FACULTY OF VETERINARY MEDICINE
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Sustainable control of gastrointestinal parasite infections in ruminants

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Laurens Zwanenburg
Student number: 01509101

Supervisor: Prof. dr. Edwin Claerebout
Supervisor: Prof. dr. Stig Thamsborg

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Content

Overview of used abbreviations

AR	Anthelmintic resistance
BZ	(Pro)benzimidazoles
CPG	Cysts per gram
dH_2O	Demineralised sterile-filtered water
ELISA	Enzyme-linked immunosorbent assay
EPG	Eggs per gram
FEC	Faecal egg count
GIN	Gastrointestinal nematodes
LMIA	Larval-migration-inhibition assay
MIC	Minimum inhibitory concentration
ML	Macrocyclic lactones
PGE	Parasitic gastroenteritis
PCR	Polymerase chain reaction
TST	Targeted selective treatment
TT	Targeted treatment

Abstract – Gastrointestinal parasite infections, particularly caused by strongyle nematodes and *Giardia*, are considered to be a major threat regarding health, animal welfare and productivity in livestock. Although drugs for treatment against gastrointestinal nematodiosis are available, the use of those anthelmintics is increasingly controversial due to the increasing public awareness for drug residues in meat and milk, potential environment impact, and not least for the development of anthelmintic resistance. Although there is no major concern about resistance in *Giardia*, no licensed anti-giardial drugs are available in Belgium for livestock, resulting in off-label use. Therefore, sustainable methods of parasite control, such as the implementation of bioactive forages, may reduce the use of anthelmintics, slow down the development of resistance and allow for a reduction of giardiosis in ruminants. This study evaluates the efficacy of 8 different bioactive compounds with a potential antiparasitic property on *Ostertagia ostertagi* and *Cooperia oncophora* L_3 larvae, using a larval-migration-inhibition assay, and on *Giardia duodenalis,* using a resazurin-reduction assay. In *O. ostertagi*, only the extracts of *Trifolium repens* inflorescence at a concentration of 500µg/ml appeared to significantly inhibit larval migration through a 28µm filter. In *G. duodenalis*, extracts from *Fucus spiralis*, *Clausena anisata*, *Pinus sylvestris* and *Trifolium repens* demonstrated a relatively potent effect on trophozoite viability with EC_{50} values of 283µg/ml, 969µg/ml, 490µg/ml and 415µg/ml, respectively. These results demonstrate how certain fractions of bioactive forages have a broad spectrum effect against multiple gastrointestinal parasites and encourages further research towards other parasites, such as *Cryptosporidium* spp. and *Eimeria* spp.

Keywords: anthelmintic resistance, *Cooperia oncophora*, *Ostertagia ostertagi*, *Giardia duodenalis*, bioactive forages, ruminants, sustainable control.

Samenvatting - **Duurzame beheersing van gastro-intestinale parasietinfecties bij herkauwers.** Parasitaire gastro-intestinale infecties, met name veroorzaakt door strongylide nematoden en *Giardia*, worden beschouwd als een grote bedreiging voor de gezondheid, het dierwelzijn en de productiviteit binnen de sector van de veehouderij. Hoewel er geneesmiddelen beschikbaar zijn voor de behandeling van gastro-intestinale nematodeninfecties, is het gebruik van zulke anthelminthica steeds controversiëler vanwege het toenemende bewustzijn voor medicatieresten in vlees, melk en het milieu en daarnaast voor de ontwikkeling van resistentie tegen deze geneesmiddelen. Wat betreft *Giardia* bestaat er geen grote bezorgdheid over resistentie, maar er zijn in België geen geregistreerde geneesmiddelen beschikbaar voor de behandeling van giardiosis in herkauwers met als gevolg het 'off-label' gebruik van producten. Door deze redenen kunnen duurzame methoden voor parasitologische controle, bijvoorbeeld door het gebruik van biologisch actieve voeders, het gebruik van anthelminthica verminderen, de ontwikkeling van resistentie vertraging en zorgen voor een vermindering van giardiosis bij herkauwers. Deze studie bestudeert het effect van 8 verschillende bioactieve stoffen op de migratiecapaciteit van *Ostertagia ostertagi* en *Cooperia oncophora* L_3 larven door gebruik te maken van een 'larval-migration-inhibition assay'. Daarnaast wordt het effect van dezelfde stoffen op *Giardia duodenalis* trofozoieten bekeken door gebruik te maken van een resazurine-reductie test. Tegen *O. ostertagi* leek enkel het extract van *Trifolium repens* aan een concentratie van 500µg/ml in staat te zijn om de migratie van larven doorheen een 28µm filter significant te remmen. Wat *G. duodenalis* betreft vertoonden de extracten van *Fucus spiralis*, *Clausena anisata*, *Pinus sylvestris* en *Trifolium repens* een effect op de levensvatbaarheid van de trofozoieten met EC_{50}-waarden van respectievelijk 283µg/ml, 969µg/ml, 490µg/ml en 415µg/ml. Deze resultaten tonen aan hoe bepaalde fracties van biologisch actieve voeders een breedspectrumeffect bezitten tegen meerdere gastro-intestinale parasieten en is een aanmoediging voor verder onderzoek naar het effect op gastro-intestinale parasieten, waaronder *Cryptosporidium* spp. en *Eimeria* spp.

Sleutelwoorden: anthelmintica resistentie, *Cooperia oncophora*, *Ostertagia ostertagi*, *Giardia duodenalis*, biologisch actieve voeders, herkauwers, duurzame beheersing

1. Literature study

Gastrointestinal parasite infections are considered to be a major threat in young cattle, generally causing growth retardation, ill thrift, diarrhea and consequently reduced animal welfare. Prior to turnout on pastures, calves come into contact with the gastrointestinal protozoan parasites *Cryptosporidium* spp., *Eimeria* spp., and *Giardia* spp., which can result in clinical signs and an initial production loss. Cryptosporidiosis only occurs within the first month of life and is treated with halofuginone lactate, although preventive use is recommended in affected herds. Coccidiosis (eimeriosis) and giardiosis on the other hand, occurs from the age of 1 month and onwards and is sometimes persistent. For coccidiosis, treatment is usually conducted with sulphonamides, toltrazuril or diclazuril. For giardiosis, however, no licensed drugs are available for cattle in Belgium resulting in the off-label use of products such as fenbendazole. After turnout, calves and adult cattle come into contact with gastrointestinal nematodes (GIN), causing more production loss and (sub)clinical signs of parasitic gastroenteritis (PGE). Currently, anthelmintics are used for therapeutic treatment and the mainstay in prevention of GIN. However, due to the lack of licenced antiparasitic drugs for *Giardia* infections in cattle and the development of anthelmintic resistance (AR) in GIN, there is a need for alternative and sustainable methods of treatments. This master thesis provides an overview of potential sustainable methods for the anti-parasitic treatment of *Giardia* and GIN infections in cattle. In addition, a selection of alternative products were tested in *in vitro* studies against both parasite groups.

1.1. *Gastrointestinal nematodes*

1.1.1. Introduction

Infections with GIN in ruminants are very common in temperate climatic regions (Agneessens et al., 2000), causing clinical PGE in severe cases with high worm burdens. However, the presence of clinical PGE has declined over the years due to intensive treatment and control measures. On the contrary, subclinical PGE is becoming more relevant due to production loss and economic consequences (Charlier et al., 2015). Moreover, intensive treatment and control measures conflict with the fact that parasite-free pastures and cattle may be unattainable and a low level of parasitism may even be preferred to allow for a positive immune response (Corwin, 1997).

Currently, treatment of GIN infections is done with anthelmintics, such as (pro)benzimidazoles (BZ), macrocyclic lactones (ML) and imidazothiazoles, represented by levamisole. However, the use of these products is increasingly controversial due to the increasing public awareness for drug residues in meat and milk, the impact on the environment and the development of resistant nematode strains. Therefore, the refinement, reduction and replacement of anthelmintic treatment is an important occupation for parasitologists worldwide. It is given that BZ, ML and levamisole have a meat withdrawal period, but it is usually not intended to slaughter animals immediately after anthelmintic treatment and thus the residue problem in meat is minimal. Moreover, BZ and levamisole have a milk withdrawal period and most MLs, except eprinomectin and moxidectin pour-on formulations, are prohibited for use in dairy cows (Jacobs et al., 2016; Taylor et al., 2016). In addition, ML residues in cattle faeces may have an adverse effect on dung-colonizing insect larvae. The impact on the ecosystem, however, remains unclear and is considered to be an acceptable risk due to the economic importance of parasite control (Wall and Beynon, 2012).

GIN species

The most important GIN species in temperate environments are presented below for cattle (Table 1.1) and for sheep and goats (Table 1.2).

Table 1.1: Most common gastrointestinal nematode species in cattle (Taylor et al., 2016).

Species	Infection site	Prepatent period
Ostertagia ostertagi	Abomasum	2-3 weeks
Haemonchus contortus	Abomasum	4 weeks
Trichostrongylus axei	Abomasum	2-3 weeks
Trichostrongylus colubriformis	Small intestines	2-3 weeks
Cooperia oncophora	Small intestines	2-3 weeks
Nematodirus helvetianus	Small intestines	2-3 weeks
Strongyloides papillosus	Small intestines	8-14 days
Toxocara vitulorum	Small intestines	3-4 weeks
Capillaria bovis	Small intestines	3-4 weeks
Oesophagostomum radiatum	Large intestines	40 days
Trichuris globulosa	Large intestines	7-10 weeks
Trichuris discolour	Large intestines	7-10 weeks

Although many different GIN species may contribute to the development of PGE, *O. ostertagi* and *C. oncophora* are considered to be the most important in cattle due to the fact that they are most present and/or pathogenic.

Table 1.2: Most common gastrointestinal nematode species in sheep and goat (Taylor et al., 2016).

Species	Infection site	Prepatent period
Haemonchus contortus	Abomasum	2-3 weeks
Teladorsagia circumcincta	Abomasum	2-3 weeks
Trichostrongylus axei	Abomasum	2-3 weeks
Trichostrongylus colubriformis	Small intestines	2-3 weeks
Trichostrongylus vitrinus	Small intestines	2-3 weeks
Cooperia punctata	Small intestines	2-3 weeks
Nematodirus spp.	Small intestines	2-3 weeks
Oesophagostomum venulosum	Large intestines	5-7 weeks
Trichuris ovis	Large intestines	7-10 weeks
Skrjabinema ovis	Large intestines	25 days

Similar to cattle, many different GIN species may play a role in the development of PGE in sheep and goats. However, *H. contortus*, *T. circumcincta*, *T. colubriformis/T. vitrinus* and *Nematodirus* spp. are considered to be the most important species in this list.

Life cycle

Most species above belong to the family of the Trichostrongylidae and thus have a direct life cycle (Figure 1.1) with a very similar pattern, although differences in egg hatching, larval development, final infection site and prepatent period are present. The eggs are passed in the faeces and develop within the faecal pat to infective third stage larvae (L_3) within a period of 2 weeks under optimal conditions. This third larval stage still contains the L_2 sheat which makes the larvae more resistant, but unable to feed. In moist circumstances, the L_3 migrates from the faecal pat onto the herbage and is ingested by grazing ruminants. After ingestion, the L_3 exsheaths in the rumen and develops further to L_4 or L_5 in the abomasal or intestinal mucosa depending on the species. Maturation to adult worms takes place on the mucosal surface or in the lumen. For most GIN, the life cycle roughly takes 3 weeks, but under certain circumstances, ingested L_3 may become arrested or inhibited in their development at the early fourth larval stage (EL_4) for a period up to 6 months and this is also referred to as hypobiosis (Armour and Duncan, 1987; Roeber et al., 2013; Taylor et al, 2016).

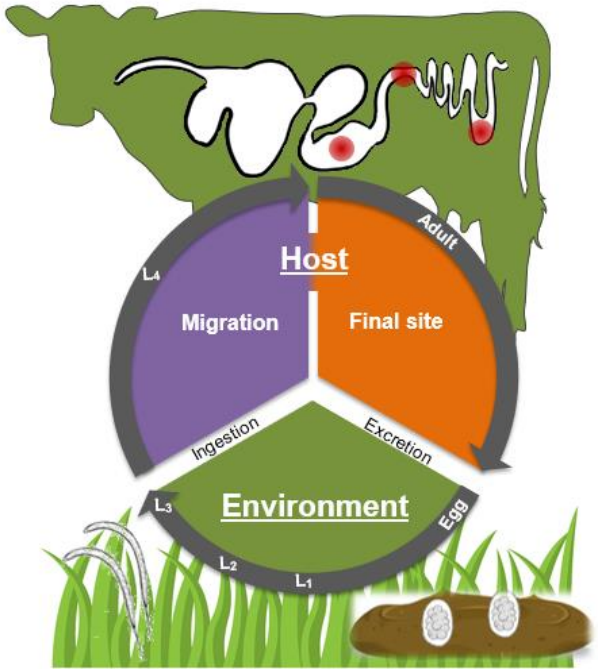


Figure 1.1: Direct life cycle of Trichostrongylidae. Eggs are excreted with the faeces and develop to infectious L_3 larvae which will migrate onto the herbage. Those larvae are taken up by grazing ruminants, migrate to their final infection site and develop to adult nematodes (Based on Taylor et al., 2016).

1.1.2. Epidemiology

First grazing season calves come in contact with GIN larvae for the first time at turnout onto pastures, usually in April-May. First, the larval population on pastures is relatively small due to last winter and is dependent on the pasture contamination of previous year. The uptake of these L_3 larvae by calves, however, leads to a rapid build-up of pasture contamination which may result in clinical signs from Juli onwards. A rapid build-up of protective immunity is mainly described in cattle infected with *C. oncophora* and *Nematodirus* spp. (Armour, 1989), but calves only develop a partial resistance against *O. ostertagi* (Klesius, 1988). According to Agneesens et al. (2000), this incomplete resistance is normally sufficient to prevent clinical signs of PGE in the second grazing season. However, this limited infection has been associated with subclinical symptoms, such as production loss (Ploeger, 1989), and may still have a big socio-economic impact on farmers due to small profit margins. Second grazing season heifers and adults cows are considered to contribute little to pasture contamination, but may still experience subclinical ostertagiosis. Regarding sheep, the development of the pasture contamination is different compared to bovine pastures, depending on the GIN species. First of all, it is important to note that an increased egg excretion is described in ewes at the end of the gestation and early lactation, referred to as peri-parturient rise. Since partus usually occurs between February-April, an initial infection peak in lambs will occur in March-May for *H. contortus*, resulting in a rapid build-up of the pasture contamination. The second infection peak in lambs will then occur in July-August and is associated with severe clinical symptoms. For *Teladorsagia* spp. and *Trichostrongylus* spp., lambs get infected earlier in the grazing season since these GIN species are able to survive on pastures during the winter, resulting on higher initial pasture contamination in addition to the peri-parturient rise. Last, *Nematodirus* spp. eggs hatch in the spring, mainly after a prolonged chill-period, and cause infections in grazing lambs (van Dijk and Morgan, 2010).

A high exposure level to GIN was described in most European countries with differences between neighbouring countries (Charlier et al., 2007a), which are likely due to differences in climate and farm management (Forbes et al., 2008; Bennema et al., 2009; Bennema et al., 2010). Moreover, only 2-6% of the *O. ostertagi* infections in first-season grazing calves are considered to be responsible for clinical

PGE. Compared to previous surveys by Ploeger et al. (2000) and Höglund et al. (2001), these infection levels remained stable over the years. This low percentage of severe infections may be explained due to high level of nematode control with anthelmintics. Additionally, risk factors such as age of calves at turnout, additional feeding, month of turnout and stabling showed clear differences between countries (Charlier et al., 2010b).

Examination of European dairy cows shows a GIN prevalence of at least 94%, which mostly concerns mixed infection of different worm genera in both the abomasum and small intestine (Armour, 1989; Agneessens et al., 2000). It has been suggested that current climate conditions, with a higher humidity and warmer days, favour the egg and larval development (Kenyon et al., 2009) and thus cause an enhanced exposure to most GIN infections (Charlier et al., 2016). Additionally, warmer temperatures during spring and summer cause an extension of the herbage growing season, allowing longer grazing seasons for livestock which may result in a greater pasture contamination with nematode eggs (Kenyon et al., 2009). Although most nematode species thrive in temperate climatic regions, *H. contortus* in sheep is mostly found in warmer areas due to the fact that it struggles to survive on pastures during cold winters. Unfavourable conditions, however, may be evaded by undergoing hypobiosis as fourth stage larvae in the abomasum of its host, whilst development to adult worms is finalized during spring when the temperature rises (Kenyon et al., 2009). Furthermore, the simultaneous occurrence of spring teladorsagiosis and nematodirosis was previously thought to be uncommon, but has now become the ordinary and is associated with climate change as well (Wilson et al., 2008; Kenyon et al., 2009).

1.1.3. Pathogenesis and clinical signs

The severity of disease caused by GIN in ruminant depends on the balance between parasite species, worm burden, animal health and immunological status of the host. Furthermore, environmental factors like climate, pasture type, stress and farm management are indirectly associated with the severity of GIN infection (Roeber et al., 2013). PGE mostly occurs in young, nonimmune animals, adult animals with an immunodeficiency, or animals exposed to very high infection levels (Zajac, 2006). However, the majority of the GIN infections in ruminants are of chronic and subclinical nature with a main impact on production efficiency (Charlier et al., 2017). Although less common, clinical signs such as diarrhea, reduced protein absorption and mortality are universal for the majority of GIN species. In addition, certain species may cause other symptoms such as anaemia in animals infected with *H. contortus* and hypersensitivity in animals infected with *Nematodirus* spp., both mainly described in sheep (Roeber et al., 2013; Charlier et al., 2017).

Underlying mechanism of production loss

According to Charlier et al. (2017), the underlying mechanism for the impact of helminthic infections in ruminants can be divided into three main components, namely: (i) reduction of feed intake; (ii) direct tissue damage; and (iii) the diversion of energy and protein resources of the host from production towards defence and immune mechanisms.

A reduced feed intake, which is a common feature for all helminth infections, is the main sign of (sub)clinical infections (Forbes et al., 2009; Charlier et al., 2017). Due to the reduced availability of nutrients, it has a direct impact on the growth rate of younger animals and it often leads to growth retardation and ill-thrift. It turned out that at the end of the first grazing season, untreated calves weigh 30 kg less on average compared to treated calves (Rehbein et al., 2013), which demonstrates the impact of PGE. In adult ruminants, reduced feed intake mainly causes weight loss and therefore economic consequences. A reduced deposition of protein (Coop et al., 1985) and fat (Bellet et al., 2016) in the carcasses of sheep and cattle infected with *Ostertagia* spp. has been described. Furthermore, it has been suggested that increased gastrin levels (Coop and Kyriazakis, 1999) and reduced leptin levels (Forbes et al., 2009) are associated with GIN infections and may be responsible for the reduced appetite. Additionally, Forbes et al. (2004) showed that the mean meal duration was extended by 11 min in cows and 38 min in heifers as a result of anthelmintic treatment and thus reinforces this substantiation about the loss of appetite in infected ruminants.

Direct tissue damage caused by GIN infections results in a decreased functioning of the infected organs, mainly the abomasum and small intestine depending on the GIN species, leading to malabsorption and maldigestion. Such infections are generally characterized by increased albumin disappearance and adverse pathophysiological changes associated with the infection site (Taylor et al., 1989). Extensive abomasal damage with mucosal hypertrophy, gastrointestinal lesions (Figure 1.2), depletion of parietal cells and mucus cell hyperplasia has been described in lambs and calves infected with *Ostertagia/Teladorsagia* spp. (Coop et al., 1985; Blanchard et al., 1986; Mihi et al., 2013), contributing to the reduced productivity of infected animals.

Figure 1.2: Lesions on the abomasal mucosa caused by ostertagiosis (Taylor et al., 2016).

The immune response that follows a GIN infection drains an important amount of energy and nutrients. According to Greer (2008a) and Greer et al. (2008b), an average production loss of 15% was observed in infected sheep due to the diversion of nutrients towards the immune system and a treatment with immunosuppressive medication led to improved energy utilization and performance. As mentioned above, immunity against *C. oncophora* in calves typically develops during the first grazing season, whilst the development of immunity against *O. ostertagi* is only partial after one grazing season (Charlier et al., 2017). This corresponds to the fact that the larger part of clinical outbreaks of PGE has been described in calves younger than 6 months, although clinical PGE is regularly found in older calves as well. Besides, calves are more prone to PGE outbreaks the younger they are at the start of the first grazing season (Shaw et al., 1998b).

1.1.4. Diagnostics

Little has changed over the last decades and the diagnosis of GIN infections in ruminants is still mainly based on clinical and coprological diagnosis. In addition, pepsinogen determination and antibody levels in milk and serum may provide valuable information, especially on herd-level.

Clinical diagnosis

Although PGE is associated with relatively clear clinical signs, such as diarrhea, reduced food uptake and production loss, it may be hard to form a diagnosis purely based on these symptoms. Therefore, a clinical examination should always be accompanied by another diagnostic method, for example coprology. In sheep, however, anaemia and death is associated with haemonchosis. The FAMACHA system for *H. contortus* specifically, which involves the assessment of the colour of conjunctival membranes, may therefore be used for the identification of animals requiring treatment on an individual basis (Besier et al., 2016). Furthermore, *post mortem* examination may provide valuable information for most GIN infections in ruminants. These infections are generally associated with direct tissue damage and adult worms may be found in the target organs. Additionally, in all situations, diagnostic protocols must take account of epidemiological factors that may influence the likelihood of GIN infections (Besier et al., 2016).

Coprological diagnosis

Coprology mainly involves performing faecal egg counts (FEC) to measure the severity of infection. These FEC are commonly used as a monitoring tool to study the relative threat of disease on herd level or pasture contamination with GIN eggs (Besier et al., 2016). The main issue with FEC, however, is that male and immature worms are not taken into account, which hinders the interpretations of the acquired eggs per gram faeces (EPG) (Besier et al., 2016). It is generally accepted that individual values above 275 EPG in calves are associated with clinical PGE, whilst an EPG of < 100 is associated with subclinical PGE (Shaw et al., 1998a). Furthermore, a herd average > 200 EPG is considered as a serious risk for future pasture contamination (Shaw et al., 1998b). One of the FEC techniques, the McMaster method, is a quantitative method used to evaluate the FEC. Due to the sensitivity of 50-100 EPG and since it is simple and quick in use, it finds a purpose in the evaluation of GIN infection in ruminants (Rinaldi et al., 2011). More recently, the diagnostic and technical performances of coprology have been improved with the development of FLOTAC (Cringoli et al., 2010) and Mini-FLOTAC (Cringoli et al., 2013), which are portable and usable by veterinarians in field practice. Compared to the McMaster, FLOTAC has a detection limit of 1-2 EPG (Cringoli, 2004) and the mini-FLOTAC has a detection limit of 5 EPG (Godber et al., 2015). An advantage of McMaster over FLOTAC, however, is the reduced time- and labour consumption (Levecke et al., 2009; Van den Putte et al., 2013). FECPAK (www.fecpak.com), which essentially is a larger version of the McMaster, allows for a sensitivity up to 20-30 EPG, but is considered to be more time and labour intensive (Demeler et al., 2009; Bosco et al., 2014). FECs are sometimes followed by faecal cultures or conventional, real-time or multiplex-tandem polymerase chain reaction (PCR) to identify the nematode species (Avramenko et al., 2015; Roeber et al., 2017). However, these two methods are considered to be time consuming and expensive, limiting their use in routine diagnosis (Charlier et al., 2017).

Pepsinogen determination

In addition to coprological diagnosis, *O. ostertagi* in cattle can be diagnosed by determining the serum pepsinogen concentration, but is limited to the use in first grazing season calves (Charlier et al., 2014; Charlier et al., 2017). Serum pepsinogen, expressed in units of tyrosine (U tyr) is a parameter for abomasal damage caused by *O. ostertagi*, resulting in an increased pH which leads to a reduced conversion of pepsinogen into pepsin. According to Dorny et al. (1999), a serum pepsinogen level of < 2 U tyr comes along with an adult *O. ostertagi* burden under 1000, whilst 4 U tyr corresponds with > 10000 adults worms. During the grazing season, it is generally assumed that U tyr values > 4.0 are associated with clinical ostertagiosis and values between 3.0 and 4.0 U tyr correspond with subclinical infections (Shaw et al., 1997; Dorny and Vercruysse, 1998). However, these levels are not standardized and may vary between different assays.

Antibody levels in milk and serum

Performing an enzyme-linked immunosorbent assay (ELISA) on bulk tank milk is a common method to determine the prevalence of GIN-specific antibodies, mainly described for *O. ostertagi* (Charlier et al., 2007a). Apart from prevalence studies, these ELISA's may find use on farm level as well, since it allows for the determination of the exposure level on a farm specifically. The results of bulk tank milk ELISA's are presented in optical density ratio (ODR) and the higher the ODR, the higher the exposure to GIN infections on a farm. Generally, one speaks of a high infection pressure if an ODR above 0.8 is detected (Charlier et al., 2007b). For its use in diagnostics, it is important that the interpretation of the ODR is always related to pasture management, deworming schedules and clinical symptoms.

Diagnostics for anthelmintic resistance

Due to the upcoming resistance of certain GIN species against commonly used anthelmintics, the development of tests for the evaluation of the AR became an important occupation for parasitologists. The most common *in vivo* test used to determine this for specific anthelmintics is a faecal egg count reduction test (FECRT), which is performed before and after anthelmintic treatment (Demeler et al., 2009). A disadvantage of this technique, however, is the fact that there is no strong correlation between worm burden and egg output by female worms (Eysker and Ploeger, 2000). Additionally, a temporary suppression of egg production due to treatment with ML has been described, which can make it seem like there is a high efficacy (De Graef et al., 2012). Apart from the *in vivo* test, there are multiple *in vitro*

methods available, such as the egg-hatch-inhibition assay (EHIA) for benzimidazoles (Johansen, 1989), larval-development-inhibition assay (LDIA) (Taylor et al., 2002) and the larval-migration-inhibition assay (LMIA) (Demeler et al., 2010b), which are considered to be more cost effective compared to the FECRT (Demeler et al., 2012). Lastly, it has been demonstrated that a qPCR could be used to determine AR in sheep nematodes (Milhes et al., 2017).

1.1.5. Current control and anthelmintic resistance

Anthelmintics in general

Based on their mechanism of action, the anthelmintics used to treat GIN infections in ruminants are divided in three major anthelmintic groups, namely (i) benzimidazoles (BZ) and pro-benzimidazoles (pro-BZ), (ii) macrocyclic lactones (ML), and (iii) imidazothiazoles (IT) and tetrahydropyrimidines (TP). Additionally, there are two new anthelmintic groups, amino-acetonitrile derivates (AAD) and spiroindoles (SI), licensed and launched onto the sheep market in some countries (Charlier et al., 2017). Between various anthelmintics, there is a difference in effectiveness against adult GIN, immature stages and inhibited or hypobiotic stages. For an anthelmintic drugs to be effective, it is supposed to have an activity of at least 90%, which currently is a concern due to the AR. In addition to the development of resistant worm strains, a reduced development of natural immunity and public concerns about drug residues in food products are possible disadvantages (Charlier et al., 2017).

The development of anthelmintic resistance

According to Coles et al. (2006), all the above anthelmintics used in livestock were considered to be very effective in reducing the adult, immature, or inhibited worm burdens, or combinations thereof. For (pro-)BZ and LEV, the efficacy used to be at least 90%, whilst the efficacy of ML, AAD and SI + ML got up to 99%. However, the first signs of anthelmintic resistance were reported for different products a couple decades ago (Figure 1.3) and now is a global concern in all major ovine nematodes, whilst it is an emerging problem in bovine nematodes, mainly involving ML resistant *C. oncophora* (Sutherland and Leathwick, 2011; Charlier et al., 2017).

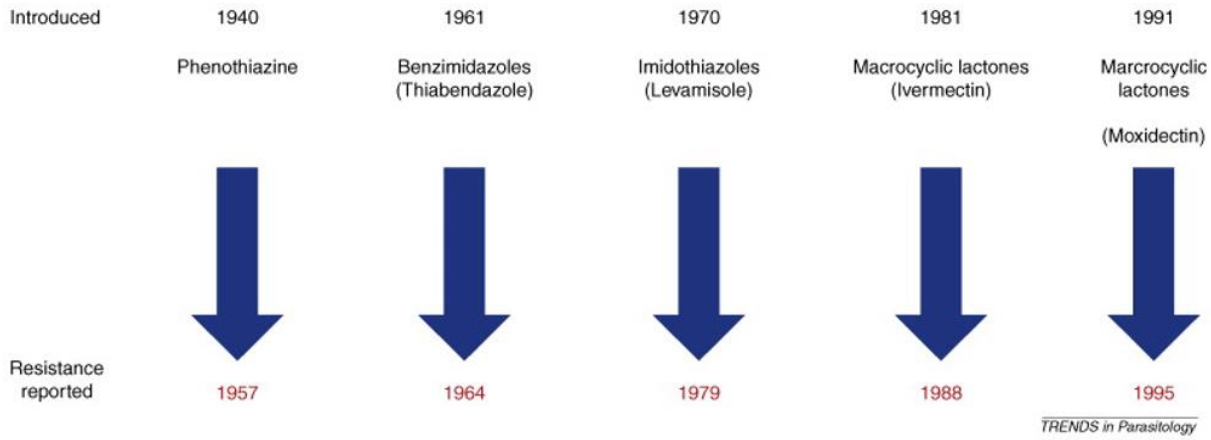


Figure 1.3: An overview of the first reported resistance against the major anthelmintic groups (James et al., 2009).

The development of AR nematode strains may be caused by many different processes. According to Gilleard (2006), there are several single-nucleotide polymorphisms associated with the development of anthelmintic resistance, which may indicate that the mutation of genes plays an important role. Furthermore, ATP-binding cassette transporters, such as P-glycoprotein and multi-drug resistance-associated proteins, resulting in changes in drug transport have been associated with AR in *H. contortus* (Lespine et al., 2008). Since anthelmintics do not have a 100% efficacy, there is always a fraction of nematodes surviving due to the 'coincidental' prevalence of resistance genes. As a result, this selection pressure will lead to a new population which is likely to be more resistant against anthelmintics. The amount of larvae not under that selective pressure, also referred to as the *in refugia* larvae, provide a

reservoir of susceptible genes and thus, the larger the refugia, the slower the AR development will be (Van Wyk, 2001; Jacobs et al., 2016).

The use of anthelmintics with a new mode of action and the implementation of sustainable control methods may reduce the spreading of AR (Martin et al., 2015). According to Charlier et al. (2017), anthelmintics will remain vital in the control of GIN infections in ruminants and should always be included in treatment programs. In addition, it may be important to maintain a large fraction of nematodes *in refugia*, whilst overuse of anthelmintics and high drenching frequencies should be avoided (Van Wyk, 2001; Leathwick et al., 2015).

Benzimidazoles

BZ are anthelmintics with a high safety margin and can be divided into (i) the pro-BZ like febantel and netobimin, which are metabolized into BZ after administration, and (ii) BZ such as oxfendazole, albendazole and fenbendazole. The mode of action of BZ is based on the binding to parasite tubulin, reducing cellular transport which results in starvation of the nematode due to inhibition of glucose uptake and protein secretion (Abongwa et al., 2017). Initial studies indicate that the pro-BZ, febantel and netobimin, are effective drugs against most GIN in sheep (Thomas, 1978; Richards et al., 1987a) and cattle (Richards et al., 1987b; Williams et al., 1988). Studies in the same time period regarding BZ, such as albendazole, indicate a very high (> 95%) efficacy against most adult and immature GIN species in calves. The effect against inhibited stadia, however, is limited to 85% (Williams et al., 1977; Borgsteede, 1979). It can therefore be concluded that (pro-)BZ mainly demonstrate a high efficacy against adults and immature stages, whilst the efficacy against inhibited stadia is limited. More recent studies, however, demonstrate a reduced efficacy due to the development of resistant GIN strains for all major nematode species in sheep (Besier and Love, 2003; Martínez-Valladares et al., 2015). Due to the fact that ML are mainly used against cattle nematodes, resistance against BZ is more limited in cattle compared to sheep (Demeler et al., 2009). Resistance against BZ in the cattle nematodes *Trichostrongylus* spp., *C. oncophora* and *O. ostertagi*, however, has been observed (Martínez-Valladares et al., 2015; Taylor et al., 2016). BZ have a meat and milk withdrawal period in cattle of 14 and 4 days respectively. According to Tsiboukis et al. (2013), the prevalence of (pro-)BZ residues in milk after treatment is relatively high (up to 27.6%), but never exceeded the maximum residue limit (MRL). Furthermore, a European study by Cooper et al. (2012) demonstrated that 2.45% of beef samples contained detectable residues of anthelmintics, such as BZ. However, the residue level was always less than 0.1% of the MRL.

Macrocyclic lactones

ML, such as ivermectin, moxidectin, doramectin and eprinomectin, are commonly used to treat GIN infections in both sheep and cattle. ML mainly act due to the cause of body wall muscle, pharyngeal muscle, and uterine muscle paralysis in parasitic nematodes (Abongwa et al., 2017). In general, ML are considered to be highly effective against adult, immature and inhibited GIN stadia. According to Williams et al. (1999), the ML above all have a very high efficacy against the major bovine nematodes and are known for their persistent effect due to the storage in fat tissue (Taylor et al., 2016). Especially sustained-release boli and pulsed-release boli provide a long persistency. Apart from their use against many different parasitic nematodes, ML are also commonly used to treat diseases caused by ectoparasites such as *Psoroptes ovis*, which may increase the development of resistant GIN strains (Vercruysse et al., 2008). Furthermore, it seemed that repeated subtherapeutic treatment with ML may lead to the increased development of more resistant nematode populations in cattle (Van Zeveren et al., 2007) and sheep (Ranjan et al., 2002). In sheep, resistance against ML has been described in all major GIN species, especially in *H. contortus* (Rinaldi et al., 2014) and *T. colubriformis* (Cringoli et al., 2007). According to Keane et al. (2014), resistance in *Nematodirus* spp. is rarely reported and not of great importance currently. Although resistance against ML is not as common as BZ, it has been described for *C. oncophora* and *Trichostrongylus* spp. in cattle and is increasingly more important over the years (Demeler et al., 2009; Edmonds et al., 2010; Geurden et al., 2015). ML resistance in *O. ostertagi* has not been described clearly, but studies suggest that it may be the case (Edmonds et al., 2010; Geurden et al., 2015). Regarding ML residues, there is a meat withdrawal period of 14-108 days, depending on the product and its formulation. In terms of residues in milk, most MLs except eprinomectin and

moxidectin pour-on formulations are prohibited for use in dairy cows (Jacobs et al., 2016; Taylor et al., 2016).

Imidazothiazoles
The most important and commonly used imidazothiazole to treat GIN infections in both sheep and cattle is levamisole. The anthelmintic effect of levamisole originates from the cholinergic stimulation by mimicking the action of acetylcholine resulting in worm paralysis (Abongwa et al., 2017). Furthermore, levamisole mainly has an effect against adult and immature GIN, whilst the efficacy against inhibited stages is very limited (Jacobs et al., 2016). Resistance against levamisole treatment in sheep has been described for all major nematodes species, mainly *T. circumcincta*, *Trichostrongylus* spp. and *H. contortus*, even dropping to an efficacy of 60% for treatment through drenching systems (Besier and Love, 2003). Similar to BZ and ML, resistance to levamisole has mainly been described in ovine GIN. Moreover, resistance has been described in bovine GIN such as *O. ostertagi*, whilst levamisole seems to be fully active against *C. oncophora* (Suarez and Cristel, 2007; Cotter et al., 2015). Levamisole has a relatively short meat- and milk withdrawal period of 4-8 days and 2-3 days respectively, depending on the formulation. According to a survey by Cooper et al. (2012), levamisole residues were found in meat, but only at 0.1% of the MRL and is considered to be negligible.

Salicylanilides and substituted phenols
The two important anthelmintics in these groups, closantel and rafoxanide, are specifically used in sheep to treat haemonchosis and are known for their persistent activity up to 4 weeks (Campbell et al., 1978). The mode of action is mainly based on causing a depletion of available energy in the parasites by reducing the availability of adenosine triphosphate (ATP) in mitochondria (Taylor et al., 2016). Similar to the anthelmintics above, resistance in *H. contortus* against both products has been described (Van Wyk et al., 1987). In terms of residues, closantel and rafoxanide are not approved for use in milk producing animals (Danaher et al., 2016).

Amino-acetonitrile derivates (AAD)
Due to the development of resistant GIN strains and the search for new anthelmintics, there has been more interest in AADs, such as monepantel. It causes paralysis (Abongwa et al., 2017) in nematodes and according to initial studies, monepantel was considered to be highly effective against all major ovine nematodes and was therefore used against resistant nematode strains (Kaminsky et al., 2008; Hosking et al., 2009). However, recent studies indicate resistance in *H. contortus* towards this product (Sales and Love, 2016; de Albuquerque et al., 2017). Despite these findings it may still have a higher efficacy against resistant GIN strains than other anthelmintics.

Spiroindoles
Derquantel is the first product within the new anthelmintic class of Spiroindoles. It is a nicotinic acetylcholine receptor antagonist and caused paralysis of the GIN (Abongwa et al., 2017). Derquantel is often combined with others anthelmintics, such as abamectin, to enlarge the spectrum and demonstrates a high efficacy against resistant ovine GIN strains (Sager et al., 2012; Geurden et al., 2014). Once more, signs of a reduced anthelmintic activity due to resistance against the derquantel/abamectin combination have been described (Lamb et al., 2017).

1.1.6. Alternative control

To prevent or counteract the fast development of AR, it is suggested that alternative methods are used which may help to reduce GIN worm burdens, nematode egg excretion and pasture contamination or strengthen the host's immunity. Figure 1.4 demonstrates where different alternative methods, including the use of anthelmintics itself, may help to reduce GIN infections in ruminants. The methods discussed below are all potentially effective to reduce the overall use of anthelmintics. Some of those methods, such as refugia management, have already been implemented in practice, while the importance and application of other methods is still being tested intensively in *in vivo* and *in vitro* studies.

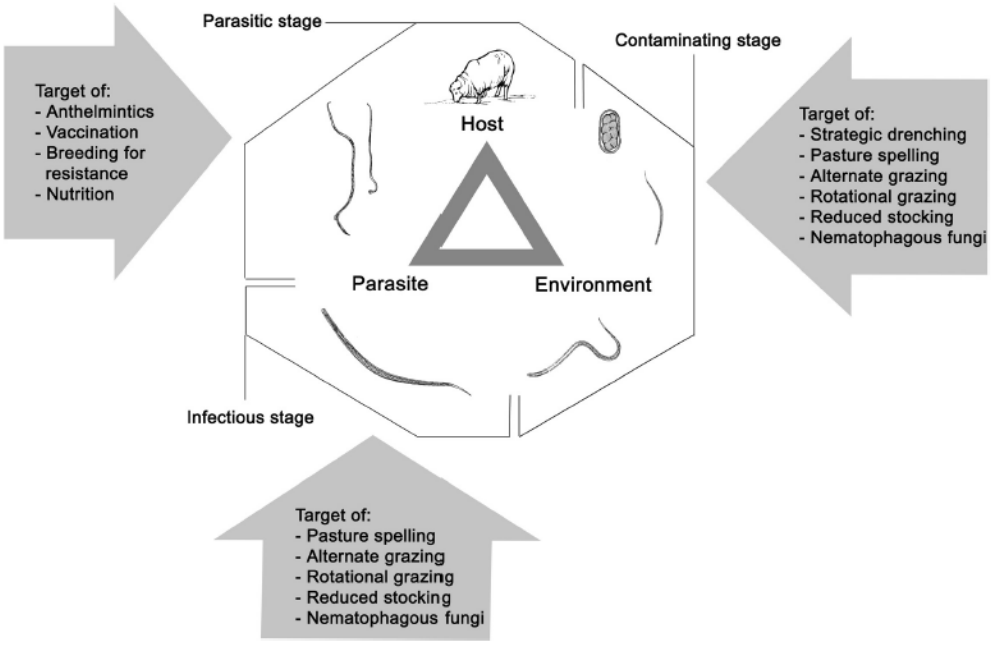


Figure 1.4: The targets of different strategies to finally reduce GIN infections in ruminants. This figure illustrates various options to reduce pasture contamination, FEC or worm burden depending on the life cycle stage of the GIN (Roeber et al., 2013).

1.1.6.1. Management systems

Refugia management

As discussed above, it is important that the selection pressure on nematodes is not too high. By only treating animals with a considerable level of infection or limiting treatment to the optimal timing or frequency, the development of resistant GIN strains may be delayed. In general, these strategies can be divided into targeted treatment (TT) and targeted selective treatment (TST). With TT, the whole herd undergoes treatment at an optimal time or frequency. This optimum is determined based on setting thresholds for specific parameters involving the risk of infection and/or production loss within the herd as a whole (Verschave et al., 2014). For example, antibody ELISAs on bulk tank milk (Charlier et al., 2010a) or mean periodical faecal sampling for FECs (Besier, 2012) are two commonly used parameters. Using TST, only selected individual animals are treated against GIN infections based on individual parameters, such as the size of worm burdens, clinical signs of PGE and/or production loss (Besier, 2012). Treating animals individually will reduce the use of anthelmintics, increase the size of the GIN population *in refugia* and delay the development of AR (Verschave et al., 2014). An obstacle in the use of TST, however, is that it may be hard to determine the threshold at which treatment is suggested. The implementation of TST is best described for *H. contortus* in sheep with the FAMACHA system, which is based on visible evidence of anaemie due to haemonchosis, but is considered very labour intensive (Van Wyk and Bath, 2002). Other systems are based on FEC, body condition score, live weight gain and/or production efficiency (Kenyon and Jackson, 2012). However, to implement these methods for common parasitic control, a major shift in farmer mentality is required according to Charlier et al. (2014).

Grazing management

Grazing management, sometimes referred to as larval-avoidance strategies, makes use of local epidemiological information and pasture rotation to limit the intake of infective nematodes and reduce pasture contamination to a minimum (Besier et al., 2016). One of the strategies is moving susceptible ruminants from contaminated to non-contaminated pastures (Eysker et al., 1998). A possible limitation in this, however, would be the fact that livestock movement between pastures is mainly determined by nutritional parameters instead of pasture contamination (Besier et al., 2016. Other possible strategies may be the (i) co-grazing of susceptible juvenile and less susceptible adult ruminants on the same pasture (Nansen et al., 1990; Thamsborg et al., 1999), (ii) alternation of susceptible and non-susceptible animal species, for example between sheep and cattle for *H. contortus* (Southcott and Barger, 1975),

and (iii) applying a later turnout time of susceptible animals and stabling earlier during the grazing season to reduce the uptake of infectious larvae.

Breeding management

Breeding management, or genetic selection, has mainly been described in sheep and aims at improving the host's resistance towards the important GIN species, resulting in a reduction of worm burden and egg excretion (Sweeney et al., 2016). Moreover, the selection of ruminants should be based on the phenotypic criteria associated with resistance, such as a low FEC (McRae et al., 2014), rather than tolerance or resilience (Sweeney et al., 2016). Various studies have demonstrated that genetic selection can result in a significant reduction of FEC and pasture contamination (Bishop and Morris, 2007; Jacquiet et al., 2009) and although the selection of resistant hosts is considered to be beneficial, it has to be combined with other sustainable methods to effectively control GIN infections. Genetic selection is considered to be a low-cost solution to reduce the impact of GIN infections on ruminants. However, highly resistant animals are usually not endowed with desirable productivity traits (Waller and Thamsborg, 2004). Furthermore, there is no evidence that nematodes evolve rapidly in response to resistant hosts, in contrast to the development of AR against anthelmintics (Zvinorova et al., 2016). A disadvantage, however, may be the fact that genetic selection is near impossible to implement in low-input/output farms due to the lack of record keeping and pedrigree data in the mating systems (Zvinorova et al., 2016).

1.1.6.2. Biological control

Vaccination

Similar to genetic selection causing resistance to GIN infection, vaccination may induce or strengthen the development of host immunity. Vaccination should not be considered as a method that could reduce GIN infections completely, but more as a tool to maintain a low pasture contamination (Smith and Zarlenga, 2006). Until recently, no commercially available vaccines were available for GIN. In 2014, however, a commercial vaccine known as Barbervax® (http://barbervax.com.au/) in Australia or Wirevax® (http://www.wirevax.co.za/) in South Africa became available for sheep and is considered to be highly (> 80%) effective against the blood-sucking stages of *H. contortus* (Emery et al., 2016). Both of these vaccines are based on H11 and H-gal-GP proteins, derived from integral gut membranes of *H. contortus* (Emery et al., 2016). For *O. ostertagi* in calves, several potential antigens for vaccine production have been evaluated and mainly the ASP-based vaccines show promising results (Rinaldi and Geldhof, 2012). In addition to *O. ostertagi*, ASP-based vaccines are also promising antigens in *C. oncophora* and may be able to cause a significant reduction in FEC (González-Hernández et al., 2018). Last, experimental vaccination with recombinant antigens against *T. circumcincta* resulted in a reduced FEC in periparturient ewes and may play an important future role in reducing *T. circumcincta* challenge in lambs (Nisbet et al., 2016).

Nematophagous fungi

The use of nematophagous fungi, such as *Duddingtonia flagrans*, has proved to be potentially useful in controlling GIN infections. After administration of *D. flagrans* spores to ruminants, the spores pass through the gastrointestinal tract and are excreted in the faeces. Once excreted, the fungi will grow and form nematode trapping structures in the faecal pat (Chartier and Pors, 2002). If *D. flagrans* spores are deposited at the same time as GIN eggs, it prevents the larval migration from faecal pats onto the pasture, significantly reducing pasture contamination of infective L_3 larvae for all major ovine GIN (Faedo et al., 2000). Furthermore, lambs and goat kids treated with *D. flagrans* showed a higher mean body weight gain and lower FEC than untreated animals, although not always significant (Epe et al., 2009). A similar reduction in pasture contamination was observed for *O. ostertagi* in cattle treated with *D. flagrans* (Fernández et al., 1999). However, there is no effect on L_3 larvae in the surrounding soil and thus *D. flagrans* is only active within the faecal pats (Faedo et al., 2000). The main disadvantages, and probably the reasons why it has not been implemented in practice yet, is the lack of a commercial source of the production of spores (Terril et al., 2012; Charlier et al., 2017) and the fact that the spores must be fed continuously in order to be effective (Epe et al., 2009).

1.1.6.3. Dietary supplementation

Protein-rich feeding

As discussed above, GIN infections can result in a reduction of food intake, digestion and utilization of nutrients such as proteins. In addition, a substantial amount of nutrients are directed towards the immune system. Especially in high production animals, this may lead to a protein scarcity, which may be resolved by adequate supplementation of high quality proteins (Coop and Kyriazakis, 2001; Sakkas et al., 2012). Moreover, supplementation during the grazing season leads to less intake of contamination pasture herbage. An increased supplementation of dietary metabolizable protein (MP) to lactating ewes leads to a reduction of the FEC up to 65% (Houdijk et al., 2001; Kidane et al., 2009). This reduction in nematode egg excretion could result in lower pasture contamination, which is mainly beneficial for lambs. Furthermore, this reduces the need for anthelmintic treatment, slowing down the development of AR (Kidane et al., 2009).

Copper-oxide wire particles

Initially, copper-oxide wire particles (COWP) were developed to function as a feed supplement in treating copper deficiency in small ruminants, but studies demonstrated the anthelmintic efficacy of COWP against GIN in sheep (Burke et al., 2004) and goats (Chartier et al., 2000). Following the administration of COWP capsules to ruminants, copper releases in the abomasum due to the low pH, resulting in elevated copper concentrations (Waller et al., 2004). A reduction of the FEC in lambs infected with *H. contortus* was described (Burke et al, 2004), but no positive effects against *T. circumcincta*, *T. colubriformis* and *O. venulosum* were demonstrated (Chartier et al., 2000). Although no signs of copper intoxication were observed in the above studies, the concentrations of copper in lamb and ewe livers was much higher than the normal range (Burke et al., 2004). The risk of copper intoxication due to COWP supplementation can therefore not be excluded. Additionally, Burke et al. (2007) suggested that *H. contortus* may become resistant to COWP.

Bioactive forages

Apart from their nutritional values, certain plants have an antiparasitic activity and may have the potential to be used as a sustainable method, reducing the overall use of anthelmintics. These plants, also referred to as bioactive forages, are generally non-toxic and cannot be overdosed, which may make it useful to integrate them into the regular diet of ruminants. Since these bioactive forages are supposed to be consumed by production animals, it is important that they comply with standard nutritional requirements, such as containing a good macronutrient profile, an acceptable digestibility, little or no negative impact on production and they must be readily consumed (Hoste et al., 2015). It has been suggested that tannins, which are one of the many bioactive compounds, are responsible for the main anthelmintic property of most bioactive forages (Hoste et al., 2015). Additionally, plant cysteine proteinases and flavonoids have been tested positively *in vitro* and *in vivo* in both sheep and cattle (Vercruysse et al., 2018). As discussed by Hoste et al. (2012), the effect of tannin on GIN infections in ruminants has been associated with three main effects. First of all, tannins cause a reduction of the worm establishment (Paolini et al., 2003). Secondly, there is a reduction in FEC up to 80% (Lange et al., 2006), which may be due to a decrease in adult worm burden (Heckendorn et al., 2006) or due to a reduced fecundity in female worms (Paolini et al., 2005). Third, tannins are associated with a reduction in the development of nematode eggs (Molan et al., 2000). In addition to these three effect, it has been suggested that tannins are able to bind free available proteins in the gastrointestinal tract, causing larval starvation (Athanasiadou et al., 2001). Due to this background knowledge, research is mainly focused on tannin-containing plants, such as sainfoin (*Onobrychis viciifolia*), sulla (*Hedysarum coronarium*), quebracho (*Schinopsis* spp.) and pine bark (*Pinus sylvestris*) (Hoste et al., 2015; Niezen et al., 2002; Athanasiadou et al., 2001; Desrues et al., 2016). Additionally, there is an interest in the anthelmintic effect of chicory (*Cichorium intybus*) and specific types of seaweed (unpublished).

Feeding sainfoin to lambs led to a decreased egg excretion by *H. contortus* and *T. colubriformis* and necropsy demonstrated a reduction in adult worm burden for both parasites (Arroyo-Lopez et al., 2014). Furthermore, a significant reduction of established *H. contortus* (Heckendorn et al., 2006) and *T. circumcincta* (Werne et al., 2013) has been described in lambs. In calves, feeding pelleted sainfoin

resulted in a reduction of *O. ostertagi* in the abomasum by 50%, although no significant reduction in FEC was observed. For *C. oncophora*, however, no significant effect of sainfoin was described. This may be due to the lack of bioactivity of condensed tannins in sainfoin against nematodes in the small intestines (Desrues et al., 2016). Although no *in vivo* effect against *C. oncophora* was demonstrated, Novobilský et al. (2011) described a reduced larval exsheathment in the presence of sainfoin extracts.

The effect of sulla has been observed in an *in vivo* study in lambs and grazing sheep and demonstrated a reduction in FEC for *T. circumcincta*, but no significant effect against *T. colubriformis* (Niezen et al., 2002; Athanasiadou et al., 2005). In addition, a significant reduction in *H. contortus* egg hatching rates was observed (Valderrábano et al., 2010). Regarding bovine GIN, no information on the efficacy of sulla is available.

One study evaluated the effect of a condensed tannin extract of the quebracho tree *in vitro* by using a larval development assay and observed a reduced viability of *H. contortus*, *T. circumcincta*, T. *colubriformis* and *T. vitrinus* L_3 larvae. Furthermore, *in vivo* studies with the same extract in sheep demonstrated a significant reduction in FEC in comparison to the untreated control group (Athanasiadou et al., 2001; Paolini et al., 2003). Another study on the effect of quebracho extracts against *H. contortus* demonstrates that parasitic burdens are not removed completely and only reduced, suggesting that it may only help to prevent severe infections from occurring (Juhnke et al., 2012). The effect of quebracho against bovine GIN has not been evaluated yet.

Pine bark (*Pinus sylvestris*), which is another tannin-rich substance, demonstrated a positive effect against *O. ostertagi* and *C. oncophora* L_1 in a feeding inhibition assay (Desrues et al., 2016). Additionally, a significant delay on the exsheathment of *H. contortus* and *T. colubriformis* has been described (Bahuaud et al., 2006).

White clover (*Trifolium repens)*, which is high in tannins during the inflorescence, fodder supplementation led to a reduction in *H. contortus* and *Teladorsagia* spp. in lambs, which may be due to a stimulation of the immune system (Ramírez-Restrepo et al., 2010). Furthermore, a feeding inhibition assay targeting L_1 and motility assay targeting adults of *O. ostertagi* and *C. oncophora* demonstrated a positive effect of two white clover extracts (Desrues et al., 2016). Compared to chicory grazing, however, white clover grazing by lambs seems to be less effective in reducing the FEC and production loss (Kidane et al., 2010).

Two tannin-rich African plants, *Clausena anisate* and *Zanthoxylum zanthxyloides*, have been positively tested against *H. contortus* in sheep (Hounzangbe-Adote et al., 2005) and *Ascaris suum* in pigs (Williams et al., 2016) and may be interesting for bovine GIN as well.

In addition to the tannin-rich forages, compounds from chicory cultivars, such as Benulite (root material) and Spadona (root material) (Peña-Espinoza et al., 2017), may have an application in the sustainable control of GIN infections in ruminants. The mode of action of chicory is less described than in tannin-rich forages, but it has been suggested that sesquiterpene lactones are responsible for the anthelmintic efficacy (Hoste et al., 2006). First of all, the consumption of chicory did not result in a significant reduction in the *T. colubriformis* establishment in grazing sheep (Athanasiadou et al., 2005). According to the authors there is evidence that chicory may not have an anthelmintic effect against intestinal nematodes in general, whilst an effect against the abomasal *T. circumcincta* has been described (Tzamaloukas et al., 2004). In calves, a significant reduction of *O. ostertagi* worm burden and FEC was described after the supplementation of chicory, whilst this was not demonstrated against *C. oncophora* (Peña-Espinoza et al., 2016). However, Peña-Espinoza et al. (2017) showed that there is an inhibitory effect of chicory on larval motility and the egg hatching of *C. oncophora*. The authors suggested that the absence of *in vivo* activity against *C. oncophora* may not be explained by an intrinsic inactivity of the compound.

1.2. *Giardia duodenalis*

1.2.1. Introduction

Giardia is an anaerobic flagellate with the ability to cause intestinal infections in a wide range of hosts, depending on the species and the genetic assemblage. This protozoan parasite contains two stages during its life cycle, i.e. a trophozoite (parasitic stage, Figure 2.1) and a cyst (environmental stage, Figure 2.2). The trophozoites are 15-20µm long, pear-shaped and contain four pairs of flagellae. In addition, they have a large anterior disc used for attachment to the intestinal wall. Cysts on the other hand are 10µm long, ovoid shaped and contain four nuclei, but do not have any flagella (Taylor et al., 2016).

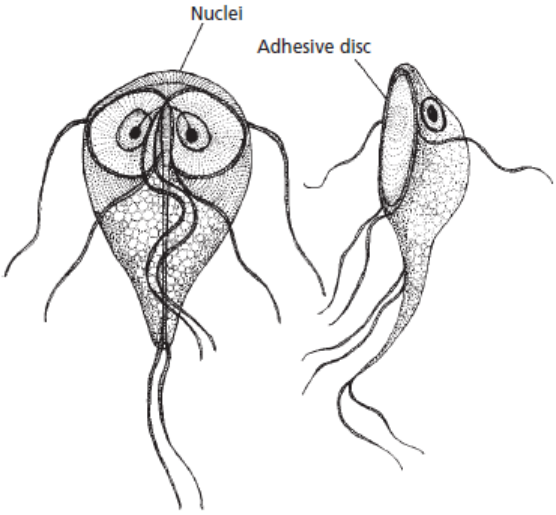


Figure 2.1: *Giardia duodenalis* trophozoites (Taylor et al., 2016).

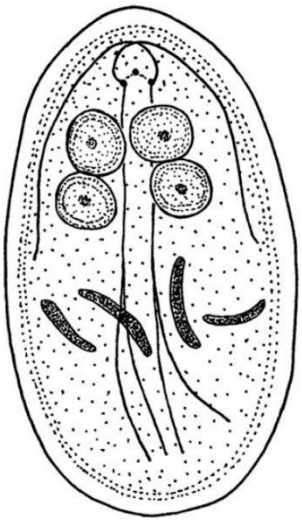


Figure 2.2: *Giardia duodenalis* cyst (Jacobs et al., 2016).

Giardia has a direct life cycle (without intermediate host) and is transmitted through faecal-oral transmission. After uptake of contaminated materials, food or water, the infectious and resistant cysts are transported through the gastrointestinal system and split into two trophozoites per cyst to enhance the chances of establishment. The pathogenic trophozoites will attach to the intestinal wall with their anterior disc and feed on intestinal content. Through binary fission within the lumen of the small intestines, the trophozoites are able to multiply. In addition, Meloni et al. (1989) suggested a sexual reproduction and more recent studies (Logsdon, 2008; Birky, 2010) agree with this statement. However, the exact mechanism of this sexual reproduction, and therefore the confirmation of it, remains unknown. A few days after infection, the trophozoites will encyst and are passed in the faeces at irregular intervals (Jacobs et al., 2016; Taylor et al., 2016). The cysts are immediately infectious after excretion and completion of the life cycle within 72 hours is possible according to Thompson et al. (1993), although the prepatent period is considered to be 4-10 days (Xiao and Herd, 1994; Olson et al., 1995).

Giardia duodenalis (synonyms *G. intestinalis* and *G. lamblia*) is a species group that contains multiple *Giardia* organisms with a similar or identical morphology and it is the only *Giardia* species with the potential to infect humans. Based on the heterogeneity, *G. duodenalis* is split up into different assemblages (Table 2.1), each containing a specific range of hosts (Heyworth, 2016).

Table 2.1: *G. duodenalis* assemblages and corresponding hosts (Heyworth, 2016)

Assemblage	Host(s)
A	Humans, dog, cat, cattle, alpaca, deer, ferret, pig, beaver, chinchilla, jaguar, horse, sheep, goat, muskox, non-human primates, cetacean(s), seals, Australian sea lion, moose, reindeer, chicken, gull
B	Humans, cattle, dog, gazelle, deer, horse, beaver, muskrat, chinchilla, ferret, rabbit, Desmarest's hutia, marsupials, guinea pig, rock hyrax, non-human primates, chicken, sheep, seals, pig, Australian sea lion, ostrich, dolphin, porpoise, gull
C	Dog, kangaroo, cattle, pig, cetacean(s)
D	Dog, chinchilla, kangaroo, cattle, fox
E	Cattle, sheep, pig, alpaca, goat, horse, yak, fox, deer, cat
F	Cat, cetacean(s), pig
G	Rat, mouse
H	Grey seal, gull

Ruminants are susceptible to multiple *G. duodenalis* assemblages. Most commonly the hoofed livestock specific assemblage E, but (co-)infections with assemblage A and B are possible. Assemblage A and B are considered to be important in humans and may contain a zoonotic property (Heyworth, 2016).

Direct contact with infected hosts and indirect contact with environmental contaminations are the two important transmission pathways. Therefore, animals held indoors are three times more likely to get infected with *Giardia* than animals kept outdoors (Ruest et al., 1998). Moreover, group housing seems to be an important factor for indoor infections, but infections can spread in individual housing as well (Ruest et al., 1998). A study by Grit et al. (2012) determined the *G. duodenalis* cyst survival in cattle slurry and showed a great reduction in the viability of cysts not until 50 days of incubation. Therefore, the spreading of manure and slurry is considered to be an infection source on farms.

1.2.2. Epidemiology and prevalence

Geurden et al. (2010a) mentions that prevalence studies around the world show a considerable variation in both animal and farm prevalence. According to the authors, however, this is mainly due to the use of different diagnostic techniques and a different study design. In addition, the age of the animals needs to be taken into consideration in order to obtain a reliable comparison. Generally, a peak prevalence in calves is seen at an age between 1 and 6 months, while a decrease in prevalence is seen in calves from the age of 6 months and onwards (Xiao et al., 1993; Nydam et al., 2001; Ralston et al., 2003; Becher et al., 2004). However, adult hosts cannot be excluded as an infection source (Cacciò et al., 2005) and a periparturient rise of cyst excretion in adult sheep, goats and cattle has been suggested (Wade et al., 2000b; Castro-Hermida et al., 2005).

Ruminants, mostly cattle, have long been considered to be an important source for *Giardia* infections in humans. The farm prevalence of *G. duodenalis* on European livestock farms is high (89.9%) for both beef and dairy calves, whilst the calf prevalence on those farms is 45.4% (Berrilli et al., 2004; Lalle et al., 2005; Geurden et al., 2012). The majority of those calves tested positive for *G. duodenalis* assemblage E, but a substantial number (43%) of calves tested positive for the zoonotic *G. duodenalis* assemblage A. Besides, the prevalence on these farms was significantly higher in calves younger than 2 months compared to older calves (Geurden et al., 2008a; Geurden et al., 2012). Additionally, there is a high farm prevalence of *Giardia* on sheep farms (100%) and goat farms (80%), whilst animal prevalence is 25.5% in lambs and 35.8% in goat kids. Similar to cattle, the host specific assemblage E was the most common cause of giardiosis in both goat kids and lambs, whilst assemblage A was identified at a lower prevalence. (Geurden et al., 2008b). Although the zoonotic assemblages are present on most livestock farms, there is no indication that ruminants should be considered to be a very important source of giardiosis in humans (Cassio et al., 2005).

1.2.3. Pathogenesis and clinical signs

General

The interest in *Giardia* within the field of veterinary medicine is mainly driven by public health concerns. Therefore, research is focussed on the prevalence and molecular characterisation of the *Giardia* assemblages with a potential zoonotic property. The clinical relevance of *Giardia* infections in production animals, however, was only studied to a limited extent. Mostly due to the vagueness of the symptoms caused by Giardia, the relevance of infection is still not widely recognised. Clinical signs associated with *Giardia* infections in ruminants have been documented by multiple studies, but the exact pathogenesis is not clearly understood. Therefore, it is not yet known if *Giardia* should be considered as a primary or secondary pathogen in ruminants, although most studies suggest that *G. duodenalis* is a primary pathogen (O'Handley et al., 1999; Aloisio et al., 2006; Geurden et al., 2006a; Geurden et al., 2010a).

Slow development of immunity

One of the major problems associated with *Giardia* infections is the delayed development of immunity in young animals. No significant increase of serum IgG is found in calves infected with *G. duodenalis* until 8 weeks post-infection (O'Handley et al., 2003). In Grit et al. 2014, an analysis of the antibody response showed an increase in the serum IgG1 and IgA levels against *G. duodenalis* assemblage A and E from week 11 post-infection. In humans, however, serum IgG and IgA increased 2 weeks post-infection, which may explain the reason why the acquisition of immunity against *Giardia* in humans is much faster than in cattle (Daniels and Belosevic, 1994; Velazques et al., 2005). A low cyst excretion due to the development of immunity was described 15 weeks post-infection (Grit et al., 2014). This slow development of immunity corresponds to the results obtained by Dreesen et al. (2012) where no intestinal immune response was seen in calves 3 weeks post-infection. According to Grit et al. (2014), interleukin(IL)-17A fulfils an important role in the development of immunity against *G. duodenalis* infections in calves. A more recent study by Paerewijck et al. (2017) states that IgA secretion in the intestinal lumen is dependent on IL-17A signalling and the upregulation of this interleukin induces the production of various antimicrobial peptides.

Histopathological changes

Most studies report histological changes in the intestinal villi and crypts. First, a trial conducted by Ruest et al. (1997) showed a reduction in villus to crypt ratio in *Giardia* infected calves. However, there is no guarantee of reliability due to the use of a limited number of calves in their trial. On top of that, the cyst excretion by the calves is unknown. Second, O'Handley et al. (2001) showed morphological improvements in the small intestines after fenbendazole treatment in *Giardia* infected calves. In addition, they described an increase in brush border surface area in the intestines of fenbendazole-treated calves compared to the untreated infected animals. These findings go hand-in-hand with the results found by Scott et al. (2000), which state that a reduction in brush border surface area is one of the major factors contributing to malabsorption and diarrhea. A third and more recent study by Cotton et al. (2011) describes how enterocyte apoptosis and epithelial barrier disruption results into villus shortening with maldigestion and malabsorption. Furthermore, their trial showed an increased chloride secretion and intestinal transit after infection, which contribute to the development of diarrhea. However, no intestinal pathologies, such as villus shortening, were observed in the intestines of infected animals (Dreesen et al., 2012). The authors state that the presence of clinical signs may depend on the infection dose. In their trial, calves excreted between 3950 and 15000 cysts per gram (CPG) of faeces, while naturally infected calves can excrete up to 10^6 CPG due to the slow development of adaptive immunity (Xiao et al., 1996; Yanke et al., 1998; O'Handley et al., 2003; Geurden et al., 2004).

An earlier trial by Oberhuber et al. (1997) showed that 96.3% of their cases had an entirely normal light-microscopic appearance of the duodenal mucosa. The other 3.7% only had mild villus shortening and mild inflammation in the duodenal mucosa. However, this study does not show the cyst excretion by the calves and it might be too low for intestinal pathologies as mentioned earlier by Dreesen et al. (2012).

According to Dreesen et al. (2012) several genes involved in lipid metabolism were also impacted by *G. duodenalis* infection, resulting in a decreased uptake of lipids in the intestines and a higher efflux of cholesterol in the intestinal lumen. This corresponds with an earlier trial by Scott et al. (2000) where they suggest a decreased activity of brush border enzymes, especially lipase, in the small intestines. It can

be concluded that it is generally accepted that *G. duodenalis* causes villus shortening resulting in maldigestion and malabsorption. Clinical signs, however, will only be present if the animals are adequately infected. Although acute symptoms can occur, chronic and intermittent diarrhea is observed more often in calves (Geurden et al., 2006a). Similar symptoms with the excretion of formless faeces have been observed in goat kids an lambs (Koudela and Vitovec, 1998; Aloisio et al., 2006).

Production loss
Economically, the consequence of a *Giardia* infection in ruminants is best described as production loss. Clinical symptoms are generally vague and chronic, but production parameters such as: a decreased feed efficiency, decreased weight gain and weight loss have been described in goat kids, lambs (Koudela and Vitovec, 1998; Aloisio et al., 2006) and calves (Geurden et al., 2006b) after infection with *G. duodenalis*. Based on these trials, it can be concluded that *Giardia* can be a cause of ill-thrift in juvenile production animals, likely due to the intestinal histopathological changes and diarrhea.

1.2.4. Diagnostics

Clinical diagnosis
As mentioned by Geurden et al. (2010a), a clinical diagnosis of *Giardia* infections in ruminants is mainly based on clinical history, data on farm management and the exclusion of other infectious diseases, such as coccidiosis. However, is it not possible to diagnose giardiosis purely based on the vague symptoms caused by infection. Therefore, the clinical diagnosis needs to be confirmed by faecal examination, either by microscopical examination, antigen detection or polymerase chain reaction (PCR). It is important to take multiple faecal samples due to the intermittent excretion of cysts and preferably samples taken from animals around 2-4 weeks of age. According to O'Handley et al. (1999), samplings should be taken from the same animal for three consecutive days or from several animals within the same housing for only one day.

Microscopical examination
Due to the increased peristalsis during diarrhea, some trophozoites will be excreted and can be detected by direct microscopy (Geurden et al., 2010a). Excreted *Giardia* cysts can be detected by microscopy directly or indirectly after concentration with sucrose or zinc sulphate. The cysts can be stained with iodine (Zajac, 1992) or trichrome (Addis et al., 1991) prior to the examination. However, the major disadvantages of microscopy are the limited sensitivity compared to immunological assays (Geurden et al., 2004), the need for a skilled and experienced microscopist, and the time consumption. On the other hand, there is only a limited cost for microscopical examination.

Immunological assays
Three commercially available techniques used are antigen immunofluorescence assay (IFA) (Xiao and Herd, 1993), ELISA (Boone et al., 1999) and rapid solid-phase qualitative immunochromatography assay (Garcia et al., 2003). Both IFA and ELISA make use of monoclonal antibodies directed against cyst wall proteins and are considered to be more sensitive and specific for the diagnosis of *Giardia* infections in calves when compared to microscopical examination (Geurden et al., 2004). The main disadvantages of these two techniques, however, is the cost and time consumption. On the other hand, the use of immunochromatography allows for a faster diagnosis and uses monoclonal antibodies directed against specific cyst or trophozoite wall proteins. However, the sensitivity of immunochromatographic tests for detection of giardiosis in calves is limited (Geurden et al., 2010c).

Nucleic acid-based detection methods
Fluorescence *in situ* hybridisation (FISH) is a technique that is based on the specific *in situ* hybridisation of fluorescently labelled probes to particular genetic loci within *Giardia* cysts. The advantages of FISH are the rapidity and the ability to determine the viability of the parasites (Adeyemo et al., 2018). Another and more frequently used method is a polymerase chain reaction (PCR), for example real-time PCR or qPCR. For the use of PCR in clinical diagnostics, 18S rDNA seems the most suitable gene to detect according to Read et al. (2004). Theoretically, PCR has a very high diagnostic sensitivity with a detection

limit of 1 cyst (Amar et al., 2002). At present, however, PCR is still considered to be an expensive method for routine diagnosis of giardiosis in production animals.

1.2.5. Current treatment

General

Currently, there are no drugs registered in Belgium for the treatment of giardiosis in ruminants and thus veterinarians are limited to the off-label use of certain products. In addition to treatment, environmental hygiene remains a very important measure to prevent re-infection (O'Handley et al., 2000). These measures are even more important when the giardiosis is treated with short term cyst-suppressing drugs and should be carried out at the end of the treatment period. However, cysts are known to be resistant to commonly used disinfectants (Geurden et al., 2006a). Alternative options with an application in calf facilities are heat (Olson et al., 1999) and disinfection with quaternary ammonium (O'Handley et al., 1997). Additional management systems, such as immediate separation of the calf and the cow after birth (Wade et al., 2000a) and a sufficient supply of colostrum (Wade et al., 2000a; O'Handley et al., 2003) could be important measures to reduce clinical giardiosis in calves.

Nitro-imidazoles

Nitro-imidazoles, such as metronidazole, tinidazole, furazolidine and quinacrine, are commonly used drugs in human medicine to treat several protozoan infections. The effect of the most thoroughly studied metronidazole is based on inflicting DNA damage after entering the trophozoite (Gillis and Wiseman, 1996; Samuelson, 1999). However, serious side-effects have been described and metronidazole is considered to be carcinogenic (Morgan et al., 1993). Besides the toxicity, resistance to drugs has been described for metronidazole and furazolidine *in vitro* and in human patients (Upcroft et al. 1990; Upcroft and Upcroft, 2001). Other studies suggest a symptomatic improvement after treatment with metronidazole in calves, but they do not provide any data regarding cyst excretion reduction (Xiao et al., 1996). On the other hand, a later study by Cedillo-Rivera et al. (2002) showed promising results for a drug called nitazoxanide. An *in vitro* trial indicates that tizoxanide, a nitazoxanide metabolite, was 2 to 8 times more active against *G. duodenalis* than metronidazole (Rossignol, 2010) and a human trial showed that nitazoxanide treatment after *Giardia* infection results in a clinical cure in 85% of the patients (Ortiz et al., 2001; Rossignol et al., 2001). A more recent study in dogs by Moron-Soto et al. (2017) documented the efficacy of nitazoxanide against *G. duodenalis* where single dose administration of 75mg/kg or 150mg/kg nitazoxanide halted the excretion of *Giardia* cysts for 13 days. Unfortunately, there is no available data on the use of nitazoxanide to treat giardiosis in ruminants.

Benzimidazoles

BZ, such as fenbendazole and albendazole, are known for their combination of a broad spectrum anthelmintic activity and a high safety margin with only a selective toxicity (Xiao et al., 1996). In addition, *in vitro* studies show that BZ have a higher efficacy against *Giardia* infections than nitro-imidazoles (Edlind et al, 1990; Meloni et al., 1990; Morgan et al., 1993). BZ interfere with the attachment of *Giardia* trophozoites to the intestinal wall by binding to the *Giardia* β-tubulin cytoskeleton (Morgan et al., 1993). In addition, BZ might bind to *Giardia*-specific proteins, called giardins, located in the ventral disc (Edlind et al., 1990; Meloni et al., 1990). Treatment of *Giardia* infected calves with fenbendazole for a period of 3 consecutive days results in a significant reduction of the cumulative cyst excretion. In addition, treated calves gain more weight than untreated calves (Geurden et al., 2010b). In lambs, a high efficacy of fenbendazole against giardiosis was observed after 3 consecutive days of oral treatment, resulting in a significant reduction in cumulative cyst excretion during 21 days (Geurden et al., 2011). According to O'Handley et al. (2000), however, the number of faecal *Giardia* cysts returns within two weeks post treatment. Therefore, environmental measures should be considered since the combination of treatment with environmental hygiene leads to a significant reduction of the cyst excretion for at least 4 weeks after treatment (Geurden et al., 2006b).

Paromomycin
Paromomycin is a member of the aminoglycoside family and has an antiprotozoal property due to the inhibition of the protein synthesis (Edlind, 1989). An early trial by Gordts et al. (1985) indicates that paromomycin has the highest minimum inhibitory concentration (MIC) amongst the following antiprotozoal drugs: tinidazole, metronidazole, chloroquine, pyrimethamine and furozolidone. This relatively high MIC was confirmed by Edlind (1989). However, they state that paromomycin is poorly absorbed from the gut. This minimal absorption makes it possible to administer a higher concentration of the drug without the risk of systemic toxicity, whereas other drugs, like hygromycin and metronidazole, are readily absorbed. Another reason to use paromomycin over metronidazole is because metronidazole has not been approved for the use in food producing animals in most countries (Xiao et al., 1996). In calves, a complete (100%) and high (≥ 93%) reduction in cyst excretion is seen until 9 days after the start of a 75 and 25mg/kg per day paromomycin treatment, respectively. The effect of the treatment, however, did not result in significantly better faecal consistency or weight gain. As with other treatments, environmental control is important to maintain low infection rates after treatment (Geurden et al., 2006a). No information is available regarding the efficacy of paromomycin to treat giardiosis in lambs or goat kids.

1.2.6. Alternative control

Resistance against nitro-imidazoles, such as metronidazole and furazolidine, has been described by multiple studies (Upcroft et al., 1990; Requena-Méndez et al., 2017; Ansell et al., 2017). However, treatment of giardiosis in ruminants is mainly with benzimidazoles and paromomycin due to the reason that metronidazole has not been approved of the use in food producing animals in most countries (Xiao et al., 1996). Although different options for alternative treatment are known, they mainly focus on the application within the field of human medicine. Therefore, the alternative options to treat giardiosis in ruminants are very limited. Only the alternatives with a potential and/or a possible application to treat giardiosis in ruminants will be mentioned below.

Vaccination
In dogs and cats, a *G. duodenalis* vaccine induced a *Giardia* specific serum IgA and IgG response leading to a significant reduction in cyst excretion, lower number of trophozoites per cm of small intestine, increased feed update and increased weight gain (Olson et al., 1996; Olson et al., 1997). To evaluate the efficacy of vaccination in preventing giardiosis in calves, Euhlinger et al. (2007) performed a trial using six 2-week old calves that were vaccinated with a subcutaneous sonicated *G. duodenalis* trophozoite vaccine, whilst six 2-week old control calves were injected with sterile phosphate-buffered-saline. The results show a significantly higher humoral immune response in the vaccinated calves compared to the controls. However, the vaccination was not efficacious in reducing the cyst excretion. In addition, there were no significant differences between vaccinated calves and controls regarding the number of trophozoites per cm small intestine. They state that the vaccine failure may have been due to the continuous exposure to cysts within the environment and the possibility that the presence of maternal antibodies in the calves could have interfered with the vaccination. In addition, the vaccine was not based on the ruminant specific *G. duodenalis* assemblage E. Therefore, antigenic differences between the assemblages could be responsible for the failure of this vaccine.

Auranofin
Auranofin originally is an antirheumatic drug approved for treatment of rheumatoid arthritis in humans. However, according to a chemical library, Iconix Biosciences, Inc. (Foster City, CA), it has an anti-giardial property as well. Tejman-Yarden et al. (2013) performed a trial *in vitro* and in mice, which revealed that auranofin was active against *G. duodenalis* assemblage A and B as it eradicated the infection. More importantly, it was active against multiple metronidazole-resistant strains. It is suggested that the anti-giardial activity is based on blocking the giardial thioredoxin oxidoreductase activity, an enzyme involved in maintaining a normal protein function and combating oxidative damage (Leitsch et al., 2011; Tejman-Yarden et al., 2013). Although there is a potential application of auranofin to treat giardiosis in humans, nothing is known about its efficacy against giardiosis in ruminants.

Vegetal substances

Geranium spp.

The first report of antiprotozoal properties of *Geranium* spp. was made by Calzada et al. (1999). In this trial, the efficacy of seven different extracts from *Geranium niveum* roots were tested against *Giardia duodenalis in vitro*. Two of these compounds, geranins A and B, seemed to have an effect on the vitality of the parasite. A later trial showed that geranin D too has a moderate antiprotozoal activity against *G. duodenalis* which is likely due to the presence of tannins (Calzada et al., 2001). Additionally, the roots of *G. mexicanum* have an anti-giardial activity *in vitro* (Calzada et al., 2005) with a higher efficacy than metronidazole according to Barbosa et al. (2007). However, only mice were used during these *in vivo* infection trials and there is no further information available regarding the effect against *G. duodenalis* infections in ruminants.

Piper longum and *Piper betle*

According to Tripathi et al. (1999), *Piper longum* fruit (long pepper) was used in traditional remedies against intestinal disorders. In their trial, it was tested for its efficacy against *G. duodenalis* infections in mice. At 250µg/ml, the extract showed a 100% giardicidal activity and an additional specific and non-specific immunostimulatory activity. A more recent study by Pecková et al. (2018) tested the effect of *Piper betle* against *G. duodenalis* infections in gerbils. Their results show a significant decline in cyst excretion after supplementation with *Piper betle* extracts, compared to a negative control. However, the reduction in cyst excretion was not as high as the positive control, a metronidazole treatment.

Yucca schidigera

A powdered preparation of *Yucca schidigera* was investigated *in vivo* in experimentally infected lambs by McAllister et al. (2001). Compared to the controls, lambs receiving 10 g of yucca powder per day in their diet had a reduced faecal cyst excretion after 5, 9, 12 and 19 days of treatment. However, a decline in prevalence of infection was not observed.

2. Issue and purpose

The aim of this study was to test the *in vitro* efficacy of different extracts of bioactive forages on the viability of *G. duodenalis* trophozoites and migration capacity of *O. ostertagi* and *C. oncophora* L_3 larvae for the purpose of finding alternative and sustainable methods against gastrointestinal parasite infections in cattle. The bioactive extracts (or compounds) used in this study were selected based on their potential activity against bovine and ovine GIN and are presented in Table 3.1. Based on preliminary studies, tannin-rich bioactive forages such as *Clausena anisate*, *Zanthoxylum zanthoxyloides*, *Pinus sylvestris* and *Trifolium repens* inflorescence are expected to have an anthelmintic affect against *O. ostertagi* and *C. oncophora* (Hounzangbe-Adote et al., 2005; Williams et al., 2016; Desrues et al., 2016). Based on a study by Peña-Espinoza et al. (2017), the efficacy of *Cichorium intybus* roots is expected to be higher against *O. ostertagi* than *C. oncophora*. Although no studies examined the efficacy of those bioactive forages on *Giardia* spp., tannin-rich forages such as *G. niveum* may have an effect on the trophozoite viability (Calzada et al., 2001).

3. Materials and methods

3.1. *Larval-migration-inhibition assay (LMIA)*

3.1.1. Parasites

Obtaining *C. oncophora* and *O. ostertagi* L_3 larvae

Bovine faecal samples containing nematode eggs were collected and incubated in a plastic jar for 10-14 days at 28°C after the addition of vermiculite, which allows for a homogenous wet mixture. The faeces were then filtered for 18-24 hours by using a Baermann apparatus with funnel system, allowing the collection of L_3 larvae afterwards. The larvae were then stored at ± 5°C in concentrations of 170-200 L_3 per ml H_2O for *O. ostertagi* and 1200-1300 L_3 per ml water for *C. oncophora*.

Exsheathment of L_3

Since the ensheathed L_3 will exsheath promptly after ingestion by a ruminant, it was the obvious step to use exsheathed L_3 in this study. To obtain these, sodium hypochlorite (20µl per ml of L_3 suspension) was added to the ensheathed L_3 suspension, followed by an incubation of 3-4 min. During this process, most of the L_3 were exsheathed and afterwards transferred to a centrifuge for 2 min at 500 g. Finally, the supernatant was discarded and replaced with PBS. These steps were repeated two more times to obtain L_3 in pure PBS suspension. Since the larvae were immediately used in the assay, there was no need for an incubation at 37°C or in a nutrient-rich environment.

3.1.2. Chemicals and compounds

To prevent difficulties while counting the L_3 larvae in the assay, Bacto Agar (agarose type II) was purchased from Sigma (A6877-500g) and used at a concentration of 1.5%. Dimethyl sulphoxide (DMSO, 100%) was ordered from Sigma (D8418-100ml) and was used for two purposes, namely as negative control at a level of 0.5% and to ensure that the DMSO levels were identical (at 0.5%) for all compound concentrations. Levamisole (2% solution in water) served as a positive control in this study and was acquired from Sigma (L9756-5g). Demineralised sterile-filtered water (dH_2O), purchased from Kruuse (340169-5L), was used to make new stock concentrations and served as main component of well-contents to obtain an end-volume of 1,8ml. Finally, Dulbecco's phosphate-buffered saline (PBS) was purchased from Sigma (D8537) and used for the incubation of exsheathed L_3 larvae to support their viability.

The different extracts of bioactive forages (Table 3.1) were selected based on preliminary studies on GIN (Ramírez-Restrepo et al., 2010; Williams et al., 2016; Desrues et al., 2016; Peña-Espinoza et al., 2017). It has been demonstrated by Desrues et al. (2016) that some of these compounds, such as pine bark and white clover, have an effect against *O. ostertag*i and *C. oncophora* adult and larval stages other than L_3.

Table 3.1: Tested extracts of bioactive forages with the corresponding plant species and references.

Extract	Plant species	Solvent	Stock concentration	References
WR1 (seaweed)	*Fucus spiralis*	DMSO	200mg/ml	Unpublished
WR2 (seaweed)	*Saccharina latissimi*	DMSO	200mg/ml	Unpublished
214A	*Clausena anisate*	DMSO	100mg/ml	Williams et al. (2016)
243A	*Zanthoxylum zanthoxyloides*	DMSO	100mg/ml	Williams et al. (2016)
Spadona Root	*Cichorium intybus*	DMSO	100mg/ml	Peña-Espinoza et al. (2017)
Benulite Root	*Cichorium intybus*	DMSO	100mg/ml	Peña-Espinoza et al. (2017)
Pine Bark F2	*Pinus sylvestris*	NA (dried solid)	100mg/ml	Desrues et al. (2016)
White Clover F2	*Trifolium repens*	NA (dried solid)	100mg/ml	Ramírez-Restrepo et al. (2010)

3.1.3. Protocol

Due to the use of *O. ostertagi* and *C. oncophora* L_3 larvae, this experiment makes use of a larval migration inhibition assay (LMIA) to determine the effect of various bioactive forages at different concentrations. This LMIA is based on the assay by Demeler et al. (2010a), but has been adapted to fit bioactive forages instead of modern anthelmintics. This assay is based on the ability of L_3 to migrate through a precision nylon filter with a defined mesh-size in the absence or presence of bioactive compounds. Due to distinct colouration of the different compounds, randomization was not very useful and thus not carried out.

Incubation phase

In the incubation phase, the ensheathed L_3 were incubated in different compound concentrations before they were transferred into the migration phase. For this phase, flat bottom 24-well culture plates (Nunc 142475) were used. Since the compounds were dissolved in DMSO, and DMSO stock concentrations were very high (100-200mg/ml), new stock concentrations for each compound had to be made beforehand in dH_2O: 50µg/ml, 100µg/ml, 250µg/ml and 500µg/ml. Furthermore, the 500µg/ml compound concentration corresponds to a DMSO level of 0.5%, which did not affect the larval migration (Figure 4.1). It was therefore decided to use this DMSO level standardized for every compound concentration and as negative control. Levamisole was used as a positive control. The wells were filled with 1690µl of dH_2O and 90µl of each compound concentration. Roughly 80-100 L_3 per well were required to obtain reliable results and therefore a suspension of 80-100 L_3 per 20µl PBS was made. Finally, 20µl of L_3 suspension was added per well to obtain an end-volume of 1800µl. All concentrations, including negative and positive controls, were tested in threefold for both GIN species (Figure 3.1). The 24-well culture plates were then incubated for 24 hours in an incubator at 22°C.

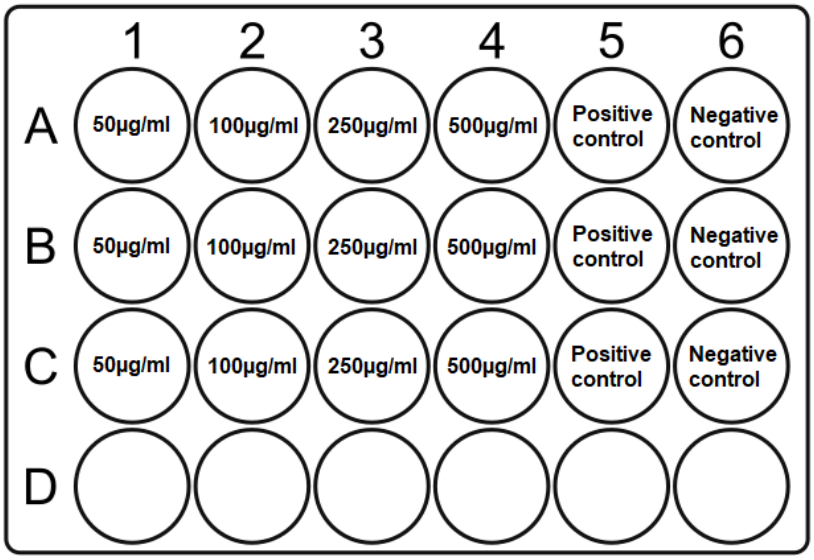


Figure 3.1: Incubation set up in threefold for each compound, including four different compound concentrations, a positive and a negative control.

Migration phase

After 24 hour incubation, the entire content of each well was transferred into migration tubes (Figure 3.2) positioned in new 24-well culture plates. Before this transfer, however, all wells must be filled with 400µl of Bacto Agar 1,5% to achieve a kind of larval bedding to stop the L_3 from migrating to the rim of the wells, which hinders counting them (Demeler et al., 2010a). Each migration tube holds a nylon filter with a defined mesh-size of 28µm, which is specifically intended for *O. ostertagi* and *C. oncophora* according to Demeler et al. (2010a) and only allows active larvae to pass through. The migration tubes must be positioned in the wells in such a way that the filters remain within the solution at all time and the volume per well must therefore be large enough. It is of importance that all filters are examined under a microscope for damage and that remaining contents of previous use are removed beforehand. Alternating tubed-rows with non-tubed-rows will make it easier to transfer non-migrated L_3 in a later part of this protocol and is demonstrated in Figure 3.3. After transferring the content of each well into the migration tubes, the new 24-well culture plates with the tubes are once again incubated for 24 hours in an incubator at 22°C.

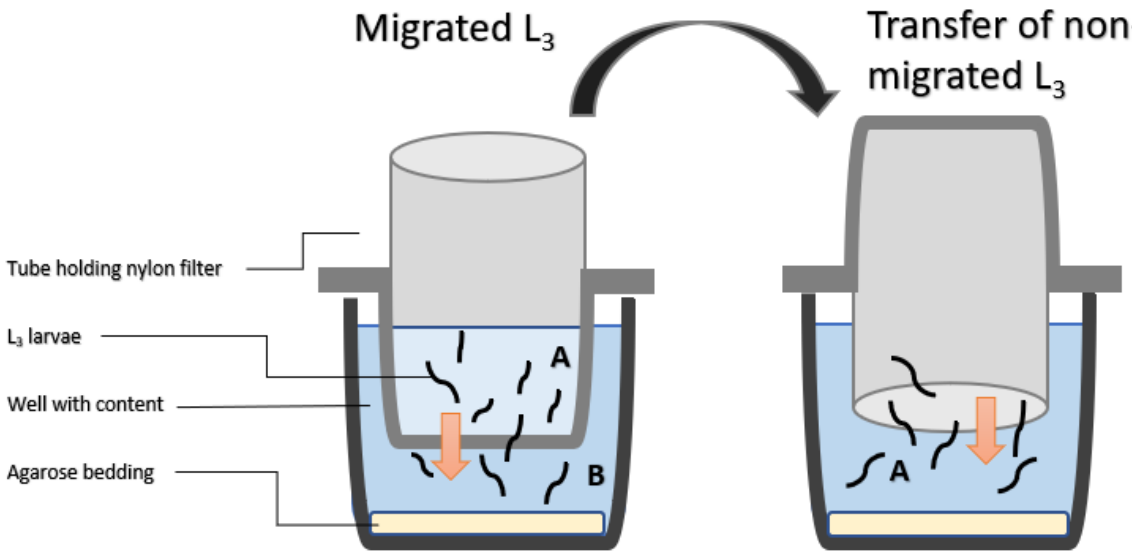


Figure 3.2: Schematic representation of the LMIA during the migration phase and the transfer of non-migrated L_3. Compartment A contains the non-migrated larvae and are transferred into the next well row (see below). Compartment B contains the migrated larvae and the orange arrows indicate the movement of larvae during the assay and during the transfer.

Counting phase

After the migration phase, the assay is stopped by lifting the sieves out of the wells. The contents on the outside of the sieves were then washed back gently into the initial row of wells with a small volume (ca. 30µl) of dH_2O. Afterwards, the sieves were flipped over into the empty row below and the internal contents were washed into the wells with ca. 2ml dH_2O as demonstrated in Figure 3.2. Finally, the L_3 were counted with a stereo microscope whereby the percentage of non-migrated L_3 was determined. In addition to the LMIA, larval motility was observed after 6 and 24 hours after the incubation at 22°C.

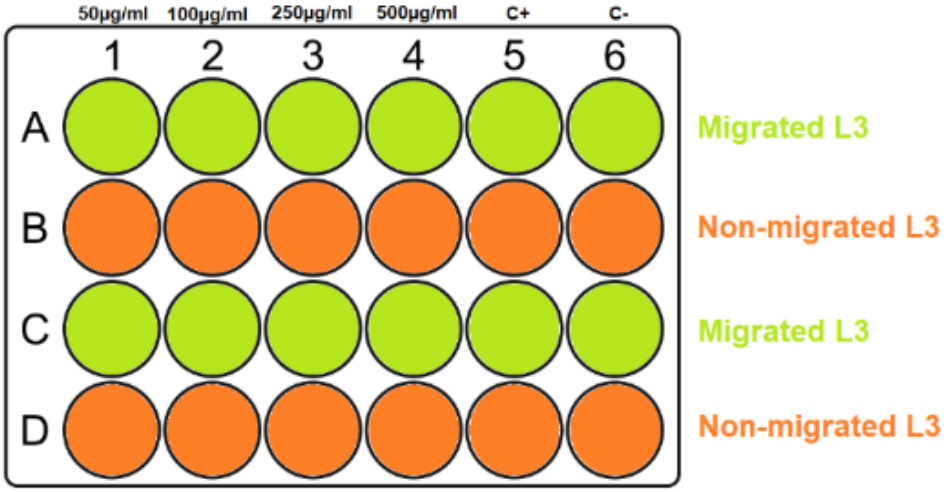


Figure 3.3: Set up of the larval-migration-inhibition assay in a 24-well culture plate. The green wells indicate the initial filters for larval migration, which were then lifted out of row A (C), turned over and flushed out in row B (D), which is indicated by the orange wells.

3.1.4. LMIA on different DMSO levels

Following the protocol discussed above, DMSO levels of 0%, 0.1%, 0.5% and 1% were examined. Since the compound concentrations of 500µg/ml for all compounds, except WR1 and WR2, automatically came with DMSO levels of 0.5%, it was decided to use this DMSO level in each compound concentration and as negative control. However, the effect of DMSO on the viability of ensheathed L_3 had to be determined beforehand to minimize the influence on the outcome of this assay.

3.1.5. LMIA on different bioactive forages

As described above, all compounds were tested at concentrations of 50µg/ml, 100µg/ml, 250µg/ml and 500µg/ml following the LMIA protocol. A total amount of 2000 L_3 was used per compound per nematode species. To determine the effect of these substances on the viability or motility of the L_3, an LD50 was calculated for compounds that show a significant inhibitory effect compared to the negative control.

3.2. *Resazurin-reduction assay*

3.2.1. Chemicals and compounds

Dulbecco's phosphate-buffered saline (PBS) was purchased from Sigma (D8537) and used for all washing steps in the assay. Dimethyl sulphoxide (DMSO, 100%) was ordered from Sigma (D8418-100ml) and was used for three purposes, namely as negative control at a level of 0.5%, positive control at 5% and to ensure that the DMSO levels were identical (at 0.5%) for all compound concentrations. TYI-S-33 medium (see below) was composed on the basis of various chemical products. Similar to the LMIA, the compounds described above in Table 3.1 were used in the resazurin-reduction assay.

3.2.2. The protocol

Although diagnostic and epidemiological studies mainly focus on the cyst form, *in vitro* culture of trophozoites remains the standard laboratory tool (Bénéré et al., 2007). This protocol is based on a study by Bénéré et al. (2007) where resazurin staining is used as quantitative method to determine the viability of *Giardia* trophozoites *in vitro*.

Cultivation of trophozoites
The stock of trophozoites was cultivated axenically in *Giardia*-specific TYI-S-33 medium supplemented with bile (Keister, 1983) at a temperature of 38°C. The medium consists of 870 ml distilled H_2O, 0.6 g KH_2PO_4, 1.31 g K_2HPO_4, 2 g NaCl, 10 g glucose, 10 g yeast extract, 20 g trypticase, 2 g L-cysteine HCl, 0.2 g L-ascorbic acid, 0.5 g bovin bile, 1 ml AFC and 100 ml inactive human serum. Prior to adding 100 ml inactive serum, the pH was corrected to 6.8. Finally, the medium was made sterile by using a 0.22µM vacuum filter (EMD Millipore™ Steritop™). For cultivation, 10 ml screw-cap culture vials (Nunc) were used to incubate the trophozoites for 2-3 days before being placed on ice for 20 minutes to reduce the adherence of trophozoites to the vial wall. Thereafter, subcultures were made for further use in the trial. It is important to cultivate the trophozoites under a reduced oxygen tension (Upcroft and Upcroft, 2001) and therefore the used vials and microplates should be air-tight and filled to 90-95% of the total volume capacity (Bénéré et al., 2007). Note that opening the vials should only be done in a sterile environment to prevent bacterial contamination. Every new vial contained a total volume of 10 ml, of which 1 ml was transferred from the incubation vial and 9 ml was new TYI-S-33 medium. Once again, after 2-3 days of incubation, the vials were placed on ice for 20 minutes. Thereupon, all the vials required for the trial were centrifuged at 800 g for 5 minutes. Nine ml of the supernatant was removed per vial to obtain a high concentration of trophozoites in a small volume. The next step was to determine this concentration by using a Bürker counting chamber and adjust it to the desired concentration. For all assays in this trial, flat-bottom 96-well microplates (Nunc) were used to incubate 2.5×10^5 trophozoites per well.

Preparation of resazurin
According to Bénéré et al. (2007) resazurin staining of trophozoites proved to be superior to other staining methods, such as 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) and 2,3-bis-(2-methoxy-4-nitro-5-sulfophenyl)-2H-tetrazolium-5-carboxanilide (XTT) (Wright et al., 1992), and was chosen for this trial due to its objectivity in quantifying the viability of the trophozoites. Resazurin (Sigma), which is blue coloured and nonfluorescent, is converted to the pink and highly fluorescent resorufin by reductase activity (O'Brien et al., 2000; Bénéré et al., 2007). According to O'Brien et al. (2000), however, it is still not known how this reduction occurs, but there appears to be a linear correlation between the viability and the reduction of resazurin to resorufin. A practical disadvantage of using resazurin is the fact that that TYI-S-33 medium and bacterial contamination will cause spontaneous reduction of the resazurin substrate (Bénéré et al., 2007). This problem was avoided by washing the trophozoites in PBS and working with sterile equipment in a sterile environment. Bénéré et al. (2007) uses ammonia as a solvent for the dilution of resazurin. However, in this trial a 2% dilution of resazurin was made in PBS since ammonia appeared to be toxic to the trophozoites. Moreover, the substitution of ammonia with distilled H_2O did not appear to have an effect on the solubility of resazurin.

Resazurin staining
After obtaining the correct trophozoite concentration, for each compound the following concentrations were made in TYI-S-33 medium: 50µg/ml, 100µg/ml, 250µg/ml and 500µg/ml. Since the compounds were dissolved in DMSO, and DMSO stock concentrations were very high (100-200mg/ml), a new diluted stock had to be made beforehand. Additionally, it was important to keep the DMSO level equal for every compound concentration. For this trial, a DMSO level of 0.5% was used based on preliminary testing with different DMSO concentrations. The decision for these concentrations are further explained below.

Afterwards, 200µl of this diluted compound and 100µl medium with 2.5×10^5 trophozoites were added per well to obtain a final culture volume of 300µl/well. Five replicates were carried out per compound concentration, plus a positive and negative control. The 96-well microplates were then sealed and incubated at 37°C for 20 hours. After the incubation, the microplates were placed on ice for 20 minutes to stimulate the detachment of trophozoites from the wall of the wells. The content of all the wells in the flat-bottom 96-well microplate were then moved to a V-bottom 96-well microplate (Nunc) which was then centrifuged at 800 g for 5 min. The supernatant was removed carefully from each well since the TYI-S-33 medium causes spontaneous reduction of the resazurin substrate (Bénéré et al., 2007). In addition, the trophozoites were washed in 200µl of PBS to minimize this reduction to a further extent. Once again, the microplate was centrifuged at 800 g for 5 min and the PBS supernatant was removed from each well. Finally, the trophozoites were resuspended in 300µl 2% resazurin in PBS per well and transferred back to a new flat-bottom 96-well plate. Finally, the microplates were sealed and further incubated at 37°C for at least 2 hours to allow conversion of resazurin.

Spectrophotometric quantification of viable trophozoites
After 2, 3 and 4 hours of incubation, the microplates were evaluated by a spectrophotometric quantifier (SpectraFluor) with an excitation and emission wavelength of 492nm and 595nm respectively. As expected, the fluorescence values increased as time progressed whereas more resazurin is converted in fluorescent resorufin over time.

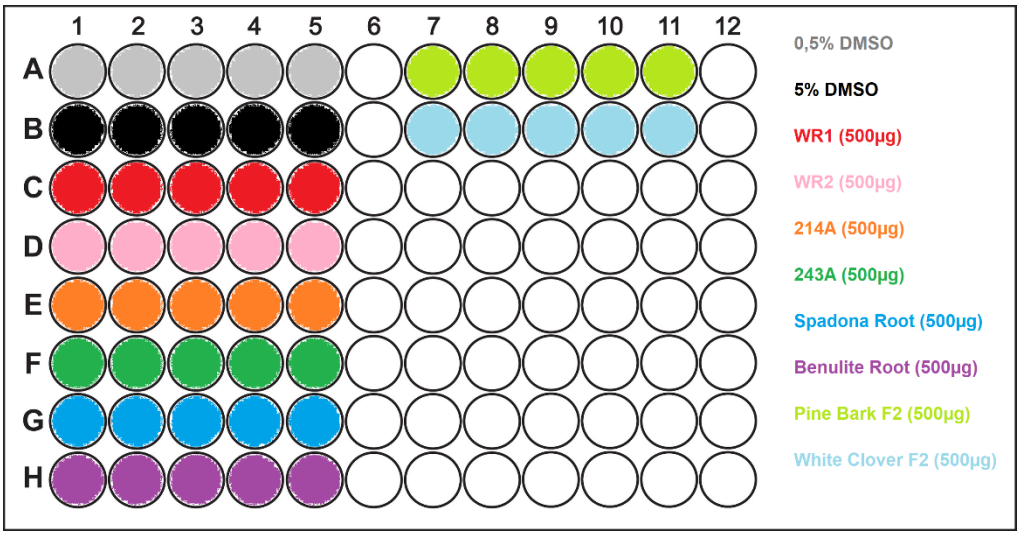
3.2.3. Evaluating different DMSO levels

The different compounds used in this trial were dissolved in DMSO at concentrations of 100-200mg/ml. Therefore, it was important to determine the effect of DMSO on the viability of *Giardia* trophozoites beforehand since no informative articles regarding this were found. In this trial, 6 different DMSO concentrations were tested: 0%, 0.1%, 0.5%, 1%, 2% and 5%. The same protocol as described above was used for this preliminary study. The highest DMSO concentration that showed no significant difference with the negative control (0% DMSO) was used in further assays as negative control for compound testing to minimize the influence of DMSO on the outcome of the tests. In addition, the DMSO concentration in further trials used in the different compound concentrations should be the same as the negative control of 0.5% DMSO. A DMSO concentration of 5% was used as positive control since it completely inhibited trophozoite growth which resulted in fluorescence values close to the standard background values.

3.2.4. Resazurin-reduction assay with different compounds

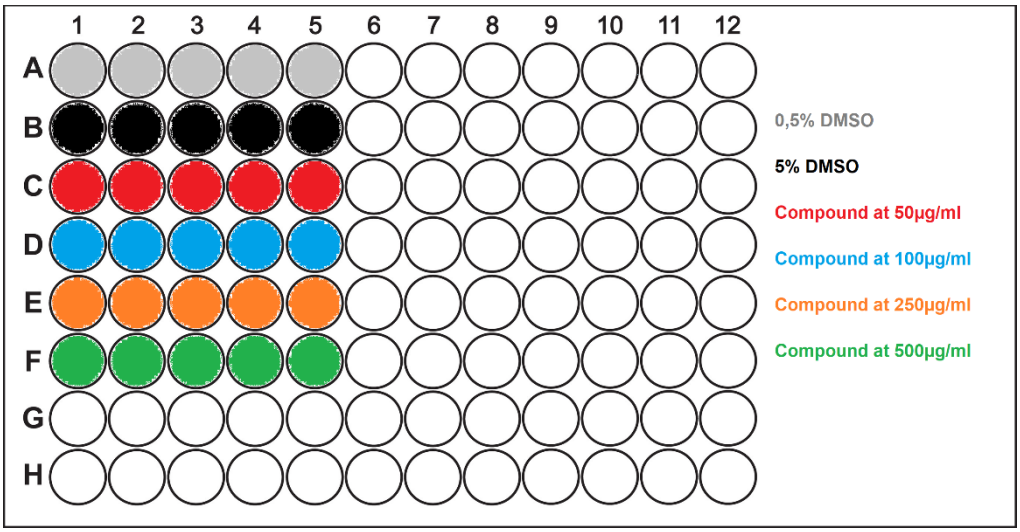
In total 8 different compounds, namely WR1, WR2, 214A, 243A, Spadona root, Benulite root, pine bark F2 and white clover F2 were used in this trial to determine their effect on the viability of *G. duodenalis in vitro* by using the same protocol as described above. All the compounds were initially tested at a concentration of 500µg/ml in fivefold for each compound (Figure 3.1).

Figure 3.1: flat-bottom 96-well microplate template for 500µg/ml compound testing.



Further testing was only done with compounds that showed a significant effect at this concentration. Those were then further tested at 50µg/ml, 100µg/ml, 250µg/ml and 500µg/ml, in fivefold for each concentration (Figure 3.2).

Figure 3.2: flat-bottom 96-well microplate template for specific compound testing.



3.3. *Statistical analysis*

The software programs R (version 3.4.3) and GraphPad Prism (version 5) were used to analyse the raw data and perform statistical tests. All p-values are based on a one-way ANOVA with multiple comparison, obtained by using a pairwise-t-test and expressed as the percentage of migration inhibition. Moreover, interactions were determined in a Two-way ANOVA model if relevant. In addition, the significance level was corrected for the multiple comparison by using the Bonferroni correction method.

4. Results

4.1. *Larval-migration-inhibition assay*

4.1.1. LMIA on exsheathed L_3

For the exsheathed L_3 assay with pine bark F2 (Figure 4.1; Appendix Figure 1.1), a very high larval migration inhibition was observed in the negative control for both *O. ostertagi* (53.5%) and *C. oncophora* (31.8%). Additionally, a very substantial nonlinear variation between different concentrations was observed for both parasites. Therefore, further assays with exsheathed larvae were not performed.

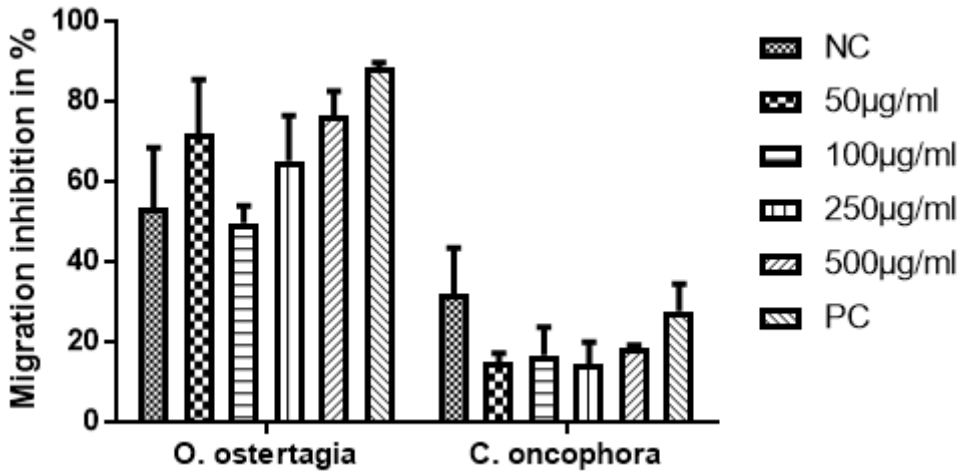


Figure 4.1: Migration inhibition of exsheathed *O. ostertagi* and *C. oncophora* L_3 larvae after 24 hours incubation with pine bark F2. Due to a strong nonlinear variation and a very high migration inhibition in the negative controls, significance was not examined statistically.

4.1.2. LMIA on ensheathed L_3

Standardization assays were performed beforehand without any bioactive compounds to evaluate the influence of different DMSO concentrations. The effect of the 0%, 0.1%, 0.5% and 1% DMSO on the migration of L_3 was evaluated and no differences were observed in both parasites (Figure 4.2; Appendix Figure 1.2). Since 0.5% DMSO was the highest concentration that would be used for testing the different compounds, no higher concentrations than 1.0% DMSO were examined in the assay. Additionally, to verify that no L_3 larvae remained onto the filters after transferring the tubes from migration to non-migration well-rows, filters were observed under a stereo-microscope during the standardization assays. There was no evidence that larvae remained on the filters after flushing them in the correct well. However, it cannot be said with certainty whether this was also the case when testing the different compounds.

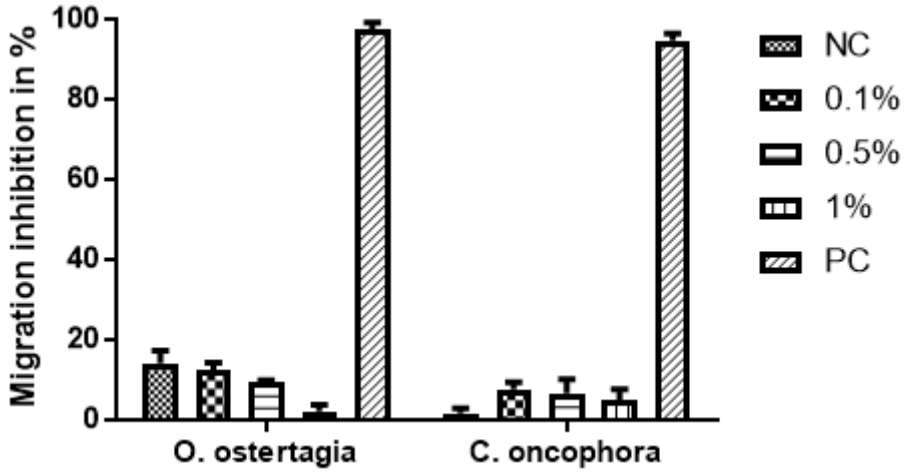


Figure 4.2: Migration inhibition of *O. ostertagi* and *C. oncophora* L_3 larvae at a negative control (NC) of 0% DMSO and at three different DMSO concentrations: 0.1%, 0.5% and 1%. Levamisole was used as a positive control (PC).

Differences between *O. ostertagi* and *C. oncophora* in migration rates were observed for all compounds, but were only significant ($p < 0.01$) for pine bark F2 and WR2 at all concentrations (Figure 4.3; Appendix Figure 1.3). Compared to the negative controls, there was only a very limited effect of the different compounds on the ability of L_3 to migrate through the 28µm filters. Only a concentration of 500µg/ml white clover F2 was able to cause a significant ($p < 0.05$) larval migration inhibition in *O. ostertagi* compared to the negative control (Figure 4.3). Furthermore, pine bark F2 was the only other compound tested close to significant for *O. ostertagi*. Although all L_3 larvae were incubated for 48 hours in total, no visual effect of the compounds on larval motility was observed after 6 and 24 hours. The positive control with levamisole, however, had a near immediate effect and no motility was observed after 6 and 24 hours. Although all compound concentrations were tested in threefold, there still was a substantial nonlinear variation between different concentrations. This was mainly observed in *C. oncophora* for both chicory compounds and in *O. ostertagi* for 243A. Due to the lack of significantly effective compounds, no dose-response curves are shown.

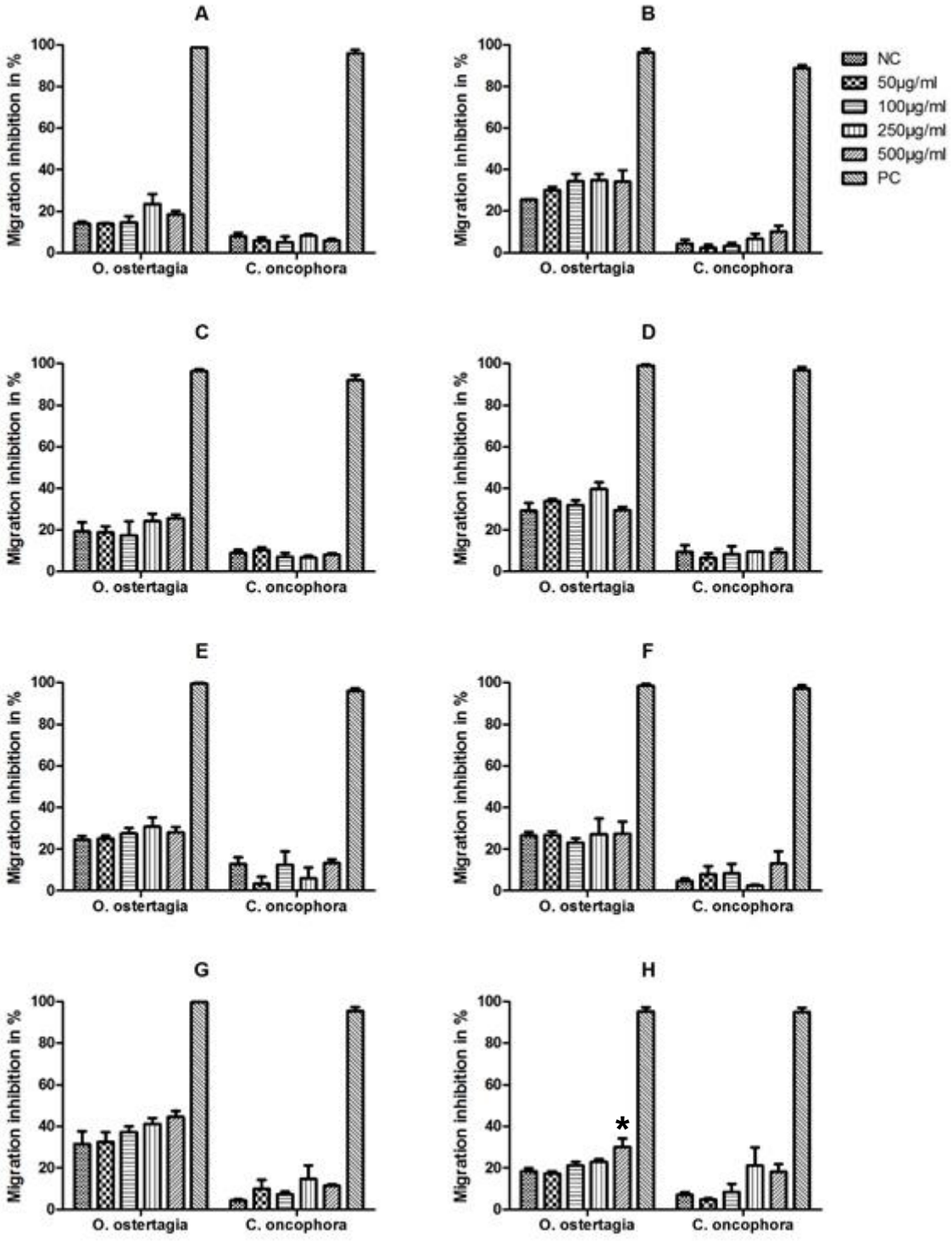


Figure 4.3: Migration inhibition of ensheathed *O. ostertagi* and *C. oncophora* L_3 larvae after 24 hours incubation in different bioactive compounds. Compared to the negative control (NC), WR1 (A), WR2 (B), 214A (C), 243A (D), Spadona root (E) and Benulite root (F) did not get close to having a significant effect on *O. ostertagi* ($p = 1$). Pine bark F2 (G) showed an effect on migration, although not significant ($p = 0.4$) and white clover F2 (H) only had a significant ($p = 0.04$) effect at a concentration of 500µg/ml. For *C. oncophora*, no compounds were active with any degree of significance ($p = 1$).

4.2. *Resazurin-reduction assay*

First, standardization assays were conducted beforehand without any bioactive compounds to evaluate the influence of 0% (NC), 0.1%, 0.5%, 1%, 2% and 5% DMSO on the viability of *G. duodenalis* trophozoites (Figure 4.4; Appendix Figure 2.2). The concentrations 0.1% and 0.5% clearly had no effect on compared to the negative control ($p > 0.3$). On the contrary, 1%, 2% and 5% DMSO showed a significant ($p < 0.01$) reduction in viability. Therefore, 0.5% DMSO was used in all compound concentrations to reduce variability between different concentrations. Due to the fact that 5% DMSO had a stronger and more significant effect on the viability of trophozoites than fenbendazole (Figure 4.5; Appendix Figure 2.3), it was used as positive control in further resazurin assays.

Secondly, the influence of TYI-S-33 medium on the reduction of resazurin into resorufin was evaluated. Outcome of these assays demonstrated that leftovers of this medium caused a strong and immediate reduction of resazurin and a high variation in the assay, making it unusable (Appendix Figure 2.1). Washing the trophozoites in PBS before adding resazurin resulted in stable replications for all assays that followed. Third, the effect of pipetting on the total number of trophozoites remained unknown during the assay. There may have been a difference of some µl per well in total content, although this did not seem to affect the stability of the assay. Last, since the reduction of resazurin into resorufin is rather fast and pipetting took a significant amount of time, resazurin was added per column instead of per row. This ensures that all wells had a very similar exposure time to resazurin.

The screening assay (Figure 4.5) was used to determine the effect of 500µg/ml of each bioactive compound on the viability of *G. duodenalis*. Only the compounds with a significant efficacy were used in further assays. The significant compounds concentrations that were selected for further testing were WR1 ($p < 0.001$), 214A ($p < 0.001$), pine bark F2 ($p < 0.001$) and white clover F2 ($p < 0.001$). Apart from 243A ($p > 0.4$), the effect of all other compounds was nonsignificant ($p = 1$).

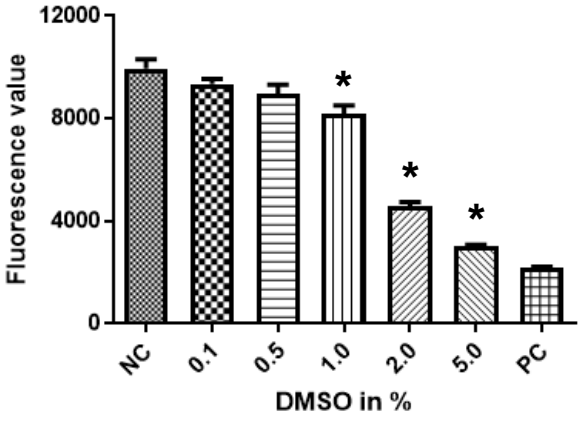


Figure 4.4: The effect of different DMSO concentrations on the viability of *G. duodenalis* trophozoites. Higher fluorescence values correlate with a higher reduction of nonfluorescent resazurin into highly fluorescent resorufin and thus represent the viability of the trophozoites. The negative control (NC) consists of 0% DMSO, whilst PC indicates the standard background fluorescence value of the wells without any content. The DMSO concentration of 5% got very close to this value and may only be higher due to minor presence of TYI-33 medium in the wells.

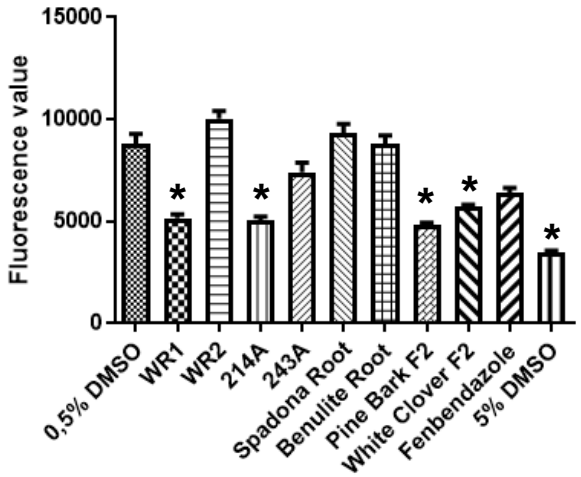


Figure 4.5: The effect of 500µg/ml compound concentrations on the viability of *G. duodenalis*. Four compounds, namely WR1, 214A, pine bark F2 and white clover F2 were tested significant ($p < 0.001$) compared to the negative control of 0.5% DMSO. All other compounds did not have a significant or close to significant effect ($p > 0.4$).

After the initial screening, the effect of WR1, 214A, pine bark F2 and white clover F2 on the viability of *G. duodenalis* was assessed at the concentrations of 50µg/ml, 100µg/ml, 250µg/ml and 500µg/ml (Figure 4.6; Appendix Figure 2.4). Compared to the negative control of 0.5% DMSO, WR1 showed a significant effect at concentrations of 250µg/ml ($p < 0.05$) and 500µg/ml ($p < 0.01$) with an EC_{50} of 283µg/ml. Second, pine bark F2 showed a significant ($p < 0.001$) effect at a concentration of 500µg/ml with an EC_{50} of 490µg/ml. Third, 214A showed a significant ($p < 0.05$) effect at a concentration of 500µg/ml with an estimated EC_{50} of 969µg/ml. Last, white clover F2 did not show a significant ($p = 1$) effect at any concentration with an estimated EC_{50} of 415µg/ml. The results in white clover may be odd since 500µg/ml tested positive in the screening assay. This is most likely due to a slight variation in the negative control that occurred during this assay.

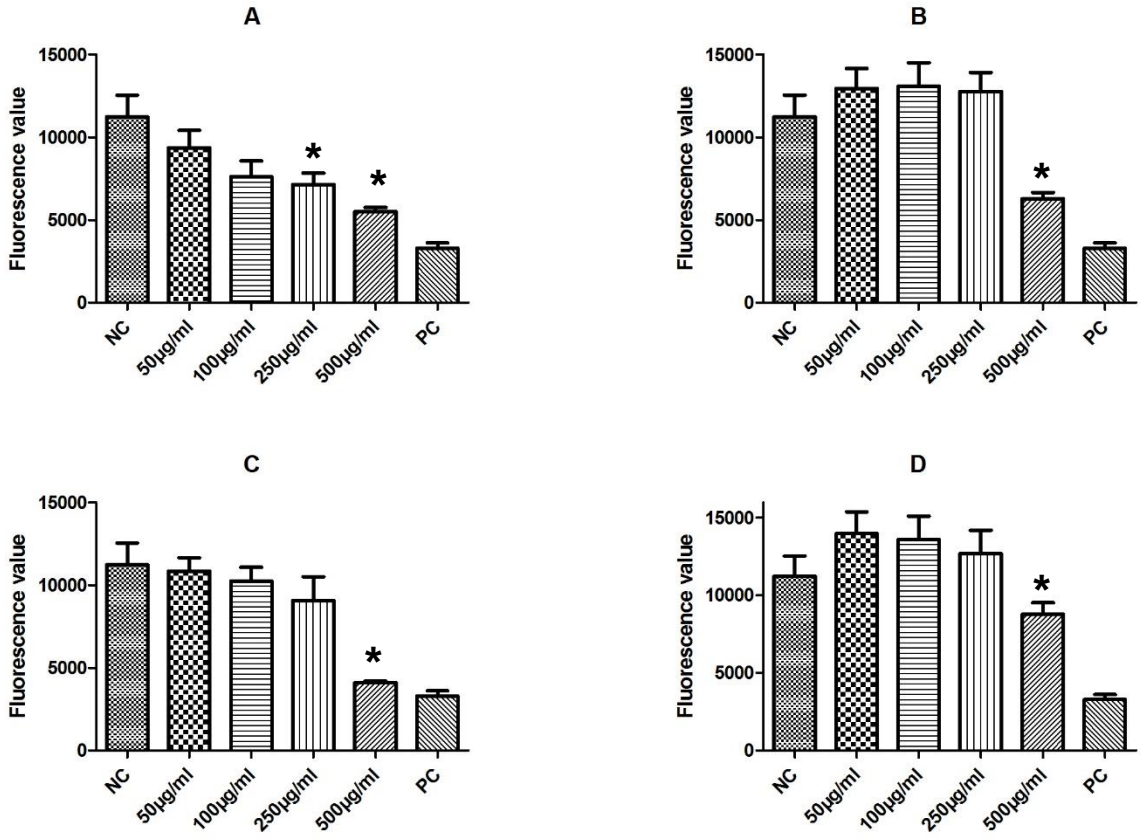


Figure 4.6: The effect of WR1 (A), 214A (B), pine bark F2 (C) and white clover F2 (D) on the viability of *G. duodenalis* at concentrations of 50µg/ml, 100µg/ml, 250µg/ml and 500µg/ml. The negative control (NC) contains 0.5% DMSO, whilst the positive control (PC) consists of 5% DMSO. Compared to the negative control, WR1 had a significant effect at concentrations of 250µg/ml ($p < 0.05$) and 500µg/ml ($p < 0.01$) Second, pine bark F2 had a significant ($p < 0.001$) effect at a concentration of 500µg/ml. Third, 214A had a significant ($p < 0.05$) effect at a concentration of 500µg/ml. Last, white clover F2 did not have a significant ($p = 1$) effect, even at 500µg/ml.

5. Discussion

Effect of bioactive compounds on *O. ostertagi* and *C. oncophora* larval motility

The LMIA is a relatively non-biased assay due to the fact that counting low numbers of larvae is rather objective, especially compared to a motility-based assay, which was opted as well. However, randomization of the different bioactive extract concentrations was not very useful due to the distinct colour differences. One of the issues that occurred during the assay was that not all wells contained 80-100 larvae. Occasionally, higher and lower numbers were observed, although never lower than 50. However, this did not seem to have any effect on the percentage of larval migration inhibition and it is unlikely that this had any effect on the validity of the assay. For the assay, a DMSO concentration of 0.5% was used in each compound concentration. This corresponds to a study by (Demeler et al., 2010a) and does not seem to affect the migration nor viability of the ensheathed L_3 larvae in any way.

Demeler et al. (2010a) stated that exsheathed larvae are significantly less viable as migration rates were inconsistent and dropped down to as low as 50%. Their trial therefore excluded exsheathed larvae from further experimentation and since similar results were seen in this study, only ensheathed L_3 were used for further experiments. However, the use of exsheathed L_3 larvae would have been a better representation since L_3 rapidly exsheath after uptake by a ruminant (Desrues et al., 2016). Therefore, the time of contact with bioactive compounds as ensheathed larvae would be very limited *in vivo*. According to Claerebout et al. (1999), the viability of the exsheathed L_3 should not be an issue in a LMIA. However, their trial used a 2 hour incubation time, whilst this study and the study by Demeler et al. (2010a) used a 24 hour incubation time which may explain the loss of viability. Additionally, the 0.5% DMSO concentration may have had an adverse effect against the exsheathed larvae which was not determined in this trial.

The main issue that may have an impact on the validity of the LMIA with ensheathed L_3 is the substantial mean larval migration inhibition of 23.7% for *O. ostertagi* and 7.3% for *C. oncophora* in the negative control groups. The positive control, however, worked appropriately and resulted in a mean migration inhibition of 98% for *O. ostertagi* and 94.7% for *C. oncophora*. Compared to the LMIA by Demeler et al. (2010a), the inhibition in the negative controls was substantially higher and the reason for this was unclear. The used filters were 28µm in mesh size, which is designed to allow for active migration of bovine GIN according to Demeler et al. (2010a) and does not explain this difference. A possible explanation may be larval mortality before the assay was conducted. The larvae were collected in Belgium and presumably vital at that time. Although it is unlikely, packaged transport to Denmark may have influenced the viability. Before the start of the assay, there was no Baermann filtration conducted to reassess the viability of the larvae and thus premature mortality cannot be excluded. Another difference with the LMIA by Demeler et al. (2010a) is the fact that the incubation and migration phases held place in an incubator of 22°C instead of 28°C. Although it seems unlikely, it may have influences the mobility of both nematodes during these phases.

Differences between *O. ostertagi* and *C. oncophora* in migration rates were observed for all compounds, but only found significant ($p < 0.01$) for pine bark F2 and WR2 (Figure 4.1). However, a trial by Demeler et al. (2010a) did not show a significant difference between both species using a 28µm mesh filter. Visually, the motility of *O. ostertagi* seemed to be much lower than *C. oncophora*. This may explain the higher migration rates observed for *C. oncophora*. In addition, *C. oncophora* may be more resilient to external influences such as the bioactive compounds. This seems likely due to the fact that other studies demonstrated that *C. oncophora* was not affected by sainfoin (Desrues et al., 2016) and chicory (Peña-Espinoza et al., 2017).

The LMIA demonstrated that white clover flower F2 at 500µg/ml caused a significant reduction in the ability of *O. ostertagi* to migrate through a 28µm filter. Since white clover inflorescence is tannin based, it might have an effect on larval motility, which corresponds to the fact that it reduces worm establishment *in vivo* (Paolini et al., 2003). White clover did not appear to have any effect against *C. oncophora*,

indicating that this intestinal nematode is less susceptible to tannin-rich extracts. Similar results were described in plenty of studies testing tannin-rich bioactive forages *in vitro* and *in vivo* against *C. oncophora*. Unexpectedly, both chicory extracts did not show any effect on larval migration for both parasites. This conflicts with other studies which describe a potent effect against adult worms and L_1 larvae (Desrues et al., 2016). Similarly, no inhibition of the larval migration was examined for the extracts of the two African plants, 214A and 243A. Other studies, however, indicate that these extracts are potent against *H. contortus* in sheep (Hounzangbe-Adote et al., 2005) and *Ascaris suum* in pigs (Williams et al., 2016). Moreover, no effect was observed in the LMIA with both seaweed extracts, WR1 and WR2. Although there is no published data available, it has been suggested that these compounds may have a potent effect against GIN (Williams, personal communication, 2018).

Pine bark F2 seemed to have a visual effect on larval motility, but it was not significant in the actual LMIA. This reduction in motility may have been influences by the high density of compound particles on the bottom of the incubation wells, limiting the larvae in their movement. Furthermore, these particles may have caused the migration filters to get clogged to a certain degree, partially preventing larval migration. The filters were always checked and washed beforehand, but may get clogged during the migration phase and a slight discolouration of the filters was noticed after using pine bark F2. What contradicts, however, is the fact that compound particles were more present in the migration wells compared to the non-migration wells, indicating that compound particles generally go through the filter instead of blocking them. Therefore, it is assumed that the impact of those particles on the assay is negligible. In 214A, another physical phenomenon occurred during the assay where the *O. ostertagi* larvae would get stuck to the bottom of the wells and could not be removed by pipetting vigorously. This assay was carried out once more and the larvae were successfully removed from the bottom of the wells by using a plastic cell scraper. This form of stickiness was not observed in any of the other compounds.

The lack of efficacy of nearly all compounds is very likely due to the presence of L_2 sheaths around the L_3 larvae. This sheath protects the L_3 from environmental influences, such as temperature and drought, but may well be capable at protecting the larvae from substances with anthelmintic potency. Compared to these bioactive compounds, however, the L_3 larvae are susceptible to most anthelmintics, such as levamisole, which was successfully used as positive control in this assay. The exact effect of the compounds is still debateable, although the tannin-rich extracts suggestively cause a reduction of the worm establishment (Paolini et al., 2003), FEC (Lange et al., 2006) and development of nematode eggs (Molan et al., 2000). Differences between pine bark F2 and white clover F2 may be due to fact that they contain a different condensed tannin fraction (Desrues et al., 2016). It cannot be said with certainty whether the effect of the compounds resulted in larval paralysis or mortality. Either way, both outcomes would result in a reduced migration of the larvae.

Although most extracts showed no reduction of the capability of *O. ostertagi* and *C. oncophora* to migrate, the LMIA itself should not be held responsible for this as its validity has been demonstrated in other studies (Demeler et al., 2010a; Williams et al., 2016). However, other methods are suggested to evaluate the effect of bioactive forages on exsheathed L_3 larvae. For an *in vitro* assay, it seems that a standard motility assay is the most suitable for this, as long as movement of the larvae is not impaired due to stickiness or high compound particle sedimentation. Furthermore, studies using higher concentrations than 500µg/ml of bioactive compounds or longer incubation times may provide additional information.

Effect of bioactive compounds on *G. duodenalis* trophozoite viability

Due to the fact that the results were gained by using a spectrophotometric quantifier, the resazurin assay is a non-biased method to determine the viability of *G. duodenalis* trophozoites. The main issue that occurred during this assay in its early stages was the presence of background fluorescence due to remaining TYI-S-33 medium and bacterial contamination, which have been confirmed as potential problem of this assay (Bénéré et al., 2007). Differences of a few µl medium per well may have been largely responsible for these variations. However, this issue was evaded by washing the trophozoites in PBS and diluting the remaining medium, which resulted in very stable replications for each

concentration. The second technical aspect of the assay was the incubation time with resazurin. Fluorescence values of > 40,000 were undetectable by the spectrophotometric quantifier and since higher fluorescence values were gained over time, the maximum incubation time was limited to 4-5 hours. It was therefore opted to use a 3 hour incubation time in combination with a fixed amount of trophozoites per well. A standard DMSO concentration of 0.5% was used in each compound concentration due to the fact that this seemed to be the highest concentration without any effect on trophozoite viability. Moreover, the highest compound concentration of 500µg/ml automatically provided a DMSO concentration of 0.5%. Fenbendazole was opted as a positive control at first at a concentration of 5%, which should be potent enough to cause a high mortality in *G. duodenalis*. However, it did not affect the trophozoites as much as 5% DMSO, which was then used as positive control for further assays. A possible explanation may be that fenbendazole is not water-soluble and thus did not obtain its full potential in the wells. The influence of different compounds on the reduction of resazurin into resorufin was not examined during this study. However, any substantial influence seems very unlikely due to the fact that they were diluted intensively together with the TYI-33 medium. It can therefore be assumed that the fluorescence values represent a realistic picture of the relative amount of surviving trophozoites.

Since the trophozoites were not visible after addition of resazurin, the results are purely based on the spectrophotometric quantification. The initial activity screening of all 8 extracts at 500µg/ml resulted in the selection of WR1, 214A, pine bark F2 and white clover F2 for dose titration. The anthelmintic property of pine bark F2 and white clover F2 has been linked to condensed tannins, although different composition (Desrues et al., 2016). It appears that *G. duodenalis* is susceptible to both of these condensed tannin fractions, but mainly to the one in pine bark F2. Although the effect of tannins on *Giardia* trophozoites has not been described clearly, it is suggested that the tannins in geranin D may affect the viability (Calzada et al., 2001). To support this, 214A and 243A are known to be rich in tannins as well. In the screening assay, both compounds seemed to affect the *Giardia* viability compared to the negative controls. Only 214A had a significant effect at 500µg/ml and thus may have a different condensed tannin composition than 243A. Due to the limited amount of research conducted within this field, more studies are required to confirm the actual anti-giardial effect of tannins. Since there is no published data available on the composition of the seaweed extracts, the background anti-giardial effect is unknown. It has been suggested that seaweed contains an anthelmintic property (Williams, personal communication, 2018), but WR1 seemed to significantly affect the *G. duodenalis* viability as well. WR2 clearly had no effect and thus may consist of different compounds than WR1, which could be explained by the fact that both extracts were derived from different parts of the plant. Lastly, it appeared that both chicory extracts did not affect the viability of the trophozoites in any way. It may therefore be so that sesquiterpene lactones, which are held responsible for the anthelmintic efficacy of chicory (Hoste et al., 2006), do not significantly affect *Giardia*. Once again, due to the lack of research within this field, more studies on sesquiterpene lactones are required to confirm this suggestion.

Regarding *G. duodenalis*, the resazurin assay may be a valuable method to examine other bioactive forages, such as the tannin-rich sainfoin, sulla and quebracho extracts. Furthermore, the suggested anti-giardial effects of some compounds may encourage further investigation on the efficacy of bioactive forages against other gastrointestinal parasites, such as *Cryptosporidium* spp. and *Eimeria* spp., which are of importance in livestock industry. The application of bioactive forages as a sustainable method for the reduction of AR development in GIN may be more straight forward. The forages could be supplemented as pasture herbage, mixed in with roughage as whole plants or provided as a supplementary pelleted feed (Thamsborg et al., 1998). On the other hand, the application against *G. duodenalis* infections, which is mainly important in calves, is more limited due to the fact that calves are raised on milk during the main period where they get in contact with *Giardia*. Even if there is an uptake of bioactive forages when calves start grazing, it is unlikely that anti-giardial concentrations will be reached. Furthermore, it may not be economically feasible if it caused a lower weight gain compared to the regular diet. Since adult cattle may be asymptomatic carriers of *G. duodenalis*, bioactive forages could potentially reduce their cyst excretion. This may indirectly reduce the cyst uptake by calves if they are housed in the same stables as the adults, which is usually not the case. For cows, it may also be

problematic to manipulate the diet and further research is required to determine the application of bioactive forages.

To conclude

This study has demonstrated that both ensheathed *O. ostertagi* and *C. oncophora* do not seem to be susceptible to any of the compounds apart from *O. ostertagi* for white clover tannins. Although the use of exsheathed larvae would have been a better representation for the *in vivo* infection model, it caused the assay to be invalid and thus other *in vitro* or *in vivo* assays may be more appropriate. *G. duodenalis* seemed to be susceptible to tannin-rich compounds, such as 214A, pine bark F2 and white clover F2, and one of the seaweed extracts.

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Appendices

Appendix 1: larval-migration-inhibition assay

Appendix Figure 1.1: LMIA on exsheathed L_3 larvae corresponding to Figure 4.1.

Nematode species	*Concentration*	*Migration inhibition in %*		
		1	2	3
O. ostertagi	50µg/ml	79,82	90,32	45,45
O. ostertagi	100µg/ml	56,92	41,89	50,00
O. ostertagi	250µg/ml	83,87	66,67	45,00
O. ostertagi	500µg/ml	63,79	81,67	83,33
O. ostertagi	Levamisole	87,76	90,74	86,67
O. ostertagi	0.5% DMSO	25,37	59,09	76,00
C. oncophora	50µg/ml	19,44	12,75	12,66
C. oncophora	100µg/ml	4,65	28,99	16,25
C. oncophora	250µg/ml	16,67	22,73	4,20
C. oncophora	500µg/ml	17,57	19,67	18,33
C. oncophora	Levamisole	32,20	14,52	36,36
C. oncophora	0.5% DMSO	16,67	54,73	24,07

Appendix Figure 1.2: LMIA with different DMSO concentrations on ensheathed L_3 larvae corresponding to Figure 4.2.

Nematode species	*Concentration*	*Migration inhibition in %*		
		1	2	3
O. ostertagi	0% DMSO	13,16	9,33	20,00
O. ostertagi	0.1% DMSO	9,26	13,11	15,28
O. ostertagi	0.5% DMSO	8,70	10,42	9,30
O. ostertagi	1% DMSO	0,00	1,28	5,26
O. ostertagi	Levamisole	98,33	100,00	94,23
C. oncophora	0% DMSO	0,00	0,88	4,05
C. oncophora	0.1% DMSO	10,29	4,35	8,42
C. oncophora	0.5% DMSO	1,96	13,46	4,62
C. oncophora	1% DMSO	0,00	9,88	4,65
C. oncophora	Levamisole	94,37	92,21	97,67

Appendix Figure 1.3: LMIA with different extracts on ensheathed L_3 larvae corresponding to Figure 4.3.

WR1

Nematode species	*Concentration*	*Migration inhibition in %*		
		1	2	3
O. ostertagi	50µg/ml	13,68	14,29	14,37
O. ostertagi	100µg/ml	19,90	14,05	9,80
O. ostertagi	250µg/ml	18,73	18,91	32,97
O. ostertagi	500µg/ml	21,39	18,55	15,57
O. ostertagi	Levamisole	98,39	98,56	99,10
O. ostertagi	0.5% DMSO	14,01	15,56	12,81
C. oncophora	50µg/ml	5,77	3,28	8,70
C. oncophora	100µg/ml	0,00	8,93	6,56
C. oncophora	250µg/ml	9,26	6,76	8,82
C. oncophora	500µg/ml	7,50	5,10	5,00
C. oncophora	Levamisole	97,22	98,18	92,42
C. oncophora	0.5% DMSO	8,51	5,00	10,71

WR2

Nematode species	*Concentration*	*Migration inhibition in %*		
		1	2	3
O. ostertagi	50µg/ml	26,82	31,48	31,76
O. ostertagi	100µg/ml	40,91	28,78	33,33
O. ostertagi	250µg/ml	40,52	33,33	30,88
O. ostertagi	500µg/ml	39,17	23,31	40,00
O. ostertagi	Levamisole	93,51	96,77	99,16
O. ostertagi	0.5% DMSO	25,40	25,98	25,26
C. oncophora	50µg/ml	0,00	5,21	1,56
C. oncophora	100µg/ml	2,08	6,15	1,15
C. oncophora	250µg/ml	3,33	11,54	5,13
C. oncophora	500µg/ml	5,05	9,43	15,58
C. oncophora	Levamisole	86,96	88,24	91,40
C. oncophora	0.5% DMSO	1,79	7,69	3,70

214A

Nematode species	*Concentration*	*Migration inhibition in %*		
		1	2	3
O. ostertagi	50µg/ml	22,89	20,27	13,48
O. ostertagi	100µg/ml	14,95	30,25	7,30
O. ostertagi	250µg/ml	29,85	17,50	25,44
O. ostertagi	500µg/ml	24,46	28,83	23,91
O. ostertagi	Levamisole	97,81	96,94	94,39
O. ostertagi	0.5% DMSO	27,78	12,50	17,60
C. oncophora	50µg/ml	13,04	8,16	9,30
C. oncophora	100µg/ml	6,25	10,53	4,29
C. oncophora	250µg/ml	6,82	8,22	5,19
C. oncophora	500µg/ml	9,38	6,90	8,00
C. oncophora	Levamisole	96,43	91,46	88,24
C. oncophora	0.5% DMSO	8,62	11,76	6,45

243A

Nematode species	*Concentration*	*Migration inhibition in %*		
		1	2	3
O. ostertagi	50µg/ml	35,76	32,54	33,33
O. ostertagi	100µg/ml	33,54	34,88	27,34
O. ostertagi	250µg/ml	45,77	38,24	34,75
O. ostertagi	500µg/ml	32,59	28,26	27,92
O. ostertagi	Levamisole	98,04	100,00	99,15
O. ostertagi	0.5% DMSO	33,81	32,09	22,22
C. oncophora	50µg/ml	6,90	10,13	2,67
C. oncophora	100µg/ml	3,57	16,05	5,15
C. oncophora	250µg/ml	9,68	9,09	9,76
C. oncophora	500µg/ml	5,97	11,48	10,14
C. oncophora	Levamisole	100,00	95,12	95,29
C. oncophora	0.5% DMSO	14,75	10,10	3,45

Spadona root

Nematode species	*Concentration*	*Migration inhibition in %*		
		1	2	3
O. ostertagi	50µg/ml	23,30	27,72	24,22
O. ostertagi	100µg/ml	32,74	25,00	25,00
O. ostertagi	250µg/ml	37,01	22,45	33,02
O. ostertagi	500µg/ml	33,03	25,20	26,17
O. ostertagi	Levamisole	98,99	100,00	100,00
O. ostertagi	0.5% DMSO	26,79	20,72	25,88
C. oncophora	50µg/ml	10,13	0,00	0,00
C. oncophora	100µg/ml	8,47	4,00	25,00
C. oncophora	250µg/ml	1,82	0,00	16,28
C. oncophora	500µg/ml	16,00	10,26	13,92
C. oncophora	Levamisole	98,46	94,44	95,24
C. oncophora	0.5% DMSO	13,33	6,98	18,18

Benulite root

Nematode species	*Concentration*	*Migration inhibition in %*		
		1	2	3
O. ostertagi	50µg/ml	27,78	29,00	23,53
O. ostertagi	100µg/ml	18,92	25,25	25,29
O. ostertagi	250µg/ml	17,24	41,76	22,61
O. ostertagi	500µg/ml	39,18	21,18	21,74
O. ostertagi	Levamisole	99,08	100,00	96,61
O. ostertagi	0.5% DMSO	27,78	28,57	23,17
C. oncophora	50µg/ml	4,92	15,56	3,30
C. oncophora	100µg/ml	16,95	1,28	6,67
C. oncophora	250µg/ml	1,27	2,53	3,28
C. oncophora	500µg/ml	1,43	21,25	16,39
C. oncophora	Levamisole	100,00	94,74	96,88
C. oncophora	0.5% DMSO	2,33	4,57	6,82

Pine bark F2

Nematode species	*Concentration*	*Migration inhibition in %*		
		1	2	3
O. ostertagi	50µg/ml	34,45	39,62	23,58
O. ostertagi	100µg/ml	38,26	41,59	31,94
O. ostertagi	250µg/ml	46,73	39,02	37,86
O. ostertagi	500µg/ml	39,17	47,83	46,75
O. ostertagi	Levamisole	100,00	99,13	100,00
O. ostertagi	0.5% DMSO	36,67	38,61	18,97
C. oncophora	50µg/ml	9,09	17,65	3,51
C. oncophora	100µg/ml	6,12	6,45	9,76
C. oncophora	250µg/ml	8,20	8,51	27,59
C. oncophora	500µg/ml	9,59	12,28	12,20
C. oncophora	Levamisole	98,18	96,26	92,21
C. oncophora	0.5% DMSO	3,13	5,36	4,55

White clover F2

Nematode species	*Concentration*	*Migration inhibition in %*		
		1	2	3
O. ostertagi	50µg/ml	18,42	18,42	14,85
O. ostertagi	100µg/ml	18,37	24,36	20,69
O. ostertagi	250µg/ml	25,23	20,97	22,73
O. ostertagi	500µg/ml	28,07	24,11	37,88
O. ostertagi	Levamisole	98,84	92,54	94,59
O. ostertagi	0.5% DMSO	17,78	21,18	16,47
C. oncophora	50µg/ml	4,69	3,77	5,88
C. oncophora	100µg/ml	5,19	4,00	16,22
C. oncophora	250µg/ml	38,89	12,28	12,12
C. oncophora	500µg/ml	18,97	11,29	24,00
C. oncophora	Levamisole	92,31	93,44	99,06
C. oncophora	0.5% DMSO	7,69	8,82	4,65

Appendix 2: resazurin-reduction assay

Appendix Figure 2.1: DMSO assay with high variation due to TYI-S-33 medium influence on the reduction of resazurin.

Compounds	*Fluorescence values*				
	1	2	3	4	5
0% DMSO	25633	31002	27918	27318	35515
0.1% DMSO	22639	28777	52435	42937	42150
0.5% DMSO	23093	51499	OVER	28796	21304
1% DMSO	15355	36201	OVER	OVER	OVER
2% DMSO	8875	17957	14198	10953	19348
5% DMSO	4235	4483	4997	4632	6582

Appendix Figure 2.2: DMSO assay corresponding to Figure 4.4.

Compounds	*Fluorescence values*				
	1	2	3	4	5
0% DMSO	10681	9548	10699	8755	10060
0.1% DMSO	9779	9693	8662	9317	9268
0.5% DMSO	9533	10071	8093	8739	8338
1% DMSO	7843	8605	8025	9209	7121
2% DMSO	3864	4714	4954	4719	4556
5% DMSO	3019	3026	3054	3135	3052

Appendix Figure 2.3: Screening assay corresponding to Figure 4.5.

Compounds	*Fluorescence values*				
	1	2	3	4	5
Fenbendazole	6992	6421	6772	5738	6166
0.5% DMSO	7718	9710	9572	9490	7495
5% DMSO	3146	3406	3469	3548	3768
*WR1 **	5886	4968	4826	5009	5085
WR2	9086	9423	9994	10849	10857
243A	6583	7661	6137	8815	7814
*214A **	4352	5062	5457	5177	5201
Spadona Root	7813	9563	9122	9947	10295
Benulite Root	10006	8294	9241	7610	8855
*Pine Bark F2 **	4783	5086	4903	4675	4895
*White Clover F2 **	5858	6017	5713	5611	5335

Appendix Figure 2.4: Definitive assays corresponding to Figure 4.6.

Compounds	*Fluorescence values*				
	1	2	3	4	5
0.5% DMSO	7435	13971	9256	11182	14239
5% DMSO	2071	3456	3614	3793	3543
WR1 50µg	6672	8109	8730	10463	12835
WR1 100µg	4300	7703	6976	8877	10102
*WR1 250µg **	6529	7223	4951	8300	8735
*WR1 500µg **	5004	5556	4890	5768	6263

Compounds	*Fluorescence values*				
	1	2	3	4	5
0.5% DMSO	7435	13971	9256	11182	14239
5% DMSO	2071	3456	3614	3793	3543
214A 50µg	11052	11867	13248	11115	17457
214A 100µg	10818	11007	11874	13252	18471
214A 250µg	10417	11669	12619	12023	17091
*214A 500µg **	5744	5742	5797	6345	7745

Compounds	*Fluorescence values*				
	1	2	3	4	5
0.5% DMSO	7435	13971	9256	11182	14239
5% DMSO	2071	3456	3614	3793	3543
Pine Bark F2 50µg	9011	9941	10322	11241	13693
Pine Bark F2 100µg	8302	9725	9236	10740	13207
Pine Bark F2 250µg	7860	8587	8294	6057	14533
*Pine Bark F2 500µg **	3851	4367	3940	4153	4176

Compounds	*Fluorescence values*				
	1	2	3	4	5
0.5% DMSO	7435	13971	9256	11182	14239
5% DMSO	2071	3456	3614	3793	3543
White Clover F2 50µg	11519	13478	12192	13357	19371
White Clover F2 100µg	11834	10958	11622	14501	19119
White Clover F2 250µg	9844	11140	10578	13760	18137
*White Clover F2 500µg **	7550	7517	7821	9679	11317