined the use of bioactive forages against GI nematodes of cattle, which may be attributed to less incentives due to limited development/diagnosis of AR in cattle nematodes and a stronger immunity of adult cattle to parasitic nematodes (compared to small ruminants), as well as higher cost of cattle as experimental animals (Sandoval-Castro et al., 2012). Nonetheless, evidence of direct anthelmintic activity of condensed tannins against free-living and parasitic stages of cattle nematodes has been confirmed *in vitro* (Novobilský et al., 2011; 2013; Desrues et al., 2016). Sievers and Nannig (2006) reported that calves with access to plantain (67.3% plantain DM in the field) during 7 h daily had significantly lower FEC of *Ostertagia* spp. and *Trichostrongylus* spp. in comparison with animals grazing ryegrass/clover and exposed to similar larval challenge from pasture. Xhomfulana et al. (2009) reported that Nguni and crossbreed cattle naturally infected with GI nematodes and supplemented with *Acacia*

karroo leaf meal (rich in CT) had lower FEC and worm burdens of *Haemonchus contortus* and *Oesophagostomum colombianum*, in comparison with non-supplemented controls. In the same study, a third group of cattle supplemented with sunflower cake (energy content not indicated) had also lower worm burdens compared to controls, thus, the anthelmintic effects of *A. karroo* leaf meal may have been a result of an improved nutrition due to supplementation rather than a direct anthelmintic activity of CT (Xhomfulana et al., 2009). Recently, Shepley et al. (2015) described that dairy heifers, naturally infected with GI nematodes and supplemented with mixed birdsfoot trefoil (32% birdsfoot trefoil DM in the mix) during 45 min daily, had significantly lower FEC compared with heifers grazing the same pasture but supplemented with alfalfa silage. Undoubtedly, substantial research is needed to further explore known and novel anthelmintic forages in cattle, with the aim to include them in integrated parasite control approaches and reduced the reliance on the still-effective anthelmintics.

1.4.1 Methods for evaluation of anthelmintic activity

Prior to on-farm use, the confirmation of the anthelmintic effects of bioactive forages and their PSM as well as their potential toxic effects must be evaluated in controlled studies (Githiori et al., 2006). Several *in vitro* assays and recommendations for *in vivo* studies have been proposed to investigate these issues, some of which are briefly described here.

1.4.1.1 *In vitro* assays

In vitro studies involve the incubation of GI nematodes with the plant extract or PSM of interest, aiming to test a direct effect on the parasite integrity or other biological processes, while excluding any host-related factors (Jackson and Hoste, 2010). *In vitro* studies can be performed as a preliminary screening of the best plant candidates for further *in vivo* trials and/or after *in vivo* studies to confirm the direct anthelmintic effects of plant compounds (Hoste et al., 2015). *In vitro* assays allow the testing of a wide range of plant compound/extract concentrations and using different nematode life stages. However, PSM concentrations tested *in vitro* may not correspond to the levels reaching the site of infection in the host, thus extrapolation of results from *in vitro* to *in vivo* conditions is complicated (Hoste et al., 2015). Detailed guidelines of *in vitro* assays have been described (Jackson and Hoste, 2010).

Several *in vitro* assays have been proposed to test the direct anthelmintic effects of plant compounds against free-living stages, most of them originally developed to investigate the anti-parasitic activity of synthetic anthelmintics (Coles et al., 1992; Demeler et al., 2010). *In vitro* assays with free-living stages are the most commonly used methods to test the anthelmintic activity of PSM and those relevant for the present PhD studies are summarised

here. The egg hatch assay (EHA) measures the ability of the compound of interest to inhibit the hatching of nematode eggs to L1, incubated under optimal conditions for hatching. The larval feeding inhibition assay (LFIA) investigates the inhibitory effects of a tested compound on the feeding behaviour of L1 exposed to fluorescein-labelled *Escherichia coli*; unaffected larvae are distinguishable by the presence of the labelled bacteria in their gut observed under fluorescence microscopy. The larval development assay (LDA) investigates the effect of a compound to inhibit the development of nematode eggs into infective L3. The larval motility inhibition assay (LMIA) measures the ability of a compound to paralyse L3 and inhibit their migration through a nylon mesh. And the larval exsheathment inhibition assay (LEIA) test the effect of a plant compound to delay the artificially-induced exsheathment of L3.

In contrast with free-living stages occurring in the environment, parasitic stages in the host have different biochemical characteristics and detoxification mechanisms, thus the same compound can exert different effects and affect distinct targets in free-living or parasitic stages (O'Grady and Kotze, 2004; Hoste et al., 2015). Moreover, parasitic (immature and adult) stages are the expected targets of dietary PSM in the host, and therefore it is of interest to confirm a direct activity of plant compound against them. The adult motility inhibition assay (AMIA) tests the effect of PSM to paralyse live adult worms, recovered from animals immediately post-mortem, and incubated in culture medium for up to 48 – 96 h (depending on the nematode species). However, the isolation of live adult nematodes requires the slaughter of infected animals, involving considerable resources that limit its routine performance. Alternative methods have been proposed to test the direct anthelmintic effect of plant compounds using the adults of the free-living nematode *Caenorhabditis elegans* (Katiki et al., 2011; Kumarasingha et al., 2014). Recently, an image-based whole-organism assay using *H. contortus* L3 and developing L4 has been explored for the low cost testing of compounds with potential anthelmintic activity (Preston et al., 2015).

1.4.1.2 Controlled *in vivo* studies

In vivo studies are essential to confirm the anthelmintic potential of bioactive forages in infected animals before on-farm use, as well to detect potential toxic effects in the animals. Based on the available evidence from CT-rich plants, it is expected that the reduction in worm burdens induced by bioactive forages is lower than following treatment with (efficacious) synthetic anthelmintics (Ketzis et al., 2006). Nevertheless, it is critical to establish the reduction levels of parasitism achieved by bioactive plants, particularly a dose-response, before on-farm studies can be designed (Githiori et al., 2006; Hoste et al., 2015). At the moment, no standardized guidelines exist for the evaluation of anthelmintic forages in controlled *in vivo* trials, although some recommendations have been proposed (Hoste et al., 2015). When testing the direct anthelmintic effect and the role of PSM it is of particular importance to exclude confounding factors such as improved nutrition (resulting from

high-protein/energy diets) which is known to enhance the immune response to helminth infections (Mansour *et al.* 1992; Van Houtert and Sykes, 1996; Coop and Kyriazakis, 2001). To overcome this, nutrient-balanced diets should be offered to the tested and control groups, particularly regarding protein and energy levels (Hoste et al., 2015).

1.5 Chicory

Chicory (*Cichorium intybus* L., *Asteraceae*) is a perennial, deep-rooting herb that can be found as a wild plant on natural grasslands or in roadsides (identifiable by its distinctive bright blue flowers; Fig 1.3) and as cultivated varieties in most temperate areas of the world, including Denmark (Barry, 1998; Li and Kemp, 2005; Nielsen et al., 2009; Wang and Cui, 2011). In Europe and other regions, chicory has traditionally been used for human consumption and as a medicinal plant (Lucchin et al., 2008; Hitova and Melzig, 2014). Nowadays, cultivated chicory can be classified in four types, based on its applications: *i)* "industrial" or "root" chicory, for the production of inulin and as a coffee substitute; *ii)* "Brussels" or "witloof" chicory, for production of etiolated leaves ("chicons" or "*julesalat*" in Danish); *iii)* "leaf" chicory for fresh or cooked consumption and *iv)* "forage" chicory for animal feeding (Street et al., 2013). Selection of forage cultivars for livestock nutrition in the 1980's culminated in the release of the first commercial forage variety, "Grassland Puna" in New Zealand (Rumball, 1986). Since then, several forage varieties have been developed (Fig. 1.3)(Rumball et al., 2003a; 2003b; Li and Kemp, 2005; Foster et al., 2006).

Fig. 1.3. Chicory flowers (left) and forage chicory cv. Spadona (middle) and cv. Puna II (right)

Cultivars of forage chicory are predominantly active in warm seasons, with plant growth occurring at >10°C (if enough precipitation) and dormant in winter. In general, forage chicory cultivars are rich in minerals (e.g. Zn, B, Mn), have variable crude protein levels (~100 – 250 g CP kg DM^{-1}), higher organic matter digestibility and higher rumen fractional disappearance rate than grasses, low NDF levels and high voluntary feed intakes (reviewed by Li and Kemp, 2005). Inclusion of forage chicory is increasingly advocated to enhance the protein, mineral and vitamin content of pastures and to sustain forage

production during warm and dry periods when ryegrass growth is low (Sanderson et al., 2003; Høgh-Jensen et al., 2006; Pirhofer-Walzl et al., 2011; Langworthy et al., 2015; Roca-Fernández et al., 2016). Similar or higher weight gains have been reported in cattle grazing pure forage chicory, compared with animals grazed on ryegrass (Fraser et al., 1988; Clark et al., 1990; Parish et al., 2012), and comparable positive effects on animal growth have been observed in sheep and deer (Scales et al., 1995; Hoskin et al., 1999b; Kidane et al., 2014). Chicory-based diets have also been reported to sustain milk production in cattle (Waugh et al., 1998; Chapman et al., 2008; Muir et al., 2014) and in sheep (Di Grigoli et al., 2012). However, the rapid growth of low-quality reproductive stems in late spring/early summer (from the second year onwards) is an agronomical shortcoming of forage chicory, while its relatively low persistency (of around 3 years) and its dormant state during winter adds further limitations to its use (Clark et al., 1990; Sanderson et al., 2003; Li and Kemp, 2005; Lee et al., 2015; Muir et al., 2015).

1.5.1 Effects of forage chicory on GI nematodes of ruminants

1.5.1.1 *In vivo* anthelmintic effects of forage chicory

Since the mid-1990's, several *in vivo* studies have explored the potential anthelmintic effects of dietary forage chicory on parasitic nematodes, mainly in sheep (Scales et al., 1995; Marley et al., 2003b; Athanasiadou et al. 2005; Tzamaloukas et al., 2005; Tzamaloukas et al., 2006; Athanasiadou et al., 2007; Heckendorn et al., 2007; Nielsen et al., 2009; Kidane et al., 2010; Miller et al., 2011) but also in deer (Hoskin et al., 1999a) and cattle (Marley et al., 2014). The main parasitological findings of these *in vivo* studies, as well as the chicory cultivar used and the percentage of chicory DM in the diets, are summarised in Table 1.1. None of these studies investigated the content of SL in forage chicory, and when CT were screened they were detected at levels <0.5% CT in DM. Scales et al. (1995) reported a reduced FEC and *Teladorsagia* worm burden in lambs grazing forage chicory. They also recovered a lower number of infective L3 from chicory swards, as compared with ryegrass pastures. These authors described three hypothetic explanations for the reduced parasitism observed in lambs grazing chicory: *i)* a direct dietary modulation of chicory on the worms, *ii)* an increased animal resilience to the infection (due to a higher nutritional quality) and/or *iii)* to an indirect reduction in pasture-borne infections (Scales et al., 1995). From the subsequent *in vivo* studies with chicory, particularly short feeding trials with experimental infections in lambs and preventing reinfection from pasture, it was strongly suggested that feeding forage chicory could directly affect abomasal worms, but not intestinal nematodes (Athanasiadou et al. 2005; Tzamaloukas et al., 2005; Heckendorn et al., 2007). Tzamaloukas et al. (2006) reported that lambs, trickle infected with *T. circumcincta* while grazing chicory, then moved to a ryegrass pasture and challenged with *T. circumcincta* L3, had lower late L4 and adult worm counts than lambs only grazing ryegrass. The authors concluded that chicory enhanced the immune response against *T.*

circumcincta, evidenced by higher levels of mucosal mast cells and globule leucocytes in chicory lambs, and they attributed these findings to the high protein content in the chicory pasture, and not to a direct anthelmintic effect.

Marley et al. (2014) reported the only *in vivo* study to date exploring the anthelmintic effects of forage chicory in grazing cattle. In their study, beef steers were naturally infected with GI nematodes and grazed a mixed chicory/ryegrass pasture (~24% chicory DM in the field) for 18 weeks which resulted in similar FEC, proportion of *O. ostertagi* or *Cooperia* spp. L3 in larval cultures, serum pepsinogen and *O. ostertagi*-antibodies levels compared to infected controls grazing ryegrass. Post-mortem worm counts were not conducted. Evidence from *in vivo* studies in sheep suggests that increased levels of chicory (≥50% chicory DM in the diet) may be necessary to affect GI parasitism in the host.

Table 1.1. Summary of published *in vivo* studies investigating the anthelmintic effects of forage chicory in ruminants.

Species	Chicory cv.	Sown	Chicory DM in diet (%)	Study design	Effects in chicory fed animals	Reference
Sheep	Grasslands Puna	pure	>85%	Grazing, exp. + nat. infections with mixed GI nematodes	Lower FEC and *Teladorsagia* worm counts ($P<0.05$)	Scales et al., 1995
Deer	Grasslands Puna	pure	>56%	Grazing, nat. infections with mixed GI nematodes and lungworms	No effect on FEC or lungworm L1 counts. Lower clinical signs associated with parasitism, requiring less anthelmintic treatment	Hoskin et al., 1999a
Sheep	Grasslands Puna	pure	n.i.	Grazing, nat. infections with mixed GI nematodes	No effect on FEC or intestinal worm counts. 19-41% lower abomasal adult counts ($P<0.001$) and 32-60% lower abomasal L4 ($P<0.01$)	Marley et al., 2003b
Sheep	n.i.	pure	80%	Grazing, exp. infections with *Trichostrongylus colubriformis*	No effect on FEC, adult or immature worm counts	Athanasiadou et al., 2005
Sheep	n.i.	pure	87%	Grazing, exp. infections with *Teladorsagia circumcincta*	No effect on FEC, female per capita fecundity or immature worm counts. Reduction in adult male worms ($P<0.01$) and lower total adult counts by 43% ($P=0.14$)	Tzamaloukas et al., 2005
Sheep	n.i.	pure	91%	Lambs trickle infected with *T. circumcincta* while grazing chicory. After anthelmintic treatment, lambs were challenged with *T. circumcincta* L3 and grazed on ryegrass	No effect on FEC. Reduced numbers of late L4 and adult worms ($P<0.05$)	Tzamaloukas et al., 2006

(continuation Table 1.1)

Sheep	Grasslands Puna	pure	~80%	Stabled lambs fed fresh-cut chicory. Exp. infections with *Haemonchus contortus* and *Cooperia curticei*	Reduced total egg output and FECDM of *H. contortus* (P<0.01). No effect on FECDM of *C. curticei*, on total FECDM or on adult worm counts	Heckendorn et al., 2007
Sheep	Grasslands Puna	pure	~80%	Grazing, nat. infections with mixed GI nematodes in ewes and lambs. Ewes were treated or not with anthelmintics	Lower FEC in lambs from untreated ewes on chicory (P<0.001). No effect on FEC of undrenched ewes or on adult worm burden in lambs	Athanasiadou et al., 2007
Sheep	Grasslands Puna	mixed	2-51% in the field; <6% intake	Grazing, nat. infections with mixed GI nematodes	No effect on FEC	Nielsen et al., 2009
Sheep	Puna II	pure	50%	Grazing, nat. infections with *T. circumcincta*	Reduced FEC (P<0.001) and lower L3 recovered from chicory swards (P=0.07)	Kidane et al., 2010
Sheep	Oasis	pure	n.i.	Grazing, nat. infections with mixed GI nematodes	Reduced FEC counts (P<0.05), lower FAMACHA scores requiring less anthelmintic treatments	Miller et al., 2011
Cattle	Puna II	mixed	~24%	Grazing, nat. infections with GI nematodes	No effect on FEC, proportion of *O. ostertagi* or *Cooperia* spp. L3 in larval cultures, serum pepsinogen or *O. ostertagi*-antibodies	Marley et al., 2014

pure = monoculture; mixed = sown as mix sward; n.i. = not indicated; exp. = experimental; nat. = natural

1.5.1.2 Effect of forage chicory on the development and survival of free-living nematode stages

Studies have also described a lower recovery of infective L3 in chicory pastures, potentially attributed to a reduced development of free-living stages. Moss and Vlassoff (1993) reported a lower recovery of total L3 from a pure chicory sward, compared with a ryegrass field, seeded with strongyle and *Nematodirus* spp. eggs. The authors attributed their findings to the markedly lower DM in the chicory field (40% less DM, compared with ryegrass) which could have changed the micro-climate in the sward and limited the development and survival of L3 (Moss and Vlassoff, 1993). Reduced herbage mass and plant cover has also been suggested to limit the development and survival of larval stages from sheep nematode (Thamsborg et al., 1996). Niezen et al. (1998) described a lower total number of L3 and reduced larval survival, particularly of *T. circumcincta*, recovered from a chicory sward seeded with GI nematode eggs, as compared with a ryegrass field. No information of the DM in the chicory field was provided. Marley et al. (2006) reported a significantly lower recovery of L3 (per kg forage DM) from chicory seeded with *C. curticei eggs* and cultivated under greenhouse conditions (as compared with ryegrass) and a significantly lower *T. circumcincta* L3 (per kg forage DM) from chicory swards (compared to ryegrass pastures). Kidane et al. (2010) recovered a lower number of L3 from a chicory sward grazed by naturally infected lambs, although not significantly different from the larval recovery from a ryegrass/clover field. These studies strongly suggest a potentially lower infection pressure on animals grazing chicory swards. Experiments have also explored a potentially reduced development of free-living nematode stages isolated from animals fed with forage chicory. Schreurs et al. (2002) reported no differences in the *in vitro* egg hatching or larval development of GI nematodes isolated from faeces of deer grazing chicory. In contrast, Marley et al. (2003a) observed a significantly lower development of *H. contortus* and *C. curticei* eggs into L3 in cultured faeces from lambs fed with forage chicory, in comparison with the larval development in faeces from animals grazing ryegrass/clover. A decreased larval development of *T. colubriformis* has also been reported in faeces from animals fed CT-rich diets (Niezen et al., 2002).

The available literature summarised here suggests that forage chicory can negatively affect infections with GI nematodes through: a) direct anthelmintic effects on parasites in the host, b) indirect effects through improved nutrition and enhancement of immunity, and c) a reduced larval intake from pasture due to a lower development and survival of infective nematode stages in chicory swards and in faeces of chicory-fed animals. The direct anthelmintic effects of chicory, which are the main subject of this PhD thesis, are believed to derive from its content of bioactive compounds or plant secondary metabolites (PSM).

1.5.2 Bioactive compounds in forage chicory

As a member of the *Asteraceae*, chicory is known to produce biologically-active sesquiterpene lactones (SL), which are also responsible for its bitter taste (Rees and Harborne, 1985; Peters and van Amerongen, 1997; Kisiel and Zielin, 2001; Zidorn, 2008). Several other phytochemicals have been described in chicory, such as chicoric, cholorogenic and caffeic acids (all derivatives of hydroxycinnamic acids), coumarins (e.g. chicoriin, aesculin) and flavonoids (e.g. several quercetin and kaempferol derivatives) (Rees and Harborne, 1985; Heimler *et al.*, 2009; Street et al., 2013; Sinkovič *et al.*, 2015). Chicory also has very low levels of condensed tannins (CT), normally <0.5% CT in DM (Jackson et al., 1996; Tzamaloukas et al., 2005; Heckendorn et al., 2007; Lombardi et al., 2015). Chicory contains sugars (fructose, glucose and sucrose) in both roots and leaves and highly soluble fructans (e.g. inulin), primarily in the roots and with only traces detectable in the leaves (Ernst et al., 1995). Forage chicory also has high levels of pectins (80 – 90 g kg DM^{-1}; Liu et al., 2013). Inulin fructans have been indicated to stimulate the human immune system, reducing the risk of GI diseases (Roberfroid, 2005), while the inclusion of chicory leaves and roots as dietary fibre can modulate the gut microbiota in pigs and chickens (Ivarsson et al., 2012; Liu et al., 2012). Chicory roots have been reported to significantly reduce the numbers of *Ascaris suum* in pigs (Jensen et al., 2011), while pigs fed with pure inulin had reduced worm burdens of *Oesophagostomum dentatum* (Petkevicius et al., 2003). These anthelmintic effects have been linked with the rapid fermentation of inulin in the large intestine and the consequent increased production of short-chain fatty acids that are known to directly affect the worms (Petkevičius et al., 2004). No similar parasitological studies with inulin have been conducted in ruminants, although the use of fructan-rich diets as prebiotics is increasingly being investigated in cattle (Samanta et al., 2013).

1.5.3 Sesquiterpene lactones

Sesquiterpene lactones are a group of extremely diverse natural terpenoids, including around 5,000 different molecules, mainly produced by plants of the family *Asteraceae* (Seaman, 1982; Schmidt, 2006). These compounds have a wide range of biological functions but are mainly associated with plant defence (Rees and Harborne, 1985; Gershenzon and Dudareva, 2007). Natural SL are classified in several groups, including: germacranolides, eudesmanolides, elemanolides, heliangolides, cadinanolides, eremophilanolides, xanthanolides, guaianolides and pseudoguaianolides, among others (Schmidt, 2006; Amorim et al., 2013). In the *Cichorieae* tribe of the *Asteraceae*, around 360 distinct SL molecules have been reported, all part of the guaianolides (~243 molecules), eudesmanolides (~73 molecules) and germacranolides (~44 molecules) groups (Zidorn, 2008). In chicory, three guaianolide SL and their 11, 13-dihydro-derivatives have been described: lactucin, 8-deoxylactucin, lactucopicrin, 11, 13-dihydrolactucin, 11, 13-dihydro-8-deoxylactucin and 11, 13-dihydrolactucopicrin (Fig. 1.4) (Rees and Harborne, 1985; Price

et al., 1990; Foster et al., 2011;Ferioli and D'Antuono, 2012; Wulfkuehler et al., 2014; Ferioli et al., 2015). Sesquiterpene lactones are present in chicory leaves and roots as free molecules or as glycosides bound to carbohydrates (Ferioli et al., 2015).

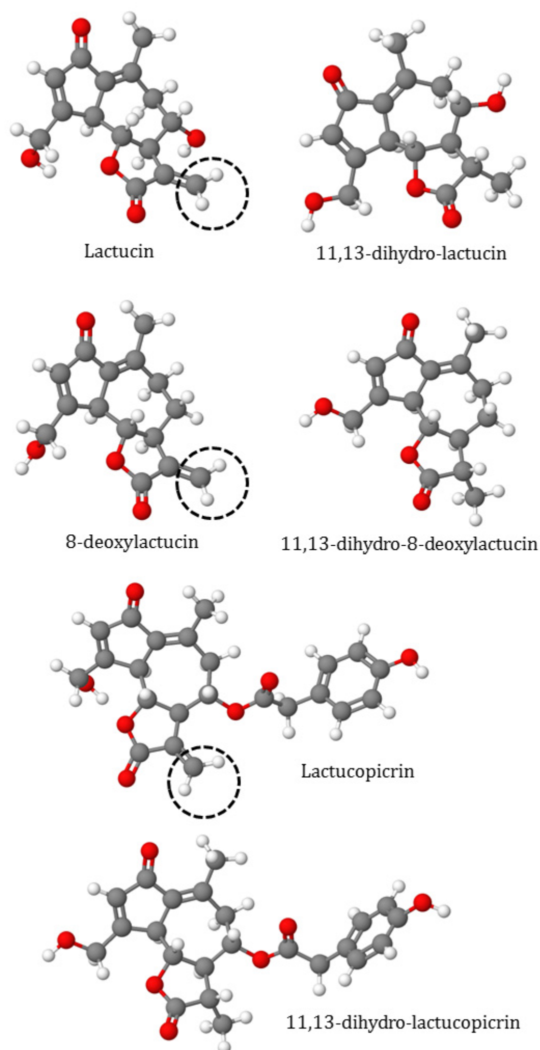


Fig. 1.4. Sesquiterpene lactones described in chicory leaves and roots. Dotted circles indicate the α-methylene (CH_2) group (absent in 11,13-dihydro-derivatives). Atoms: Grey = C; Red = O; White = H (structures designed in MolView.org).

1.5.3.1 Biological activities of sesquiterpene lactones

Numerous studies have demonstrated substantial biological activities exerted by SL, including antibiotic, antiprotozoan, antioxidant and anticancerigenous properties (Bischoff et al., 2004; Cavin et al., 2005; Schmidt, 2006; Barrera et al., 2013; Chadwick et al., 2013). As an example, artemisinin (and its derivatives), a SL originally isolated from sweet wormwood (*Artemisia annua*), is currently one of the major antimalarial drugs used worldwide (Meshnick, 2002; Gershenzon and Dudareva, 2007). Guaianolide and other SL are known to exert potent cytotoxic activities, mainly related to the presence of an α-methylene (CH_2) group attached to the γ-lactone in the SL molecule (Fig. 1.4) (Simonsen et al., 2013). This α-methylene group reacts with sulfhydryl (thiol) groups in any cysteine and cysteine-containing peptide, enzyme or other protein by a Michael-type addition, leading to an alkylation of cellular macromolecules and disruption of cellular functions (e.g. impairing cell signalling, cell replication and mitochondrial respiration) (Schmidt, 2006; Simonsen et al., 2013). As an example, SL were reported to reduce the intracellular concentration of free glutathione in *Leishmania mexicana mexicana*, which led to a toxic intracellular accumulation of reactive oxygen species (ROS) and blocked cell proliferation (Barrera et al., 2013). However, dihydro-derivatives of SL (also present in chicory) lack this α-methylene group but still can exert a strong biological activity (Lee et al., 1977; Schmidt, 2006; Ghantous et al., 2010), indicating that there is not only one biological mechanism of action for all SL.

1.5.3.2 Toxicity of sesquiterpene lactones

The unspecificity of the α-methylene–cysteine reaction induced by most SL, which is responsible for the broad biological activities of these molecules, can potentially induce toxic effects in humans and animals exposed (Amorim et al., 2013). As an example in veterinary medicine, SL have been described as the toxic compounds behind the noxious effects to animals of *Helenium* spp. and *Hymenoxys* spp. plants, which contain pseudoguaianolides, helenanolides and secohelenanolides (Burrows and Tyrl, 2004). However, the long tradition of growing and consumption of chicory by humans and the increasing use of chicory for animal feeding suggest that chicory and its guaianolide SL are non-toxic at the concentrations present in human and animal diets. To the knowledge of the author, only one study has investigated the toxicity of chicory in mammals; Schmidt et al. (2007) evaluated the toxicity of chicory root extracts containing 60% of guaianolide SL in rats dosed orally for 28 days (top dose 1000 mg extract BW kg^{-1} day^{-1}). The authors did not detect any clinical, post-mortem or histopathological signs of treatment-related effects in animals from any dosage group. However, the same chicory extract induced an inhibition in colony numbers of *Salmonella typhimurium* and *E. coli* when incubated *in vitro* at doses of 5 mg/plate (Schmidt et al., 2007). These results suggest a marked difference in

susceptibility to dietary SL from chicory in mammals and other organisms, and similar effects may be induced in GI nematodes.

1.5.3.3 Sesquiterpene lactones as anthelmintic compounds in forage chicory

The role of SL in the anthelmintic activity of forage chicory was first suggested by Schreurs et al. (2002), who reported a reduced *in vitro* motility of lungworms L1 when incubated in rumen and abomasal fluid from deer grazing chicory. The authors speculated that SL present in rumen and abomasal fluid of chicory-grazed deer were involved in the lower motility of lungworm L1; however, no detection of SL was performed. Molan et al. (2003) further explored the anthelmintic effects of SL from chicory and reported direct inhibitory effects of crude SL-extracts from chicory roots on the motility of lungworm L1 and L3 and of GI nematode L3 from deer. Still, chemical confirmation of SL in the tested extracts was not performed. The strongest evidence of the anthelmintic effect of SL from chicory was reported by Foster et al. (2011), who observed a dose-dependent inhibition in the egg hatching in *H. contortus* by extracts containing free SL isolated from forage chicory cv. Grasslands Puna and cv. Forage Feast. The study also characterised the three main SL of chicory in the tested extracts (LAC, 8-DOL and LCP) and detected an increased anthelmintic effect of the Grasslands Puna-extract, which was linked with its higher content of 8-DOL (Foster et al., 2011). Further, the authors suggested that dihydro-derivatives of the three main SL could have been present in their extracts, although their detection was not performed and the contribution of these compounds to the anthelmintic activity of forage chicory is unknown (Foster et al., 2011). Moreover, no studies have examined the direct effect of SL or SL-containing extracts from forage chicory against parasitic nematode stages, which are the expected primary targets of dietary SL in the host, or towards key mechanisms in the nematode life cycle that may clarify the *in vivo* anthelmintic effects of forage chicory.

Chapter 2. The present investigations

2.1 Objectives:

The overall objective of the present investigations was to contribute towards the integrated control of bovine GI nematodes by exploring the efficacy of the most common drug used for anthelmintic treatment in Danish cattle and the use of forage chicory as a potential complementary parasite control strategy.

The specific objectives were:

I. To assess the efficacy of ivermectin against GI nematodes in naturally infected Danish cattle by the FECRT and examining different statistical approaches for estimation of drug efficacy, and to report the general use of anthelmintics in selected farms (Manuscript I).

II. To investigate the anthelmintic effects of forage chicory-rich diets against experimental infections with GI nematodes in stabled and grazing calves, in relation with the PSM content in chicory (Manuscript II).

III. To study the direct effects of sesquiterpene lactone-containing extracts isolated from two forage chicory cultivars on key biological processes of free-living and parasitic stages of the cattle nematode *Ostertagia ostertagi* (Manuscript III).

2.2 Materials and methods

A brief summary of the materials and methods used in the present investigations (including unpublished pilot studies) is outlined in this section and further details can be found in the attached Manuscripts I – III.

2.2.1. Field efficacy of ivermectin and prescription of anthelmintics in Danish cattle

2.2.1.1 Efficacy of ivermectin against GI nematodes evaluated by different methods for analysis of faecal egg count reduction data (Manuscript I)

The field efficacy of ivermectin against GI nematodes in Danish cattle was investigated by the FECRT. Cattle farms (~50) were contacted during spring 2013 and 2014 through local veterinarians across Denmark. A total of 19 farmers accepted the invitation and 20 FSG calves were sampled in each farm. Animals were not treated with anthelmintics 8 weeks prior to sampling and had ≥ 4 weeks of access to pastures. Due to very low FEC in most of the properties only six farms were selected for the final study. All six farms were visited during June – September of 2013/2014 to conduct the FECRT (Fig. 2.1). In each farm, 10 FSG calves were treated with recommended dose of injectable IVM (IVM group) while 10 calves remained untreated as controls (CTL group). Faecal samples were collected from all animals at day of treatment (Day 0) and 14 days post-treatment (Day 14) and transported to the laboratory for FEC analysis. Pooled larval cultures per group were prepared on Day 0 and 14 for the species-specific diagnosis of *O. ostertagi* and *C. oncophora* L3 by real-time PCR (Höglund et al., 2013).

Estimation of drug efficacy was performed by calculating the arithmetic mean FEC reduction percentage (FECR%) post-IVM treatment according to WAAVP guidelines (Coles et al. 1992). Furthermore, we used two Bayesian modelling approaches to evaluate the efficacy of IVM treatments; the "Bayescount" method modified from Denwood et al. (2010) and Geurden et al. (2015) and the "EggCounts" method described by Torgerson et al. (2014). Both MCMC methods model the observed pre-and post-treatment FEC data as separate gamma-Poisson (negative binomial) distributions; the difference of the means between these two distributions is the FECR%. These methods also account for the FEC aggregation within animal groups and the Poisson errors of the egg counting process. However, the major difference between these Bayesian methods is in the prior distributions used in the modelling. The EggCounts method defines a single prior to the dispersion parameter (over-dispersion), and as a consequence, assumes that no variation in drug efficacy exists between animals in the same treated group. In contrast, the Bayescount relaxes this assumption and allows such variation in the modelling. Treatment efficacies

estimated by the WAAVP and Bayescount methods were calculated using the FEC variation in the IVM and CTL groups (with CTL) or the FEC variation only in the IVM group (without CTL) between Day 0 and 14, whereas the analysis by the EggCounts only included the FEC data from the IVM group at Day 0 and 14.

The efficacy of IVM treatments by each method was interpreted based on the mean FECR% and lower 95% CI as recommended by Coles et al. (1992) as well as the upper 95% CI as suggested by Lyndal-Murphy et al. (2014), from which we categorised three conditions:

i) Efficacious, when mean FECR% and upper CI ≥ 95% and lower CI ≥ 90%;
ii) Reduced efficacy, when mean FECR% and upper CI was < 95% and lower CI < 90%;
iii) Inconclusive, when none of the above conditions were met.

In addition, a simulation study was performed to compare the performance of the WAAVP method and the two Bayesian approaches (without CTL) to estimate drug efficacy in datasets based on the observed field data from the FECRT. Datasets were simulated based on treatment groups of 10 animals, with simulated FECR = 85% or 97% and varying levels of FEC aggregation values representing those observed in the FECRT studies. A total of 6,000 datasets were generated and analysed by all methods of calculation. The performance of the methods was investigated by: i) studying the probability of the simulated FECR% being included in the 95% CI provided by each method (i.e. coverage probability of the 95% CI), and ii) the classification probability that the methods would correctly detect a reduced efficacy (for a simulated FECR = 85%), efficacious treatment (for a simulated FECR = 97%) or inconclusive results using the interpretation described above. Further, detailed information about prescriptions of anthelmintics in the six selected farms between 2002 and 2012 was retrieved from the Danish system for surveillance of the veterinary use of drugs for production animals, VetStat, as described in Section 2.2.1.2.

Fig. 2.1. First-season grazing calves included in faecal egg count reduction tests performed in Danish cattle farms during 2013 and 2014.

2.2.1.2. Prescription of anthelmintics in Danish cattle: preliminary study

In parallel to the study of the field efficacy of IVM in the selected farms, we briefly examined the prescription of anthelmintics for the full Danish cattle population over the period between 2010 and 2014. Data were retrieved from the Danish system for surveillance of the veterinary use of drugs for production animals, VetStat[1]. The selected drugs with their active compounds, administration routes and indications are presented in Table 2.1 and correspond to all anthelmintics registered for use in cattle in Denmark during the period. For each prescription, data retrieved from VetStat included: *a)* the total amount of drug prescribed, *b)* farm identity, *c)* the target body system for which the treatment was prescribed (i.e. GI, respiratory, integumentary or other) and *d)* the targeted age group. In VetStat, cattle are divided into three age groups: *i)* calves < 12 months-old (heifer and bull calves); *ii)* young cattle ≥ 12 months-old (heifers until first calving and steers until slaughter) and *iii)* adult cattle (cows after first calving). The exact number of treatments performed based on each prescription is not recorded in VetStat. Thus, we used the total amount of a prescribed drug, the recommended dose of a given anthelmintic (considering its formulation) and a defined BW for each cattle age group to estimate the number of "defined doses" (DD) for each prescription. The defined BWs for the age groups were estimated from national databases on Danish cattle (Danmap, 2012) and were defined as: 200 kg BW for calves, 450 kg BW for young cattle and 620 kg BW for adult cattle. Due to the defined BW and the likely variation in the actual amount of active compound used for different animals following each prescription, the calculated DD is only a proxy of the number of treated animals. In addition, it was assumed that whenever an anthelmintic was prescribed and sold to a farmer, the drug was used within a month, which may not always have been the case. However, this potential bias is assumed to be consistent across the different drugs and age groups. Data on the total number of cattle and cattle farms in Denmark were extracted from Statistics Denmark (www.statbank.dk, visited on 15 December 2015).

[1] https://www.foedevarestyrelsen.dk/Leksikon/Sider/VetStat.aspx#

Table 2.1. Anthelmintics registered for use in cattle in Denmark between 2010 and 2014. The prescription data were retrieved from the Danish system for surveillance of the veterinary use of drugs for production animals, VetStat.

Active compounds	Administration routes	Indicated for parasites of:[a]
Albendazole (ALB)	oral	GI (including liverflukes); RES
Doramectin (DOR)	injectable	GI; RES; IG
Eprinomectin (EPR)	pour-on	GI; RES; IG
Fenbendazole (FEB)	oral	GI; RES
Ivermectin (IVM)	injectable; pour-on	GI; RES; IG
Ivermectin + Clorsulon (IVM + CLR)[b]	injectable	GI (including liverflukes); RES; IG
Ivermectin + Closantel (IVM + CLO)	pour-on	GI (including liverflukes); RES; IG
Levamisole (LEV)	injectable	GI; RES
Moxidectin (MOX)	injectable; oral; pour-on	GI; RES; IG
Oxfenbendazole (OXF)	bolus	GI; RES
Triclabendazole (TRI)[c]	oral	Liverflukes only

[a]Indications are based on the efficacy claims of each drug against particular parasite species and summarised to match the prescription registration of the VetStat system by target body system: GI= gastrointestinal system; RES=respiratory system; IG = integumentary system. [b]Introduced in Denmark in 2011.
[c]Not labelled for veterinary use in DK and only available for treatment in individual farms following laboratory diagnosis and dispensation from the general rules issued by the veterinary authorities.

2.2.2. *In vivo* studies of the anthelmintic effects of forage chicory against GI nematodes in experimentally infected cattle (Manuscript II)

Two independent and consecutive experiments were conducted to investigate the direct anthelmintic effects of forage chicory against GI nematodes in cattle. Calves were experimentally infected with laboratory parasite strains in two trials (indoor = Experiment 1 and outdoor = Experiment 2) with similar study design. Re-infections from pasture (Experiment 2) were prevented, thus excluding any potential effect of a lower parasite challenge from chicory pastures as previously described (Moss and Vlassoff, 1993; Marley et al., 2003). In Experiment 1, protein and energy intakes were balanced between feeding groups to relate the possible anthelmintic activity of chicory to its content of PSM and to exclude any indirect effect due an improved nutritional status. Moreover, short infection periods (≤42 days) were designed to avoid any potential development of natural immunity during the studies. The two major differences between the trials were:

i) Experiment 1 was performed during October – December 2013 as an indoor trial with calves fed either chicory silage or ryegrass/clover hay during 56 days (Fig 2.2). Throughout Experiment 1, the fodder consumption of calves was monitored to balance protein and energy intakes between groups using concentrates. Experiment 2 in contrast was conducted during August – September 2014 as a grazing trial with calves allocated to a pure chicory sward or to ryegrass/clover pasture for 42 days (Fig. 2.3). In Experiment 2 calves were not supplemented with concentrates and the protein/energy intakes between groups were not controlled.

ii) Experiment 1 tested the anthelmintic effects of chicory silage against mixed-infections with *O. ostertagi* and *C. oncophora* and calves were infected 16 days after the start of the feeding trial. Experiment 2 was conducted to primarily confirm results from Experiment 1 by investigating the effect of fresh chicory and chicory silage on mono-infections with *O. ostertagi* and the animals were infected 7 days after the beginning of the grazing trial.

Fig. 2.2. Experiment 1: calves fed with chicory silage (n=9, left) and control calves fed with ryegrass/clover hay (n=6, right). Calves were penned in subgroups of three animals throughout the study (3 chicory subgroups and 2 control subgroups)

Fig 2.3. Experiment 2: calves grazing a second-year pure chicory sward progressing into reproductive (flowering) stage (n=10, left) and control calves grazing a ryegrass/clover field (n=10, right).

2.2.3. *In vitro* studies of the anthelmintic activity of plant secondary metabolites of forage chicory

2.2.3.1. Studies of the direct anthelmintic effects of purified forage chicory extracts against *Cooperia oncophora*: preliminary investigations

Previous to the commencement of *in vivo* and *in vitro* studies with forage chicory described in Manuscripts II and III, preliminary investigations studied the direct anthelmintic effect of purified extracts from chicory leaves (cv. Spadona) against free-living and parasitic stages of *C. oncophora*. Chicory leaves were collected from an organic dairy farm in Årre, Denmark, on December 2013. Purified chicory extracts were extracted following the method described by Foster et al. (2011), which is a modification of the method reported by Tamaki et al. (1995) to extract and purify free SL from plant material. Briefly, dried leaf tissue was extracted in a Soxhlet apparatus using methanol. The resulting extract was fractionated using solid-phase extraction cartridges to enhance isolation of PSM, such as SL. The obtained eluate was dried, dissolved in 100% dimethyl sulfoxide (DMSO) and serially diluted to obtain concentrations ranging from 30 to 5000 μg dry extract mL^{-1}. Two calves mono-infected with *C. oncophora* (Section 2.2.4) were used for collection of nematode eggs from faeces and for isolation of live adult worms post-mortem. Isolated eggs and adults were used in an EHA or AMIA, respectively. Eggs or adult worms were incubated at the described concentrations of chicory extract (final DMSO concentration of 2% in Mili-Q-H_20) or 2% DMSO in Mili-Q-H_20 (negative controls).

2.2.3.2. Studies of the direct anthelmintic effects of SL-containing extracts from forage chicory against *Ostertagia ostertagi* (Manuscript III)

Following Experiment 1 (indoor trial), the potential role of SL in the anthelmintic activity of forage chicory against *O. ostertagi* was investigated using SL-extracts from chicory leaves. At the moment of the experiments, only two of the three guaianolide SL from chicory were available as pure standards (lactucin and lactucopicrin) but their high costs made them inaccessible in high enough quantities for *in vitro* studies. Therefore, these pure SL were only used as pure standards for HPLC-MS analyses. In addition, none of the SL dihydro-derivatives from chicory were commercially available. Hence, purified SL-extracts were prepared for the *in vitro* studies. Sesquiterpene lactones were extracted from leaves of chicory Spadona and Puna II (Fig. 2.4) following the procedure described by Ferioli and D'Antuono (2012) with the modifications detailed in Manuscript III. This method selectively fractionates total (free and unbound) SL from phenols and other plant compounds. The resulting dried extracts were not readily dissolved in PBS but were successfully dissolved with 100% DMSO. Stock solutions of both purified extracts in DMSO were prepared for the *in vitro* assays. Purified extracts dissolved in 100% methanol were used for characterization of SL by HPLC-MS. *Ostertagia ostertagi* L1 or L3 were used for

LFIA or LEIA, respectively, as described by Jackson and Hoste (2010) and Novobilský et al. (2011), with the modifications described in Manuscript III. In the LFIA, final concentrations of Spadona and Puna II extracts ranged from 10 to 500 µg dry extract mL^{-1}. In the LFIA, final concentrations ranged from 250 to 1000 µg dry extract mL^{-1}. Adult (male and female) worms immediately collected post-mortem were used in two independent adult motility inhibition assays (AMIA), following the methods described by Paolini et al. (2004) and Jackson and Hoste (2010), with the modifications described in Manuscript III. In the AMIA, adult worms were exposed to Spadona and Puna II extracts tested at final concentrations ranging from 10 to 1000 µg dry extract mL^{-1}. In addition, adult worms exposed to 1% DMSO (negative controls) and to the highest concentration of Spadona and Puna II extracts for 24 h were selected for scanning electron microscopy (SEM) investigations.

2.2.4 Parasite material and animals

First-season grazing calves in the field trials acquired natural infections with GI nematodes in the six investigated farms (Manuscript I). For the *in vivo* studies with forage chicory (Manuscript II) anthelmintic-susceptible strains from two sources were used: In Experiment 1, experimental infections were performed with *O. ostertagi* and *C. oncophora* strains kindly provided by Prof. Dr. Janina Demeler (Freie Universität Berlin, Germany; "Berlin strains"). While in Experiment 2, experimental infections were conducted with an IVM-susceptible *O. ostertagi* strain obtained from Ridgeway Research, UK (Batch number: OOSG10; "Ridgeway strain"), which was also used for *in vitro* studies (Manuscript III).

Heifer and bull calves (Manuscript I) or only bull calves (Manuscript II – III) between 3 – 12 months-old were used. First-season grazing calves included in the field trials in Danish cattle farms (n= 120; Manuscript I) were Danish Holstein, Danish Holstein crossbreeds and Danish Jersey. Bull nematode-naïve calves (reared indoor since birth) enrolled in the *in vivo* trials of forage chicory (Manuscript II) were Danish Jersey (n=15; Experiment 1) and Danish Holstein (n=20; Experiment 2). Danish Jersey bull-calves (n=4) were used as donor calves for propagation of infective strains of *O. ostertagi* and *C. oncophora* ("Berlin strains") for Experiment 1 (Manuscript II); two calves were infected with pure *C. oncophora*, while two animals had a mixed infection composed of 30% *O. ostertagi* and 70% *C. oncophora* (according to morphological identification and qPCR). Danish Jersey bull-caves (n=2) were infected with pure *O. ostertagi* ("Ridgeway strain") and acted as donor calves for propagation of infective L3 for Experiment 2 (Manuscript II) and as a source of nematode eggs for culture of L1 and L3 and of live adult worms for *in vitro* studies (Manuscript III).

2.2.5 Experimental feeds for *in vivo* studies (Manuscript II) and plant material for *in vitro* studies (Manuscript III)

Two chicory fields were cultivated for our investigations; a chicory cv. Spadona field cultivated as a pure sward (7.8 kg seeds ha^{-1}) and a chicory cv. Puna II pasture mixed with timothy (*Phleum pratense,* 6 kg chicory seeds + 6 kg timothy seeds ha^{-1}). Both chicory fields were sown on early May 2013 at the Experimental Farm of the University of Copenhagen, Tåstrup, Denmark. The chicory Spadona field was cultivated to provide forage for *in vivo* studies (Manuscript II) and the Puna II field was cultivated as a back-up in case of poor establishment of the chicory Spadona. Leaves from both cultivars were collected on 23 July 2013 for extraction of SL for *in vitro* studies (Manuscript III). At the time of collection chicory plants were at the vegetative stage (Fig. 2.4).

Fig. 2.4. Chicory cv. Spadona (left) and cv. Puna II (right) leaves at the time of collection for extraction of sesquiterpene lactones (Manuscript III).

When the chicory Spadona field was harvested on September 2013 to prepare chicory silage for *in vivo* Experiment 1 (Manuscript II) approximately 45% of the DM in the field corresponded to unsown species, mainly chamomile (*Matricaria recutita*), with some mugwort (*Artemisia vulgaris*) and shepherd's purse (*Capsella bursa-pastoris*) (Fig. 2.5). For Experiment 1 (Manuscript II), ryegrass/clover (*Lolium perenne*/*Trifolium repens*) hay was used as control feed and all animals were supplemented with a commercial concentrate for calves (Grønmix®, Danish Agro, Denmark) to balance protein and energy intakes between the groups.

Fig. 2.5. Chicory cv. Spadona field two weeks before the cut for ensiling (left), wrapped chicory leaves at ensiling on September 2013 (middle) and chicory silage offered to calves in Experiment 1 (right).

For Experiment 2 (Manuscript II), the chicory Spadona field was allowed to regrow from September 2013 until May 2014, when it was cut for ensiling to provide potentially supplementary feed for calves allocated to the chicory group during the grazing trial. At the start of Experiment 2, chicory plants were progressing into their reproductive (flowering) stage and calves quickly consumed the chicory leaves and the upper parts of stems and flowers, which, in addition to poor leaf regrowth during the study, resulted in a low amount of chicory on the pasture by the second half of the trial (Fig. 2.6, left). Consequently, chicory calves were supplemented *ad libitum* with chicory silage prepared in May 2014 (Fig. 2.6, right) during the last 19 days of the trial. As a control pasture for Experiment 2, a 0.8 ha ryegrass/clover field, left ungrazed during the 2013 and mowed on mid-June 2014, was used. Calves allocated to control pastures were not supplemented during Experiment 2. All experimental feeds used in both *in vivo* studies (Manuscript II) were screened for CT using the acetone-butanol-HCl method reported by Grabber *et al.* (2013) and for SL following the method described by Ferioli and D'Antuono (2012) with the modifications detailed in Manuscript III.

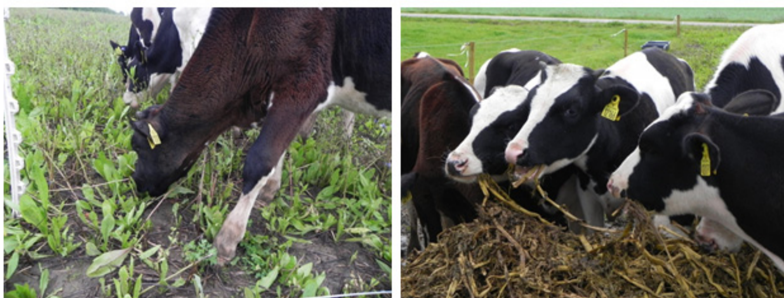


Fig. 2.6. Experiment 2; Calves grazing forage chicory (left) and supplemented with chicory silage in the field (right).

2.2.6 Parasitological analyses

Faecal egg counts in naturally-infected calves (Manuscript I) and experimentally infected animals (Manuscript II) were analysed using a modified McMaster technique with a sensitivity of 5 EPG (Henriksen and Aagard, 1975). Faecal larval cultures were prepared according to Roepstorff and Nansen (1998) and used to isolate infective L3 from naturally-infected cattle (Manuscript I) and from experimentally infected animals (Manuscript II and III). All L3 recovered from naturally-infected cattle before and after IVM treatment (Manuscript I) were analysed by real-time qPCR for species-specific detection and quantification of *O. ostertagi* and *C. oncophora* (Höglund et al., 2013).

In the *in vivo* chicory trials (Manuscript II), adult nematodes were recovered immediately after slaughter for worm counting from the abomasum and small intestine (Experiment 1) or only abomasum (Experiment 2) of experimental calves. Organs were opened into individual buckets, thoroughly washed with warm saline solution (0.9% NaCl, 38°C) and subsamples of diluted digesta were passed through a 25 µm sieve for collection of adult nematodes. Live adult worms from donor calves for *in vitro* assays (AMIA, Manuscript III) were recovered immediately after slaughter using the agar-migration method described by Slotved et al. (1996), in which worms actively migrate from a digesta-agar mixture, adhered to cloths, into warm saline (Fig. 2.7). Migrated live worms were collected using a 20 µm sieve, washed and immediately used in the AMIA.

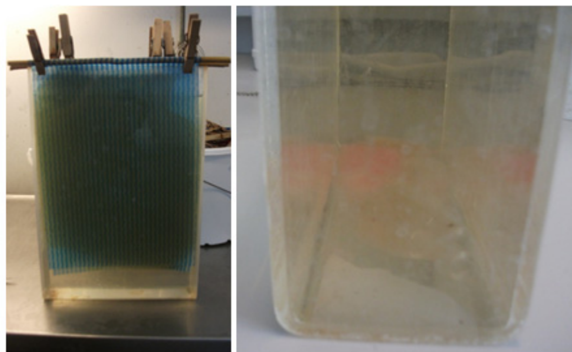


Fig. 2.7. Agar-migration method for isolation of live worms: digesta-agar mixture adhered to three cloths and placed in a container with warm saline (left) and *Ostertagia ostertagi* adult collected in the bottom of the container (red pellets) after 3 h incubation at 37°C (right).

Chapter 3: Manuscripts and preliminary studies – results and discussion

The main results of the present investigations and their significance are briefly summarised in this chapter and the detailed descriptions of our findings can be found in the attached Manuscripts I – III, with the exception of the preliminary studies presented here.

3.1. Field efficacy of anthelmintics in Danish cattle

3.1.1 Prescription of anthelmintics in Danish cattle: preliminary results

Between 2010 and 2014 (5 years), an estimated 534,858 defined doses with anthelmintics were prescribed for the entire Danish cattle population, with at least one prescription registered in 6,142 different farms (corresponding to 48% of all Danish cattle farms at the time of the investigations). The estimated number of defined doses per active compound, year and age cattle group is presented in Fig. 3.1. Considering the total Danish cattle population for each age group between 2010 and 2014, the estimated defined doses presented in Fig. 3.1 corresponded to the treatment of approximately 4.8% of all calves, 8.4% of all young cattle and 3.3% of all adult cattle once per year in Denmark. Considering all the investigated drugs (in all formulations), treatments with ML accounted for 85% of all prescriptions, and of these 72.1% were performed with IVM alone or in combination with CLR or CLO. Topical anthelmintics were the predominant formulations used for the treatment of calves (71.3%), young cattle (73.7%) and adult cattle (80.2%). Based on the number of estimated defined doses for each indication ("target system"), calves and young cattle were primarily treated against infections of the GI system (45.3% of all treatments), followed by infections of the respiratory system (32.2%) and the integumentary system (20.1%). In comparison, adult cattle were predominantly treated against infestations of the integumentary (39.1% of all treatments), followed by infections of the GI (36.3%) and respiratory systems (20.1%). Whether the commonly-used topical treatments are still efficacious against GI in Danish cattle need to be assessed, but poor efficacy of pour-ons has been previously reported under field conditions in Northern Europe (Sargison et al., 2009; Areskog et al., 2013). Moreover, the selection for AR in nematode populations exposed to variable and sub-therapeutic drug concentrations in cattle treated with pour-on ML has been suggested (Sargison et al., 2009; Sutherland and Bullen, 2015) but this remains to be confirmed.

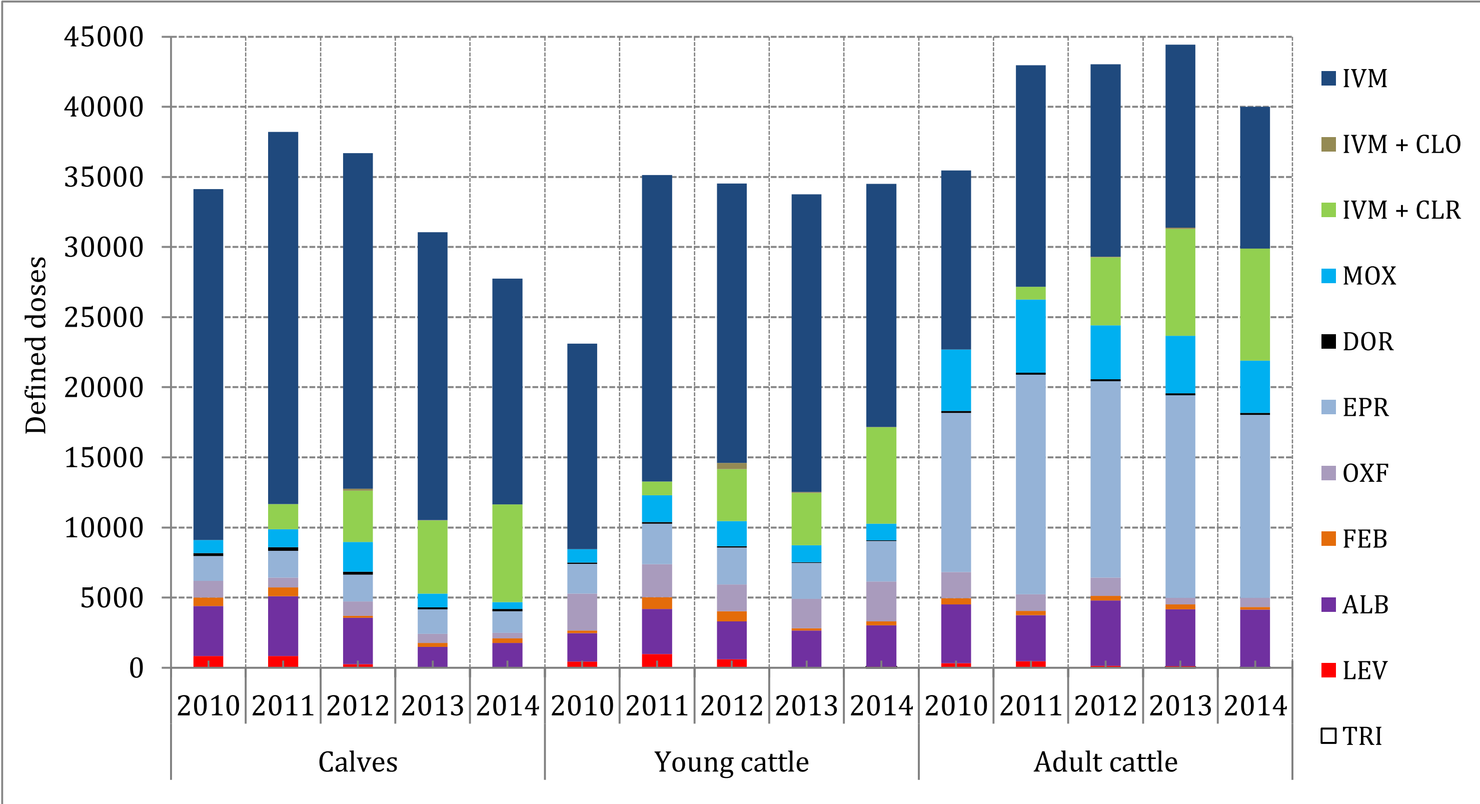


Fig. 3.1. Defined doses of anthelmintics prescribed for all indications in Danish cattle between 2010 and 2014. Data was extracted from the Danish system for surveillance of the veterinary use of drugs for production animals, VetStat. Details on the calculation of defined doses are presented in Section 2.2.1.2. ALB = albendazole; CLR= clorsulon; CLO = closantel; DOR = doramectin; EPR = eprinomectin; FEB = fenbendazole; IVM = ivermectin; LEV = levamisole; MOX = moxidectin; OXF = oxfenbendazole; TRI = triclabendazole.

3.1.2 Reduced efficacy of ivermectin against GI nematodes in Danish cattle and comparison of methods to analyse FECR data (Manuscript I)

In the initial screening, faecal samples from a total of 380 FSG calves in 19 farms were analysed for FEC during mid-June and early September in 2013 and 2014. Strongyle eggs were detected in all properties, but very low egg excretion levels (mean FEC ≤ 44 epg) were seen in most farms (63%). As a result, six farms with mean FEC ≥ 75 EPG were selected for FECRT.

In the FECRT, the WAAVP and the Bayescount methods including the FEC of IVM and CTL groups (with CTL) declared reduced treatment efficacy in farm #2, while reduced efficacy was declared in farm #1 only by the WAAVP method. Further, the WAAVP method (with CTL) declared the treatment to be efficacious in farm #5, while IVM treatments in the remaining farms were inconclusive as determined by both methods (with CTL). In comparison, the WAAVP, Bayescount and EggCounts analysing FEC data only from IVM groups (no CTL) unanimously declared reduced IVM efficacy in farms #1, #2 and #4. Additionally in farm #1, five CTL calves with high FEC at Day 14 were treated with injectable moxidectin (0.2 mg kg^{-1} BW s.c., Cydectin® inj. 10 mg/mL) and FEC at 21 days post-treatment revealed a mean FECR = 89% (95% CI = 82 – 93) by the EggCounts method (data not shown). All methods (no CTL) declared inconclusive results in farm #5 and in two farms the methods were in disagreement: in farm #6 only the two Bayesian methods declared reduced efficacy, while in farm #3 reduced IVM efficacy was only detected by the EggCounts method. Post-treatment, qPCR analyses of L3 from pooled larval cultures revealed that *C. oncophora* was the predominant species surviving injectable IVM in all farms detected, which is in agreement with previous European reports (Demeler et al., 2009; Geurden et al., 2015). The more pathogenic *O. ostertagi* was detected post-treatment in IVM groups from two farms Yet, reduced efficacy by all methods (no CTL) was only detected in one of these farms (#4) and therefore the potential presence of an *O. ostertagi*-strain less susceptible to IVM in this herd should be further investigated.

The interpretation of FECRT results was affected by the method of calculation used to estimate treatment efficacy. Methods including the FEC of untreated animals estimated a higher drug efficacy when the FEC of control calves increased between Day 0 and 14 (farms #3, #4, # 5 and #6) compared with methods not including FEC of CTL groups. Nevertheless, similar drug efficacies were detected by all methods when the FEC of CTL animals slightly decreased between samplings (farms #1 and #2). Inclusion of untreated controls in a FECRT has been suggested to allow the detection of such fluctuations in FEC not related with the treatment, which could affect the interpretation of the test (Coles et al., 1992; Lyndal-Murphy et al., 2014). Yet, dynamics of FEC excretion in untreated cattle are not directly comparable with the egg excretion in treated animals due to density-dependent control of female fecundity and poor correlation between worm burdens and FEC. The FEC

of untreated animals may for example be reduced in response to new infections, and FEC of treated animals may increase the egg excretion of females due to lower worm burden and competition (Michel, 1967; 1969; Dobson et al., 2012). Moreover, when ≤ 20 infected animals are available for FECRT in a farm (as observed in most farms in our study) the inclusion of untreated controls will reduce the size of the treatment group and consequently increase uncertainty of the estimated efficacy (Denwood et al., 2010). Therefore, our results suggest that under Danish conditions inclusion of untreated cattle in the FECRT is not recommended. Instead, it may be advisable to include more animals in the treatment group (n ≥ 15), which is likely to increase the certainty of the estimated treatment efficacy (Denwood et al., 2010; Levecke et al., 2012).

Similarly to the observations at the national level, the study of the anti-parasitic prescriptions in the six farms between 2002 and 2012 showed that 98% of all treatments were ML, mostly IVM pour-on products, regardless of age group. However, we did not aim to directly correlate anthelmintic use and development of AR to IVM given the extensive use of this drug in Danish cattle since the 1990's. Nevertheless, the widespread selection of IVM as the drug of choice in most of the farms where a reduced efficacy was confirmed in the FECRT needs close monitoring to prevent further deterioration of the situation and to identify drugs that are still effective in these farms. Evidence from research on small ruminant nematodes suggest that treatment of selected animals in a group (TST approach), leaving some (or most) of the animals untreated to guarantee a *refugia* of unselected worms, is perhaps the most important strategy to slow the development of AR in nematode populations. Still, it is critical that the anthelmintic used in a TST approach is highly efficacious (Knox et al., 2012). This is also particularly relevant under Danish regulations of prescription-only use of anthelmintics, in which therapeutic treatments should have a high efficacy to cure clinically-affected animals.

In the simulation study, highly aggregated FEC data were poorly analysed by the WAAVP and EggCounts methods, which were unable to optimally include the simulated FECR% in the 95% CI provided by the methods. This was further reflected in the higher frequency of "false negatives" (i.e. declaring efficacious treatment when the simulated FECR = 85%) provided by the EggCounts and the WAAVP methods with highly aggregated simulated FEC data, suggesting that drug efficacy estimates calculated by these two methods with over-dispersed FECR data should be interpreted with caution. The poor coverage probability of 95% CI by the EggCounts derives from the distribution priors, which exclude the possibility of extremely high over-dispersion. In contrast, at low and moderate FEC aggregations, the EggCounts provided the higher rate of "true positive" (i.e. declaring reduced efficacy for simulated FECR = 85) and "true negative' (i.e. declaring efficacious treatment for simulate FECR = 97%) results for the simulated sample size values, followed by the WAAVP and the Bayescount. The Bayescount method, on the other hand, provided no false negative results despite varying FEC aggregation levels, but it provided the highest

rate of inconclusive results in comparison with the other two methods. This is a limitation of the Bayescount as a sample size of 10 animals was too small to provide conclusive results and to correctly distinguish between a FECR of 85% and 97% in the presence of extreme over-dispersion. Given the difficulties associated with the EggCounts method when FEC are highly over-dispersed, and the Bayescount method when the number of samples is low, we suggest that the use of both Bayesian methods requires the selection of appropriate prior distributions assigned in the models (to accurately include in the model the [over] dispersion parameters of the data) and an increased sample size. Nonetheless, there is an urgent need for standardised international guidelines for the design and evaluation of FECRT in cattle that can be used in different farming contexts and considering the characteristics of FEC excretion in cattle, e.g. low FEC and high aggregation. Furthermore, molecular methods for detection and quantification of resistant alleles are undoubtedly needed to detect AR before it reaches clinical levels and become apparent in tests like the FECRT.

Notwithstanding, reasons other than AR may result in a lower than expected efficacy in the FECRT, such as under dosing (e.g. due to inaccurate estimation of BW) and/or altered drug pharmacokinetics and pharmacodynamics (e.g. due to nutritionally related variations in fat body reserves that may affect the persistent efficacy of ML, erratic absorption of the drug from the site of s.c. injection and/or due to interactions with other co-administered drugs) (González Canga et al., 2008; El-Abdellati et al., 2010; Areskog et al., 2012; De Graef et al., 2013; Areskog et al., 2014). All these factors may impact the correct estimation of drug efficacy and detection of AR, particularly in the dose-limiting species *C. oncophora*. At the moment, the controlled efficacy test (CET) is the gold standard method to confirm drug-resistant nematode strains initially reported by the FECRT. In general, a good correlation exists in cattle between CET and FECRT when FECR ≥ 98% or < 80%, i.e. when the treatment is highly effective or when a high level of resistance is likely to be present, respectively (Mejía et al., 2003; Neves et al., 2014). However, when the FECR is 80 – 95% (as in the present study), the confirmation of AR in the CET has proved more challenging. For example, *C. oncophora* populations declared resistant to the recommended dose of injectable IVM by FECRT in two Swedish cattle farms (using girth tape for weight estimation and with FECR% [upper CI] = 78% [97%] and 79% [98%] in each farm; Demeler et al., 2009) were declared IVM-susceptible when tested in calves under controlled conditions and weighed using scales (Areskog et al., 2014). Due to the absence of weight scales in all of our investigated farms, the BW of the animals was estimated based on girth measurements. Therefore, the reduced efficacy declared by FECRT in the present study may not be related to phenotypic resistance but could have originated from under dosing or changes in the pharmacokinetics of the drug under field conditions, and therefore our findings require confirmation by CET or at least a repeated FECRT. Nevertheless, the evidence of reduced IVM efficacy in three farms and the widespread use of ML in Denmark emphasises the importance of increased surveillance of anthelmintic efficacy in Danish

cattle and to develop novel control strategies that can lower the reliance on anthelmintics and the selection for AR in cattle nematodes.

3.2 Forage chicory selectively reduces *O. ostertagi* worm burdens in two independent experiments, while *C. oncophora* is unaffected (Manuscript II)

The anthelmintic effects of forage chicory against GI nematodes of cattle were investigated in two independent experiments. In Experiment 1, calves infected with *O. ostertagi* and *C. oncophora* and fed with chicory silage (≥70% chicory in the DM intake) had largely a similar faecal egg excretion pattern throughout the trial compared with infected control calves fed a standard balanced protein/energy diet. At Day 19 p.i., *O. ostertagi* and *C. oncophora* L3 were present at a similar ratio in pooled larval cultures from chicory and control groups; however, on Day 26 and 33 p.i., a markedly lower number of *O. ostertagi* L3 was observed in larval cultures from the chicory-fed calves. Post-mortem nematode recovery revealed that calves fed with chicory silage had significantly lower worm burdens of *O. ostertagi* in the abomasum (arithmetic/geometric mean reduction of 52/60%; $P < 0.01$) in comparison with control animals. In contrast, adult counts of *C. oncophora* in the small intestine were not statistically different between groups ($P = 0.12$). Chicory and control calves had comparable protein and energy intakes throughout the trial and similar daily weight gain until Day 19 p.i. (chicory = 383 [± 116] vs. control = 325 [± 236] g day^{-1}). From Day 20 p.i. until slaughter, chicory and control groups had mean growth rates of 811 [± 59] and 391 [± 132] g day^{-1}, respectively.

In Experiment 2, calves mono-infected with *O. ostertagi* and grazing a pure-chicory sward or ryegrass/clover (controls) had a similar faecal egg excretion until Day 20 p.i. ($P = 0.7$). From Day 22 p.i. a rapid and significant reduction in faecal egg excretion was observed in chicory-fed calves until the end of the trial, and at Day 35 p.i. chicory calves had a 65% lower mean FECDM compared with control animals ($P < 0.01$). Cumulative FECDM during the entire experiment were significantly reduced in the chicory group (mean [± S.D.] chicory = 12,870 [± 8,136] vs. control = 22,260 [± 5,671] EPG of faecal DM; $P < 0.01$). Post-mortem worm recovery revealed that calves fed pure forage chicory (fresh and silage) had highly significant reductions in the adult worm burdens of *O. ostertagi* (arithmetic/geometric mean reduction of 57/66%; $P < 0.001$) in comparison with control animals. The estimated per capita fecundity of *O. ostertagi* females (FECDM at slaughter/total number of females recovered) was not statistically different between groups ($P = 0.24$). Based on sward measurements, chicory calves had an estimated mean daily intake of 5.7 kg DM $calf^{-1}$ during the trial, with increasing consumption of chicory silage from Day 16 p.i. onwards; by Day 34 p.i. ~70% of the DM intake in this group was chicory silage. In contrast, control animals had an estimated daily intake of 6.9 kg DM

ryegrass/clover calf^{-1} during the entire experiment. Calves fed with pure chicory and control animals had growth rates of 374 (±160) g day^{-1} and 783 (±229) g day^{-1} in CHI_2 and CTL_2 calves, respectively. Thus, close monitoring of the DM available in pure chicory swards grazed by parasitized calves, particularly during periods of rapid reproductive plant growth, seems critical to secure that animal production goals are met.

Our study only considered the direct anthelmintic effects of chicory against experimental infections, while we prevented any reinfection from pasture in Experiment 2, thus excluding the potential reduced infection pressure due to lower number of L3 in chicory pastures as previously reported (Moss and Vlassoff, 1993; Marley et al., 2003). However, with the study designs of Experiment 1 and 2 it is not possible to determine if the reduced *O. ostertagi* counts in chicory-fed animals were a result of reduced worm establishment, due to the expulsion of established adult worms or both. Nevertheless, the similar faecal egg excretion until Day 20 p.i. in chicory and control calves in Experiment 2 suggests that dietary chicory may not prevent the establishment and development of *O. ostertagi* into egg-laying adults; rather, the marked drop in FECDM observed from Day 22 p.i. in chicory-fed calves indicates that dietary chicory may selectively (and rapidly) affect the survival of adult worms. Sesquiterpene lactones were identified in both experiments only in forage chicory (fresh and silage), while CT were not detected in any of the experimental feeds. In Experiment 1, chicory silage had a concentration of 12.3 g total SL kg DM^{-1}, with 11,13-dihydro-8-deoxylactucin (DI-8-DOL) and 11,13-dihydro-lactucopicrin (DI-LCP) as the main SL. In Experiment 2, fresh chicory had a concentration of 22.5 g total SL kg DM^{-1} and chicory silage of 16.8 g total SL kg DM^{-1}; 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. Considering the SL content in fresh/silage chicory, the DM intake per calf in both trials and assuming that dietary SL were not inactivated or metabolised in the forestomach, the concentration of SL reaching the abomasum (assuming an abomasum volume of 7 – 8 L) would roughly correspond to 5.37 mg SL mL^{-1} (Experiment 1) and 13.25 mg SL mL^{-1} (Experiment 2) per animal. Undoubtedly, further research is required to directly quantify the concentration of dietary SL reaching different gut compartments, which may influence the anthelmintic activity of SL *in vivo*.

Feeding forage chicory-rich diets resulted in a significant reduction of adult worm burdens of *O. ostertagi* in infected calves in two independent experiments, while no apparent activity was observed against *C. oncophora*. The markedly lower number of *O. ostertagi* L3 detected in chicory calves in the Experiment 1, was likely a result of the reduced worm burden in these animals. This contrasts a previous study reporting that infected steers grazing a mixed chicory/ryegrass pasture (~24% of chicory DM in the field) and animals grazing pure ryegrass had similar numbers of *O. ostertagi* L3 in larval cultures, while worm counts were not reported (Marley et al., 2014). Our results therefore indicate that a higher amount of chicory in the diet (≥ 70% of chicory DM in the diet) may be necessary to exert

an anthelmintic effect against *O. ostertagi in vivo*. Sesquiterpene lactones were identified in fresh chicory and silage, suggesting that these PSM may contribute to the observed anthelmintic effects of chicory. Forage chicory (fresh and ensiled) was readily consumed by calves and we demonstrated that the anthelmintic effects observed were preserved despite ensiling. Essential requirements for any novel approach for parasite control is that farmers are able to access, adopt, adapt and implement these methods to their production systems (Vercruysse and Dorny, 1999; Ketzis et al., 2006). In this regard, the preservation of chicory as silage may be a practical strategy for farmers to use forage chicory as a selective anti-*Ostertagia* feed, independent of seasonal availability of chicory in the field. It is interesting to notice that, in comparison with fresh forage, chicory silage prepared for both experiments (in two consecutive years) contained increased concentrations of DI-LCP and DI-8-DOL. In the *in vitro* studies (Manuscript III), DI-8-DOL was linked with higher anthelmintic potency against *O. ostertagi*. Consequently, further research is needed to clarify how ensiling affects the SL profile and anthelmintic activity of chicory silage in comparison with fresh leaves, as well as the optimal ensiling procedures to preserve the crop's nutritional quality.

3.3 *In vitro* studies of the anthelmintic activity of plant secondary metabolites of forage chicory

3.3.1 *In vitro* studies of the direct anthelmintic effects of purified forage chicory extracts against *Cooperia oncophora*: preliminary investigations

In the preliminary *in vitro* studies using chicory leaves (cv. Spadona) collected in an organic dairy farm, incubation with chicory extracts in the EHA induced a dose-dependent inhibition in the hatching of *C. oncophora* eggs, with more than 95% inhibition at concentrations ≥ 2500 µg dry extract mL^{-1} and an EC_{50} of 640 µg dry extract mL^{-1} (95% CI = 490 – 830). In the AMIA, after 12 h of incubation in the chicory extract a total inhibition in worm motility was detected at concentrations > 500 µg dry extract mL^{-1} (EC_{50} = 190 µg dry extract mL^{-1}; 95% CI = 170-230), and after 48 h of incubation a complete inhibition of worm motility was observed at concentrations > 250 µg dry extract mL^{-1} (EC_{50} = 70 µg dry extract mL^{-1}; 95% CI = 60-90). Adult worms incubated at 2% DMSO (negative controls) had 100% motility until 48 h of incubation. Chromatographic analyses by HPLC-MS revealed no clear molecular profile and this may be explained, to some extent, by the potential presence of undetectable free SL in this extract, in comparison with unbound ("released") SL detected in extracts used in Manuscript III and isolated with a different procedure. These direct anthelmintic effects contrast the lack of activity of forage chicory towards *C. oncophora* adults observed in Experiment 1 (Manuscript II), and could reflect that active compounds present in forage chicory do not reach the small intestine in concentrations sufficient to exert their activity against *C. oncophora*.

3.3.2 Sesquiterpene lactone-containing extracts from two chicory cultivars show distinct chemical profiles and direct anthelmintic effects against *O. ostertagi* L1 and adult worms (Manuscript III)

Chemical analyses by HPLC-MS of chicory Spadona and Puna II extracts confirmed the presence of the three well-known guaianolide SL of chicory: lactucin (LAC), 8-deoxylactucin (8-DOL) and lactucopicrin (LCP). In addition, the 11, 13-dihydro (DI) derivatives of the mentioned SL were also detected: DI-LAC, DI-8-DOL and DI-LCP. Between 60 – 65% of the tested extracts was composed of SL, while the remaining (unknown) compounds could not be determined. Comparable levels of total and individual SL and unknown compounds were detected in both extracts, with the exception of DI-LAC and DI-8-DOL which were present in significantly higher concentrations in Puna II and Spadona extracts, respectively.

In the LFIA, Spadona and Puna II extracts induced a dose-dependent inhibition of larval feeding with increasing concentrations; however, Spadona extract exerted a remarkably higher potency than Puna II-extract when tested at equal concentrations, illustrated by significantly lower EC_{50} values ($P < 0.0001$). In the LEIA, no differences were observed in exsheathment rates of *O. ostertagi* L3 exposed to SL-containing extracts (at all tested concentrations) or negative controls ($P > 0.05$). In the AMIA, both SL-containing extracts exerted a rapid and dose-dependent paralysis of *O. ostertagi* adults with increasing concentrations. After only 6 h of incubation with the highest extract concentrations, Spadona and Puna II extracts induced worm paralysis in 100 and 92% of the incubated worms, respectively. However, at lower concentrations Spadona extract showed a higher potency reflected in significantly lower EC_{50} values after 6, 24 and 48 h of incubation, in comparison with Puna II extracts ($P < 0.0001$). Adult worms exposed to 1000 μg dry Spadona or Puna II extract mL^{-1} for 24 h (dead) and examined by SEM showed no obvious structural damage in the buccal opening or cuticle and appeared similar to worms incubated with 1% DMSO (live negative controls) (Fig. 3.2). These findings suggest that the mode of anthelmintic action of SL may be different from that of CT or cysteine proteinases, which are known to induce marked disruptions in the cuticle of exposed worms. Based on results from the *in vitro* assays, our data suggest that the increased concentration of DI-8-DOL in Spadona-extract (3.6-fold increase) coincides, to some extent, with the increased potency and lower EC_{50} values exerted by Spadona-extract in the LFIA (3.3-fold lower EC_{50}) and in the AMIA (2.6-fold lower EC_{50}), in comparison with Puna II-extract. These findings suggest that DI-8-DOL is a likely candidate to explain the higher anthelmintic effects of the Spadona-extract and this molecule has not been previously described to have an anthelmintic effect. Nevertheless, the higher potency of Spadona-extract may also have been related with a synergistic effect of all the compounds present in the extract rather than being caused by a single molecule. As purified DI-8-DOL is not commercially available, subsequent studies were carried out in an attempt to fractionate and isolate DI-8-DOL from

Spadona-extracts and to confirm its anthelmintic activity *in vitro*. Fractionation of purified extracts prepared from chicory cv. Spadona leaves was performed using preparative HPLC, and DI-8-DOL was isolated as pure fractions but at extremely low quantities (<0.5 mg), not sufficient to accurately measure its weight or to perform *in vitro* assays, and therefore additional fractionation work was required. Unfortunately, further work was not possible within the time frame of the present PhD study.

Sesquiterpene lactone-containing extracts from forage chicory inhibited larval feeding of L1 and exerted a lethal effect towards *O. ostertagi* adults, inducing a rapid paralysis, whereas no effects were observed on the exsheathment of L3. We observed substantial differences in the anti-parasitic activity between extracts of two forage chicory cultivars and this may be related with their distinct content of SL. To confirm these findings, further fractionation, isolation and testing of individual SL are needed to identify the most active compound(s).

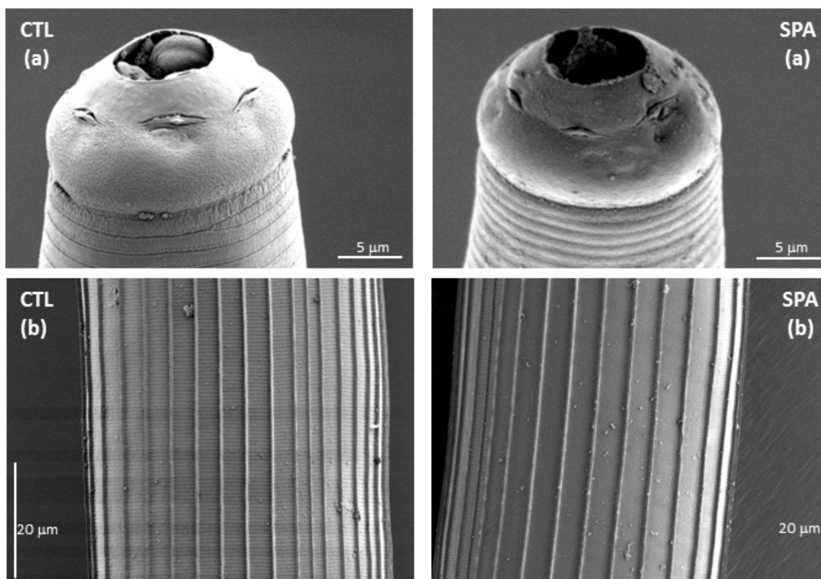


Fig. 3.2. Scanning electron microscopy images of *Ostertagia ostertagi* adults incubated during 24 hours with 1% DMSO in PBS (CTL) or 1000 µg Spadona extract/mL in PBS (SPA). Note the intact mouth opening and circular striations close to the mouth opening (a) and intact prominent longitudinal ridges (b) in all worms.

Chapter 4: Conclusions

The constant risk posed by GI parasites in grazing cattle, the development of AR in bovine nematodes and the continuous pressure to reduce the use of synthetic chemicals in food animals require integrated approaches that combine the field monitoring of AR and the use of novel control methods to safeguard animal health and productivity in a sustainable manner. In the present investigations, a range of field, controlled and laboratory studies were performed to advance our knowledge on the efficacy of the most common anthelmintic drug used in Danish cattle, the potential use of forage chicory as a complementary control of GI nematodes and the role of SL in the anthelmintic activity of chicory.

Field studies (Manuscript I) revealed a reduced efficacy of injectable IVM to control GI nematodes in FSG calves in three of six Danish farms investigated. *Cooperia oncophora* was the main species surviving IVM treatment in all farms, but *O. ostertagi* was also identified post-treatment in one farm with confirmed reduced treatment efficacy. Yet, due to the low number of farms enrolled in the FECRT, this study may not represent the general situation in Denmark. Moreover, the presence of IVM-resistant nematodes suggested by the FECRT in three farms with reduced treatment efficacy should preferably be confirmed by CET. High FEC aggregation markedly affects the performance of the statistical methods used to estimate drug efficacy and results provided by such methods for highly over-dispersed datasets and with low sample size ($n \leq 10$) should be interpreted with caution and standardised guidelines for the design and evaluation of FECRT in cattle are pressingly needed. More than 98% of all anthelmintic treatments performed in the six farms investigated during the 11 years prior to our FECRT were ML, mostly IVM pour-on products. Accordingly, preliminary studies revealed that ML were the predominant anthelmintic drugs in Danish cattle production during 2010 to 2014 (unpublished data). The reduced efficacy of IVM detected in this study and the widespread use of ML in Danish cattle, suggest that farmers and their advisors should be aware of potential lack of efficacy and preferably closely monitor the efficacy of their anthelmintic treatments. Also other initiatives should be implemented in order to slow-down the selection of resistance to IVM and other ML e.g., use of other drug groups or targeted selective treatments with the still effective compounds. In addition, novel parasite control methods are required to reduce the reliance on anthelmintics while decreasing parasite burdens in grazing cattle.

Two independent controlled experiments (Manuscript II) demonstrated that calves fed with ≥ 70% of chicory DM in the diet had significant reductions in the adult worm burden of the pathogenic *O. ostertagi* in the abomasum (60 – 66% reduction), while *C. oncophora* in the small intestine was unaffected. Forage chicory (fresh and silage) was readily consumed by calves and the reduced burden of *O. ostertagi* in these animals was also

associated with a decrease in FEC from Day 22 p.i. onwards, which suggests that dietary chicory selectively affects the adult worms but not the preceding larval stages. Stabled animals fed chicory silage had higher weight gains compared with controls. In contrast calves allocated to a pure chicory pasture in its second year showed lower growth rates due to poor regrowth and low nutritional quality of the chicory crop. Therefore, grazing of calves on pure chicory under Danish conditions seems to require careful planning and monitoring to secure acceptable production gains. Sesquiterpene lactones were identified only in fresh chicory and silage and these PSM may contribute to the observed anthelmintic effects. In addition, the anthelmintic effects of chicory were preserved despite ensiling, and this may be a practical way to conserve and use chicory in farms, independent of season and pasture availability. Pelleting may be another option, which, however, was not tested in the current studies. Furthermore, chicory is well-suited for cultivation in Denmark and other Northern temperate climates and thus show promise as anti-*Ostertagia* feed for use in cattle that may reduce infection levels and the need of drug treatments.

The observed *in vivo* effects of forage chicory against *O. ostertagi* were confirmed by *in vitro* studies. These studies (Manuscript III), demonstrated direct anthelmintic effects of SL-containing extracts from two chicory cultivars against *O. ostertagi* L1 and adults, but not towards L3. Interestingly, chicory extracts from the tested cultivars showed distinctly different potencies and SL profiles; the more potent extract isolated from chicory Spadona had a significantly higher concentration of only one compound, DI-8-DOL. Whether the increased activity of Spadona extract is related only with the higher concentration of DI-8-DOL or to the synergistic effect of all SL in chicory Spadona is not clear. In addition, other molecules with anti-parasitic activity and not detected by chromatographic analyses (i.e. due to poor absorption of UV light) may have been present in the chicory extracts. Moreover, the differences in potency between chicory cultivars *in vitro* should be confirmed *in vivo*. In this context, our results can help to target the responsible compound(s) and to select cultivars with higher anthelmintic activity for cattle and potentially other herbivores.

Chapter 5: Perspectives and future research

Current and future changes in the management of cattle production systems (e.g. due to economic pressure or legislative changes) can have profound impact on the infection dynamics of GI nematodes and the required control interventions. Furthermore, the effects of climate change are expected to affect parasite biology, grassland growth, length of the grazing season and animal husbandry management in very specific and complex interplays that will require a sound understanding of parasite epidemiology and farming practices to maintain optimal parasite control (Morgan and Wall, 2009; van Dijk et al., 2010; Phelan et al., 2016). In Denmark, grazing is usually associated with lower profits and milk production (compared to indoor, loose-housing systems), and despite the lack of official statistics, educated guesses indicate that approximately 35% of all Danish cattle farms still apply grazing in one or more age groups (Reijs et al., 2013). Most of these are organic farms, which by law must provide cattle with access to pasture from mid-April until November (Naturerhvervstyrelsen, 2014). Organic farms are encouraged to apply other means of parasite control than (preventive) use of veterinary drugs. However, due to lack of documentation of other effective parasite control methods, most farms, whether organic or not, still rely on commercial anti-parasitic drugs.

Our results revealed a lower than expected efficacy of injectable IVM confirmed in three of six studied cattle farms, while inconclusive results were observed in the other three farms. Although our results cannot be directly interpreted as cases of IVM-resistance in cattle nematodes and may not reflect the IVM efficacy at the country level, they should stir up further AR surveys in Denmark. This is particularly relevant considering the widespread use of ML in Danish cattle. In this context, it seems relevant to reduce the selection pressure for AR on the still effective anthelmintics, but first those effective drugs should be identified. The selection of suitable cattle farms for the performance of the FECRT proved to be challenging. At the same time, standardise methods for the design and evaluation of FECRT in cattle are required. Once such guidelines are available, the monitoring of the treatment efficacies on a particular farm may be combined with the treatments routinely performed by veterinarians.

One potential alternative to anthelmintics for the control of GI nematodes explored in the present investigations, was the use of forage chicory-rich diets. The selective activity of forage chicory against *O. ostertagi*, the most pathogenic cattle nematode in Denmark and other temperate regions, observed in our controlled experiments is encouraging. However, several issues still need to be investigated before the use of forage chicory can be recommended for on-farm use (see future research below). Grazing cattle of all ages acquire infections with mixed species in the field (although older animals are predominantly infected with *O. ostertagi*), and therefore, it is expected that not all GI nematodes will be

affected by chicory feeding, as observed with *C. oncophora* in our study. Moreover, the economic benefit of including chicory-rich diets in production systems, in terms of animal growth/milk production as a consequence of its anthelmintic effects, needs to be assessed and compared with the extra costs associated with the production and use of chicory. The benefits are expected to vary largely according to different management systems (e.g. availability of [extra] land for cultivation of pure chicory, length of the grazing season and exposure to GI nematodes, etc.) and perhaps age groups (calves, heifers or adult cattle). However, forage chicory, as any other nutraceutical or bioactive forage, is first a feed and therefore both its nutritional and anthelmintic value should be evaluated. In Denmark, chicory is increasingly popular as a component of mixed pastures for cattle grazing (Smidt and Brimer, 2005; Pirhofer-Walzl et al., 2011), although usually sown at a very low rate which may be related to the risk of off-flavour in milk. Our findings indicate that levels of 70% chicory DM in the diet will exert an anthelmintic effect but the lower effective intake is currently not established. Whether sufficiently high concentrations of chicory can be achieved in Danish cattle systems needs further research, but the evaluation of pure swards of chicory for cattle grazing is progressing in other countries (Chapman et al., 2008; Parish et al., 2012; Muir et al., 2014). Moreover, the potential use of chicory silage, which seems to preserve the anthelmintic activity of chicory, should be further explored as a delivery strategy that could be appealing for farmers, even as supplementary feeding for grazing cattle.

Results of the *in vitro* studies testing SL-containing extracts from chicory suggest that different chicory cultivars may have distinct anthelmintic potencies, which may be linked to their particular SL profile, but this remain to be tested *in vivo*. Interestingly, the higher anthelmintic activity of the chicory Spadona extract (rich in DI-8-DOL) compared with the chicory Puna extract against *O. ostertagi* has recently been confirmed by our group against pig nematodes (Williams et al., 2016), suggesting that the molecules in this particular extract have a broad-spectrum anthelmintic activity that warrants further research. It suggests also that the lack of effect against *C. oncophora* may perhaps relate to pharmacokinetics and not pharmacodynamics.

Based on the findings of this PhD thesis, there is a need to further investigate the presence of AR in cattle nematodes in Denmark and to advance our knowledge on the anthelmintic activity of forage chicory and its bioactive compounds. This could be done by:

- Examining the field efficacy of different anthelmintics against GI nematodes infecting Danish cattle and the extent of AR in cattle nematodes through field studies including a larger number of farms across Denmark. However, this requires the development of standardised guidelines for the performance and analysis of FECRT in cattle, which are currently not available.

- Defining the life stage(s) of *O. ostertagi* affected by forage chicory diets and the length of chicory feeding required to exert an anti-*Ostertagia* activity in infected cattle. More specifically, the effects of short-term feeding with pure chicory (e.g. 1, 2 or 5 days) could be studied in animals infected with *O. ostertagi* and slaughtered at different time points to detect the numbers of larval and adult stages. This may allow detecting if chicory primarily exerts its effect by preventing the establishment of *O. ostertagi* L3 or by eliminating established adult worm populations. In addition, the effect of forage chicory on the total egg output of *O. ostertagi*, not only on FEC/FECDM, should be addressed by studying the total faecal output of chicory-fed animals. Moreover, to determine the full potential of chicory as an anthelmintic forage for cattle there is a need to address whether chicory can affect other abomasal nematodes of cattle (e.g. *Haemonchus placei, Trichostrongylus axei*).

- Studying whether the ensiling affects the presence of certain SL and/or the anthelmintic potency of forage chicory by comparing the SL profile and *in vivo* anthelmintic activity of fresh and ensiled chicory. Further, there is a need to examine if chicory can be preserved as pellets or by other means of conservation. Before recommending the use of forage chicory as an anthelmintic strategy in cattle farms, the effect of chicory diets against GI nematode should be studied in the field using animals naturally infected with mixed species, and the cost-effectiveness of including chicory in the production system should be addressed. Finally, it would be interesting to explore whether infected cattle voluntarily graze/feed on chicory as a self-medication strategy.

- With the aim to elucidate the role of SL in the anthelmintic activity of chicory and whether SL can reach the abomasum and other compartments of the GI tract to exert their anthelmintic activity, the pharmacokinetics and pharmacodynamics of SL from chicory should be explored. This can be achieved by detecting SL in the feed and in different body compartments and fluids (e.g. plasma, rumen, abomasum, small intestine and faeces) of animals fed with chicory. The molecular mechanisms of the direct activity of SL and their dihydro-derivatives against parasitic nematodes are not known and should be elucidated by exploring the potential inhibitory effects on nematode vital processes, like enzymatic pathways. Furthermore, it is needed to scrutinize if anthelmintic compounds other than SL are present in chicory by studying the nematocidal activity of different fractions from chicory extracts and by describing their components.

- Finally, the potential adaptation of GI nematodes to the activity of SL and other PSM from chicory should be further investigated e.g. by exposing several nematode generations to sub-therapeutic dose levels, leading to a possible “resistance” of nematodes to bioactive plant compounds, or by comparing the activity of chicory PSM against different nematode strains with potentially distinct susceptibilities to bioactive compounds, as earlier described (Calderón-Quintal et al., 2010).

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Chapter 7: Manuscripts

Manuscript I

Miguel Peña-Espinoza, Stig M. Thamsborg, Matthew J. Denwood, Markus Drag, Tina V. Hansen, Vibeke F. Jensen, Heidi L. Enemark. 2016. Efficacy of ivermectin against gastrointestinal nematodes of cattle in Denmark evaluated by different methods for analysis of faecal egg count reduction. International Journal for Parasitology: Drugs and Drug Resistance (*in press*), http://dx.doi.org/10.1016/j.ijpddr.2016.10.004

Manuscript II

Miguel Peña-Espinoza, Stig M. Thamsborg, Olivier Desrues, Tina V. A. Hansen, Heidi L. Enemark. 2016. Anthelmintic effects of forage chicory (*Cichorium intybus*) against gastrointestinal nematode parasites in experimentally infected cattle. Parasitology 143, 1279–1293, http://dx.doi.org/10.1017/S0031182016000706

Manuscript III

Miguel Peña-Espinoza, Ulrik Boas, Andrew R. Williams, Stig M. Thamsborg, Henrik T. Simonsen, Heidi L. Enemark. 2015. Sesquiterpene lactone containing extracts from two cultivars of forage chicory (*Cichorium intybus*) show distinctive chemical profiles and *in vitro* activity against *Ostertagia ostertagi*. International Journal for Parasitology: Drugs and Drug Resistance 5, 191–200, http://dx.doi.org/10.1016/j.ijpddr.2015.10.002

7.1 Manuscript I

Efficacy of ivermectin against gastrointestinal nematodes of cattle in Denmark evaluated by different methods for analysis of faecal egg count reduction

Miguel Peña-Espinoza, Stig M. Thamsborg, Matthew J. Denwood, Markus Drag, Tina V. Hansen, Vibeke F. Jensen, Heidi L. Enemark. 2016. International Journal for Parasitology: Drugs and Drug Resistance (*in press*),

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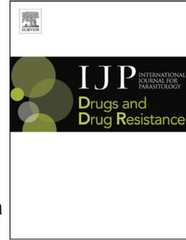
Accepted Manuscript



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1 **Efficacy of ivermectin against gastrointestinal nematodes of cattle in Denmark evaluated by**
2 **different methods for analysis of faecal egg count reduction**

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25 Note: Supplementary data associated with this article

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26 **Abstract**

27 The efficacy of ivermectin (IVM) against gastrointestinal nematodes in Danish cattle was assessed
28 by faecal egg count reduction test (FECRT). Six cattle farms with history of clinical parasitism and
29 avermectin use were included. On the day of treatment (Day 0), 20 naturally infected calves per
30 farm (total n = 120) were stratified by initial faecal egg counts (FEC) and randomly allocated to a
31 treatment group dosed with 0.2 mg IVM kg^{-1} body weight s.c. (IVM; n = 10) or an untreated control
32 group (CTL; n = 10). Individual FEC were obtained at Day 0 and Day 14 post-treatment and pooled
33 faeces by group were cultured to isolate L3 for detection of *Ostertagia ostertagi* and *Cooperia*
34 *oncophora* by qPCR. Treatment efficacies were analysed using the recommended WAAVP method
35 and two open-source statistical procedures based on Bayesian modelling: 'eggCounts' and
36 'Bayescount'. A simulation study evaluated the performance of the different procedures to correctly
37 identify FEC reduction percentages of simulated bovine FEC data representing the observed real
38 data. In the FECRT, reduced IVM efficacy was detected in three farms by all procedures using data
39 from treated animals only, and in one farm according to the procedures including data from treated
40 and untreated cattle. Post-treatment, *O. ostertagi* and *C. oncophora* L3 were detected by qPCR in
41 faeces of treated animals from one and three herds with declared reduced IVM efficacy,
42 respectively. Based on the simulation study, all methods showed a reduced performance when FEC
43 aggregation increased post-treatment and suggested that a treatment group of 10 animals is
44 insufficient for the FECRT in cattle. This is the first report of reduced anthelmintic efficacy in
45 Danish cattle and warrants the implementation of larger surveys. Advantages and caveats regarding
46 the use of Bayesian modelling and the relevance of including untreated cattle in the FECRT are
47 discussed.

48

49 **Keywords**: gastrointestinal nematodes, cattle, ivermectin, faecal egg count reduction test, real-time
50 PCR, Bayesian modelling

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51 **1. Introduction**

52

53 Grazing cattle are continuously exposed to infection with gastrointestinal nematodes (GIN) that can
54 severely impair the health and productivity of pasture-based livestock systems (Corwin, 1997; Shaw
55 et al., 1998; Charlier et al., 2014). In practice, the control of GIN in cattle largely relies on the
56 routine use of anthelmintic drugs, mainly from the macrocyclic lactone (ML) family (Vercruysse
57 and Rew, 2002; Geurden et al., 2015). As a consequence, worm populations resistant to MLs have
58 been selected, and anthelmintic resistance (AR) is now becoming a serious threat to the control of
59 bovine nematodes in several countries (Sutherland and Leathwick, 2011; Gasbarre, 2014;
60 Sutherland and Bullen, 2015). Coinciding with the development of AR, concerns regarding the
61 prophylactic use of veterinary drugs and chemical residues in both food and environment have led
62 to stricter regulations on the use of anthelmintics in some nations (Thamsborg et al., 1999). In 1999,
63 Denmark became the first country to introduce prescription-only use of anthelmintics in livestock,
64 requiring a mandatory veterinary diagnosis before treatment in both organic and conventional farms
65 (Anonymous, 1998; 2013). Since 2000, there has been an additional requirement for all
66 prescriptions in production animals to be registered in 'VetStat' − the Danish system for
67 surveillance of the veterinary use of drugs (Stege et al., 2003). Preliminary analyses in VetStat
68 indicate that MLs accounted for ~85% of all anthelmintics prescribed for Danish cattle between
69 2010 and 2012, with ivermectin (alone or in combination) representing 72% of all ML prescribed
70 (Peña-Espinoza et al, unpublished data). However, and despite the significance of ivermectin for
71 current parasite control strategies in cattle, its field efficacy against GIN has not been investigated
72 in Denmark.

73

74 In the absence of quantitative molecular techniques for the detection of ML-resistance, and the high
75 cost of the controlled efficacy test (the current gold standard method for verification of anthelmintic

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76 activity; Wood et al., 1995), the only readily available technique for investigating field drug
77 efficacy is the faecal egg count reduction test (FECRT). This technique estimates the efficacy of an
78 anthelmintic to reduce the faecal egg counts (FEC) of infected animals based on measurements pre-
79 and post-treatment, or between treated and untreated individuals. The major advantages of the
80 FECRT are that all drugs can be tested regardless of active compounds or formulation and that it
81 relies on FEC detection methods readily available in most diagnostic laboratories. The current
82 recommendations to conduct and analyse FECRT in cattle derive from guidelines by the World
83 Association for Advancement of Veterinary Parasitology (WAAVP), which were originally
84 developed for detection of AR in sheep nematodes (Coles et al., 1992). However, potential
85 limitations have been highlighted concerning the use of FECRT with bovine nematodes, mainly due
86 to the lower faecal egg excretion of cattle, compared to sheep, and the highly aggregated
87 distribution of FEC in cattle groups (Coles, 2002; Coles et al., 2006; Demeler et al., 2010; El-
88 Abdellati et al., 2010; Sutherland and Leathwick, 2011). These factors may limit the correct
89 analysis of FECRT data and inference of drug efficacy in cattle using the WAAVP guidelines. More
90 recently, Bayesian modelling using Markov chain Monte Carlo (MCMC) methods have been
91 advocated as robust statistical analyses to cope with low and aggregated FEC data (Denwood et al.,
92 2010; Torgerson et al., 2014). These MCMC-based procedures, available as open-source R
93 packages or web-interface software, are being increasingly used to infer drug efficacy and to
94 monitor AR in horse nematodes (Denwood et al., 2010; Fischer et al., 2015) and cattle helminths
95 (Neves et al., 2014; O'Shaughnessy et al., 2014; Geurden et al., 2015; Novobilský and Höglund,
96 2015; Ramos et al., 2016). However, the performance of these MCMC procedures with the low
97 mean FEC and parasite aggregation levels commonly found in cattle has not yet been evaluated. In
98 addition, sensitive and species-specific tests to detect which GIN species survive treatment are
99 critical for the surveillance of AR and are urgently required for cattle (Coles, 2002; Sutherland and
100 Leathwick, 2011).

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101 The objectives of the present study were: 1) to assess the efficacy of ivermectin (IVM) against GIN
102 in naturally infected Danish cattle by FECRT, and 2) to evaluate the performance of different
103 statistical approaches for estimating drug efficacy using simulated bovine FEC data of similar
104 characteristics to those observed in Danish cattle. In addition, we investigated the prescription
105 patterns of anthelmintics in the study farms in order to examine a possible relationship between
106 previous use of avermectins and IVM efficacy in the FECRT.
107
108 **2. Materials and methods**
109 *2.1. Selection of farms*
110 Cattle farms (~50) with a history of clinical parasitism were contacted through local veterinarians
111 across Denmark during spring 2013 and 2014. The farmers were offered free FEC analyses and
112 evaluation of anthelmintic efficacy by FECRT. Farms were selected based on the following criteria:
113 herd size ≥ 20 first-season grazing (FSG) calves with ≥ 4 weeks of grazing (before the initial
114 screening) and not treated with anthelmintics within 8 weeks prior to sampling. In addition, the
115 availability of a cattle crush or barn was required for the handling of animals. A total of 19 farms (8
116 in 2013 and 11 in 2014) that fulfilled these criteria accepted the invitation. Individual faecal
117 samples were collected from 20 FSG calves in each farm between mid-June and early September of
118 2013 and 2014 for analysis of FEC (initial screening). Due to a low number of farms with mean
119 FEC > 150 strongyle eggs per g (epg) of faeces (as recommended by Coles et al., 1992), farms with
120 a mean FEC ≥ 75 epg were selected for the FECRT. Of the six farms finally included in the study,
121 one herd was a conventional beef farm (farm #1), three were organic dairy farms (#2, #4 and #6),
122 one was an organic beef farm (#5) and one was a conventional dairy farm (#3). In Denmark,
123 organic cattle farms should by law provide access to pasture from 15 April until 1 November
124 (Anonymous, 2016), while conventional farms do not have to comply with this rule. The cattle
125 breeds in the investigated farms were Danish Holstein crossbreeds (#1 and #5), Danish Holstein

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126 (#2, #3, and #6) and Danish Jersey (#4). All the selected farms were located in the Jutland Peninsula
127 and the FECRT was conducted within one to four weeks after the initial screening.
128

129 *2.2 Faecal egg count reduction test (FECRT)*

130 The FECRT was performed to test the efficacy of IVM based on WAAVP recommendations (Coles
131 et al., 1992). Pre- and post-treatment faecal samples from treated and untreated animals were
132 included, and a total of 120 FSG calves were enrolled in the FECRT studies. On the day of
133 treatment (Day 0), 20 FSG animals from each farm were stratified by FEC (based on the initial
134 screening) and randomly allocated to a treatment group (IVM; n=10) or an untreated control group
135 (CTL; n=10) of similar (initial) mean FEC. Due to a limited number of animals available in farms
136 #4 and #6 at the start of the FECRT, the CTL groups at these properties consisted of nine calves.
137 Oral formulations of IVM are not registered for use in cattle in Denmark, thus injectable IVM was
138 used. At Day 0, individual body weights (BW) were estimated in the IVM group using a girth tape
139 for cattle (Rondo combi®, Kruuse, Denmark), and the calves in the treatment group were injected
140 with the recommended dose of IVM (0.2 mg kg^{-1} BW s.c., Ivomec® 10 mg/mL, Merial Norden
141 A/S). A comparison of BW estimations between girth tape and electronic scale in a group of 30
142 FSG calves (BW range= 84 − 172 kg) was performed prior to the study and demonstrated a very
143 high correlation between the methods (Pearson's correlation = 0.98). Faecal samples were collected
144 rectally from all animals on Day 0 and 14 days post-treatment (Day 14). Immediately after
145 collection, the faecal samples were vacuum packed (Freshield Touch, CSE Co, Gyeonggi-do,
146 Korea) to create anaerobic conditions and transported to the laboratory in a cooling box. On all
147 farms, animals in the IVM and CTL groups grazed together on the same pastures until Day 14,
148 when all control calves were treated with the recommended dose of injectable IVM as described
149 above.
150

151 *2.3 Parasitological analyses*

152 Upon arrival at the laboratory, faecal samples were refrigerated at 5°C until analysis. Individual
153 FEC were determined using an accredited, modified McMaster technique with a sensitivity of 5 epg
154 (Henriksen and Aagard, 1975). At Day 0 and Day 14, pooled larval cultures were prepared from the
155 IVM and CTL groups by mixing 10 g of faeces from each animal of the same group into a pool,
156 which was then cultured according to Roepstorff and Nansen (1998). After 14 days of incubation at
157 20°C, nematode L3 were recovered by Baermannisation and stored at 12°C. A small number of L3
158 were harvested in the post-treatment larval cultures from farms #1, #2, #5 and #6 (< 40 larvae per
159 group). All pooled L3 were used for molecular detection of *Ostertagia ostertagi* and *Cooperia*
160 *oncophora* by real-time quantitative PCR (qPCR).

161

162 *2.4 Species-specific identification of nematode larvae by qPCR*

163 Molecular detection of *O. ostertagi* and *C. oncophora* in the pooled L3 suspensions was performed
164 using the qPCR method described by Höglund et al. (2013), with modifications. Briefly, all L3
165 pooled per group were concentrated by centrifugation and transferred into a 2 mL cryotube. Larvae
166 (in 200 µl of tap water) were mixed with 1 mL buffer ATL (QIAGEN, Germany) and 600 µl of
167 0.5 mm Zirconia beads (BioSpec Products, USA) and homogenised by bead-beating for 1 min at
168 6.5 m/s (FastPrep®-24, MP Biomedicals, USA). Subsequently, the suspension was digested at 56°C
169 for 60 min using 20 µl of Proteinase K (20 mg/ml, QIAGEN, Germany) following manufacturer's
170 instructions. Genomic DNA was extracted from the digested larval homogenate by QIAamp® DNA
171 Mini Kit (QIAGEN, Germany). For qPCR analyses, primers and probes targeting the second
172 internal transcribed spacer (ITS-2) of the ribosomal DNA of *O. ostertagi* and *C. oncophora* were
173 used (Höglund et al., 2013). ITS-2 copies of both nematode species were quantified by correlating
174 cycle threshold (Ct) values to a standard curve with 2×10^7, 2×10^6, 10^5, 10^4 and 10^3 molecules μl^{-1}
175 of stock plasmid DNA. The plasmid DNA was made from a pCR® 2.1 vector (Thermo-Fischer

176 Scientific, USA) that comprised ITS-2 sequences of *C. oncophora* (GenBank® accession no.
177 AB245040.2, position 651-729) and *O. ostertagi* (GenBank® accession no. AB245021.2, position
178 1036-1126) (Höglund et al., 2013). The reactions were run in a Rotor-Gene Q RG-6000®
179 (QIAGEN, Germany) in total volumes of 25 µL using 2 µl DNA as a template. The PCR mix
180 contained 0.65 U Taq2000® polymerase (Agilent Technologies, USA), 0.3 µM forward and reverse
181 primers, 0.2 µM probe, 200 µM dNTP and 5.5 mM $MgCl_2$. Rotor-Gene Q® series software
182 (QIAGEN, Germany) was used to determine Ct values for each run. The cycling conditions were
183 95°C for 10 min and 50 cycles of amplification (95°C, 15 sec, 62°C, 60 sec). All samples and
184 standards were carried out in technical duplicates, with exception of the standard curves which were
185 carried out in triplicates. Sensitivity and specificity of the qPCR method was 97.2% (95%
186 confidence interval [CI] = 83.8 – 100%) and 83.3% (95% CI = 36.4 – 99%), respectively, based on
187 analyses of spiked larval samples (n=42) containing known numbers of mixed or pure *O. ostertagi*
188 and/or *C. oncophora* L3 (unpublished results).
189
190 *2.5 Use of anthelmintics in the selected farms between 2002 and 2012*
191 Recordings of all anthelmintics prescribed between 2002 and 2012 in the six farms selected for the
192 FECRT were extracted from the VetStat database (Stege et al., 2003). In Denmark, every veterinary
193 drug prescribed for production animals is dispensed to farmers by official pharmacies or by
194 veterinary practitioners. The dispensing pharmacy or veterinarian must register in VetStat the total
195 amount of a specific drug sold to the farmer, the farm identity, the animal species and the age group
196 which receive the prescribed treatment. In VetStat, cattle are divided into three age groups: 1)
197 calves < 12 months old (heifer and bull calves); 2) young cattle ≥ 12 months old (heifers until first
198 calving and steers until slaughter) and 3) adult cattle (cows after first calving). For the study of
199 anthelmintic use, data retrieved included the name and active compound of the prescribed
200 anthelmintic, the formulation and total amount (in total mL or g) of the drug prescribed, and the

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201 targeted cattle age group. However, the exact number of treatments actually performed in each
202 prescription is not recorded in VetStat. We therefore estimated the number of treatments for each
203 prescription in the 'targeted' group (i.e. calves, young cattle or adult cattle) within each farm using
204 the total amount of a prescribed drug (exact data from VetStat), the recommended dose of a given
205 anthelmintic (considering its formulation) and a defined BW for each cattle age group. The defined
206 BW for each age group was estimated based on data from the Danish Cattle Association (Danmap,
207 2012) and considering common anthelmintic treatment practices in each group as: 200 kg BW for
208 calves, 450 kg BW for young cattle and 620 kg BW for adult cattle. Due to this defined BWs and
209 the likely variation in the actual amount of active compound used for different animals following
210 each prescription, the calculated number of treatments is only a proxy of the real number of animals
211 treated. In addition, it was assumed that anthelmintics delivered to farmers were used within a
212 month, which may not always have been the case; however, this potential bias is presumably
213 consistent across the different drugs and age groups. The number of animals per age group in each
214 farm at the prescription date was retrieved from the Central Husbandry Register (Ministry of
215 Environment and Food of Denmark, http://chr.fvst.dk, accessed on 15 March 2016). The efficacy of
216 previously used anthelmintics had not been tested in any of the six farms prior to the study.
217
218 *2.6. Estimation of treatment efficacy*
219 The efficacy of IVM in the FECRT was analysed by calculating the arithmetic mean FEC reduction
220 percentage (FECR%) with 95% CI using the recommended WAAVP method and two procedures
221 using Bayesian MCMC methods:
222
223 a) *WAAVP*: Following recommended WAAVP guidelines (Coles et al., 1992) as: *i)* FECR% (*With*
224 CTL) = $100 \times (1-[T_2/C_2])$, where T_2 and C_2 are the arithmetic mean FEC of the IVM and CTL
225 group at Day 14, respectively, and *ii)* FECR% (*No* CTL) = $100 \times (1-[T_2/T_1])$, where T_1 and T_2 are

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226 the arithmetic mean FEC of the IVM group at Day 0 and 14, correspondingly. The calculations of
227 FECR% and 95% CI with the WAAVP method were performed according to Coles et al. (1992) in
228 Microsoft Excel® 2010.
229
230 b) *eggCounts*: Using the Bayesian MCMC procedure implemented in 'eggCounts' (version 1.1-1)
231 described by Torgerson et al. (2014). The analyses were performed via the freely available web
232 interface of the procedure (available at: http://shiny.math.uzh.ch/user/furrer/shinyas/shiny-
233 eggCounts/; visited on 01 September 2016). This software is also available as an R package (Wang
234 et al., 2016). The eggCounts procedure uses MCMC to fit a model using a gamma-Poisson
235 distribution for pre- and post-treatment FEC data, thus accounting for aggregation in FEC data and
236 the Poisson errors of the egg counting process, to generate the FECR% with 95% CI (Torgerson et
237 al., 2014). The procedure uses a single prior for the over-dispersion parameter (aggregation). Two
238 model options are available using the eggCounts web interface: *i)* the unpaired model, which
239 models pre- and post-treatment data as independent gamma-Poisson distributions, with a scaled
240 mean and common aggregation parameters, and *ii)* the paired model, which fits a gamma-Poisson
241 distribution to the pre-treatment data only and scales the means of the Poisson processes from each
242 individual by the same constant to model the mean of the Poisson processes representing the post-
243 treatment data. Therefore in the paired model, pre- and post-treatment mean FEC come from the
244 same Poisson distribution (i.e. this assumes that the degree of FEC aggregation does not change
245 post-treatment relative to the pre-treatment). Both models are available with or without a zero-
246 inflation component. For comparison, we used both paired and unpaired models to analyse our
247 FECRT data, with the default moderately informative prior distributions as described by Paul et al.
248 (2014) and Wang et al. (2016), without zero-inflation. A correction factor of 5 epg was applied to
249 the data (to obtain the number of eggs counted) and FEC data from only the IVM group at Day 0

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250 and 14 were used (*No* CTL), as the software currently does not incorporate the FEC variation of a
251 separated CTL group.
252
253 c) *Modified Bayescount*: Using a model based on that implemented by the Bayesian MCMC
254 'Bayescount' procedure described by Denwood et al. (2010) and Geurden et al. (2015). The
255 Bayescount paired model describes the pre-treatment FEC data as a compound gamma-gamma-
256 Poisson distribution, with the first gamma distribution reflecting the variation between animals and
257 the second gamma-Poisson (negative binomial) distribution describing the variation in observed
258 FEC that would be expected with repeated samples from the same animal. The post-treatment FEC
259 data is modelled as a separate gamma-Poisson (negative binomial) distribution based on the
260 estimated mean for that animal, and scaled by the FECR%. The procedure provides the 95% CI of
261 the FECR%, while accounting for the FEC aggregation between individuals and for the Poisson
262 errors of the egg counting method. The model is also able to separate the between- and within-
263 animal FEC variation, and allows for changes in the FEC aggregation of post-treatment data relative
264 to the corresponding pre-treatment observation from the same animal. This increases the uncertainty
265 in the estimates compared with assuming that FEC aggregation is identical at Day 0 and 14, but
266 allows for potential differences in drug efficacy between animals to result in a higher FEC
267 aggregation post-treatment. Further description of the method can be found in the appendix of
268 Geurden et al. (2015). Furthermore, for the present study the Bayescount paired model was
269 modified to incorporate the FEC data from the initial screening in each farm and to model the data
270 from all six herds simultaneously, therefore allowing for pooling of one or more variance
271 parameters between farms. As a result, the procedure allows inference to be made on three FEC
272 variability (aggregation) estimates (k) separately: *i)* the expected variability between the
273 unobserved true mean of the animals (between-animal pre-treatment k); *ii)* the expected variability
274 between pre-treatment samples from the same animal (within-animal pre-treatment k) and *iii)* the

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275 expected variability between post-treatment samples from the same animal (within-animal post-
276 treatment *k*, which also captures variation in the true efficacy between animals).
277 The mean FEC from each time point was modelled independently in each farm, so that no pooling
278 of mean FEC or FECR% parameters took place between herds. Minimally informative DuMouchel
279 priors (Denwood, 2016) were used for the mean FEC and various *k* parameters, and a Beta(1,1)
280 prior was used for the FECR% and 95% CI estimates. Using this modified Bayescount model, the
281 efficacy of IVM in the FECRT studies was evaluated considering *i)* the FEC variation in the IVM
282 and CTL groups between Day 0 and 14 (*With* CTL), and *ii)* the FEC variation only in the IVM
283 group between Day 0 and 14 (*No* CTL). All FEC data were transformed to the number of eggs
284 counted (FEC divided by 5) for analysis. All modelling was performed in JAGS (Plummer, 2003)
285 using the 'runjags' package (Denwood, 2016) in R version 3.2.2 (R Core Team, 2015), with
286 convergence assessed both visually and using the Gelman-Rubin statistic. Full model code can be
287 obtained from the corresponding author.
288
289 The fit of all MCMC models to the FECRT data was assessed using the Deviance Information
290 Criterion (DIC) (Spiegelhalter et al, 2002). For the modified Bayescount procedure, DIC was used
291 to compare the fit of models with between- and within-animal *k* parameters estimated separately or
292 pooled between farms. For the eggCounts procedure, DIC was obtained using JAGS models with
293 identical formulations to those used by eggCounts (Wang et al., 2016) to compare the fit of the
294 paired vs. unpaired models.
295
296 *2.7 Interpretation of treatment efficacy*
297 Results with the methods described in Section 2.6 were used to estimate the efficacy of IVM
298 treatments based on the obtained FECR% and lower 95% CI as recommended by Coles et al.

299 (1992), as well as the upper 95% CI as suggested by Lyndal-Murphy et al. (2014), from which we
300 categorised three conditions:
301
302 *i)* Efficacious treatment, when mean FECR% and upper CI ≥ 95% and lower CI ≥ 90%;
303 *ii)* Reduced efficacy, when mean FECR% and upper CI < 95% and lower CI < 90%;
304 *iii)* Inconclusive, when none of the above conditions were met.
305
306 *2.8 Simulation study*
307 A simulation study was carried out to compare the performance of the methods outlined in Section
308 2.6. For this study, we analysed the FECR% between Day 0 and 14 in simulated treatment groups
309 (without untreated animals) with different levels of FEC aggregation. Datasets were simulated
310 based on hierarchical gamma-gamma-Poisson distributions with parameter estimates obtained from
311 the modified Bayescount procedure as described in Section 2.6 and applied to the real FECRT data.
312 Based on the fit of these models, we assumed that pre-treatment k is fixed between farms (i.e. not
313 herd dependent), but that post-treatment k varies (independently) between herds. As a result, a total
314 pre-treatment $k = 0.8$ (divided into between-animal pre-treatment $k = 1.3$ and within-animal pre-
315 treatment $k = 4.1$) were used for all simulations. Three different total post-treatment k parameters
316 were simulated: $k = 0.8$ (no change of FEC aggregation between Day 0 and 14), $k = 0.3$ (moderately
317 increased FEC aggregation between Day 0 and Day 14) and $k = 0.1$ (substantially increased FEC
318 aggregation between Day 0 and Day 14). Pre-treatment (Day 0) FEC datasets were simulated with
319 sample sizes of n =10, 20, 30 and 40 and a mean FEC of 34 eggs counted (equal to 170 epg using a
320 FEC method with a detection limit of 5 epg), using the same paired model as described for the
321 modified Bayescount procedure. Post-treatment (Day 14) data were simulated using a FECR% of
322 85% or 97%. This procedure was repeated 500 times for each combination of the (two) simulated
323 FECR%, (three) total post-treatment k levels and (four) sample sizes described (i.e. representing the

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324 results of 500 different treatment groups for each set of parameters). Each of the simulated datasets
325 were then analysed using *i)* the WAAVP procedure, *ii)* the paired and unpaired eggCounts
326 procedures implemented using the eggCounts package version 1.1-1 (Torgerson et al, 2014), and *iii)*
327 the standard (unmodified) Bayescount paired model procedure using equivalent (moderately
328 informative) priors to those used by eggCounts in order to facilitate comparison. The mean FECR%
329 and 95% CI obtained for a given simulated dataset using each procedure were recorded, and the
330 performance of the procedures was investigated by studying the probability of the simulated
331 FECR% being included in the 95% CI (i.e. coverage probability of the 95% CI) along with the
332 uncertainty of the 95% CI (i.e. the relative size of the 95% CI) provided by each procedure. All data
333 simulation and analysis procedures were performed in R version 3.2.2 (R Core Team, 2015).
334
335 **3. Results**
336 *3.1. Initial screening of farms*
337 Faecal egg count data observed in groups of 20 FSG calves from the 19 farms initially screened are
338 summarised in Table 1. A total of 380 animals were sampled during mid-June and early September
339 of 2013 and 2014. Strongyle eggs were detected on all properties but very low egg excretion levels
340 (mean FEC ≤ 44 epg) were seen in 12 farms (63%). All six farms selected for the FECRT had an
341 initial arithmetic mean FEC ≥ 75 epg.
342
343 *3.2 FECRT*
344 At Day 0, IVM groups in farms #5 and #6 had a very low mean FEC (≤ 55 epg). In farm #6, three
345 calves in the IVM group could not be sampled at Day 14 and were excluded from the analyses. In
346 the model fitting analyses, the eggCounts unpaired and the modified Bayescount with pooled pre-
347 treatment *k* between farms (herd dependent) and separate post-treatment *k* between farms (herd

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348 independent) had the lowest DIC values (data not shown), offering the best fit for the FECRT data,
349 and were therefore selected for the FECR% analyses.
350 Results of the FECRT analyses using the WAAVP method and the selected eggCounts and
351 modified Bayescount procedures are presented in Table 2. The modified Bayescount procedure also
352 provided inference on the FEC aggregation estimates (k). The pre-treatment k was modelled as
353 fixed between farms, with an estimate of 0.8 (95% CI 0.6 − 0.9) for all six herds. In contrast, the
354 post-treatment k was allowed to vary between farms and there was evidence for a decrease in post-
355 treatment k (i.e. increased FEC aggregation) in Farms #3, #4 and #6 (Table 2).
356 The WAAVP and the modified Bayescount procedures including the FEC of treated and untreated
357 animals (*With CTL*) declared reduced IVM efficacy in farm #2, whereas only the modified
358 Bayescount stated reduced drug efficacy in farm #1 (Table 2). Furthermore, the WAAVP and
359 modified Bayescount procedures (*With CTL*) indicated efficacious treatment in farm #5, while all
360 other results were inconclusive. While in analyses including the FEC fluctuations in the treated
361 groups only (*No CTL*), the WAAVP, modified Bayescount and eggCounts procedures unanimously
362 indicated reduced IVM efficacy in farms #1, #2 and #4. All methods (*No CTL*) yielded inconclusive
363 results in farms #3, #5 and #6 (Table 2).
364
365 *3.4 Real-time qPCR for detection of O. ostertagi and C. oncophora*
366 Proportions of ITS-2 copies of *O. ostertagi* and *C. oncophora* detected in pre- and post-treatment
367 pooled larval cultures in the FECRT are presented in Table 3. At Day 0, *O. ostertagi* and *C.*
368 *oncophora* ITS-2 copies were detected in both groups from all farms, with the exception of the CTL
369 group in farm #2, where only *C. oncophora* was identified. Post-treatment, *O. ostertagi* ITS-2
370 copies were detected only in IVM groups of farms #3 and #4, while *C. oncophora* ITS-2 copies
371 were identified in treated groups from all six farms. Amplification efficiencies of the standard

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372 curves were 96% and 100% for *C. oncophora* and *O. ostertagi*, respectively, with correlation
373 coefficients (R^2) of 0.99 for both species.
374
375 *3.5 Anthelmintic prescriptions in the farms selected for FECRT*
376 The anthelmintic prescriptions between 2002 and 2012 in the six farms selected for FECRT and the
377 estimated number of animals treated are shown in Supplementary Table 1. Prescriptions with
378 anthelmintics other than MLs were rarely observed (only in farm #2 with albendazole and farm #3
379 with levamisole). In calves from all six farms, IVM was the most commonly used ML (87.2% of all
380 prescriptions), followed by moxidectin (12.8%). Similarly, IVM was the predominant ML used in
381 young cattle (84.7% of all prescriptions), followed by moxidectin (14.7%). In adult cattle, IVM
382 constituted 61.5% of all prescriptions in farms #2, #3, #4 and #6, followed by eprinomectin (23.8%)
383 and moxidectin (12.5%). No adult cattle were recorded in farms #1 and #5 during the period.
384 Topical (pour-on) drugs were the predominant formulations used in all six farms (98.6%, 95.1%
385 and 88.4% of all prescribed treatments in calves, young and adult cattle, respectively). In general,
386 treatments were prescribed at irregular intervals in all farms throughout the decade preceding the
387 FECRT, and 61% of all prescriptions were estimated to have been targeted to less than 50% of the
388 animals in a given age group. Nevertheless, evidence suggesting whole-group treatments was
389 observed at all farms, particularly farm #1 (the only conventional beef herd in the study) and farm
390 #6 (organic dairy farm). There were no marked differences in the prescription patterns between beef
391 and dairy or conventional and organic farms.
392
393 *3.6 Simulation study*
394 The performance of the different procedures used to estimate drug efficacy in the FECRT is
395 presented in Table 4. The coverage probability of the 95% CI describes the ability of each
396 procedure to correctly include the simulated FECR% in the generated CI under varying degrees of

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397 FEC aggregation (simulated total post-treatment k). The target coverage of the 95% CI is expected
398 to be close to 95%. Whereas the uncertainty of the 95% CI is defined as the average difference
399 between the upper and lower 95% CI generated by the procedure with each dataset, with a smaller
400 uncertainty representing (on average) a narrower 95% CI, and therefore the dataset is less likely to
401 be classified as inconclusive. The WAAVP method had good coverage for most parameter sets,
402 although this was lower for the datasets with very low post-treatment k, regardless of sample size
403 and FECR% (Table 4). The eggCounts paired model had consistently lower coverage probabilities
404 compared with the unpaired model, at all levels of post-treatment k, sample size and FECR% within
405 the conditions simulated. The unpaired eggCounts procedure had good coverage for most datasets
406 and reduced uncertainty compared to the Bayescount procedure, which reflects the assumption that
407 FEC aggregation in post-treatment data is the same as that in the pre-treatment data. However,
408 where this assumption was strongly violated by high post-treatment aggregation (post-treatment k =
409 0.1), then coverage of the unpaired eggCounts method dropped to 76% for the highest sample size.
410 The 95% CI provided by the modified Bayescount procedure had good coverage for all parameter
411 sets, although generally higher uncertainty than the other procedures for the 97% simulated
412 FECR%.

413 Coverage and uncertainty of the 95% CI generated by all procedures increased or decreased,
414 respectively, following the increase in group size in both simulated FECR% scenarios (Table 4).
415 Based on results with the WAAVP method (which generally gives a good balance between
416 coverage and uncertainty), and applying the classification criteria described in Section 2.7, the
417 minimum sample size that is required before 80% of datasets with a FECR = 97% can be correctly
418 classified as efficacious treatment is n = 20 for moderately increased FEC aggregation (pre and
419 post-treatment k = 0.8 and 0.3, respectively) or n = 40 for extremely increased FEC aggregation (pre
420 and post-treatment k = 0.8 and = 0.1, correspondingly; data not shown). Similarly, to give an 80%
421 chance of correctly classifying datasets with a FECR = 85% as reduced efficacy, a minimum of n =

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422 20 is required for no increase in post-treatment aggregation, or n = 30 for moderately increased
423 aggregation. With extreme increases in aggregation, even a sample size of n = 40 may be expected
424 to give inconclusive results approximately 50% of the time (data not shown).

425

426 **4. Discussion**

427 In the FECRT, reduced IVM efficacy was detected in three farms by all analyses based on the FEC
428 of treated cattle only, and in one farm according to both procedures including the FEC from treated
429 and untreated animals. Post-treatment, *O. ostertagi* and *C. oncophora* L3 were identified in faeces
430 of treated animals in one and three farms with declared reduced IVM efficacy, respectively. This is
431 the first report of reduced anthelmintic efficacy in Danish cattle.

432

433 During the initial screening prior to the FECRT, FSG calves with mean FEC < 100 epg were
434 detected in most farms. Similar observations were reported in untreated FSG calves from Belgium,
435 Germany (El-Abdellati et al., 2010) and France (Geurden et al., 2015). At Day 0 of the FECRT,
436 three farms had a mean FEC < 150 epg (farms #4, #5 and #6), which was a consequence of our
437 initial selection of farms with mean FEC ≥ 75 epg. Although the use of a FEC method with high
438 sensitivity (5 epg in the present study) reduces the diagnostic uncertainty in samples with low FEC
439 (El-Abdellati et al., 2010; Levecke et al., 2011), a low mean FEC pre-treatment will likely affect the
440 outcome of a FECRT, particularly when group size ≤ 10 and FEC are highly aggregated (Levecke
441 et al., 2012). Therefore, the reduced drug efficacy detected in Danish farms with initial mean FEC
442 < 150 epg should preferably be confirmed by new FECRTs including cattle with higher egg
443 excretion.

444

445 The qPCR method applied in the present study was able to detect ITS-2 copies of *O. ostertagi* and
446 *C. oncophora* in pooled larval cultures from naturally infected cattle, as earlier reported by Areskog

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447 et al. (2013). In agreement with previous European reports (Demeler et al., 2009; Geurden et al.,
448 2015), *C. oncophora* was the predominant species surviving IVM treatment in all farms with
449 reduced drug efficacy. This result was expected as *C. oncophora* is the dose-limiting GIN for IVM
450 (Egerton et al., 1981). The more pathogenic *O. ostertagi* was detected in IVM groups from all six
451 farms at Day 0 and from two farms post-treatment. However, reduced efficacy by all methods (*No*
452 CTL) was only detected in one of these farms (#4), therefore the possible presence of an IVM-
453 resistant *O. ostertagi* in this herd should be further confirmed. The absence of *O. ostertagi* L3 in
454 post-treatment cultures from most farms reflects the expected high efficacy of injectable IVM
455 against this abomasal species (Egerton et al., 1981; Lifschitz et al., 2000). Nevertheless, the
456 examination of pooled L3, the low FEC of samples and the poor yield of L3 following culture may
457 have been masking the true ratio of the species surviving treatment. Individual larval cultures could
458 have increased the sensitivity of the test and resulted in a different outcome, however, this was not
459 possible within the timeframe of the study. A practical limitation for the routine use of infective
460 larvae for species detection is the time required to culture L3 and the well-known variability in
461 developmental requirements of different nematodes in larval cultures (Roeber and Kahn, 2014). As
462 alternative, other nematode stages could be used for species-specific detection by molecular
463 techniques (Harmon et al., 2006), and recent studies from our group have explored this by
464 effectively quantifying ITS-2 copies in eggs and first-stage larvae of *O. ostertagi* (Drag et al.,
465 2016).
466
467 In the FECRT, methods of calculation that included the FEC of untreated animals resulted in a
468 higher number of inconclusive results, in comparison with methods excluding the CTL group. The
469 inclusion of untreated animals in a FECRT has been recommended to detect changes in the FEC of
470 a herd not related with the treatment, and for the correction of such fluctuations in the estimation of
471 drug efficacy (Coles et al., 1992; Lyndal-Murphy et al., 2014). However, the inclusion of a control

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472 group is based on the assumption that treated and untreated calves with comparable FEC share
473 similar worm burdens, which may not be always the case (Michel, 1967; 1969). Moreover, the
474 density-dependent control of fecundity in some cattle nematodes, such as in *O. ostertagi*, may
475 reduce the FEC in untreated animals due to newly acquired infections and could increase the egg
476 excretion of female worms in treated groups due to lower worm burden and competition (Dobson et
477 al., 2012). In practice, when few animals are available for a FECRT, the inclusion of a control
478 group will limit the size of the treatment group and consequently increase uncertainty of the
479 estimated efficacy (Denwood et al., 2010). The low number of FSG calves with positive FEC that
480 could be included in our study suggests that in these circumstances, the inclusion of untreated cattle
481 in the FECRT should not be recommended. Instead, it may be advisable to include more animals in
482 the treatment group in order to increase the certainty of the calculated FECR% (Denwood et al.,
483 2010; Levecke et al., 2012).
484
485 The simulation study showed the difficulties of analysing FECRT data, even with sophisticated
486 methods such as that implemented in eggCounts and Bayescount. The poor coverage of 95% CI
487 produced by the paired eggCounts procedure suggests (based on our simulations) that the
488 eggCounts unpaired model should be preferred, even when analysing paired data such as that
489 simulated here. Both eggCounts models were also tested with the use of the zero-inflation option,
490 but results were qualitatively similar to those without zero-inflation (data shown). The relatively
491 simple WAAVP procedure showed a better coverage probability than most of the other procedures,
492 but does have the limitation of not providing a 95% CI when post-treatment FEC are zero
493 (Denwood et al., 2010, Torgerson et al., 2014; Geurden et al., 2015). Only the modified Bayescount
494 procedure was able to consistently identify the simulated FECR% within the 95% CI produced,
495 although this comes at the cost of increased uncertainty. The results also suggest that a treatment
496 group of n = 10 is inadequate for cattle, and also support the observations of Gill et al (1986) that

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497 the minimum required sample size depends strongly on the degree of aggregation that is assumed.
498 Given these complications, new statistical methods are needed to quickly determine the prospective
499 study power of a given animal group size, mean FEC and aggregation parameters. However, despite
500 the limited sample size available, we were able to modify the paired Bayescount model to make use
501 of the available data from the initial screening and use of pooled variance parameters to bolster the
502 inference of the model. This produced substantially reduced 95% CI relative to the independently
503 modelled datasets using the same procedure (data not shown), and is therefore highly recommended
504 as a way to maximise the utility of MCMC for these types of data. A second benefit of this
505 approach is that the variability parameters themselves may give useful information; in this case,
506 there is a strong suggestion that the change in FEC aggregation is more substantial in some farms
507 than others. If this is due to variation in efficacy between animals, then a large post-treatment
508 change in variability could itself indicate the early signs of developing AR. It is also possible to
509 incorporate moderately informative priors into models fit using Bayesian MCMC as a way of
510 maximising the inference from the data. Informative priors were explored as part of the modified
511 Bayescount model presented, but ultimately did not provide any more information than the
512 minimally informative priors. Moderately informative priors are used by eggCounts, and these are
513 certainly valid in the context given by Paul et al. (2014), but care should be taken when using these
514 models for different datasets to ensure that the priors are appropriate in the situation at hand. If the
515 use of these methods is attempted without some understanding of the theory and application of
516 MCMC, then the potential for erroneous inference is extremely large due to either selection of
517 inappropriate model formulations (including prior selection) or errors introduced by poor
518 convergence and/or high autocorrelation (Brooks and Roberts 1998; Kass et al 1998; Toft et al
519 2007). We therefore strongly recommend that users without the necessary statistical experience
520 seek assistance with implementation and interpretation of both the eggCounts and Bayescount
521 procedures, although when correctly applied these methods can be used to maximise the

522 information available from the data by incorporating additional data sources and using techniques
523 such as partial pooling. There is also substantial scope for the development of a procedure that is
524 simpler in application and interpretation than MCMC, but until such method is available, our results
525 support the continued use of the WAAVP method to analyse FECRT in cattle where mean post-
526 treatment FEC are greater than zero and statistical expertise is not available.
527

528 At the recommended dose of 0.2 mg kg^{-1} BW, injectable IVM is expected to reduce susceptible *O.*
529 *ostertagi* adults and L4 stages by ≥ 99% and susceptible *C. oncophora* adults and L4 stages by
530 ≥ 97% (Egerton et al., 1981). However, the current WAAVP guidelines suggests that any treatment
531 with a FECR > 90% in cattle should not be considered a case of drug resistance (Coles et al., 1992).
532 In practice, most studies evaluating anthelmintic efficacy in cattle declare AR when FECR < 95%
533 and lower 95% CI < 90, as proposed for sheep. Nevertheless, it has been suggested that this criteria
534 is biased towards declaration of AR when there is none, particularly if the mean FECR% is between
535 90 − 95% and the CI is wide (Lyndal-Murphy et al., 2014). In the present study, we included the
536 upper 95% CI in the interpretation of the FECRT to increase the certainty of detecting true cases of
537 IVM inefficacy. A similar interpretation for FECRT studies in cattle has been reported in recent
538 investigations by Geurden et al. (2015) and Ramos et al. (2016). However, the effect of including
539 the upper 95% CI in the interpretation for estimating drug efficacy using a FECRT, and how this
540 correlates with an actual resistant phenotype confirmed by controlled efficacy tests, warrant further
541 investigation. It is also important to note that a reduced FECR% may not necessarily be caused by
542 AR. A lower-than-expected *in vivo* efficacy, or varying drug response between animals, could be
543 the result of under dosing (e.g. due to inaccurate estimation of BW) and/or altered drug
544 pharmacokinetics and pharmacodynamics in different animals (e.g. due to nutrition-related
545 variations in fat reserves that may affect the persistent efficacy of ML, erratic absorption of drugs
546 from the site of injection and/or interactions with other co-administered drugs) (González Canga et

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547 al., 2008; El-Abdellati et al., 2010; Areskog et al., 2012; De Graef et al., 2013; Areskog et al.,
548 2014). These factors can impair the correct estimation of drug efficacy and detection of AR,
549 particularly in the dose-limiting species *C. oncophora*. Recently, *C. oncophora* populations that
550 were declared resistant to the recommended dose of injectable IVM by FECRT in two Swedish
551 cattle farms (using girth tape for weight estimation and with FECR% [upper CI] = 78% [97%] and
552 79% [98%] in each farm; Demeler et al., 2009) were declared IVM-susceptible when tested in
553 calves under controlled conditions (Areskog et al., 2014). Therefore, the presence of IVM-resistant
554 nematodes suggested by our FECRT in three farms, as well as the AR status in the farms with
555 inconclusive results and low initial FEC, should be confirmed by controlled efficacy test.
556
557 The use of anthelmintics in the farms included in our FECRT was investigated to potentially detect
558 trends in drug use and the extent of treatments with avermectins. Data was retrieved from the
559 VetStat database and used to estimate the number of animals treated with a given anthelmintic at
560 each prescription. However, the actual number of cattle treated at each investigated prescription is
561 unknown and our analysis aimed only to offer a rough estimate of the anthelmintic use in these
562 farms. Furthermore, VetStat does not register whether adult cattle are lactating or not at the time of
563 treatment, and therefore the prescription of drugs not allowed for treatment of animals in lactation
564 (e.g. IVM, levamisole) recorded in some of the studied farms deserves further investigation. Based
565 on the data retrieved from VetStat, most of the anthelmintics prescribed in the six farms between
566 2002 and 2012 were avermectins, mostly topical IVM products. A similar reliance on avermectins
567 has been preliminarily detected in the entire Danish cattle population in the period 2010 − 2014,
568 constituting ~80% of all treatments – of which 79% were IVM, mainly in topical formulations
569 (Peña-Espinoza et al., unpublished data). The irregular prescription of anthelmintics observed in the
570 study farms correlates with the prescription-only regulations in Denmark, illustrated by the
571 treatment of single animals or selected groups of animals in the herds. However, prescription

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572 patterns suggesting whole-group treatments in some farms indicate that these may be recommended
573 by veterinarians under certain conditions (e.g. during outbreaks of dictyocaulosis), and the effect of
574 this practice on the selection for AR needs further investigation. All treatments against GIN in
575 Denmark should be based on a clinical and/or laboratory examination, and preventive/strategic
576 anthelmintic treatments without such diagnosis are illegal. Organic farms are further encouraged to
577 apply other means of parasite control than use of anthelmintics; however, due to limited knowledge
578 of alternative and effective parasite control methods, most farms (whether organic or not) still rely
579 on anthelmintic drugs. Therefore, and considering the relevance of IVM and other anthelmintics for
580 nematode control in Danish cattle, the true extent of AR in bovine nematodes in Denmark needs to
581 be assessed in larger surveys. Until then, producers and veterinarians should be aware of potentially
582 ineffective treatments against GIN in cattle, while reducing the reliance on anthelmintics by
583 including other parasite control strategies with documented efficacy, such as grazing management
584 and feeding with bioactive forages (Nansen et al., 1987; Peña-Espinoza et al., 2016).
585
586 In conclusion, reduced IVM efficacy was detected by all methods for analysis of FECRT data
587 excluding untreated controls in three of six Danish cattle farms investigated. *Cooperia oncophora*
588 was the main species surviving IVM treatment in the three farms with confirmed reduced drug
589 efficacy, while *O. ostertagi* was also identified post-treatment by qPCR in one farm with reduced
590 IVM efficacy. Nevertheless, the presence of IVM-resistant nematode strains suggested by the
591 FECRT should preferably be confirmed by controlled efficacy test. Nevertheless, the reduced
592 efficacy of IVM detected in this study and the widespread use of ML drugs in Danish cattle suggest
593 that farmers and their advisors should be aware of potentially ineffective treatments and larger
594 surveys are warranted to describe the true extent of the problem. However, further validation of the
595 design and analysis of the FECRT in cattle are urgently needed before such surveys can be
596 implemented in cattle farms.

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597 **Conflicts of interests**

598 The authors declare that they have no conflicts of interests.

599

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607

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Table 1. Faecal egg counts (FEC) of 20 first-season grazing calves for each of the 19 Danish farms sampled during initial screening in the 2013 and 2014 grazing seasons. A total of 380 first-season grazers were examined. Data are presented as arithmetic mean FEC, range (in egg per gram of faeces, epg) and the date of sampling. Six farms (farms #1 – 6) were included in the subsequent faecal egg count reduction tests (FECRT).

Farm	Mean FEC (epg)	Range (epg)	Date of sampling[b]
1	438	85 – 975	16 June 2013
2	101	5 – 205	24 June 2013
3	184	5 – 730	20 August 2013
4	273	20 – 960	27 June 2014
5	75	10 – 225	09 July 2014
6	75	15 – 215	14 August 2014
7	8	0 – 40	02 July 2013
8	37	0 – 135	04 July 2013
9	44	0 – 135	23 July 2013
10	3	0 – 30	05 August 2013
11	17	0 – 80	14 August 2013
12	17	0 – 60	24 June 2014
13	221[a]	5 – 1165	02 July 2014
14	18	0 – 55	22 July 2014
15	8	0 – 40	23 July 2014
16	6	0 – 25	15 August 2014
17	3	0 – 20	25 August 2014
18	2	0 – 10	26 August 2014
19	11	0 – 40	07 September 2014

[a]Despite high FEC this farmer did not want to participate in the FECRT
[b]Turn-out of grazing cattle in Denmark is usually around late April/early May

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Table 2. Faecal egg count reduction test in calves naturally infected with gastrointestinal nematodes in six Danish cattle farms. A total of 115 first-season grazers were sampled on the day of treatment (Day 0) and 14 days post-treatment (Day 14). The calves were treated with ivermectin (IVM, 0.2 mg kg^{-1} body weight s.c.) or left untreated (CTL). Treatment efficacies were calculated including the variation of faecal egg counts (FEC) in treated and control groups (**With CTL**) or in treated groups only (**No CTL**). Total post-treatment FEC aggregation estimates (**k**) for each farm are shown according to the modified Bayescount.

	Farm #1 (Beef, conv.)		Farm #2 (Dairy, org.)		Farm #3 (Dairy, conv.)		Farm #4 (Dairy, org.)		Farm #5 (Beef, org.)		Farm #6 (Dairy, org.)	
Group	IVM	CTL	IVM	CTL	IVM	CTL	IVM	CTL	IVM	CTL	IVM	CTL
n	10	10	10	10	10	10	10	9	10	10	7	9
FEC Day 0	333	325	76	69	173	209	354	351	55	39	31	77
FEC Day 14	46	311	14	55	15	286	51	562	4	98	4	126
k	0.7 (0.4 – 0.9)		0.7 (0.1 – 1.0)		0.3 (0.1 – 0.6)		0.3 (0.2 – 0.5)		1.0 (0.6 – 1.4)		0.2 (0.1 – 0.4)	
With CTL	FECR%	[CI]	FECR%	[CI]	FECR%	[CI]	FECR%	[CI]	FECR%	[CI]	FECR%	[CI]
WAAVP[a]	85[I]	[67 – 95]	75[R]	[27 – 92]	95[I]	[56 – 99]	91[I]	[74 – 97]	96[E]	[92 – 99]	97[I]	[65 – 99]
Bayescount[b]	87[R]	[80 – 94]	78[R]	[61 – 92]	93[I]	[70 – 99]	91[I]	[73 – 99]	96[E]	[92 – 99]	90[I]	[52 – 99]
No CTL	FECR%	[CI]	FECR%	[CI]	FECR%	[CI]	FECR%	[CI]	FECR%	[CI]	FECR%	[CI]
WAAVP[a]	86[R]	[66 – 94]	82[R]	[47 – 94]	92[I]	[30 – 99]	86[R]	[67 – 94]	94[I]	[87 – 97]	83[I]	[−50 – 98]
Bayescount[b]	87[R]	[81 – 93]	83[R]	[72 – 92]	90[I]	[62 – 98]	81[R]	[50 – 94]	92[I]	[84 – 98]	81[I]	[25 – 99]
eggCounts[c]	84[R]	[55 – 94]	80[R]	[50 – 91]	86[I]	[21 – 98]	83[R]	[47 – 94]	93[I]	[82 – 97]	83[I]	[22 – 97]

FEC = arithmetic mean faecal egg count; **k** = total post-treatment FEC aggregation estimate; FECR% = FEC reduction percentage; CI = 95% confidence interval

[a]Coles et al. (1992); [b]Modified Bayescount (paired model with pooled between-animal and within-animal pre-treatment **k**); [c]eggCounts unpaired model (Torgerson et al., 2014); [E]Efficacious; [R]Reduced efficacy; [I]Inconclusive; conv. = conventional; org. = organic

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Table 3. Proportion (percentage) of the second internal transcriber space (ITS-2) copy numbers of **Ostertagia ostertagi** and **Cooperia oncophora** detected by real-time quantitative PCR in pooled L3 cultured from faeces of naturally infected calves. The calves were treated with the recommended dose of injectable ivermectin (IVM) or left untreated (CTL). The samples were collected on the day of treatment (Day 0) and 14 days post-treatment (Day 14).

Farm	Group	Day 0		Day 14	
		O.o.	**C.o.**	**O.o.**	**C.o.**
#1	IVM	42	58	0	100
	CTL	39	61	57	43
#2	IVM	79	21	0	100
	CTL	0	100	2	98
#3	IVM	45	55	85	15
	CTL	78	22	74	26
#4	IVM	8	92	0.5	99.5
	CTL	17	83	6	94
#5	IVM	42	58	0	100
	CTL	84	16	0	100
#6	IVM	3	97	0	100
	CTL	6	94	0	100

O.o. = Ostertagia ostertagi; C.o. = Cooperia oncophora

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Table 4. Coverage probabilities and uncertainty of 95% confidence intervals) provided by the WAAVP, eggCounts (paired and unpaired model) and modified Bayescount procedures with datasets of simulated FECR = 85% or 97% and with varying levels of group sizes (n) and total post-treatment aggregation estimate (**k**). Results are presented as the percentage of iterations in which the simulated FECR% was correctly included within the 95% CI provided by the procedure. A total of 500 iterations were performed for each combination of true FECR% and **k**. See text for further details on the simulated parameters.

		Coverage 95% CI				Uncertainty 95% CI			
FECR% = 85%		WAAVP[a]	eggCounts[b]		Modified Bayescount	WAAVP[a]	eggCounts[b]		Modified Bayescount
n	**k**		paired	unpaired			paired	unpaired	
10	0.1	82	23	80	95	65	8	45	74
	0.3	95	40	96	99	49	9	45	51
	0.8	98	63	99	100	34	9	36	32
20	0.1	88	26	82	97	45	6	34	60
	0.3	94	39	94	95	31	6	27	30
	0.8	100	64	99	99	23	6	22	17
30	0.1	90	22	84	96	40	5	29	50
	0.3	96	44	95	95	25	5	21	20
	0.8	100	67	100	99	18	5	17	13
40	0.1	91	22	81	97	34	4	23	40
	0.3	97	39	94	96	22	4	18	17
	0.8	100	64	99	97	16	4	15	10
FECR% = 97%		WAAVP[a]	eggCounts[b]		Modified Bayescount	WAAVP[a]	eggCounts[b]		Modified Bayescount
n	**k**		paired	unpaired			paired	unpaired	
10	0.1	84	49	85	99	15	4	14	68
	0.3	93	67	93	100	10	4	11	38
	0.8	98	85	99	100	9	4	10	18
20	0.1	87	46	79	97	11	3	7	43
	0.3	96	74	96	99	7	3	6	11
	0.8	98	83	99	99	5	3	5	5
30	0.1	89	43	78	95	8	2	5	25
	0.3	96	68	94	97	5	2	4	6
	0.8	99	83	99	98	4	2	4	4
40	0.1	90	44	76	95	7	2	4	15
	0.3	97	70	94	95	5	2	4	4
	0.8	98	82	98	97	4	2	3	3

aColes et al. (1992); [b]Torgerson et al. (2014);CI = confidence interval; FECR% = Simulated faecal egg count reduction; n = simulated group size; **k** = simulated total post-treatment FEC aggregation estimate

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Highlights:

- Reduced IVM efficacy in three out of six Danish cattle farms declared by FECRT
- **Cooperia oncophora** was identified by qPCR in three herds with reduced IVM efficacy
- **Ostertagia ostertagi** was detected in one farm with reduced IVM efficacy
- Increased FEC aggregation post-treatment affects estimation of drug efficacy
- Further validation of design and analysis of FECRT in cattle are urgently needed

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7.2 Manuscript II

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

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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_1; *n* = 9) or ryegrass/clover hay (CTL_1; *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_2; *n*=10) or ryegrass/clover (CTL_2; *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_2 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_1 by 60% (geometric mean; $P < 0{\cdot}01$), whereas *C. oncophora* burdens were unaffected ($P = 0{\cdot}12$). In Exp. 2, FEC were markedly lowered in CHI_2 from D22 p.i onwards ($P < 0{\cdot}01$). *Ostertagia ostertagi* adult burdens were significantly reduced in CHI_2 by 66% ($P < 0{\cdot}001$). 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*. 2011*a*; 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⩾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 baermannized 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 (±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 (CHI_1, $n = 9$; three subgroups of three calves) or control (CTL_1, $n = 6$; two subgroups of three calves) feeding groups. The calves were fed twice daily *ad libitum* with chicory silage (CHI_1) or ryegrass/clover hay (CTL_1) 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

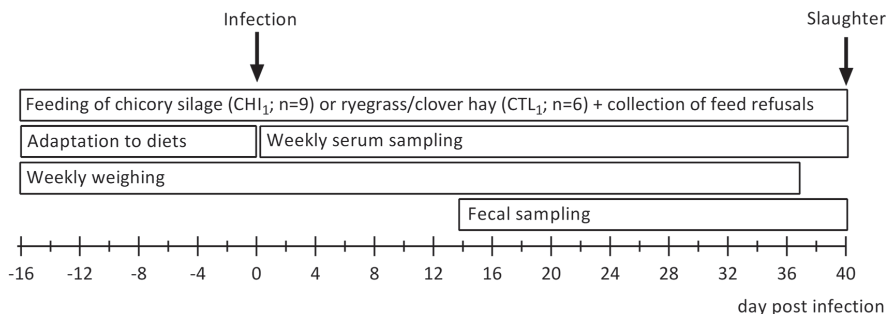

Fig. 1. Study design of Experiment 1 investigating the course of experimental infections with *Ostertagia ostertagi* and *Cooperia oncophora* in stabled calves fed with chicory silage and concentrate (CHI_1) or ryegrass/clover hay and concentrate (CTL_1) during 56 days.

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_2) or a control (CTL_2) group of ten animals each. The CHI_2 and CTL_2 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_2 and CTL_2 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_2 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_2 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_2 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 (55°67′48″N, 12°29′75″E). The soil

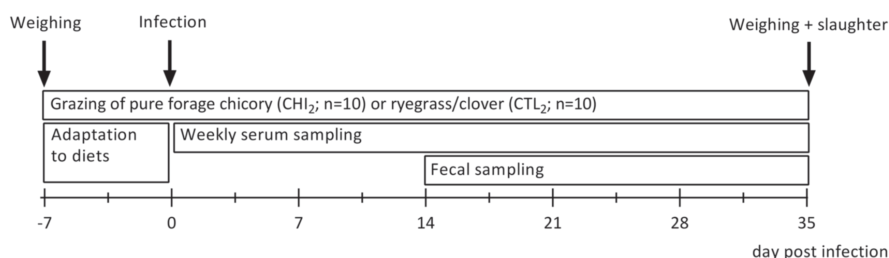

Fig. 2. Study design of Experiment 2 investigating the course of experimental infections with *Ostertagia ostertagi* in calves grazing pure forage chicory (CHI_2) or ryegrass/clover (CTL_2) during 42 days.

(moraine clay loam) was fertilized with 50 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.

Experiment 2. In early March 2014, the same forage chicory sward used for Exp. 1 was fertilized with 80 kg N ha^{-1}. 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_1 group consumed hay uniformly, whereas CHI_1 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^2, 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_2 and CTL_2 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_2 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 × *in vitro* OMD%, (b) fresh chicory *in vivo* OMD% = 4·1 + 0·959 × *in vitro* OMD%, (c) concentrate *in vivo* OMD% = 5·38 + 0·867 × *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 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 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:

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

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_1 and two CTL_1 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 (*.nb*) distribution and testing the effect of feeding group (CHI_1 or CTL_1) 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

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[a]
DM (%)	35·7	86·5	89·6
CP (g kg^{-1} DM)	93	84	204
ME (MJ kg^{-1} DM)	6·5	7·2	10·5
AAT (g kg^{-1} DM)	54	63	114
OMD (% of DM)	62	59	83
Crude ash (g kg^{-1} DM)	207	49	70
Sand (g kg^{-1} DM)	72	0·0	0·0
NDF (g kg^{-1} DM)	326	577	286
CT[b] (% DM)	n.d.	n.d.	n.d.
SL[c] (g kg^{-1} 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.

[a] Grønmix® (Danish Agro).

[b] According to Grabber *et al*. (2013; estimated detection limit = 0·2% of CT in DM).

[c] 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 (±S.D.). Percentage of *O. ostertagi* and *C. 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 (CHI_2 or CTL_2) 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 $P < 0{\cdot}05$ 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^{-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-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	Daily mean (±S.D.) intake 100 kg BW^{-1}	Day post infection						
		0	7	14	21	28	35	40
Chicory ($n=9$)	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)	192·7 (±26·5)	264·9 (±10·4)	278·8 (±2·9)	356·2 (±17·6)	369·5 (±2·9)	375·3 (±12·7)	362·6 (±12·1)
	ME (MJ)	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 ($n=6$)	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)	236·9 (±5·2)	278·6 (±3·4)	298·0 (±31·0)	343·7 (±18·0)	375·6 (±20·9)	372·0 (±17·4)	366·7 (±14·0)
	ME (MJ)	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.

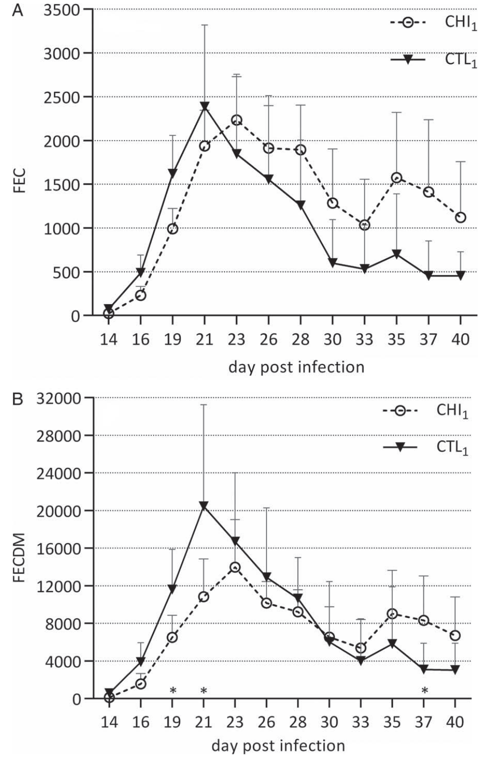

Fig. 3. Arithmetic mean fecal egg counts (FEC; A) and FEC per g of fecal dry matter (FECDM; B) in stabled calves experimentally infected with *Ostertagia ostertagi* and *Cooperia oncophora* and fed with chicory silage and concentrate (CHI_1) or ryegrass/clover hay and concentrate (CTL_1) in Experiment 1. Error bars indicate S.D. $*P<0{\cdot}05$.

A comparable increase in DM intake was recorded in CHI_1 and CTL_1 animals during the trial. At D0 p.i., the DM intake in the CHI_1 group was composed of 76% chicory silage, whereas the CTL_1 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_1 and CTL_1 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_1 calves, but with comparable levels between groups throughout the experiment.

Parasitology. Fecal DM% was not significantly affected by feeding group, time or their interaction ($P>0{\cdot}10$), but a significant effect of initial values (covariate) was confirmed ($P<0{\cdot}001$). 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,

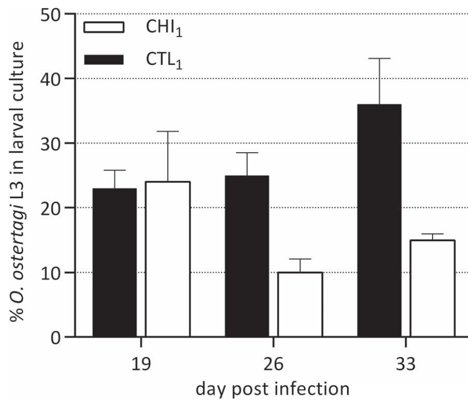

Fig. 4. Arithmetic mean percentage of *Ostertagia ostertagi* third-stage larvae (L3) identified in pooled fecal larval cultures of stabled calves experimentally infected with *O. ostertagi* and *Cooperia oncophora* and fed with chicory silage and concentrate (CHI_1) or ryegrass/clover hay and concentrate (CTL_1) in Experiment 1. Error bars indicate S.D.

D21 and D37 p.i. Higher FECDM was observed initially in CTL_1 (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_1 throughout the study period. However, the cumulative FECDM during the entire experiment was not significantly different between groups [mean (± S.D.) CHI_1 = 204 280 (±65 264) *vs* CTL_1 = 225 128 (±94 501) epg of fecal DM; $P = 0{\cdot}6$]. 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_1 and CTL_1 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_1 calves.

Post-mortem worm recovery in CHI_1 and CTL_1 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_1 animals ($P < 0{\cdot}01$). In contrast, adult counts of *C. oncophora* were not statistically different between groups ($P = 0{\cdot}12$). In CTL_1 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_1 and CTL_1 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

	O. ostertagi adult worm counts			*C. oncophora* adult worm counts		
Group	Males	Females	Total	Males	Females	Total
Chicory ($n = 9$)	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 ($n = 6$)	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).
*$P < 0{\cdot}05$; **$P < 0{\cdot}01$.

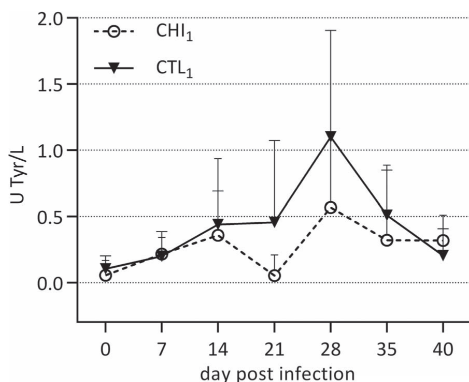

Fig. 5. Arithmetic mean serum pepsinogen levels (in units tyrosine; U Tyr/L) in stabled calves experimentally infected with *Ostertagia ostertagi* and *Cooperia oncophora* and fed with chicory silage and concentrate (CHI_1) or ryegrass/clover hay and concentrate (CTL_1) in Experiment 1. Error bars indicate S.D.

serum pepsinogen levels from D7 until D40 p.i. in CHI_1 and CTL_1 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 affected pepsinogen values ($P > 0·18$). Serum TP and ALB levels were not affected by group or 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 influenced by time ($P < 0·001$), but additionally group × 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_1 group (data not shown).

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 CHI_1 calves. Similar mean (±S.D.) daily growth rates were observed in both groups until D19 p.i. [$CHI_1 = 383$ (±116) *vs* $CTL_1 = 325$ (±236) g day^{-1}]. In contrast, from D19 until D37 p.i., CHI_1 and CTL_1 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, CHI_2 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 CTL_2 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 CTL_2 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 CHI_2 and CTL_2 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_2 and CTL_2 were 1589 and 1474 epg, respectively ($P = 0·7$). From this point, mean FECDM in the CHI_2 group was rapidly and significantly reduced until the end of the trial: at D35 p.i., CHI_2 animals had a 65% lower mean FECDM compared with CTL_2 calves ($P < 0·01$). Cumulative FECDM during the entire experiment was significantly reduced in the CHI_2 group [mean (± S.D.) $CHI_2 = 12\,870$ (±8136) *vs* $CTL_2 = 22\,260$ (±5671) epg of fecal DM; $P < 0·01$].

Post-mortem worm recovery of CHI_2 calves revealed significantly lower numbers of *O. ostertagi* males [mean (range) $CHI_2 = 2106$ (283–5425) *vs* $CTL_2 = 4692$ (2775–6800) male worms; $P < 0·01$] and females [$CHI_2 = 2256$ (617–4100) *vs* $CTL_2 = 5386$ (3700–6700) female worms; $P < 0·001$], compared with CTL_2 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) $CHI_2 = 4362$ (900–9525) *vs* $CTL_2 = 10\,078$ (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^{-1} DM)	98·2 (±32·1)	117·2	159·8 (±4·1)
ME (MJ kg^{-1} DM)	7·2 (±0·9)	8·6	8·3 (±0·5)
AAT (g kg^{-1} 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^{-1} DM)	150·2 (±30·7)	147·2	108·6 (±5·2)
Sand (g kg^{-1} DM)	17·0 (±1·4)	5·0	22·0 (±11·3)
NDF (g kg^{-1} DM)	260·5 (±54·5)	326·4	430·2 (±24·3)
CT[a] (% DM)	n.d.	n.d.	n.d.
SL[b] (g kg^{-1} 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.
[a] According to Grabber *et al.* (2013; estimated detection limit = 0·2% of CT in DM).
[b] 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) $CHI_2 = 0·19$ (0·05–0·39) *vs* $CTL_2 = 0·25$ (0·12–0·39) eggs female^{-1} 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.) $CHI_2 = 1·02 \pm 0·4$ U Tyr/L; $CTL_2 = 1·01 \pm 0·2$ 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_2 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^{-1} and 783 (±229) g day^{-1} in CHI_2 and CTL_2 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_1 and CTL_1 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

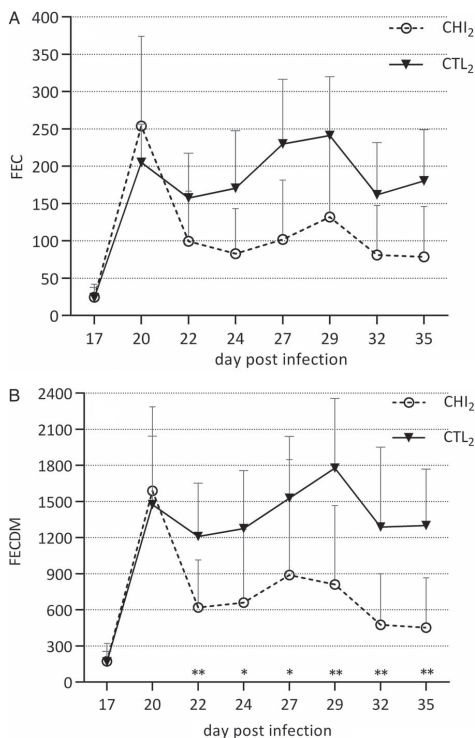

Fig. 6. Arithmetic mean fecal egg counts (FEC; A) and FEC per g of fecal dry matter (FECDM; B) in calves experimentally infected with *Ostertagia ostertagi* and grazing pure forage chicory (CHI_2) or ryegrass/clover (CTL_2) in Experiment 2. Error bars indicate S.D. $*P < 0{\cdot}05$; $**P < 0{\cdot}01$.

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_1 calves had a lower mean FECDM compared with CHI_1 animals (only significant at D37 p.i.). Post-mortem analyses demonstrated lower mean *C. oncophora* counts in CTL_1 animals compared with CHI_1 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_2 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

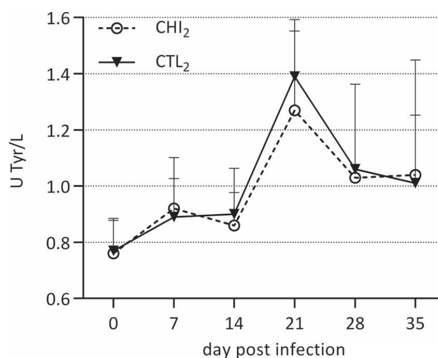

Fig. 7. Arithmetic mean serum pepsinogen levels (in units tyrosine; U Tyr/L) in calves experimentally infected with *Ostertagia ostertagi* and grazing pure forage chicory (CHI_2) or ryegrass/clover (CTL_2) in Experiment 2. Error bars indicate S.D.

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 (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-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 (⩾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, 2011*b*). 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*. 2011*b*; 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, cholorogenic 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_1 and CTL_1 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^{-1} for CHI_2 and CTL_2, 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_2 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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7.3 Manuscript III

Sesquiterpene lactone containing extracts from two cultivars of forage chicory (*Cichorium intybus*) show distinctive chemical profiles and *in vitro* activity against *Ostertagia ostertagi*.

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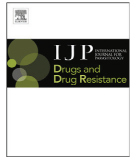

Sesquiterpene lactone containing extracts from two cultivars of forage chicory (*Cichorium intybus*) show distinctive chemical profiles and *in vitro* activity against *Ostertagia ostertagi*

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A B S T R A C T

The study investigated direct anthelmintic effects of sesquiterpene lactones (SL)-containing extracts from forage chicory against free-living and parasitic stages of *Ostertagia ostertagi*. Freeze-dried leaves from chicory cultivars 'Spadona' and 'Puna II' were extracted using methanol/water. Total SL were further fractionated by solid-phase extraction and resulting extracts were characterised by high-performance liquid chromatography (HPLC). *O. ostertagi* eggs from faeces of mono-infected calves were hatched and L1 were used in a larval feeding inhibition assay (LFIA), while cultured L3 were used in a larval exsheathment inhibition assay (LEIA). Adult worms were immediately recovered after slaughter and used for motility inhibition assays (AMIA). Electron microscopy (EM) was performed on adult *O. ostertagi* exposed to 1000 μg extract mL^{-1} of both chicory cultivars. In all assays, decreasing concentrations of SL-containing extracts in PBS (1% DMSO) were tested in replicates with 1% DMSO in PBS as negative controls. HPLC demonstrated similar concentrations of most SL in both extracts. However, Spadona-extract contained significantly higher concentrations of 11, 13-dihydro-8-deoxylactucin ($P = 0.01$), while Puna II-extract had increased levels of 11, 13-dihydrolactucin ($P < 0.0001$). In the LFIA, both extracts reduced larval feeding at increasing concentrations, but Spadona-extract showed higher potency confirmed by significantly lower EC_{50} ($P < 0.0001$). In the LEIA, neither of the two extracts interfered with the exsheathment of L3 ($P > 0.05$). In the AMIA, both SL-containing extracts induced a dose-dependent effect but Spadona-extract showed greater activity and exerted faster worm paralysis than Puna II-extract with significantly lower EC_{50} ($P < 0.0001$). No cuticular damage was observed by EM in worms exposed to any of the extracts. We have demonstrated that SL-containing extracts from forage chicory can inhibit feeding of free-living larvae and exert direct effects against parasitic stages of *O. ostertagi*. Our results may contribute to the identification of natural anti-parasitic compounds and to interpret the *in vivo* anthelmintic effects of forage chicory.
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1. Introduction

In the face of increasing anthelmintic resistance in parasitic nematodes of ruminants, the development of efficacious novel control strategies that can reduce the reliance on anthelmintics are urgently needed (Waller and Thamsborg, 2004). Feeding of animals with certain plants has shown much promise as a complementary

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parasite control option, with *in vivo* results for several forages demonstrating anthelmintic efficacy against a number of gastrointestinal nematode species (Githiori et al., 2006; Hoste et al., 2015). Most of the anti-parasitic activity of these plants is believed to derive from the presence of biologically active compounds, designated as plant secondary metabolites (PSM). Consequently, a comprehensive study of PSM can help to understand the anthelmintic effects of bioactive crops in the field and to potentially detect novel anti-parasitic molecules. One of the plants investigated as a potential anthelmintic forage for ruminants is chicory (*Cichorium intybus*), which has been linked with significant reductions in adult worm burdens and egg excretion of abomasal nematodes in sheep (Scales et al., 1994; Marley et al., 2003; Tzamaloukas et al., 2005; Heckendorn et al., 2007). Chicory is known to produce a group of biologically active terpenoids, the sesquiterpene lactones (SL), in both leaves and roots (Rees and Harborne, 1985; Price et al., 1990; Foster et al., 2011; Graziani et al., 2015). In chicory leaves, SL are present as glycosides (bound to carbohydrates) and as free molecules (Ferioli et al., 2015). Sesquiterpene lactones are PSM thought to be involved in the protection of plants against herbivore attacks and for signalling between plants (Gershenzon and Dudareva, 2007). In biological systems, SL have demonstrated antioxidant, antibiotic, anticancerigenous and antiprotozoan properties (Bischoff et al., 2004; Cavin et al., 2005; Schmidt, 2006; Barrera et al., 2013).

To date, two studies have provided preliminary evidence of *in vitro* activity of SL from chicory against free-living stages of parasitic nematodes of ruminants. Molan et al. (2003) reported inhibitory effects of crude SL-extracts from chicory roots on free-living larvae of *Dictyocaulus viviparus* and mixed-species of gastrointestinal nematodes isolated from red deer, however, detection and characterisation of SL in the tested extracts was not performed. Foster et al. (2011) analysed extracts containing free SL from two chicory cultivars and found differences in the concentration of individual SL and inhibitory effects towards the eclosion of a predominantly *Haemonchus contortus* egg population. In contrast with free-living stages occurring in the environment, parasitic stages in the host have different biochemical characteristics and detoxification mechanisms, thus the same compound(s)/PSM can exert different effects and affect distinct targets in free-living or adult stages (O'Grady and Kotze, 2004; Hoste et al., 2015). As yet, no studies have explored the direct effects of well-characterised SL-containing extracts from forage chicory on parasitic stages, which are the expected primary targets of dietary SL in the host.

The main objective of our study was to investigate and compare the direct effects of SL-containing extracts isolated from two forage chicory cultivars on key biological processes of free-living and parasitic stages of *Ostertagia ostertagi*. This abomasal nematode is considered to be the most pathogenic and economically important parasite infecting grazing cattle in temperate areas of the world (Nansen, 1993; Fox, 1993; Charlier et al., 2014). Two chicory cultivars were included in the study in order to account for variations in the activity of extracts derived from different plant cultivars, previously described in chicory (Foster et al., 2011). In relation, a detailed characterisation of the SL profile in the tested chicory extracts was performed.

2. Materials and methods

2.1. Plant materials

Chicory cultivar (cv.) 'Spadona' and cv. 'Puna II' were sown on 7 May 2013 in two separate fields at the experimental farm of the University of Copenhagen (Tåstrup, Denmark, 55°67′48″N, 12°29′75″E). Both fields were fertilized with 50 kg N/ha in spring (early May). The soil type in both fields was moraine clay loam. Chicory cv. Spadona was sown as a pure sward (7.8 kg seeds/ha) whereas cv. Puna II was mixed with timothy (*Phleum pratense*; seed rate: 6 kg chicory + 6 kg timothy seeds/ha). Leaves from both chicory cultivars were hand-picked on 23 July 2013 and stored at −20 °C in the dark until extraction of SL. At the moment of collection, all chicory plants were at the vegetative stage.

2.2. Extraction of sesquiterpene lactones from forage chicory

Sesquiterpene lactones were isolated and purified from chicory leaves according to Ferioli and D'Antuono (2012), with some modifications. This extraction method selectively separates total (free and unbound) SL from phenols and other plant compounds. Chicory leaves were freeze-dried overnight, ground into powder, after which pulverized leaf material (2 g) from each cultivar was weighed separately into two 50 mL tubes. Extraction solvent (30 mL) of methanol/Milli-Q-H_2O (4/1; v/v) containing 2% formic acid (v/v) was added to each tube. The tubes were vortexed for 1 min, sonicated in a water bath at room temperature for 10 min and centrifuged (10 min, 1540 *g*). After centrifugation, supernatants from the same cultivar were pooled in a 200 mL round-bottomed glass flask (1 flask per cultivar). Remaining leaf material in the 50 mL tubes was extracted three more times as described above and supernatants were pooled in the corresponding round-bottomed flask. Collected supernatants were concentrated under reduced pressure at 35 °C to evaporate methanol and formic acid and freeze-dried overnight to remove Milli-Q-H_2O. The resulting crude extracts were resuspended in methanol (5 mL) and dissolved with 70 mL of a cellulase enzyme solution (10 mg cellulase from *Aspergillus niger* [Sigma 22178]/mL Milli-Q-H_2O) in order to release glycoside (bound) SL. The dissolved extracts with cellulase enzyme solution were then transferred to 15 mL tubes and incubated in a water bath for 2 h at 40 °C. After enzymatic treatment, extracts were distributed into 50 mL tubes (3 tubes per cultivar) and ethyl acetate (25 mL) was added to each tube. The tubes were centrifuged (10 min, 1540 *g*) and the supernatants (ethyl acetate-extracts) were collected in round-bottomed glass flasks (1 flask per cultivar). Remaining sedimented material in the 50 mL tubes was extracted with ethyl acetate (2 × 25 mL) as described above. The collected ethyl acetate-extracts were evaporated to dryness under reduced pressure (35 °C), redissolved in methanol (8 mL) and transferred to 50 mL tubes (1 tube per cultivar). Final purification of SL from phenols and other plant compounds was performed by solid-phase extraction (SPE). Dichloromethane (28 mL) was added to each tube and centrifuged (10 min, 1540 *g*). A SPE vacuum manifold was equipped with 12 × 6 mL SPE tubes (Supelclean® LC-Si SPE tubes, Supelco 505374). SPE tubes were conditioned with dichloromethane/i-propanol (6 mL, 1/1; v/v) and equilibrated with dichloromethane (6 mL). Clean 12 × 15 mL tubes (6 tubes per cultivar) were set in the vacuum manifold for collection of SL. Extract mixed with dichloromethane (6 mL) was loaded in each SPE tube and the obtained liquid fractions were transferred into one tared 50 mL glass flask per chicory cultivar. Collected fractions were dried under reduced pressure (35 °C) and resulting purified extracts were weighed. Obtained extracts were highly viscous with low solubility in Milli-Q-H_2O or PBS. These purified chicory extracts were therefore resuspended in 100% DMSO at a concentration of 100 mg dry weight extract/mL DMSO (stock solution) and stored at −20 °C until use for *in vitro* assays and chemical analyses. High-performance liquid chromatography (HPLC) analyses were performed with stock solution of purified chicory extracts after removal of DMSO by freeze-drying and resuspension in methanol (10 mg dry weight extract/mL methanol). Two additional extractions from the same plant material were performed as described

above and confirmed the repeatability of the extraction method (data not shown).

2.3. Chemical analyses of purified chicory extracts

Compounds present in the obtained purified extracts were characterised by HPLC with mass spectrometry (MS) carried out in a Shimadzu Nexera X2 equipment and a Bruker MicrOTOF-Q III mass spectrometer, using an Supelco, Ascentis Express Peptide ES-C18 column (2.7 μm, 160 Å). A 1 mL/min linear gradient from 0 to 100% buffer B over 10 min was employed (buffer A: 0.025% trifluroacetic acid [TFA] in 10% aqueous acetonitrile-MeCN; buffer B: 0.025% TFA in 90% aqueous MeCN). Molecular mass analysis was carried out in conjunction with HPLC on a quadruple mass spectrometer in positive and negative electron spray ionization modes. Individual SL were identified by comparison of the main fragment ions (m/z) detected in all major peaks and retention times from published reports of SL profiles in chicory (Sessa et al., 2000; Ferioli and D'Antuono, 2012; Graziani et al., 2015). To quantify individual SL, HPLC was carried out at a wavelength where the UV absorptions of the different compounds were approximately the same (i.e. isosbetic point). This approximate isosbestic point was determined from the UV absorption spectra of all compounds detected in the purified extracts and occurred at wavelengths of 271 nm for cv. Spadona and 275 nm for cv. Puna II (Supplementary Figs. 1 and 2, respectively). Consequently, each extract was analysed at the mentioned wavelength for determination of individual peak areas. Of the SL known to be present in chicory, only lactucopicrin was commercially available as a pure standard when the experiments were conducted. Therefore, all detected peak areas were quantified by comparison with an external standard calibration curve using pure lactucopicrin. Here, a two-fold dilution of pure lactucopicrin (Extrasynthese 3813) was prepared in methanol (1000–15.6 μg lactucopicrin/mL) and analysed by HPLC-MS at 271 nm and 275 nm under the same conditions as the purified chicory extracts. Two separate SL quantifications were performed on each purified chicory extract.

2.4. Parasite material

Two Jersey calves, aged 5–6 months, were infected with 10,000 or 20,000 third-stage larvae (L3) of an ivermectin-susceptible *O. ostertagi* strain (Ref Label: OOSG10, Ridgeway Research, UK). The study was approved by the Animal Experiment Inspectorate, Ministry of Food, Agriculture and Fisheries of Denmark (j. No. 2013-15-2934-00763). Nematode eggs were detectable in faeces from both animals at day 16 post-infection. The eggs were recovered and hatched to obtain first-stage larvae (L1) according to Novobilský et al. (2011) and were used immediately after collection for a larval feeding inhibition assay. L3 were obtained from culture of eggs in faeces mixed with vermiculite for 14 days at 25 °C as described by Roepstorff and Nansen (1998). After recovery by baermannisation, L3 were maintained at 12 °C until use in a larval exsheathment inhibition assay. Calves were necropsied at two consecutive days in order to isolate live adult worms for motility inhibition assays. After captive bolt stunning and bleeding, the abomasum was immediately recovered and live *O. ostertagi* adults were isolated using the agar-migration method (Christensen et al., 1995). Adult worms were allowed to actively migrate from digesta into warm saline for 3 h at 37 °C and were then collected using a 20 μm sieve. Subsequently, worms were transferred into a 50 mL tube in warm saline solution (37 °C) and were washed three times in warm, sterile incubation medium (RPMI 1640 with L-glutamine, Gibco 11875-085), supplemented with 200 U/mL penicillin, 200 μg/mL streptomycin and 2 μg/mL of amphotericin B. After the last wash, worms were transferred to a Petri dish from which they were immediately collected for the assays.

2.5. In vitro assays

2.5.1. Larval feeding inhibition assay (LFIA)

The objective of the LFIA was to investigate the effect of SL-containing extracts on the feeding behaviour of *O. ostertagi* L1 and performed according with Jackson and Hoste (2010) and Novobilský et al. (2011), with modifications. Fluorescein isothiocyanate (FITC)-labelled *Escherichia coli* was prepared according to Jackson and Hoste (2010) and preserved at −20 °C until use. After isolation, L1 were diluted in PBS (0.05 M NaCl in Milli-Q-H_20, pH 6.9) until achieving a suspension of approximately 100 L1/500 μL of PBS. Stock solutions of purified chicory extracts were serially dissolved in PBS and five concentrations (in triplicates) were prepared for each extract in 1.5 mL Eppendorf tubes (500 μL of diluted extract per tube). Approximately 100 L1 (500 μl of larval suspension) were added to each tube, reaching final concentrations of 500, 250, 100, 50, 10 μg dry extract/mL PBS (final concentration 1% DMSO; total volume in tube = 1000 μL). L1 incubated with ivermectin (IVM, Sigma I8898, 1 mg/mL) and 1% DMSO in PBS were run in triplicates as positive and negative controls, respectively. All tubes were gently shaken and pre-incubated horizontally for 2 h at 25 °C. After pre-incubation, 10 μL of FITC-labelled *E. coli* were added to each tube. Then, the tubes were gently shaken and incubated horizontally for 18 h at 25 °C. After incubation, the tubes were centrifuged for 1 min at 6000 *g* and 800 mL of the supernatant was removed. Sediment with L1 was transferred into microscope slides and observed under a fluorescent microscope with a blue filter (Leica DMR A2, band pass filter 450–490 nm, long pass filter 515 nm) at 200× and 400× magnifications. All L1 in each tube were counted and classified as: a) fed larvae, with the presence of green fluorescent FITC-labelled *E. coli* in the larval oesophagus and/or intestine or b) unfed larvae, lacking FITC-labelled *E. coli* in the gut or presence of green fluorescence only outside the larval body or around the buccal opening. The number of fed/unfed larvae was used to calculate larval feeding percentages in each replicate, as: % larval feeding = 100 × [(number of fed L1)/(total number of L1)].

2.5.2. Larval exsheathment inhibition assay (LEIA)

This assay measured the potential inhibitory effect of SL-containing extracts on the chemically-induced exsheatment of *O. ostertagi* L3 and was conducted as described by Jackson and Hoste (2010) with minor modifications. Briefly, ensheathed L3 (2–3 months after isolation) were baermannised for 3 h using a 28 μm nylon mesh in order to isolate only live larvae. Obtained L3 were resuspended in PBS (pH 6.9) until a larval suspension of approximately 1650 L3/mL PBS was achieved. Stock solutions of Spadona and Puna II extracts were serially diluted in 100% DMSO and three decreasing concentrations were prepared for each chicory extract in 1.5 mL Eppendorf tubes (10 μL of each decreasing extract concentration per tube). Extra tubes were prepared with 10 μL of 100% DMSO as negative controls. Approximately 1600 L3 larvae (990 μl of the L3 suspension in PBS) were added to each tube, achieving final concentrations of 1000, 500 and 250 μg dry extract/mL PBS (1% DMSO) and 1% DMSO (negative controls). All tubes were vortex agitated for 10 s and pre-incubated horizontally for 3 h at 22 °C. After pre-incubation, the tubes were centrifuged at 6000 *g* for 2 min and the supernatants were removed. One mL of PBS was added to each tube and the washing procedure was repeated two more times before L3 were finally resuspended in 1 mL of PBS. A set of 24-well plates were prepared for the artificially induced larval exsheathment by adding 1940 μL of an exsheathment solution per well. Exsheathment solution was prepared by dilution of sodium

hypochlorite 10–15% (Sigma 425044) in PBS (1/500; v/v, pH 6.9). Approximately 100 L3 from each pre-incubation tube were then transferred into each well of the 24-well plate containing exsheathment solution (1 well plate per tube). This chemically-induced exsheathment was sequentially stopped by addition of 100 μL of Lugol iodine per well at 15, 30, 45 and 60 min after start of incubation. Exsheathment was replicated four times for each chicory extract concentration and 1% DMSO (negative control) and for each time point. Ensheathed or exsheathed larvae in all wells at each time point were quantified using an inverted microscope at 200 × magnification. Exsheathment rates were calculated for each well using the formula: % exsheathment = 100 × [(number of exsheathed larvae per well)/(total number of larvae per well)].

2.5.3. Adult motility inhibition assay (AMIA)

The AMIAs were designed to investigate the inhibitory effect of SL-containing extracts on the motility of adult *O. ostertagi*. The assays were carried out following the methods described by Paolini et al. (2004) and Jackson and Hoste (2010), with some modifications. Two independent AMIAs were performed testing decreasing concentrations of each purified chicory extract (four assays in total) using freshly isolated adult worms collected after slaughter (see Section 2.4). For each assay, 48 well-plates were prepared with six decreasing concentrations of Spadona or Puna II extracts dissolved in the incubation medium for adult worms described in Section 2.4. The following concentrations were tested: 1000, 500, 250, 100, 50 and 10 μg dry extract/mL incubation medium (final concentration 1% DMSO; total volume in well = 1000 μL). Every concentration was tested in four replicates per plate. Wells containing IVM (1 mg/mL) and 1% DMSO dissolved in incubation medium were also prepared in four replicates in each plate as positive and negative controls, respectively. Four additional replicate wells were prepared for incubation of worms in 1000 μg dry extract/mL or 1% DMSO for electron microscopy (EM). Immediately after collection from digesta and washing (section 2.4), four to six adult *O. ostertagi* (males and females) were carefully added to each well. The plates were then placed in an incubator at 37 °C and worm motility was checked after 6, 24 and 48 h of incubation using a stereomicroscope. At each time point, the plates were gently hand-agitated to stimulate worm movement and the number of motile and non-motile (no movement detected during 10 s) individuals in each well were recorded. All observations were made by the same person. After 24 h of incubation, the incubation medium was removed from all wells and replaced by fresh incubation medium (1000 μL; 37 °C) containing identical Spadona or Puna II-extract concentrations. Further, at 24 h worms incubated in the extra replicates at 1000 μg dry extract/mL and 1% DMSO were collected, carefully washed in PBS and fixed in 2% glutaraldehyde in PBS for EM (section 2.6). Motility scores obtained in each AMIA were used to calculate worm motility percentage in each well as: % worm motility = 100 × [(number of motile worms per well)/(total number of worms per well)].

2.6. Electron microscopy

Scanning electron microscopy (SEM) and transmission electron microscopy (TEM) investigations were performed to detect ultrastructural damage in adult *O. ostertagi* exposed to the highest concentration of SL-containing extracts during 24 h. Adult worms incubated with both chicory extracts and 1% DMSO were selected for SEM, while worms exposed to Spadona-extract and 1% DMSO were selected for TEM. Adult worms for SEM were fixed as described by Williams et al. (2014a) and after fixation worms were placed into 99% ethanol and then coated with gold using a Low Vacuum Coater Leica EM ACE200. Scanning electron microscopies were performed using a Phillips XL 30 FEG SEM and image processing was done with Scandium software. Adult worms for TEM were fixed, processed and analysed as described by Williams et al. (2014a).

2.7. Statistical analysis

Concentrations of individual and total compounds, detected in two separate quantifications on each purified extract, were compared between Spadona and Puna II extracts using a pairwise t-test with Bonferroni's correction. In the AMIA and the LFIA the effective concentration of each SL-containing extract able to inhibit the motility or feeding in 50% of adults or L1 (EC_{50}), respectively, was calculated. Tested extract concentrations were log-transformed and motility/feeding percentages obtained in the assays (eight replicates per concentration for AMIA and three replicates per concentration for LFIA) were analysed by non-linear (least squares) regression using the model *log (inhibitor) vs. response—variable slope* in GraphPad Prism® version 6.05, as described by Demeler et al. (2010). In the AMIA and LFIA the highest extract concentration tested from both extracts achieved a total inhibition (0%) of motility/feeding, with the sole exception of Puna II-extract in the AMIA after 6 h of incubation. Consequently, bottom values were set as 0% and top values were set as the mean motility/feeding percentage observed in negative control replicates. The R squared measure of goodness of fit (R^2) was calculated for each dose—response curve. Statistical differences between EC_{50s} obtained with both purified extracts were analysed by extra sum-of-squares F test with a null hypothesis of equal EC_{50}. In the LEIA, the mean exsheathment percentages were calculated for each concentration and time point and were compared with the exsheathment rates obtained in the negative control wells (1% DMSO) by two-way ANOVA. A value of $P < 0.05$ was considered significant.

3. Results

3.1. Chemical analyses of purified chicory extracts

Chromatograms of the purified chicory extracts analysed by HPLC-MS are presented in Fig. 1. Chemical characterisations of detected molecules in two separate quantifications of each chicory extract are presented in Table 1. All peaks in both extracts were detected before 4 min. Identification of *m/z* ion fragments (positive and negative mode) by MS confirmed that a certain degree of hydrolysis occurred in the SL during the extraction process (Supplementary Table 1). Lactucin (LAC), 8-deoxylactucin (8-DOL) and lactucopicrin (LCP) were detected in extracts from both chicory cultivars, as well as their 11, 13-dihydro (DI)-derivatives: DI-LAC, DI-8-DOL and DI-LCP (Table 1, Fig. 2). In addition, four unknown compounds were detected in both chicory extracts. From the original leaf material, mean (±S.D.) total SL recovered were 9.4 (±1.2) g total SL/kg of dried leaves in Spadona and 11.7 (±1.3) g SL/kg of dried leaves in Puna II. Similar levels of total and individual SL and unknown compounds were detected between extracts, with the exception of DI-LAC and DI-8-DOL which were found in significantly higher concentrations in Puna II and Spadona extracts, respectively (Table 1).

3.2. LFIA

In negative controls, 55.2% (±3.3%) of L1 incubated with 1% DMSO had fed at the end of the incubation. All fed larvae of the negative controls were motile while all unfed larvae were dead. In contrast, all L1 incubated with IVM (positive control) were dead and unfed. Larvae exposed to SL-containing extracts, however,

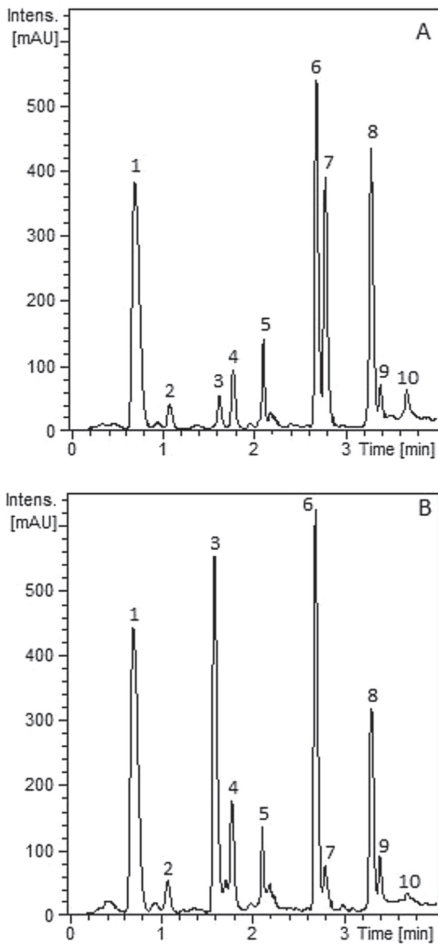

Fig. 1. Chromatograms obtained of purified extracts from chicory cv. Spadona (A) and cv. Puna II (B) used for *in vitro* assays with free-living and parasitic stages of *Ostertagia ostertagi*. Chromatograms are presented at the wavelength where isosbestic points were detected for each extract (271 nm for Spadona and 275 nm for Puna II). 3 = 11, 13-dihydro-lactucin; 4 = lactucin; 6 = 8-deoxy-lactucin; **7** = 11, 13-dihydro-8-deoxylactucin; 8 = 11, 13-dihydro-lactucopicrin; 9 = lactucopicrin. Peaks 1, 2, 5 and 10 = unknown.

showed a distinct pattern as all fed L1 were motile while unfed larvae were either motile or dead. No morphological damage was observed in L1 exposed to the SL-containing extracts at any tested concentration. Dose-response curves obtained in the LFIA with the tested extracts are depicted in Fig. 3. Larvae incubated with the lowest concentration of SL-containing extracts had similar feeding percentage as observed in the negative controls, while the highest concentration tested (500 μg dry extract/mL) of both extracts induced a total suppression of larval feeding. Nevertheless, at intermediate concentrations, Spadona-extract exerted a remarkably higher potency than Puna II-extract, tested at equal concentrations, illustrated by significantly lower EC_{50} values (EC_{50} [95% confidence interval] in μg dry extract/mL: Spadona = 26.9 [22.3–32.4] and Puna II = 108.7 [80.9–146.2], $P < 0.0001$).

3.3. LEIA

No significant differences were observed in the exsheathment rates between SL-containing extracts at all tested concentrations and negative controls ($P > 0.05$). All negative control L3 (pre-incubated with 1% DMSO) were exsheathed after 45 min of incubation with the exsheathment solution. Similarly, >99% of L3 exposed to any of the SL-containing extracts were exsheathed after 45 min of incubation.

3.4. AMIA

In negative controls, a mean worm motility of 100% was observed at 6 and 24 h of incubation with 1% DMSO. After 48 h of incubation, mean (±S.D.) worm motility percentage in the negative controls was 94.8% (±2.6%). Dose-response curves obtained in the AMIAs at 6, 24 and 48 h of incubation are presented in Fig. 4. Mean motility of worms incubated with the lowest concentration of both SL-containing extracts was not different from negative controls at any time point. In contrast, and after 6 h of incubation with the highest concentration, Spadona and Puna II extracts induced a paralysis in 100 and 92% of the incubated worms, respectively. However, at intermediate concentrations Spadona-extract induced a greater paralysis in exposed worms, reflected in significantly lower EC_{50} values at all time points, as compared with worms incubated with the same concentrations of Puna II-extract ($P < 0.0001$, Table 2). In addition, a time-dependent effect of the inhibitory activity of the SL-containing extracts was observed, represented as decreasing EC_{50} values for both extracts as the incubation period progressed.

3.5. Electron microscopy

At the time of collection for electron microscopy (after 24 h of incubation) all worms incubated at 1% DMSO were motile and all worms incubated with the SL-containing extracts were dead. Examinations by SEM showed no noticeable differences between negative control nematodes and worms exposed to SL-containing extracts, with no obvious structural damage in the buccal opening or cuticle of worms exposed to Spadona-extract (Supplementary Fig. 3a, b) or Puna II-extract. Ultrastructural examination of thin sections of *O. ostertagi* adults by TEM did not demonstrate substantial differences between nematodes. Negative control nematodes and nematodes incubated with Spadona-extract had integral exterior borders of the cuticle and an intact basal layer and hypodermis. The only apparent difference was a slight decolouration and subtle erosion of the epicuticle in worms incubated with Spadona-extract (Supplementary Fig. 3c).

4. Discussion

Here, we have demonstrated direct and dose-dependent anti-parasitic effects of SL-containing extracts of forage chicory on the motility and feeding of *O. ostertagi* adults and L1, respectively. In contrast, no effects were observed on the exsheathment of L3 at any of the tested concentrations. Marked differences in anthelmintic activity were detected in SL-containing extracts from two forage chicory cultivars, with Spadona-extract exerting higher anti-parasitic effects compared with Puna II-extract. Despite the similar content of total SL and unknown molecules in the two tested extracts, the increased potency of Spadona-extract may be associated with its distinctive composition of individual compounds, which is further discussed below.

In the AMIA, nonetheless the rapid lethal effect of high concentrations of SL-containing extracts on adult worms, EM investigations did not reveal any morphological damage of SL-

Table 1
Chemical characterisation by high-performance liquid chromatography-mass spectrometry of purified extracts from leaves of chicory cv. Spadona and cv. Puna II used for *in vitro* assays with free-living and parasitic stages of *Ostertagia ostertagi*.

Extract		Spadona			Puna II			Pairwise t-test
Peak	Compound	% Mean total peak area[a]	μg Compound/mg dry extract		% Mean total peak area[a]	μg Compound/mg dry extract		
			Q1[b]	Q2[b]		Q1[b]	Q2[b]	
1	U1	27.4	143.0	117.0	26.4	137.7	106.7	N.S.
2	U2	2.1	9.6	9.4	2.4	17.7	17.3	N.S.
3	DI-LAC	2.3	10.7	11.4	19.9	105.3	87.2	****
4	LAC	4.0	19.7	18.5	6.3	37.3	29.8	N.S.
5	U3	5.1	25.7	23.9	5.0	30.5	23.9	N.S.
6	8-DOL	20.4	105.7	81.2	20.6	108.6	84.2	N.S.
7	DI-8-DOL	14.8	76.5	61.6	3.0	20.4	17.4	***
8	DI-LCP	17.0	87.8	74.8	11.9	65.2	50.9	N.S.
9	LCP	2.4	11.1	12.2	2.8	19.5	23.5	N.S.
10	U4	4.5	22.3	21.6	1.7	13.5	12.7	N.S.
Total SL		60.9	311.5	259.7	64.5	356.3	293.0	N.S.
Unknown		39.1	200.6	171.9	35.5	199.4	160.6	N.S.

Q: quantification; U: Unknown; SL: sesquiterpene lactones; LAC: Lactucin; DI-LAC: 11, 13-dihydrolactucin; 8-DOL: 8-deoxylactucin; DI-8-DOL: 11, 13-dihydro-8-deoxylactucin; LCP: Lactucopicrin; DI-LCP: 11, 13-dihydro-lactucopicrin.
N.S: $p > 0.05$; $^{***}P = 0.01$; $^{****}P < 0.0001$.
[a] Area percentage of the respective peak of the total peak area in the chromatograms presented in Fig. 1.
[b] Quantification of individual compounds was performed using an external standard curve with a pure lactucopicrin standard.

exposed worms, with the exception of a decoloured and slightly eroded epicuticle, suggesting a possible early stage of cuticle degeneration. These results are in contrast with studies describing marked disruptions in the cuticle and ultrastructure of nematodes exposed to other bioactive plant compounds like cysteine proteinases and condensed tannins (Stepek et al., 2004; Brunet et al., 2011; Martínez-Ortíz-de-Montellano et al., 2013; Williams et al., 2014a, 2014b). Our findings therefore suggest that the mode of action of SL is different from that of the above mentioned PSM and this needs to be further explored.

In the LFIA, mean percentage of fed larvae in the negative controls (1% DMSO in PBS) were similar to published reports of LFIA with *O. ostertagi* L1 using 100% PBS as negative control medium (Novobilský et al., 2011). All unfed larvae in the negative controls were dead and similar low control values were observed by the authors in preliminary studies with L1 from the same *O. ostertagi* strain, but the reasons for this are unknown. In larvae exposed to SL, both extracts demonstrated a dose-dependent inhibitory effect on the feeding of *O. ostertagi* L1. Nonetheless, Spadona-extract inhibited larval feeding at much lower concentrations than Puna II-extract, which correlates with results from the AMIA. EC_{50} values obtained in the LFIA with both chicory extracts were much lower than those observed in the AMIA, confirming that inhibition of larval feeding required a much lower concentration than the inhibition of adult worm motility, as observed previously in studies with IVM (Geary et al., 1993; Gill et al., 1995). This indicates that the inhibition of the pharyngeal muscles appears to occur at a much lower extract concentration than the inhibition of motility, suggesting that pharyngeal muscles could be a more sensitive target for SL.

In the LEIA, SL-containing extracts did not affect the exsheathment kinetics of exposed *O. ostertagi* L3. Exsheathment is a precondition for the later embedding of L3 in the abomasal mucosa and our results agree with *in vivo* findings that showed no effect of forage chicory (cv. Grasslands Puna) on the establishment of *Teladorsagia circumcincta* L3 in infected lambs (Tzamaloukas et al., 2005). In the LEIA, all L3 incubated with the highest concentration of both chicory extracts were alive and motile at the end of the assay. These results are further supported by preliminary data from our group indicating that there is no effect of the same SL-containing extracts on the motility of *O. ostertagi* L3, even after incubation for 24–48 h with very high concentrations (2000 μg dry extract/mL, data not published).

At sampling, chicory plants were at the vegetative stage and leaves from both cultivars were hand-picked on the same occasion in order to standardise the collection procedure. Early findings by Rees and Harborne (1985) described that free SL are present in the latex of chicory roots and leaves and that levels of individual SL vary in different parts of the chicory plant according to the growth stage. Nevertheless, these findings need to be confirmed in different chicory cultivars as well as by screening of other sections of the aerial part of the plant (i.e. stems and flowers), which can also be grazed by cattle (Peña-Espinoza et al., unpublished observations). With respect to the quantification of compounds in the extracts, this was performed using an external calibration curve with pure lactucopicrin, the only SL of chicory commercially available as a pure standard at the time of the experiments. Our approach was a quantification of the compounds in the extracts (unknown concentrations) by comparison with the calibration curve (known concentrations), using peak areas and assuming similar chromatographic properties in increasing concentrations of compounds in the extract and in the standard. A quantification using pure standards of all SL and their dihydro-derivatives would have increased the sensitivity of the method. However, this was an initial attempt to understand the SL profile in these particular extracts using, as a standard, a compound actually present in the extracts (lactucopicrin). For comparison, previous studies have added santonin (another SL not present in chicory) as internal standard for quantification of SL in chicory (Ferioli and D'Antuono, 2012; Graziani et al., 2015).

Our results are the first evidence of anthelmintic activity of SL-containing extracts against larval feeding and adult motility, contributing to expand our knowledge on the role of SL in the anti-parasitic properties of chicory. Previously, Molan et al. (2003) were the first to suggest the potential anthelmintic effect of SL in chicory and reported the inhibitory effects of a crude extract from chicory roots against free-living larvae. However, these authors did not report the SL characterisation of the tested extract or the chicory cultivar used. More recently, Foster et al. (2011) described the dose-dependent inhibition of nematode egg hatching by free SL-containing extracts from forage chicory cv. Grasslands Puna and cv. Forage Feast. These authors reported the presence of only the three major SL of chicory in the tested extracts and detected an increased anthelmintic effect of Grasslands Puna-extract. In contrast to the present investigations, Foster et al. (2011) used much larger test concentrations (0–10 mg dry extract/mL). In our

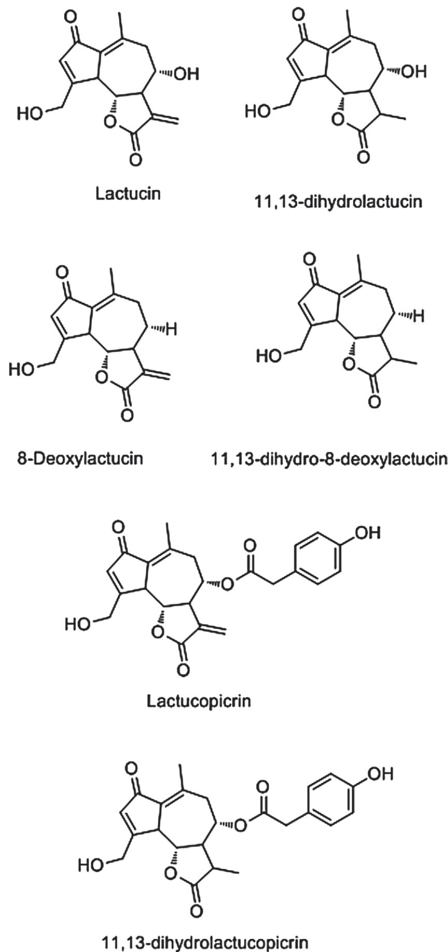

Fig. 2. Structures of sesquiterpene lactones detected in purified extracts from chicory cv. Spadona and cv. Puna II and tested *in vitro* against free-living and parasitic stages of *Ostertagia ostertagi*.

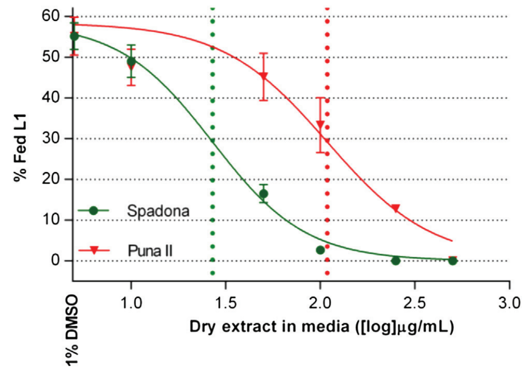

Fig. 3. Dose–response curves obtained in the larval feeding inhibition assay with *Ostertagia ostertagi* L1 incubated at different concentrations of SL-containing extracts ([log] µg dry extract/ml) from two chicory cultivars. Error bars represent S. D. between replicates (n = 3 X concentration per extract). Data points with no error bars indicate that the variation among values was 0 or close to 0. Doted vertical lines represent EC_{50} values for each extract.

study the range of tested concentrations was based on preliminary *in vitro* studies with *O. ostertagi* by the authors (data not published) and was selected with the aim to detect a dose-dependent response. Scarce literature exists on the metabolism of SL from chicory in the digestive tract of ruminants and therefore, at the moment, it is difficult to accurately estimate the concentration of dietary SL that could reach the abomasum, the predilection site of *O. ostertagi* in cattle. However, in a preliminary study we have observed that 4 months-old calves are able to consume daily up to 3 kg dry matter of chicory silage (with 5.3 g SL/kg dry matter), and assuming an abomasum volume of 5 L (Nickel et al., 1979) and that SL are not inactivated in the forestomach, this would roughly correspond to a maximum of 3.18 mg SL/mL in the abomasum (Peña-Espinoza et al., unpublished results).

In our study, the three well-known guaianolide SL of chicory were also detected in leaf extracts of cv. Spadona and cv. Puna II: lactucin, 8-deoxylactucin and lactucopicrin, as reported for other chicory cultivars (Rees and Harborne, 1985; Price et al., 1990; Foster et al., 2011; Ferioli and D'Antuono, 2012; Graziani et al., 2015). Furthermore, the 11, 13-dihydro-derivatives of the mentioned SL were also detected: 11, 13-dihydro-lactucin, 11, 13-dihydro-8-deoxylactucin and 11, 13-dihydro-lactucopicrin, which have been also described in salad chicory cultivars (Ferioli and D'Antuono, 2012; Wulfkuehler et al., 2014; Ferioli et al., 2015). Spadona and Puna II extracts induced dose-dependent anthelmintic effects in the LFIA and the AMIA, suggesting that anti-parasitic compounds were present in both extracts. In addition, unknown compounds were also detected in both chicory extracts, which may have contributed to the observed anthelmintic effects. The HPLC-MS analyses revealed similar quantities of unknown compounds and of total and individual SL between the tested extracts, except for 11, 13-dihydro-lactucin and 11, 13-dihydro-8-deoxylactucin, which were found in significantly higher concentrations in Puna II and Spadona extracts, respectively. Based on results from the *in vitro* assays, our data suggest that the increased concentration of 11, 13-dihydro-8-deoxylactucin in Spadona-extract (3.6-fold increase) correlates, to some extent, with the increased potency and lower EC_{50} values exerted by Spadona-extract in the LFIA (3.3-fold lower EC_{50}) and in the AMIA (2.6-fold lower EC_{50}), in comparison with Puna II-extract. These findings suggest that 11, 13-dihydro-8-deoxylactucin is a likely candidate to explain the higher anthelmintic effects of the Spadona-extract. In contrast, 11, 13-dihydro-lactucin, with increased levels in the less-potent Puna II-extract, seems not to be particularly related with an anthelmintic effect. However, unknown compounds were also present in the extract and no pure SL were tested in our study. Therefore we cannot confirm a direct relation between concentration of a particular SL and anthelmintic effect at the moment. To confirm these findings, further fractionation, isolation and testing of individual molecules in the extracts would identify the most active compound(s). Nevertheless, and to the best of our knowledge, this is the first report of the SL profiles of forage chicory cv. Spadona and cv. Puna II and the first study describing the *in vitro* anti-parasitic activity of forage chicory extracts containing a distinctive profile of dihydro-derivatives of SL. Previously, Foster et al. (2011) linked the higher *in vitro* activity of Grassland Puna-extract to the higher content of 8-deoxylactucin, which in our study was present in similar quantities in Spadona and Puna II extracts. This suggests that, if SL are

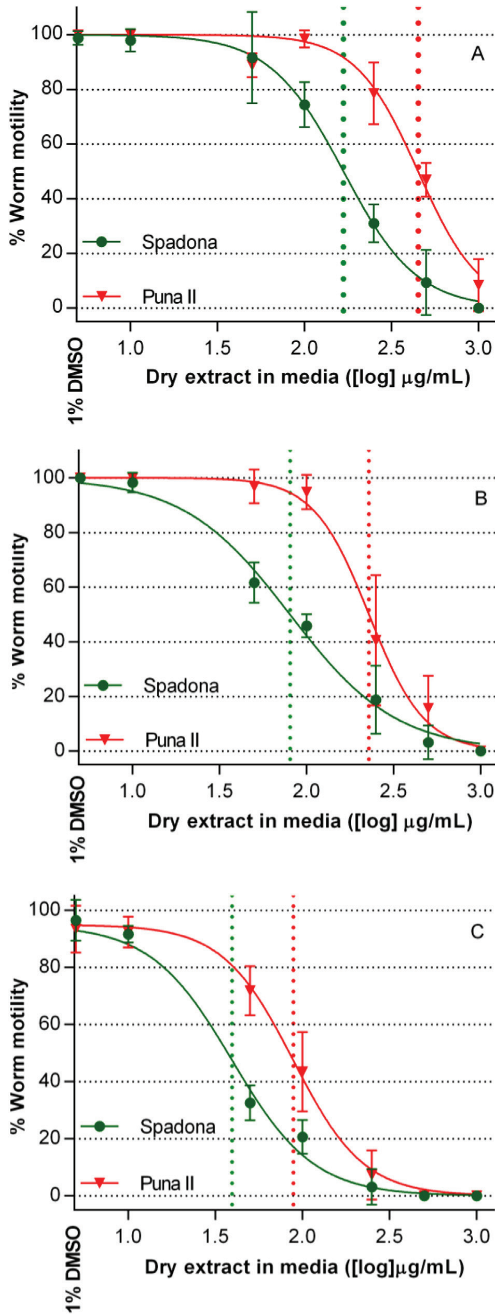

Fig. 4. Dose–response curves obtained in adult motility inhibition assays with *Ostertagia ostertagi* at 6 h (A), 24 h (B) and 48 h (C) after incubation with different concentrations of SL-containing extracts ([log] µg dry extract/ml) from two chicory cultivars. Each data point in the graphs represents the mean motility percentage of 8 replicates (n = 4–6 adult worms per replicate) for each concentration obtained in two independent assays. Error bars represent S.D. between replicates. Data points with no error bars indicate that the variation among values was 0 or close to 0. Doted vertical lines represent EC_{50} values for each chicory extract.

responsible for the anti-parasitic effects of chicory *in vivo*, only some SL may be considered as candidates for novel anthelmintic compounds and for selection of forage chicory cultivars with increased anthelmintic activity.

It is known that guaianolide SL can exert potent cytotoxic activities, which have been mainly attributed to the presence of an α-methylene (CH_2) functional group linked to the γ-lactone in the SL molecule (Simonsen et al., 2013). This α-methylene group is known to react with sulfhydryl (thiol) groups in cysteine and cysteine-containing peptides by a Michael-type addition, affecting cell signalling, cell replication, apoptosis and mitochondrial respiration (Schmidt, 2006; Simonsen et al., 2013). As an example, it has been reported that natural SL can reduce the intracellular concentration of free glutathione in *Leishmania mexicana mexicana*, which led to a toxic intra-cellular accumulation of reactive oxygen species and blocked cell proliferation (Barrera et al., 2013). However, dihydro-derivatives of SL lack this α-methylene group and still can exert biological effects (Lee et al., 1977; Schmidt, 2006; Ghantous et al., 2010), which clearly indicates that there is not just one single biological mechanism of effect for all SL. Ren et al. (2005) reported that 11, 13-β-dihydro-lactucopicrin and 11, 13-β-dihydro-lactucin from *Mulgedium tatarica*, missing the α-methylene group but having an ester group at C-8 instead, exerted a higher toxicity towards human nasopharyngeal and liver cancer cells. Currently, the molecular mechanisms of action of SL and their dihydro-derivatives towards nematodes are unknown and need to be further investigated. Moreover, it is uncertain whether the anti-parasitic effects observed in our study correspond to the action of single (known and/or unknown) molecules or a synergistic effect between different plant compounds, as indicated by recent findings with condensed tannins and flavonoids (Klongsiriwet et al., 2015).

Our study indicates that the tested compounds isolated from forage chicory could affect *O. ostertagi* populations in cattle by reducing the feeding and motility/survival of worms. In addition, the higher anti-parasitic effects exerted by Spadona-extract, and the dose-dependent effect of both extracts, suggest that potential *in vivo* effects could be dependent on the chicory cultivar under investigation and the concentration of active molecules that reach the abomasum. However, compounds present in the tested extracts may not be the same molecules available at the site of infection of *O. ostertagi*. Although little is known about the pharmacokinetics of SL in ruminants, Ferreira and Gonzalez (2008) demonstrated that artemisinin, a purified SL widely used to treat malaria in humans, given to goats as an oral capsule (23 mg artemisinin/kg body weight), was partly metabolised in the rumen and was detected as dihydroartemisinin in plasma after 4 h of treatment. Moreover, *in vivo* trials in sheep indicate that the active compounds do not seem to be degraded or inactivated in the rumen and can reach the abomasum to exert their anti-parasitic activity (Scales et al., 1994; Tzamaloukas et al., 2005). Yet, extensive research is needed to elucidate the fate of dietary SL and other bioactive plant compounds in the digestive tract of ruminants.

In conclusion, we have demonstrated that SL-containing extracts from forage chicory can inhibit larval feeding and exert direct anti-parasitic effects against adult stages of *O. ostertagi*, whereas no effects were observed on larval exsheathment. We observed substantial differences in the anti-parasitic activity between extracts of two forage chicory cultivars and this may be related to variations in the concentration of individual SL. The distinctive anti-parasitic activities of extracts from different cultivars with particular molecular profiles can help to target the identification of the responsible compound(s). However, further studies are warranted not only to confirm the PSM/compound(s) responsible for the anti-

Table 2
Effective concentration able to induce 50% inhibition in worm motility (EC_{50}) by purified extracts from leaves of chicory cv. Spadona and cv. Puna II at different incubation times in adult motility inhibition assays with *Ostertagia ostertagi*.

Incubation time	6 h		24 h		48 h	
Extract	Spadona	Puna II	Spadona	Puna II	Spadona	Puna II
EC_{50} (μg dry extract/mL)	167.6****	449.4	80.3****	228.2	35.7****	82.4
95% CI	146.7–191.3	406.1–497.4	70.5–91.6	198.6–262.1	31.5–40.3	72.3–93.4
R^2	0.96	0.95	0.97	0.95	0.98	0.96

CI = confidence interval; R^2 = goodness-of-fit; ****$P < 0.0001$.

parasitic activity in the tested chicory extracts, but also to assess the *in vivo* anthelmintic effects of a chicory-based diet in cattle infected with gastrointestinal nematodes.

Conflicts of interest

The authors declare that they have no conflicts of interest.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.ijpddr.2015.10.002.

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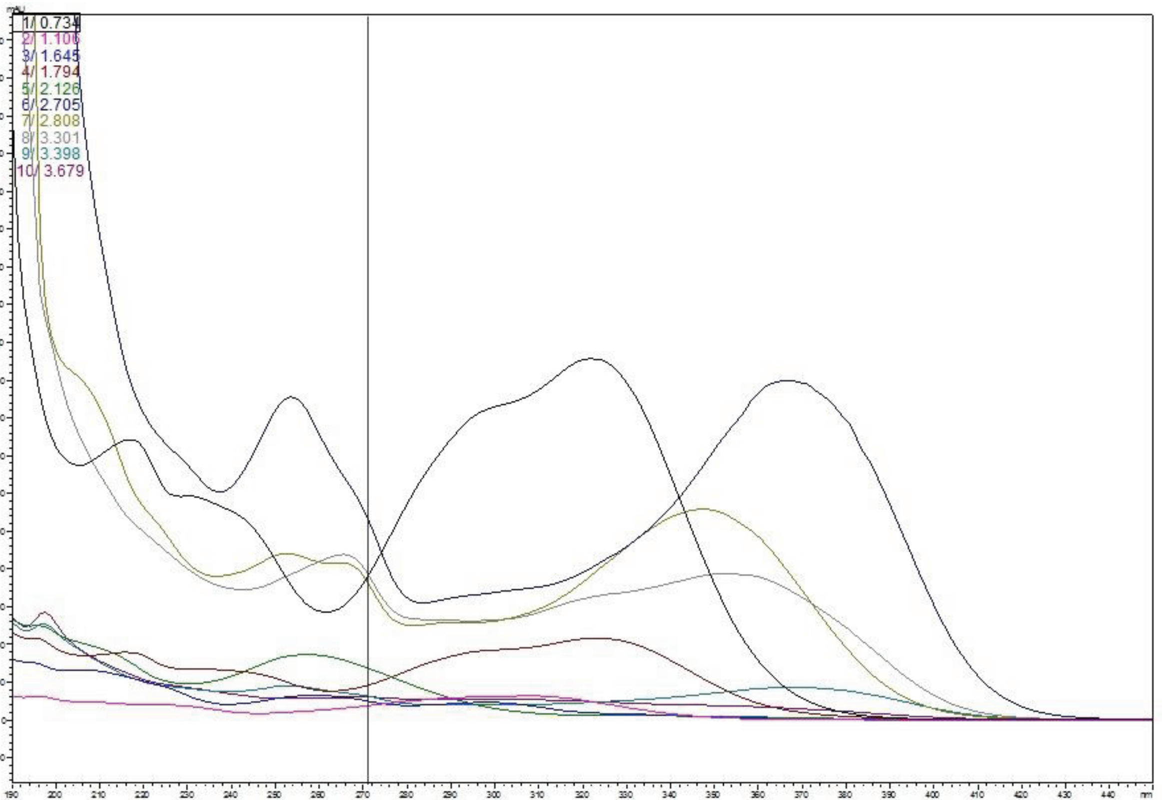
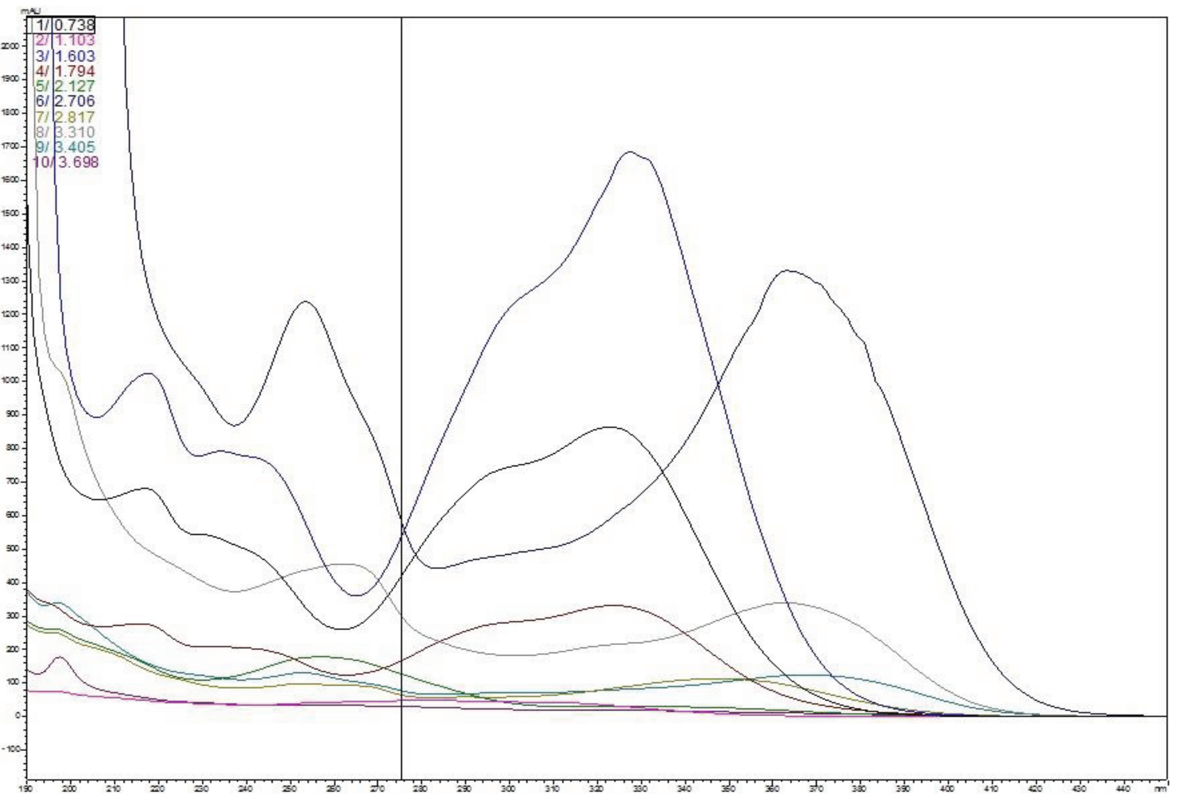
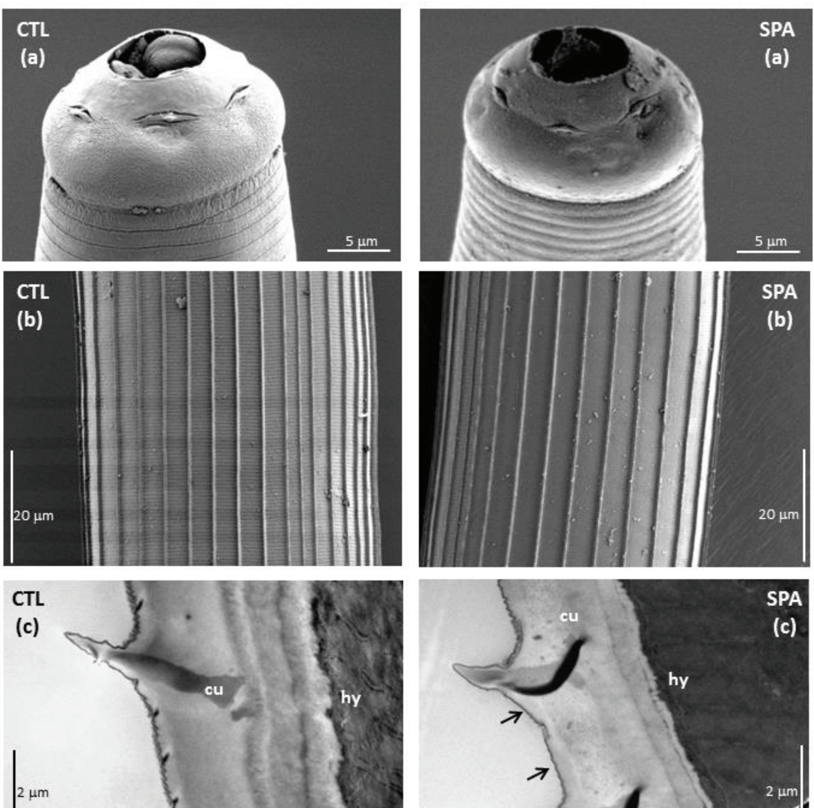
Supplementary material



Supplementary Figure 1. UV absorption patterns of the compounds detected in the Spadona-extract and determination of isosbestic point (271 nm, black line). Numbers represent the molecules presented in Table 1.



Supplementary Figure 2. UV absorption patterns of the compounds detected in the Puna II-extract and determination of isosbestic point (275 nm, black line). Numbers represent the molecules presented in Table 1.



Supplementary Figure 3. Electron microscopy images of *Ostertagia ostertagi* adults incubated during 24 hours with 1% DMSO in PBS (CTL) or 1000 µg Spadona-extract/mL in PBS (SPA). In the scanning electron micrographs (a, b) note the intact mouth opening, circular striations (close to the mouth opening) and intact prominent longitudinal ridges in all worms. In transmission electron micrographs (c) worms exposed to Spadona-extract had a slight decolouration in the cuticule (cu) and a subtle erosion of the epicuticle (black arrows), in comparison with negative controls. Note the intact basal layer separating (cu) and the underlying intact hypodermis (hy) in all worms.

Supplementary Table 1. Molecular fragments (m/z) of sesquiterpene lactones detected by high-performance liquid chromatography – mass spectrometry (positive and negative-ion modes) in selected peaks

Peak	Compound	Molecular weight	Major ion fragments (m/z)	
			(+)	(-)
3	11, 13-dihydrolactucin	278	303 $[M+Na+2H]^+$; 351$[M+MeCN+MeOH]^+$	311$[M+MeOH+H]^-$
4	Lactucin	276	294 [M+H2O]+; 295 [M+H2O+H]+; 315 [M+K]+; 351 [M+MeCN+MeOH+2H]+; 413 [M+TFA+Na]+	473 [2M-2K-H]-; 520 [2M-MeOH]-
6	8-deoxylactucin	261	303 $[M+MeCN+H]^+$	285$[M+Na+H]^-$; 301$[M+MeCN-H]^-$; 415 $[M+TFA+Na+H]^-$
7	11, 13-dihydro-8-deoxylactucin	263	287 $[M+Na+H]^+$; 303 $[M+K+H]^+$	285$[M+Na-H]^-$; 301 $[M+K-H]^-$; 415 $[M+TFA+Na-H]^-$
8	11 , 13-dihydrolactucopricin	410	317 $[M-2HCO_2H-H]$+; 411 [M+H]+	399$[M-HCO_2+2H+MeOH]$-; 429 [M+H20+H]-; 523 [M+TFA-H]-; 593[M+pHPA+MeOH]-
9	Lactucopricin	412	411 $[M-H]^+$	399 $[M-HCO_2+MeOH]^-$

HCO_2: formate ion (from formic acid); MeCN: acetonitrile; K: potasium; Na: sodium; MeOH: methanol; TFA: trifluroacetic acid; pHPA:p-hydroxyphenilacetate

Parasitism with gastrointestinal nematodes is ubiquitous in grazing cattle and its control is critical to sustain satisfactory levels of animal health, welfare and productivity. Conventional control strategies that rely exclusively on anthelmintic drugs are severely threatened by the development of anthelmintic resistance in bovine nematodes. Therefore, alternative control approaches to minimize selection for drug-resistance and that can be combined with the still effective anthelmintics in integrated control strategies are urgently needed. This PhD thesis aims to contribute towards the integrated control of bovine gastrointestinal nematodes by evaluating the field efficacy of ivermectin in naturally-infected Danish cattle and by exploring the direct anthelmintic activity of forage chicory (Cichorium intybus) and its bioactive compounds against cattle nematodes.

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Anthelmintic activity of chicory and efficacy of ivermectin against GI nematodes in cattle

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