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The dual role of *Haemonchus contortus* ABC transporters in macrocyclic lactone resistance and their extrusion activity on the parasite's lipidomics

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Ce mémoire intitulé

The dual role of *Haemonchus contortus* ABC transporters in macrocyclic lactone resistance and their extrusion activity on the parasite's lipidomics

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Résumé

La résistance aux lactones macrocycliques (LM) constitue une préoccupation croissante dans le contrôle des nématodes parasitaires, notamment l'*Haemonchus contortus* chez les ruminants. Parmi les mécanismes étudiés dans la résistance aux LM chez les nématodes d'importance en santé animale, il y a les pompes ABC, principalement les glycoprotéines-p, connues pour leur rôle dans la détoxification des LM chez les strongles. Il n'existe toutefois aucune étude sur l'extrusion des lipides par les pompes ABC en tant que produits excrétoires/sécrétoires provenant d'*H. contortus* (Hc-PES). Nous émettons l'hypothèse que les pompes ABC chez *H. contortus* sont à la fois impliquées dans l'extrusion de LM (contribuant à la résistance aux antihelminthiques) et dans l'efflux de lipides secrétés par le parasite. Notre objectif était de caractériser le rôle des pompes ABC chez *H. contortus* dans le contexte de la résistance aux LM et de l'extrusion des lipides. L'efficacité de l'ivermectine, un membre de LM, a été évaluée dans 8 fermes étudiées par un test de réduction de la numération des œufs dans les selles (TRNOS). Les niveaux d'expression des pompes ABC ont été évalués dans des isolats de champ d'*H. contortus* avec des résultats TRNOS faibles (présumé souches résistantes). D'ailleurs, des vers adultes d'*H. contortus* ont été incubés avec trois inhibiteurs de pompes ABC, dont le Fumitremorgin C, le Kétoconazole et le Mk-571 à concentrations différentes. Les lipides ont été identifiés par CL/SM dans les milieux de culture récupérés à 2 h, à 4 h et à 8 h après l'incubation d'*H. contortus* dans les groupes contrôle et traités. L'expression des gènes *Hco-pgp-2* et *Hco-pgp-3* était augmentée chez les isolats de champ d'*H. contortus*. Nous avons identifié 1045 lipides appartenant à diverses catégories. L'extrusion des lipides en Hc-PES a changé en présence d'inhibiteurs de pompes ABC, en particulier pour les lipides composés de structures correspondant à celles pour le transport par les pompes ABC. Nous avons donc conclu que les pompes ABC chez *H. contortus* représentent un système de multi-extrusion et sont impliquées dans la sécrétion de lipides avec importance dans l'interaction avec l'hôte, mais aussi dans la résistance aux LM chez le nématode.

Mots-clés : *Haemonchus contortus*, pompes ABC, résistance aux vermifuges, lipidomique, lactones macrocycliques, ivermectine, produits d'excrétion/sécrétion, nématodes parasitaires.

Abstract

Macrocyclic lactones (MLs) resistance is a growing concern in controlling parasitic nematodes, particularly *Haemonchus contortus* in the ruminants' industry. ABC transporters are known to participate in translocating various lipophilic molecules, including MLs and lipids. Some ABC transporters, mostly P-glycoproteins are known to be involved in MLs detoxification in parasitic nematodes; but there is no data about extrusion of lipids by ABC transporters as Excretory/Secretory Products in *H. contortus* (Hc-ESP). We hypothesize that ABC transporters in *H. contortus* have a dual role participating in the efflux of MLs, thus contributing to anthelmintic resistance, and in the extrusion of lipids out of the parasite. This study aimed to characterize the role of *H. contortus* ABC transporters in the context of ML resistance and the extrusion of lipids. Ivermectin (a member of MLs) efficacy was evaluated in 8 studied farms by the fecal egg count reduction test (FECRT). The expression levels of ABC transporters were evaluated in field isolates of *H. contortus* with low FECRT results (suspected of resistance). *H. contortus* adult worms were incubated with three ABC inhibitors, such as Fumitremorgin C, Ketoconazole and Mk-571 with different concentrations. Lipids were identified by LC/MS in culture media at 2h, 4h and 8h post incubation with *H. contortus* in control and treated groups. *Hco-pgp-2* and *Hco-pgp-3* were found upregulated in *H. contortus* field isolates. We identified 1045 lipid molecules belonging to different categories. Interestingly, the lipid profile in Hc-ESP was altered in the presence of ABC transporter inhibitors, which shows structural features compatible as substrates for nematode transporters' activity. Therefore, ABC transporters in *H. contortus* participate in extrusion of lipids and also may help in detoxification of MLs, becoming a multipurpose pumping system involved in ML resistance and secretion of lipids at the interplay with the host and among nematodes.

Keywords: *Haemonchus contortus*, ABC transporters, anthelmintic resistance, lipidomic, Macrocyclic lactones, Ivermectin, Excretory/secretory products, gastrointestinal parasites.

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°C: degree Celsius

ABC: ATP-binding cassette

AR: anthelmintic resistance

ATP: Adenosine triphosphate

Bp: base pair (s)

BZ: benzimidazole

cDNA: complementary deoxyribonucleic acid

Ce: *Caenorhabditis elegans*

COX-1: cytochrome c oxidase subunit 1 (gene)

DMSO: dimethyl sulfoxide

DNA: deoxyribonucleic acid

EPG: egg per gram

ESP: excretory/secretory product

EV: extracellular vesicles

FC: fold change

FEC: fecal egg count

FECRT: fecal egg count reduction test

FW: forward primer

GABA: γ-amino-butyric acid

GAPDH: glyceraldehyde 3-phophate dehydrogenase (gene)

GIN: gastro-intestinal nematode

GluCls: glutamine-gated chloride channels

h: hour (s)

Hc-ESP: *Haemonchus contortus* excretory/secretory products

Hco: *Haemonchus contortus*

ITS-2: internal transcribed spacer 2 (gene)

IVM: ivermectin

L1, L2, L3, L4: larvae stage 1, 2, 3 and 4, respectively

LC: liquid chromatography

LDA: larval developmental assay

LEV: levamisole

min: minute

miRNA: micro-RNA

ML: macrocyclic lactone

mM: milli molar

MOX: moxidectin

mRNA: messenger ribonucleic acid

MRP: multidrug resistance protein

mrp: multidrug resistance protein (gene)

MS/MS: tandem mass spectrometry

MS: mass spectrometry

MW: molecular weight

NA: not applicable

nAChR: nicotinic acetylcholine receptor

NBD: nucleotide binding domain

PBS: phosphate buffered saline

PCR: polymerase chain reaction

P-gp: p-glycoprotein

pgp: p-glycoprotein (gene)

Post-tx: post treatment

Pre-tx: pretreatment

qPCR: quantitative real-time PCR

RNA: ribonucleic acid

RV: reverse primer

sIPM: sustainable integrated parasite management

SNP: single nucleotide polymorphism

spp.: species

TMD: transmembrane domain

Wk: week

ηM: nano molar

µl: micro litre

µM: micro molar

To my beloved wife; for your support, love, and motivation.

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Chapter 1 – Introduction and Literature review

1.1. Parasitism in domestic animals

Parasitism is a relationship between two organisms in which one organism (called parasite) benefits another organism (called host) and is generally harmful for the host [1]. Parasites are ubiquitous organisms that can be found living outside or inside of the host. This can be a big threat for the livestock industry worldwide, resulting in notable economic losses [2-4]. A recent study shows significant economic losses in Europe due to parasitic diseases, amounting to 1-2.7 billion euros annually [5]. These losses were primarily due to the lost production (81%) and treatment costs (19%) [5]. Parasitic diseases of veterinary importance are divided into main three groups as below:

- Ectoparasites (external parasites)
- Endoparasites (internal parasites)
- Protozoa

1.1.1. Ectoparasites (external parasites)

Ectoparasites belong to a wide group of invertebrates termed "arthropods" including the main two groups: insects and arachnids [1]. External parasites can have negative impact on livestock production by causing irritation, stress and impairing productivity throughout the world [6]. Moreover, some of the ectoparasites might act as vectors for transmitting the disease such as pinkeye or anaplasmosis to the other animals [1, 7]. Some of the vector-borne pathogens such as West Nile disease or Lymph disease are zoonotic diseases [8]. Arthropods particularly insects (mosquitoes, lice, flies etc.), and arachnids such as mites and ticks are the most important ectoparasites that cause economical loss in livestock production [1, 6-8]. Furthermore, there are several fly species which can cause myiasis in various animal species [1]. Myiasis is due to the damage produced by maggots of some fly species residing in open wounds and may lead to disease, discomfort and eventually death of these animals, if left untreated [1, 7]. Some of myiasis, including New World and Old-World screwworms are notifiable diseases by the World

Organization for Animal Health (OIE) [7]. Most of the lice or mites live on the host permanently and can be controlled easily; however, flies and some other insects live temporary on the host, that makes controlling strategies much more complicated. The life cycle of most ectoparasites is seasonal and depends on environmental factors, such as temperature [1, 7, 8].

1.1.2. Endoparasites or internal parasites

1.1.2.1. Nematoda (roundworms)

The Nematoda phylum, also called roundworms, consist of sixteen superfamilies which are grouped in six orders and two classes, as shown in Table 1. The most common features of nematodes include their cylindrical unsegmented form, tubular digestive system and the cuticle, a non-cellular colourless layer for covering body [1, 9]. In nematodes, the digestive tract is composed of an oral cavity followed by a muscular oesophagus continued by the intestine and anal pore (Figure 1) [1, 9]. Several nematodes possess an opening mouth; however, some species have a developed mouth consisting of a leaf crown or a buccal capsule [1]. Also, nematodes have an excretory pore, which is an opening structure for the excretory system and morphologically varies among nematode species [1, 9].

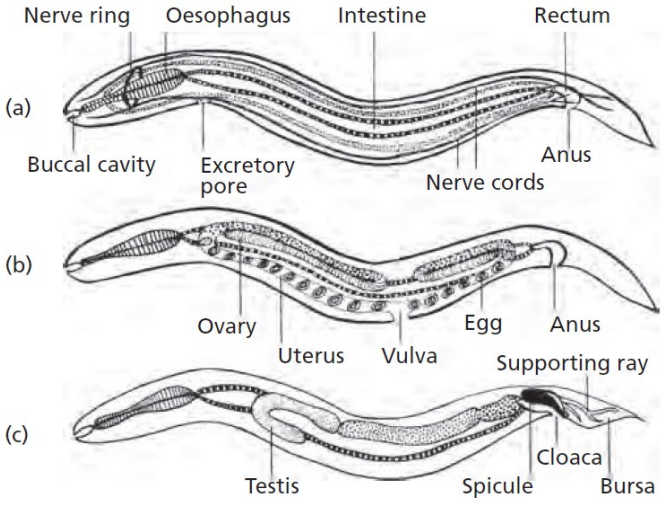


Figure 1. – Generalized nematode morphology [1].

(a) Digestive, excretory and nervous systems. (b) Reproductive system of a female nematode. (c) Reproductive system of a male nematode.

Female and male worms are separated by sexual dimorphism [1, 9]. Usually, female worms have bigger size than males and are able to lay on eggs or larvae [1, 9]. Size, shape and thickness of nematode eggs vary notably between different species [1, 9]. In nematodes, the eggs are embedded in a shell and commonly composed of three layers consisting of proteins in outer layer, chitinous in middle and impermeable lipid in inner side [1, 9]. The thickness of the eggshell might have an important role for the eggs to survive in harsh conditions of environment [1, 9].

In terms of biology, a vast number of the parasitic nematodes have a direct life cycle, some members of this large phylum have shown indirect lifecycles requiring one or more host [1, 10].

Class	Order	Superfamily	Life cycle	Infection
Secernentea	Strongylida	Trichostrongyloidea	Direct	L3
		Strongyloidea	Direct	L3
		Ancylostomatoidea	Direct	L3
		Diaphanocephaloidea	Direct	-
		Metastrongyloidea	Indirect	L3 in intermediate host
	Rhabditida	Rhabditoidea	Direct	L3
	Ascaridida	Ascaridoidea	Direct	L2 in egg
		Dioctophymatoidea	Indirect	L3 in aquatic annelids
	Oxyurida	Oxyuroidea	Direct	L3 in egg
	Spirurida	Spiruroidea	Indirect	L3 from insects
		Subuluroidea	Indirect	L3 in intermediate host
		Dracunculoidea	Direct or Indirect	-
		Acuarioidea	Indirect	L3 in intermediate host
		Filarioidea	Indirect	L3 from insects
Adenophorea	Enoplida	Trichuroidea	Direct or Indirect	L1
		Trichinelloidea	Direct or Indirect	L1

Table 1. – Classification and life cycle characteristics of parasitic nematodes in phylum Nematoda [1]

The major difference between the direct and indirect lifecycles is in the site of the two first moults [1]. First stage larvae (L1) moults to second stage larvae (L2) and then third stage larvae (L3) which

is the infectious stage in most of the parasitic nematodes [1]. These two first moults occur in the environment and inside the intermediate hosts for the direct and indirect lifecycles, respectively [1, 9]. Furthermore, many species of parasitic nematodes migrate through the body before reaching to the final site, however, movements in some species such as gastrointestinal nematodes (GINs), are bounded [1].

Among parasitic nematodes that affect livestock, some species are the major causes of important parasitic diseases in ruminants, with a significant economic impact worldwide as well as in Canada [3-5, 10, 11]. In ruminants, the most important parasitic nematodes belong to the Trichostrongyloidea superfamily, distinguished by their bursa morphology (Figure 2) [1, 5, 10, 11]. Bursate nematodes are categorized based on the presence of copulatory bursa in males for embracing female worms during mating [1].

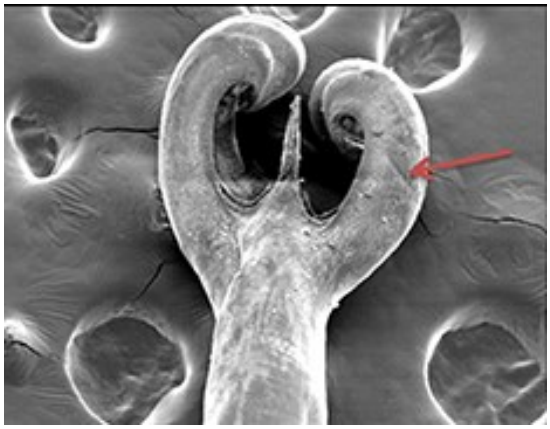


Figure 2. – The copulatory bursa (arrow) in the tail region of male *Haemonchus spp*. [12].

1.1.2.2. Platyhelminthes (flatworms)

There are three classes in this phylum however, only two classes are parasitic worms of veterinary importance, including Trematoda and Cestoda [1].

1.1.2.2.1. Trematoda (flukes)

The subclass Digenea of the Trematoda class contains important flukes in domestic animals which have an indirect lifecycle [1]. Unlike nematodes, flukes have distinctive features such as flat shape, blind alimentary tract, suckers for attachment and hermaphrodite reproduction [1]. The most

important flukes of ruminants are *Fasciola hepatica* and *F. gigantica* which are widespread in different areas of the world [1, 11]. *F. hepatica* can cause chronic, subacute and acute disease in different species and has an indirect life cycle and it takes several weeks, where snails and slugs act as intermediate host [1, 11]. The final site for the adult stage of *F. hepatica* is the liver and flukes migrate from the duodenum by penetrating the intestinal wall and locating in the bile ducts, where they start egg shedding [1]. *F. gigantica* is similar to *F. hepatica* but bigger in size [1]. There are other flukes that are less important including *Dicrocoelium dendriticum*, *Fascioloides magna* and *Paramphistomum spp.* [1, 11, 13].

1.1.2.2.2 Cestoda (tapeworms)

Known as tapeworms due to their tape-like body shape and having some differences with other helminths [1]. Cestodes vary widely in size and have a segmented body that makes proglottids and they do not have a body cavity or alimentary canal [1]. Cestodes generally have a complex lifecycle and one or two intermediate hosts may participate in the development of larvae [1]. The most common parasitic disease associated with tapeworms in ruminants are caused by *Moniezia* species. *M. expansa* and *M. benedeni* are found in cattle, sheep and goats, generally in young animals [1, 10, 11]. Presence of *Moniezia* species in high populations cause gastrointestinal impairments [11]. Moreover, infection varies in different seasons due to presence of a mite as an intermediate host in the life cycle of *Moniezia* species [1, 11]. Furthermore, *Echinococcus spp.* are important tapeworms worldwide that are responsible for zoonotic disease in which sheep and cattle act as an intermediate host [1, 14]. *E. granulosus* is the most important species worldwide causing cystic echinococcosis [14].

1.1.3. Protozoa

Protozoa are unicellular eukaryotic organisms that affect animals including ruminants [15]. These parasites are mainly located in the gastro-intestinal tract [1]. Some genders such as *Eimeria* spp. (known as coccidia) are the most important protozoa worldwide, including Canada and causes notable economic losses in cattle, sheep, goats, pigs, poultry and less common in other species [1, 10, 11, 15]. Some species of *Eimeria* and *Isospora* are highly pathogenic, especially in young animals, and cause coccidiosis by acute invasion to intestinal mucosa [1, 11, 15]. Generally young

animals are sensitive to coccidiosis under certain conditions (weaning, unfavorable weather and nutrition), and is uncommon in adults due to strong immunity [10, 11]. Other important protozoa in livestock are the *Cryptosporidium* and *Giardia* species that are less common comparing with coccidiosis [11, 15].

1.1.4. Important GINs of small ruminants/camelids

1.1.4.1. *Haemonchus contortus*

H. contortus is a hematophagous gastrointestinal parasitic nematode, also known as barber's pole worm, typified as one of the most pathogenic parasitic nematodes of small ruminants worldwide [16, 17]. *H. contortus* imposes huge economic losses globally especially in small ruminants' industry in tropical and subtropical regions, with a great impact on animal health [16, 18-20].

H. contortus have a direct life cycle like other parasitic nematodes, and female worms are able to lay thousands eggs daily [1, 17]. Eggs hatch to L1 in feces and moult two times to get to the infective L3 stage in external environment [17]. Moreover, L3 larvae are able to pass through feces onto pasture and animals on grazing serve as hosts, becoming infected by ingestion of the L3 larvae (infective stage) [21].

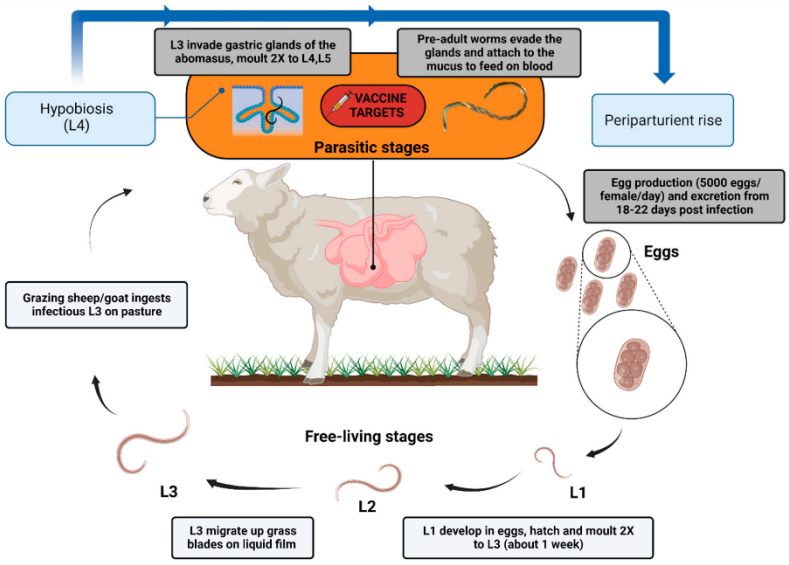


Figure 3. – Life cycle of *H. contortus* [17].

Once in the host's digestive tract, exsheathment of L3 in rumen and moulting to the L4 stage result in attachment of the parasite to the abomasum mucosa via buccal tooth and start to feed on the host's blood for the first time [1, 21]. Finally, L4 moults to adult male (10-22 mm length) and female (20-30 mm length) worms mating and producing offspring, to complete the life cycle [1]. *H. contortus* worms display a marked blood feeding activity, leading to haemonchosis, characterized by anemia, hypoproteinemia, oedema (most notably a submandibular or "bottle jaw") and even death [1, 21].

The life cycle of other GINs of veterinary importance with direct life cycle is very similar to *H. contortus* as shown in Figure 3, however existence of some differences is undeniable [1].

1.1.4.2. *Teladorsagia circumcincta*

Previously known as *Ostertagia circumcincta*, this GIN species has similarities with the bovine nematode *O. ostertagi* in many features including their morphology [1, 21]. In addition to their morphological similarities, they resemble each other in terms of pathogenesis and pathology [1, 21]. *T. circumcincta,* known as the brown stomach worm, is 6-10 mm long [1] and is one of the main GINs of small ruminants especially in temperate regions [22]. As other GINs, the L4 stage larvae from *T. circumcincta* may be arrested inside the host at the rumen and duodenum, as *O. ostertagi* [23]. Duration of hipobiosis (developmental arresting) is variable and resuming on larval development will happen in appropriate environmental conditions [11]. Heavy parasitism by the brown stomach worm may produce lethargy and collapse, diarrhea, weight loss, gut inflammation, and death [1, 11, 22]. The most specific feature of *T. circumcincta* is the presence of a "Moroccan leather" appearance in abomasum as well as *O. ostertagi* [1].

1.1.4.3. *Cooperia* species

C. curticei is major species of sheep and goats comparing with other *Cooperia* species [1, 13, 24]. *C. oncophora* and *C. punctata* are one of the most prevalent intestinal parasites of cattle and less common in sheep and goats in tropical regions globally, including Canada [10, 13, 25]. *Cooperia* species inhabit in the first part of the small intestine and have 4.5-9 mm length [1]. They have direct life cycle and prepatent period is 2-3 weeks [1, 13]. Male worms have large bursa [1].

Cooperia species do not feed on blood but cause damages to intestinal mucosa and lead to diarrhea, anorexia and emaciation without anemia [1].

1.1.4.4. *Oesophagostomum venulosum*

The final site of *O. venulosum*, known as the large bowl worm, is the large intestine of sheep, goats and other ruminants which usually is non-pathogenic [1]. The adults are 11-24 mm long, and females are longer than males [1]. The L4 stage hypobiosis occurs in sheep during the autumn and winter seasons and the L3 is capable to survive on pasture during winter season [1]. Unlike *O. radiatum* and *O. columbianum* (nodular worms), nodules are rarely formed in the large intestine due to migration of L3s deep in the mucosa; and even when nodules are formed, they are small [1].

1.1.4.5. *Chabertia ovina*

C. ovina, also known as the large-mouthed bowel worm [11], is the largest nematode present in the colon of sheep, goats, and occasionally other ruminants worldwide [1]. The ingested L3 first penetrate the mucosa of the small intestine and are capable to arrest as L4s to overwinter; later emerge and pass to colon [1, 11]. The adult worms (1.3 – 2.0 cm) cause local hemorrhage and loss of protein by damaging the mucosa [1]. The infection is usually moderate, asymptomatic and recognized at necropsy; therefore clinical chabertiosis is very rare [1, 11].

1.1.4.6. *Trichostrongylus* species

Trichostrongylus species are small hair-like worms in ruminants and some other species [1]. They have direct life cycle and prepatent period is about 2-3 weeks [1]. *T. axei,* also called stomach hairworm, is one of the common GINs in sheep, goats, cattle and some other animals [1, 11]. The final site of *T. axei* is the abomasum, while other *Trichostrongylus* species inhabit in the first part of the small intestine [1]. Furthermore, *T. colubriformis* and *T. vitrinus* are the major parasitic nematodes of sheep and goats so-called black scour worms [1, 11]. Also, *T. rugatus* is common parasite of small ruminants in some regions [1]. Trichostrongylosis is more common in younger animals and cause damage in small intestine by villous atrophy and consequently result in

malabsorption and digestion impairment [1]. Other main signs of black scour worms are anorexia, diarrhea, etc. [1, 11].

1.1.4.7. *Nematodirus* species

These have direct life cycle and the prepatent period is about 3 weeks [1]. The eggs are larger compared with Trichostrongylus species, ovoid, colorless and resistant to environmental conditions [1, 11]. Younger animals are more susceptible and most clinical infections happen in lambs and kids [11]. Infection with *Nematodirus* species causes intestinal disorders similar to that in *Cooperia* species and results in diarrhea and anorexia [1, 13].

1.1.4.8. *Trichuris* species

The infection with *Trichuris spp.* usually is not severe and results in mild and asymptomatic disease; however, youngers are more susceptible to heavier infection, especially in the presence of many worms [1, 11]. The eggs have a specific shape and are very resistant [1, 11]. *T. ovis* (3.5-8 cm in length) is the most important species and is distributed worldwide, mainly among sheep, goats, and occasionally other ruminants [1, 11]. The final site of adult worms in the host is caecum [1].

1.1.4.9. *Camelostrongylus mentulatus*

C. mentulatus is a GIN predominantly from camelids, seizing 6.5 – 10 mm long and the predilection site is the abomasum as well as the small intestine [1, 26, 27]. With a usually low pathogenicity, severe infections can result in abomasomal pH increase and gastric hyperplasia [1]. *C. mentulatus* can infect sheep, goats, llamas and alpacas [1, 26].

1.1.4.10. *Marshallagia marshalli*

M. marshalli infects sheep, goats and other ruminants and the final site is abomasum [1, 28]. The adult males are 10-13 mm, and females are 15-20 mm in length. Also, hypobiosis can occur in larval stage [1].

1.1.4.11. *Lamanema chavezi*

L. chavezi is 9-18 mm long, and its specific host is alpacas [1]. Predilection site for adults is the small intestine, while the immature stages are found in the liver and lungs [1, 26]. Therefore, severe infections can result in respiratory and liver failures [26].

1.2. Control strategies against GINs

Controlling parasitic infections induced by roundworms, especially GINs, is an integral part of efficient livestock production [29, 30]. However, GINs impose noticeable adverse effects on ruminants' herd profitability, affecting animal health and productivity [31]. Furthermore, the burden of GINs in small ruminants is higher comparing with large ruminants, particularly cattle. This difference is due to largely subclinical manifestations of GINs in cattle [32].

Currently, effective management of GINs consists of various approaches known as "sustainable integrated parasite management (sIPM)" including:

- Monitoring GINs [1, 11, 33]
- Vaccination [3, 34]
- Biosecurity [10, 11]
- Grazing management [11, 29]
- Genetic resistance and resilience [29, 35]
- Management of refugia [11, 36]
- Biological approaches [29, 34, 35]
- Anthelmintics (appropriate usage) [3, 11, 29]

1.2.1. Monitoring GINs

One of the valuable methods to determine GINs load is coprology analysis [33]. Fecal egg counts (FEC) is a simple, cheap and widely available method around the world useful in estimating the parasitic infection burden in the host by counting GIN eggs and also in evaluating total egg shedding [1, 11]. The coprological analysis however may have different results when run on individual animals, therefore, pooled sampling would be more accurate to apply the FEC as a

method to estimate anthelmintic efficacy [1]. In addition, FEC results before and after anthelmintic treatment are used to calculate fecal egg count reduction test (FECRT) [37] . FECRT is a “gold standard” method in the determination of field efficacy of anthelmintics [1, 11, 30, 37]. Moreover, there are other *in vitro* methods including egg hatch assay (EHA), larval development assay (LDA) and larval migration inhibition assay (LMIA) to evaluate anthelmintics efficacy and also to detect the potential resistance among GINs population [1]. Although coprological assays are time-consuming and less sensitive, the lack of genetical markers in most of the anthelmintic groups, still identify these assays as the main available methods to detect resistant parasitic populations [33].

Since anemia is the most predominant clinical sign in GIN infections, particularly in *H. contortus*, the animals' anemia level control would help estimate the parasite burden in the individual animals [38].

There is a standard method for the determination of anemia level in haemonchosis of sheep and goats named the FAMACHA© system [38-40]. This system works on the basis of differentiation of color of ocular mucosa with the standard chart to categorize the anemia level in sheep and goats and also has been extended to camelids affected by *H. contortus [41]*. Regarding this standard chart, animals that are classified in levels one and two do not need to be treated. However, animals in categories three to five should receive treatment which is then called targeted selective treatment [41, 42]. The simplicity and affordability are the advantages of the FAMACHA© system as a helpful method in controlling *H. contortus* [11, 38, 40].

1.2.1.1. Microscopic identification of GIN species

Some GIN eggs with specific morphological features such as size, shape, etc., (Figure 4) can be distinguished from others by microscope [1]. *Nematodirus* and *Trichuris* eggs can be identified by microscope due to their specific size and shape [1, 43, 44].

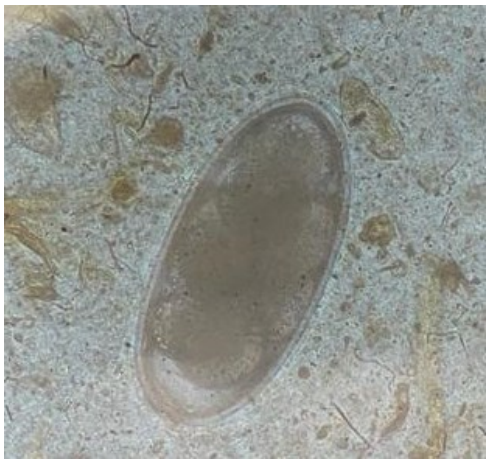



Figure 4. – *Trichuris spp.* (left) and *Nematodirus spp.* (right)

1.2.1.2. Molecular identification of GIN species

Since the size of most GIN eggs is very similar, it is complex to differentiate and identify them [1]. Therefore, molecular approaches by amplifying species-specific regions of specific reference genes, are used to identify the GIN species even in very low numbers [43].

1.2.1.2.1. Internal transcribed spacer-2 (ITS-2)

The *ITS-2* region is located between 18S and 28S ribosomal DNA genes; and is used among eukaryotes to construct phylogenic trees [45]. Plenty of rDNA copies in the genome make them sensitive genetic targets in molecular approaches [43, 45]. Furthermore, *ITS-2* region is a small (usually <800 bp) part of the *ITS* region, which is also repetitive and easier to amplify [46]. Therefore, The *ITS-2* ribosomal DNA is an important and valuable genetic marker for identifying nematodes [43, 45, 47].

1.2.1.2.2. Cytochrome c oxidase subunit 1 (COX-1)

The *COX-1* mitochondrial gene is another genetic marker for identifying nematode species. There are no introns in mitochondrial DNA, and they are less exposed to recombination compared with the nuclear genome [48, 49].

1.2.2. Vaccination

Several efforts in the development of vaccines as control tools against pathogens such as GINs have been tested with variable outcomes [50]. However, immunization through successful

vaccines is strongly recommended nowadays due to growing concerns about drug residues [3]. Moreover, anthelmintic resistance is likely a massive objection in control of GINs as well as other parasitic nematodes of veterinary importance [31, 51]. Thus far, many attempts have been made in vaccine development for GINs in ruminants [52]. However, the only successful commercial vaccine against GINs in ruminants is *H. contortus* vaccine for sheep; Barbervax® and Wirevax® have been registered in Australia and South Africa, respectively [50]. The administration of these vaccines is also possible in the United Kingdom in some specific circumstances [50]. Vaccination with Barbervax® and Wirevax® in sheep farms reduced 93-95% and 72-94% of FEC and worm load, respectively [50]. Nonetheless, there is limited data on the outcome of vaccines in the development of immunity against barber's pole worm in goats, and Barbervax® could be used off-label for goat [17, 50].

Currently, gut membrane antigen mixtures as native antigens of *H. contortus* have been used to produce the commercial vaccines (Barbervax® and Wirevax®) against this helminth in sheep [50]. Further research efforts are trying to identify recombinantly expressed antigens instead of native ones as a potential strategy for vaccine development [52]. Moreover, some desirable results have been obtained by the identification of a somatic antigen in adult *H. contortus* (Hc23) that results in an 80% reduction in FECs and worm load [53]. However, further studies are required to establish and commercialize this antigen as a vaccine against barber's pole worm. Finally, besides vaccination, other complementing strategies should be implemented in an integrated program to achieve considerable results in the control of GIN infections [3].

There are still growing studies characterizing candidate antigens on behalf of developing effective vaccines in other important GINs, such as *T. circumcincta*, *O. ostertagi*, and *C. oncophora* [3, 50].

1.2.3. Biosecurity

As a part of a successful sIPM, in the case of adding new animals to the herd, the new arrivals should be isolated for 1-3 days and treated with appropriate anthelmintic drugs as they may be a source of resistant worms to the farm [10, 11]. In addition, coprology analysis should be performed to evaluate FEC in order to determine the GINs burden and egg shedding [11].

1.2.4. Grazing management

Pasture contamination is an essential part of the sIPM that should be well managed in ruminants feeding on paddocks, particularly small ruminants [1, 11]. In almost all GINs of ruminants, third-stage larvae (L3) are the source of infection that develops outside the host [1]. During grazing season, intensive use of paddocks leads to recontamination, thus exposing ruminants, including susceptible ones to a higher number of L3, followed by a vast number of adult worms. To reduce contamination, the usage of paddocks should therefore be managed, and this could be done best by using paddocks every two years to allow the larvae to be killed [11, 29, 34]. Also, co-grazing of sheep and goats should be avoided due to low immunity and higher egg shedding in goats [11, 29]. Furthermore, larvae mostly reside near the base of herbage to have access to more moisture, so that it would be helpful to harrow the paddocks in hot and dry seasons [11]. In addition, some studies have shown different loads of L3 in various herbage species [10, 11, 29].

Other factors are the temperature and humidity, which are crucial in developing L3s on the pasture [29]. Therefore, in a country like Canada with cold weather conditions, GIN L3s boost in spring by increasing the temperature, which might vary in different regions [11]. A second peak in pasture L3s occurs during mid to late grazing season (July/August), in which lambs and kids are responsible [11]. Since lambs and kids are more susceptible to infections, using low-risk pastures for grazing is very important; and if possible, it is recommended not to graze lambs and kids [11]. Short-term grazing (< 7-14 days) and changing paddocks in these time points may help reduce animal exposure to L3s and help control parasitism on the farm [11, 29, 30]. Moreover, rotating livestock species like horses will help reduce pasture infectivity for sheep and goats; camelids are not helpful as they share GINs [11].

1.2.5. Genetic resistance and resilience

Developing genetically resistant and resilient breeds will result in animals with low worm burdens and lower FEC [54, 55]. In this approach, genetically resistant rams are included in the breeding program to achieve breeds with more resistance to worms during long periods [29]. This approach has been followed well based on Australian sheep breeding values as there is more resistance to anthelmintic compounds in sheep herds of Australia [55]. Moreover, other countries have started

genetic selection for breeds resistant to endoparasites, including but not limited to France, Germany, South Africa, etc. [30, 54].

1.2.6. Management of refugia

Management of refugia is a critical method in controlling GINs infection by improving the anthelmintics efficacy and ultimately, delay the development of anthelmintic resistance [11, 36, 56]. The refugia term is related to the free-living stages (untreated populations) with susceptible phenotype of the GINs, including eggs, L1, L2, and L3. Typically, refugia is constitutive 80% of the total parasite on the farm; The remaining 20% is related to L4 and adult worms inside the host [11, 36]. By using refugia, cross-host transmission between susceptible and resistant parasite populations lead to dilution of resistant phenotypes, resulting in an increased anthelmintic efficacy [36]. Consequently, replacement of parasite populations followed by the management of refugia has been proven to help in recovering the anthelmintic efficacy in resistant GIN populations [56]. In addition, the FAMACHA© system which was previously explained, is known to be useful in refugia management by applying targeted anthelmintic treatment; in which only individual severe infected animals are treated [57].

1.2.7. Biological approaches

Some other methods are helpful in controlling GINs, such as bioactive forages, copper oxide wires, and nematophagous fungi [1, 35]. There are commercial products containing *Duddingtonia flagrans* fungus species used as feed additives in ruminants [29, 34]. Fungal spores pass through feces and kill the first-stage larvae in feces outside the host [34] .

1.2.8. Anthelmintics

Despite growing concerns about using anthelmintics worldwide, they are still the main control strategy of GINs in ruminants [3]. There are several groups of anthelmintics against GINs used in ruminants around the world [1]. Moreover, some commercial products containing a combination of two dewormers are available to achieve maximum efficacy following administration. However, only some of them are licensed for ruminants and are presently available for use in Canada [10, 11]. Additionally, the use of anthelmintics to control GINs in goats is more complicated due to the

lack of any licensed drug for use in Canada [11]. For this reason, licensed veterinarians can prescribe other anthelmintic drugs as off-label use. On the other hand, off-label use of drugs is always risky, primarily due to the constraints to use the recommended dosage and ideal route of administration in animal species where there is no official reference [10, 11]. Also, the unknown withdrawal times for milk and meat, for instance in meat-goat farms should limit the use of off-label anthelmintic formulations [11]. In addition, discovering new anthelmintic drugs has always been a long-term process including challenging drug screenings and trials, involving costly budgets ranging around 50–100 million USD$ [58]. Therefore, the current anthelmintic drugs available should be appropriately administered by choosing the best compound with the correct dose and the best time for the related animal species to avoid treatment failure [3, 11].

1.2.8.1. Benzimidazoles (BZs)

BZs, also known as white drenches, are the most frequently used anthelmintics to control GINs in ruminants worldwide as well as in Canada [10, 11]. Albendazole and fenbendazole are the licensed members of this group in Canada to control GINs in cattle [10, 11]. However, they may be used off-label in sheep and goats by prescription of a licensed veterinarian. BZs have broad-spectrum activity against most nematodes, including L4 stage larvae, and also have ovicidal activity [11, 59]. The mechanism of action of BZs is by binding to β-tubulin protein of the targeted parasite and inhibition of microtubule polymerization [60]. Thus, cell structure is destroyed and leads to parasite death. Furthermore, BZs have high safety and very low adverse effects [59].

1.2.8.2. Imidazothiazoles

Currently, levamisole is the only commercially available member of this anthelmintic class but is no longer available in Canada [11, 60]. Levamisole binds to the nicotinic acetylcholine receptors (nAChR) that are expressed in the wall muscles of nematodes, producing a spastic paralysis of the parasite and death [60]. Levamisole acts against most of the GIN adult worms, and to a lower degree, it is effective against L4 larvae [60]. Very little difference in toxic and treatment dose of levamisole is the major problem, especially when administered in goats [11]. Since 2005, the use of levamisole to control GINs in ruminants is prohibited in Canada but still is available in some countries [11].

1.2.8.3. Tetrahydro pyrimidines

Morantel, pyrantel, and oxantel are the members of this anthelmintic class that have a mode of action similar to imidazothiazoles by binding to the nAChRs [60]. None of the tetrahydro pyrimidines are licensed in Canada to use in small ruminants for controlling GINs [10, 11, 60].

1.2.8.4. Closantel

Closantel is a synthetic narrow-spectrum anthelmintic drug licensed in Canada, originally developed as a first-choice compound against *F. hepatica* and lately used for the control of *H. contortus* in sheep [11, 61]. Its mechanism of action is by disrupting the energy metabolism pathways [11]. More specifically, closantel inhibits ATP synthesis in the parasite's mitochondria and also binds actively to albumins in the host's plasma to directly be delivered to the blood-feeding nematode [11]. However, closantel is not effective against immature non-feeding stages of *H. contortus [11]*. A remarkable point in the use of closantel is the meat withdrawal time that is 49 days in sheep [61]. Furthermore, administrating closantel in dairy ewes producing milk for human consumption is prohibited [61].

1.2.8.5. Amino-Acetonitrile derivates (AADs)

Monepantel, the first member of this new anthelmintic class, was introduced in 2009 and licensed in the UK and New Zealand for use in sheep [11]. It binds to the nematode-specific class of acetylcholine receptor subunits (nAChR) and greatly affects multidrug-resistant GINs, including *H. contortus* [62, 63]. However, the presence of resistance in small ruminants has been reported in recent years [11, 63].

1.2.8.6. Macrocyclic lactones (MLs)

MLs are large hydrophobic molecules and broad-spectrum endectocides, divided into two major groups termed avermectins and milbemycins [64]. Ivermectin is the first member of the MLs, which was introduced in 1980 in animal health [65]. Structurally, the MLs share a core macrocyclic lactone ring as a common feature of members in this group (Figure 5) [66]. In contrast, avermectins possess a disaccharide moiety, in addition to benzofuran and spiroketal moieties, with some differences among members (Table 2). The main difference between avermectins and

milbemycins is the absence of a disaccharide moiety in the latter group [66]. The benzofuran moiety is responsible for binding to the glutamate-gated chloride channels (GluCls), unique receptors present in nematodes [65, 66]. In addition, variations in the size of the spiroketal group in MLs, affect their binding ability to the allosteric site of ion channels [66].

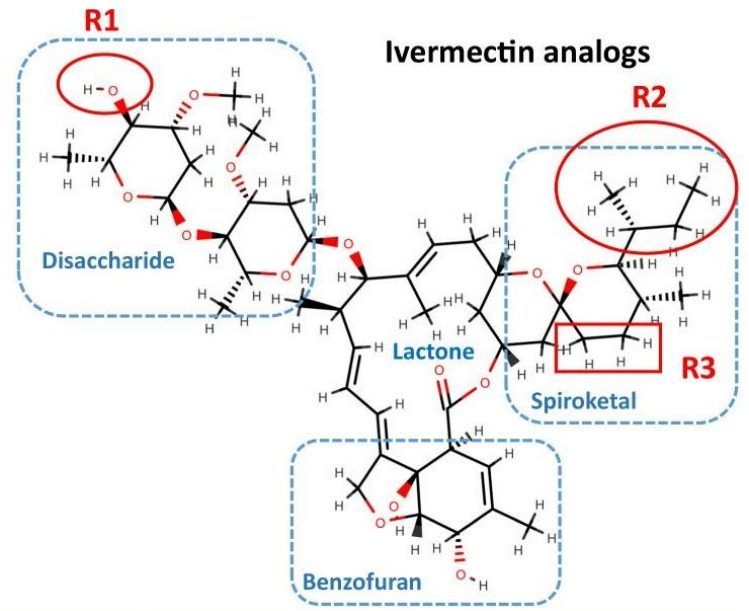


Figure 5. – Structure of IVM and analogs [66].

Drug	R1	R2	R3
Ivermectin	-OH	-Ch(CH3)2 and -CH(CH3)C2H5	-CH2-CH2-
Abamectin	-OH	-Ch(CH3)2 and -CH(CH3)C2H5	-CH=CH-
Moxidectin	Aglycon	-CH=CHCH2(CH3)2	-C=C(NOCH3)-

Table 2. – The structural differences among IVM analogs [66].

All MLs have a similar mechanism of action in nematodes (Figure 6). MLs bind selectively to allosteric binding sites of GluCls with high affinity [60, 66]. GluCls are expressed in the cell membrane of different organs in nematodes, such as the pharyngeal muscles, motor nerves, female reproductive tracts, and the excretory/secretory pores [66]. Binding of MLs to the GluCls in these organs leads to the permanent opening of these channels, allowing the chloride ions to

pass to the post synaptic membrane on the connecting neuron cell [66]. This process induces the hyperpolarization of synaptic neuro-muscular cells, ending up in flaccid paralysis and death of the nematodes [65, 66]. Moreover, avermectins act as antagonists of 4-aminobutyric acid (GABA) available on muscle and nerve cells of nematodes and other invertebrates [60, 66].

MLs are effective against most nematodes, including migrating L4 stage larvae, and also have activity against some insects and other arthropods [11]. However, MLs do not have ovicidal activity [11]. Some pharmacokinetic features of the MLs, are their storage on fat tissue following administration resulting in longer half-life compared to other anthelmintic classes [11, 65]. This characteristic has advantages and drawbacks: on one side, the longer circulation of MLs released from adipose tissue, allow for an optimal concentration of the compounds to reach different tissues in the host, thus targeting larval and adult stages of parasitic nematodes [11]. Nonetheless, the longer half-life of the MLs implies extended milk and meat withdrawal times compared with other anthelmintics [11, 65]. Furthermore, the type of administration also affects withdrawal time for MLs. For instance, the meat withdrawal time for ivermectin in injectable and drench forms in sheep is 35 and 15 days, respectively [11].

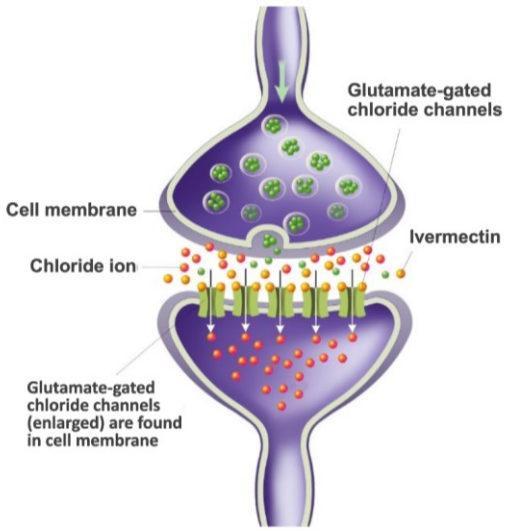


Figure 6. – Ivermectin mode of action [67].

The available MLs for controlling ruminant GINs in Canada are abamectin, ivermectin, doramectin, eprinomectin belonging to avermectins, and moxidectin as a member of milbemycins

[10, 11]. Among commercially available ML products, ivermectin (injectable and drench forms) and abamectin in combination with derquantel (Startect®) as drench form, are licensed for sheep [11]. Abamectin is an ML that belongs to the avermectins group [66], and derquantel (2-deoxy-paraherquamide), a member of spiroindoles, is a new anthelmintic drugs class [60]. Derquantel has an indole or oxindole moiety with a carbocycle group and is a nicotinic cholinergic antagonist that was introduced in 2010 [68]. Therefore, derquantel leads to blockage in cholinergic neuromuscular transmission and result in paralysis and death of nematodes. Both MLs and spiroindoles result in flaccid paralysis in nematodes. StartectTM helps control GINs in sheep, especially when resistance to other anthelmintics exists [68]. In addition, eprinomectin can be used as an off-label drug by prescription of a licensed veterinarian [11].

Finally, it should be noted that being successful in sIPM to control GINs depends on the implementation of all related approaches [31, 35]. Therefore, the application of a few approaches in herd management and, in some cases, just on the basis of chemical therapeutics will lead to undesirable results [10, 11, 30]. This would be more tangible when anthelmintic drugs, as a fundamental approach in controlling GINs, will become less effective [31].

1.3. Anthelmintic resistance (AR)

Anthelmintics are still the prime strategy to control parasitic nematodes in ruminants [3]. Therefore, different effective anthelmintic drugs have been discovered for controlling parasitic nematodes for decades [69]. However, after a short while, resistance has been reported almost in all anthelmintic classes (Table 3) [31, 51]. Parasitic nematodes develop some mechanisms, which are still poorly understood, to reduce the efficacy of anthelmintic compounds. Briefly, anthelmintic resistance (AR) development results in the survival of the parasitic nematodes after anthelmintic therapy [69]. As a result, AR is an emerging concern nowadays that also affects new anthelmintic drug discovery as a big challenge [58].

Class	Anthelmintic drug	Introduction	Report of resistance
Heterocyclic compounds	Phenothiazine	1940	1957
Benzimidazoles	Thiabendazole	1961	1964
Imidazothiazoles	Levamisole	1970	1979

Benzimidazoles	Albendazole	1972	1983
	Fenbendazole	1975	1982
Macrocyclic lactones	Abamectin	Late 1970's	2001
	Ivermectin	1981	1988
	Moxidectin	1991	1995
	Doramectin	1993	2007
	Eprinomectin	1996	2003
Amino-acetonitrile derivative	Monepantel	2009	2013

Table 3. – Resistance reports of various anthelmintic drugs during decades [31, 51].

The inheritable essence of AR makes it more threatening as the population of susceptible parasitic nematodes could be substituted quickly by resistant ones. Although successful treatment of ruminants with anthelmintics can lead to the death of many parasites, some resistant ones can survive. This results in resistant parasites continuing their lifecycle and transferring their genetic profile to their offspring, leading to appearance of plenty of worms with resistance phenotypes to appear on the farm [11, 69]. Previously used anthelmintic drugs will consequently not have the same effect as before, at least with the same dosage. Furthermore, the development of AR in parasitic nematodes can result in single or multidrug resistance [31, 70]. As a result, continuous and improper usage of anthelmintics in farms allows AR development in GINs such as *H. contortus* [51].

1.3.1. Mechanisms of AR development

Various studies have been done to find out the mechanisms of resistance against Benzimidazoles (BZs), Macrocyclic lactones (MLs), Imidazothiazoles, Tetrahydro pyrimidines, and other anthelmintic compounds. Among the latter, the only proven AR mechanism is reported in BZs, and some limited data is available about AR development in other classes [69, 71-74].

1.3.2. Benzimidazoles resistance

A mutation designated single nucleotide polymorphism (SNP) in the β tubulin isotype one gene is the proven mechanism for BZs resistance in parasitic nematodes [57, 74]. Since β tubulin is the target of BZs to inhibit parasitic nematodes, a modification in the structure of β tubulin by SNPs

leads to the inability of BZs to bind on their targets, thus, failing the BZs effect to kill parasitic nematodes [57]. The first report of SNPs regarding BZs resistance was made in 1994. Kwa et al., (1994) reported a SNP at position 200 on β tubulin-encoding gene on *H. contortus*, describing an amino acid change from TTC codon (phenylalanine) in susceptible parasites substituted by a TAC residue (Tyrosine) in resistant ones [71]. Later, Kwa et al. (1995) modulated BZ resistance in a free-living nematode by transferring BZ resistance alleles (SNP at position 200) from *H. contortus* to *C. elegans* and changed its phenotype to BZ resistant [75]. Furthermore, two other SNPs at positions 167 and 198 were reported in 2002 and 2007, respectively, in *H. contortus* and other GINs [72, 73]. The mutation at position 167 is similar to SNP at position 200 (TTC-TAC) [72]. However, a later report of SNP at position 198 is related to the substitution of GAA with GCA in resistant nematodes [73]. Although SNP at position 198 was identified from *in vitro* assays of *H. contortus* strains selection for BZ resistance but is rarely reported from field isolates [76].

1.3.3. Macrocyclic lactones resistance

Numerous studies have been done after first reports of resistance against MLs either in parasitic nematodes or free-living nematode *C. elegans* [66]. However, the exact underlying mechanism of ML resistance in parasitic nematodes, including *H. contortus*, is still not clearly elucidated [66, 69]. Some mechanisms have been reported to relate to ML resistance development, as discussed below. A whole body of data regarding ML resistance may indicate that it corresponds to a multigenic phenomenon in parasitic nematodes. Here below are described the most studied genes and pathways that may explain ML resistance in parasitic nematodes.

1.3.3.1. MLs targets modification

Research to understand ML resistance has focused on the glutamine-gated chloride channels (GluCls), which are the main targets of MLs in parasitic nematodes [66, 74]. Therefore, any changes in the structure of these channels can result in unbinding MLs to allosteric sites, and consequently, the desired efficacy will not be achieved [66]. GluCls receptors in nematodes are constituted by a complex array of heteroamorous subunits such as glc-(1-6) and avr-(14-15), which are some of the most studied genes linked to ML resistance that encode for GluCls subunits across nematodes [66]. It has been proven that mutations in these genes could result in the

development of a moderate to severe ML resistance phenotype in free-living and parasitic nematodes, including *H. contortus* [66, 74, 77].

1.3.3.2. Amphidial structure

Amphids are sensory neuronal structures in the cephalic end of nematodes and known as primary olfactory, chemoreceptive, and thermoreceptive organs that help the parasite in contacting with the external environment [78, 79]. Comparing resistant and susceptible strains of *H. contortus*, considerable microscopic differences have been demonstrated in amphid structures. It has been reported that some amphidial neuron dendrites have been shortened in *H. contortus* resistant strains comparing with susceptible ones [78]. Similar conclusions have been reported in free-living nematode *C. elegans* [80]. These findings persist on the importance of amphids in the survival of nematodes, which might be correlated with drugs, including MLs uptake [80]. Only one report of overexpression in amphidal neuron genes, including *osm-1*, *osm-5*, *dyf-7*, *dyf-11*, and *che-3*, describe the correlation with resistance development against MLs [78].

1.3.3.3. Micro RNAs (miRNAs)

Extracellular vesicles containing miRNAs are part of the nematode-derived molecules, extruded by esophageal glands in the excretory/secretory pore of nematodes [66, 81]. miRNAs are small non-coding RNA that can alter the expression of specific genes in nematodes affecting their messenger RNAs (mRNA) sequences [82]. As other mechanisms of MLs resistance, *C. elegans* was the first nematode where miRNAs were linked to an ML resistant phenotype [83, 84].

Although many miRNAs have been identified among various nematodes, the exact targets of most miRNAs are still undiscovered. The miRNA *miR-9551* is one of the proven miRNAs in resistant backcrosses of *H. contortus* as well as in resistant *T. circumcincta* [82]. It has been reported that upregulation of *miR-9551* can affect detoxification pathways [82].

1.3.3.5. ABC transporters

ABC transporters are one of the largest protein families that are involved in the extrusion of various substrates and xenobiotics, including anthelmintics [85, 86]. Almost all the ABC transporters are exporters, and only one recognized ABC transporter participates in importing

substrates [87, 88]. Therefore, this specific feature of ABC transporters has illustrated their potential role in resistance development against MLs in parasitic nematodes [74]. Also, the high affinity of ABC transporters to translocate hydrophobic or neutral drugs proposes the MLs as desirable candidates for extrusion [87]. Among MLs, avermectins are more suitable substrates for ABC transporters to be translocated compared with milbemycins. This relies on the disaccharide moiety's attached to the ML core ring present in avermectins [66]. This structural difference may explain the efficacy of moxidectin to act as an alternative in case of lack of efficacy on avermectins against resistant GIN isolates [65]. Parasitic nematodes overexpressing specific ABC transporters genes during exposure to MLs, have been studied over the last 25 years [89].

In this context, assorted studies have been conducted to establish the relationship between overexpression of ABC transporters genes and the development of ML resistance in nematodes. Among 60 identified ABC transporter genes in *C. elegans* as a model, only some of them have been studied in parasitic nematodes. P-glycoproteins (P-gps), were the first identified members of the ABC transporters family, linked to ML resistance `[90, 91]. Moreover, some of the half ABC transporters, multidrug resistance proteins (MRPs), and ABC transporters class F (ABCFs) have also been examined through different studies [90]. Consequently, it has been reported that *pgp-2* [19], and *pgp-9* [19, 20] have been overexpressed considerably in resistant strains of *H. contortus*. Also, in resistant isolates of *T. circumcincta*, expression levels of *pgp-9* [92] and *pgp-11* [93] have been upregulated. In *C. oncophora*, another important parasitic nematode of ruminants, *pgp-11*, has been overexpressed during exposure to higher concentrations of ivermectin [94].

Many efforts have been made to investigate the exact pathway of ML resistance development among various nematodes [66]. However, scientists could not find a major mechanism associated with ML resistance, similar to those proven findings in BZs resistance. Simultaneously, huge genetic diversity among field isolates of *H. contortus* in different parts of the world makes it much more complicated to consistently find the same genetic markers associated with ML resistance in field isolates [69, 95]. Finally, further research is required for detecting reliable biomarker for ML resistance in field isolates from different GIN species including *H. contortus*.

1-3-4 Prevalence of AR in *H. contortus*

The occurrence of AR in veterinary nematodes such as *H. contortus*, has been reported worldwide [95]. However, Australia, New Zealand, and South Africa have the highest challenges regarding AR in small ruminants [51, 58]. The same problem is happening in the United States regarding AR in *H. contortus*. Based on Table 4, it has been predicted that AR is an emerging and growing concern nowadays in animal health. Furthermore, studies in Canada, Europe, Australia, and Brazil about AR in *H. contortus* are consistent with results in the United States [96].

Study location and period	BZ	LVM	IVM	MOX	All three anthelmintics
Southern US (Sheep & Goat farms) (2002-2006)	98%	54%	76%	24%	48%
Eastern US (Goat farms) (2011-2016)	100%	44%	94%	56%	30%
Eastern US (Sheep farms) (2011-2016)	97%	21%	81%	40%	-
Western US (Sheep farms) (2011-2016)	91%	13%	38%	3%	-

Table 4. – Prevalence of AR in *H. contortus* in the United States [89].

BZ: Benzimidazole, LVM: Levamisole, IVM: Ivermectin, MOX: Moxidectin.

Also, in Canada, several studies have been done to investigate the prevalence rate of AR in *H. contortus*. In studies conducted in Quebec and Ontario, authors have reported an estimated resistance rate of 77.7% and 68.5% against BZs, respectively, by genetically analysis in *H. contortus* field isolates of sheep farms [97, 98]. In a recent study done in sheep flocks of western Canada including four provinces (Alberta, British Columbia, Manitoba, and Saskatchewan), the authors reported anthelmintic efficacy under optimal range (95%) for *H. contortus* populations in 9 of 10 farms treated with BZ and 11 of 17 farms treated with MLs [99].

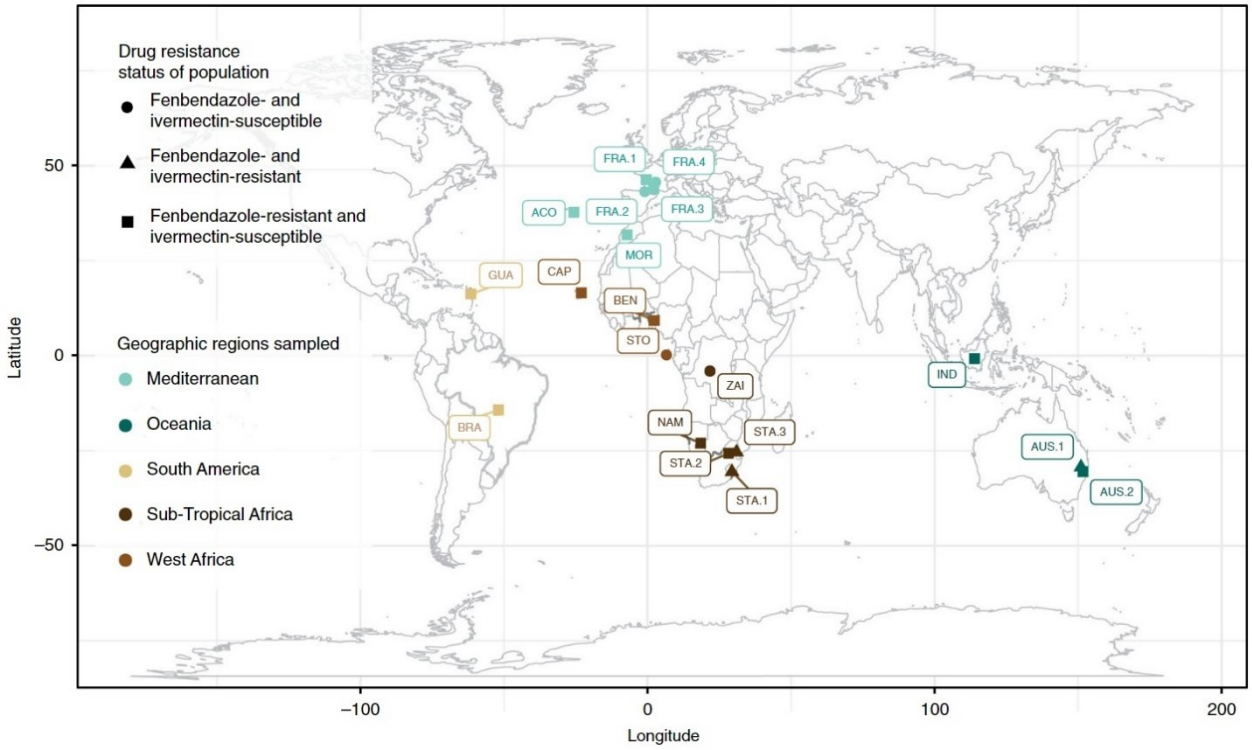


Figure 7. – Distribution of *H. contortus* isolates with different AR status worldwide [95].

1.4. Excretory secretory products (ESPs) of *H. contortus*

Nematodes, including *H. contortus*, produce excretory secretory products (ESPs) which consist of a large repertoire of biomolecules including proteins, lipids, amino acids, nuclei acids, small signaling molecules, secondary metabolites, as free entities or as cargo material in extracellular vesicles (EVs) [100, 101]. *H. contortus* ESPs interact with the host mainly to promote the infection process as well as modulate host immune responses [102]. Since some ESPs actively participate in the pathogenesis of haemonchosis, they are potential targets to focus on vaccine development and biomarkers for AR [100, 103, 104].

1.4.1. Proteins

Proteins are part of Hc-ESPs which have different functions. Some ESP proteins are known to participate in tissue penetration and host protein degradation processes [105]. Schallig et al. (1997) reported that sheep vaccination with proteins derived from Hc-ESPs induced immunity against *H. contortus* infection in sheep by resulting in more than 70% decrease in FECs and

abomasal worm burden [106]. Moreover, Hcgp55, a 55kDa glycoprotein, is known to modulate the host immunity by blocking neutrophil migration, in order to facilitate *H. contortus* attachment and feeding in host's abomasum [107]. In addition, Hc15, Hc24 and Hc40 proteins are identified In Hc-ESP and reported to be involved in the host-parasite interactions including infectious process and immune evasion [104].

1.4.2. Lipids

Lipids are a diverse group of biomolecules characterized by their hydrophobicity [108]. The eight main categories of lipids, based on their chemical structure and biosynthetic origins, are fatty acids, glycerolipids, glycerophospholipids, sphingolipids, sterol lipids, prenol lipids, saccharolipids and polyketides [108]. In eukaryotes, lipids have an important role in cell biology of organisms by forming the cellular membranes, as energy storage and also as signaling molecules [103]. Furthermore, lipids play a crucial role in pathogenesis of parasites, including invasion processes and host-parasite interactions, and interestingly, the abundance of lipids varies by lifecycle [101, 103]. Yet there is no published data regarding the lipidomic profile of ESPs in *H. contortus*; however, few studies have been done to identify the composition of lipids in whole adult worms and some specific organs [103, 109].

Translocating various lipids by ABC transporters is proven in plasma and intracellular membranes of human cells (Figure 8), and disruption in these ABC transporters leads to lipid-associated disease [87, 110]. In the meantime, ABC transporters in *C. elegans*, a free-living nematode, are expressed in different organs, including reproductive, digestive and excretory/secretory system, which help the parasite to efflux the xenobiotics outside of its body [111, 112]. Moreover, MLs and lipids have some common features including planar structures and hydroxyl groups (Figure 9), making them suitable substrates to be extruded with ABC transporters [87]. In addition, the detoxification activity of ABC transporters has been modified by lipids in *H. contortus* [113].

In this study, our general goal was to characterize the role of *H. contortus* ABC transporters in the context of ML resistance and the extrusion of lipids.

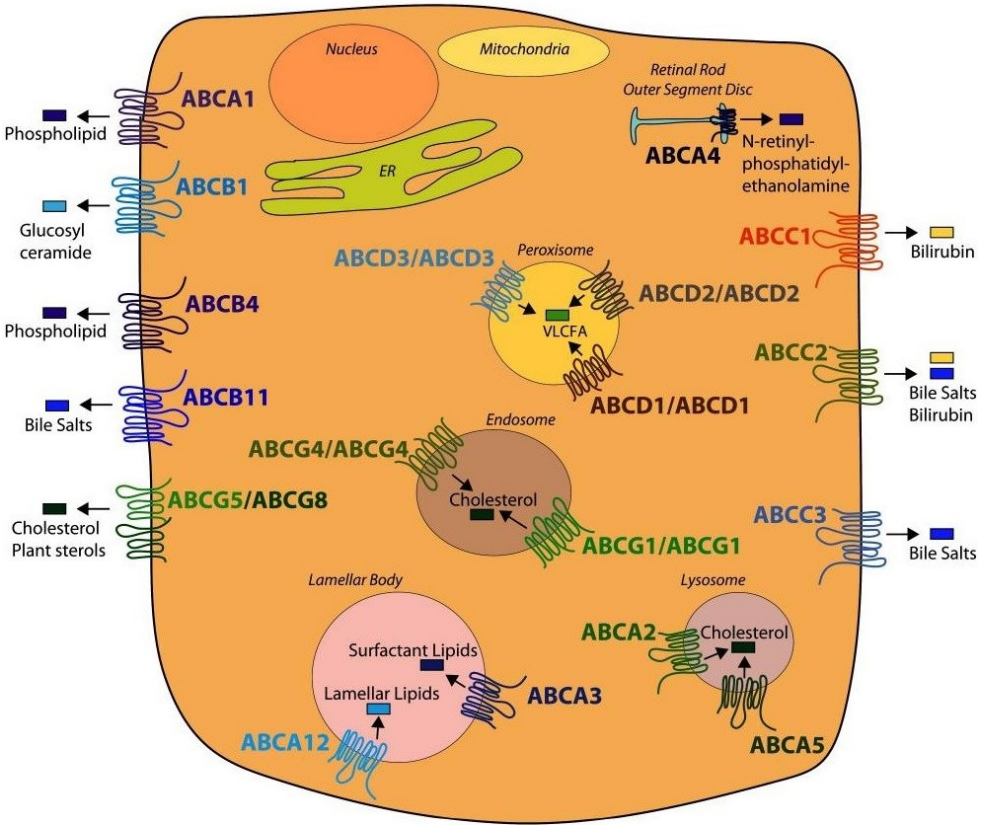


Figure 8. – ABC lipid transporters localization in human cell [110].

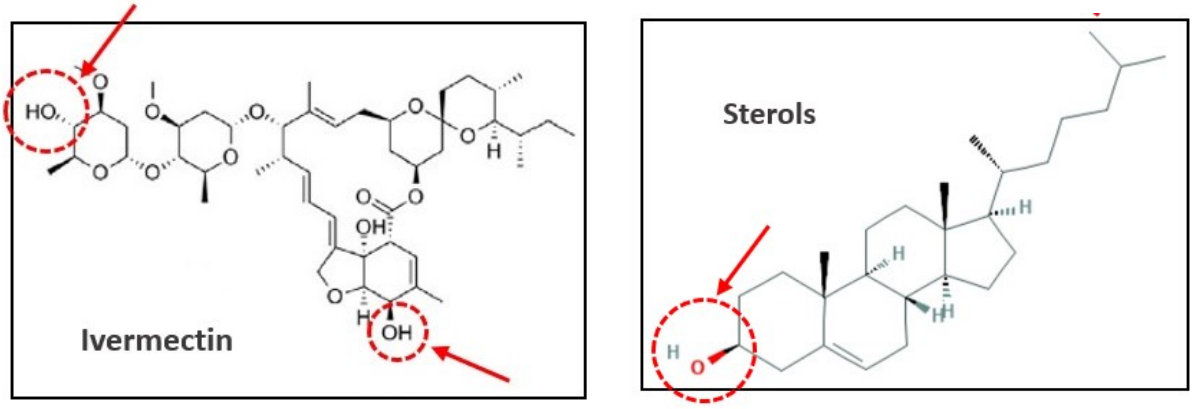


Figure 9. – Structural similarities among MLs (ivermectin) and lipids (sterols) [65, 114].

Hypothesis

ABC transporters in *H. contortus* act as a multipurpose extrusion system and get involved in the MLs detoxification and extrusion of lipids.

Objectives

1. To determine the ML efficacy against GINs, including *H. contortus* isolates from different ruminant farms
2. To establish the ABC transporters gene expression profile from *H. contortus* isolates at larval stage
3. To identify lipids secreted by *H. contortus* and link their extrusion to the ABC transporters' activity

Chapter 2 – Material and Methods

2.1. IVM efficacy and gastrointestinal nematode (GIN) species identification

2.1.1. IVM efficacy

In order to evaluate the IVM efficacy, coprology analysis, FECRT, and LDA were conducted in different ruminant farms, including sheep, goats and camelids.

2.1.1.1. Sampling

Pre-treatment (pre-tx) and 14 days post-treatment (post-tx) with IVM fecal samples were collected from different ruminant farms [33, 115]. In total, fecal samples from four sheep, one goat, one Llama and four alpaca farms were collected between April 2021 and September 2022 (Table 5). Samples collection was with the pooling method in most farms; however, individual samples were collected in three alpaca farms.

Farm	Specie	Number of pre-tx samples	Number of post-tx samples
#1	Sheep*	19	9
	Llama*	3	1
#2	Sheep*	1	1
#3	Sheep*	2	0
	Goat*	1	0
#4	Sheep*	1	0
#5	Alpaca**	4	4
#6	Alpaca**	4	0
#7	Alpaca*	1	0
#8	Alpaca**	7	0

Table 5. – Number of various pool samples from different ruminant farms

* pool sampling; ** individual sampling

2.1.1.2. Fecal egg count (FEC)

FEC was performed with two methods to determine the GINs presence in groups of animals or farms.

2.1.1.2.1. Wisconsin

For collected samples from each farm included in this study, three replicates of 3 gr (± 0.1 gr) of feces were weighed and dissolved entirely in about 20 ml of water in a small plastic beaker; then stirred in a 50 ml Falcon and water added up to 45 ml [98]. The prepared mix was centrifuged at 850×g for five mins, and the supernatant was discarded [98]. The remaining pellet was resuspended in 5 ml saturated sugar solution (specific gravity: 1,30) and transferred to a 15 ml Falcon tube, followed by centrifugation at 350×g for two mins [98]. Finally, a saturated sugar solution was added to the tubes until the formation of a positive meniscus at the top of the tube and a coverslip was fixed on the tubes. After one h of incubation at RT, the coverslip was picked up and placed on the glass slide for FEC under the compound microscope at 10X magnification. The total number of counted eggs in each slide was divided into three (the weight of feces) to achieve each replicate's egg per gram (EPG), and the mean EPG of three replicates represented the EPG of the sample.

2.1.1.2.2. Mini-Flotac

The mini-Flotac system [116] was applied in parallel to assess FEC from each ruminant farm. Briefly, 2.5 grams of feces were weighed and placed in the conical collector of the fill-Flotac. Then, 47.5 ml of a saturated salt solution (specific gravity: 1.2) was added to the fill-Flotac container and closed the screw. By gently pumping and circulating the homogenizer pole of the fill-Flotac, the sample was homogenized without the formation of bubbles. Immediately after homogenization, the two one ml chambers of the mini-Flotac were filled gently without bubbles [117]. After, the sample was incubated for ten mins, the reading disk of mini-Flotac was turned clockwise (90°) gently and placed under a microscope to count the GIN eggs. The total FEC in two chambers was multiplied by ten to achieve EPG, and finally, the mean EPG of three replicates was identified as the final mean EPG of the sample [116, 117].

2.1.1.3. Fecal egg count reduction test (FECRT)

FECRT was carried out by using the formula below to evaluate the IVM efficacy in the treated animals as a first approach to finding potentially resistant GIN populations, including *H. contortus* [33, 115]. Based on the results of this test, we categorized the IVM efficacy into three groups: optimal (>95%), sub-optimal (90-95%), and inefficient or indicative of resistance (<90%) [115].

$$FECRT(\%) = 100 \times \left\{1 - \frac{(FEC\ (pre\_tx) - FEC\ (post\_tx)}{FEC\ (pre\_tx)}\right\}$$

2.1.1.4. Larval Development assay (LDA)

In order to conduct an in-vitro analysis of the IVM efficacy, the LDA was performed based on the protocol described below [118].

2.1.1.4.1. GIN eggs recovery

GIN eggs were recovered with the same method described above in the Wisconsin FEC method. However, instead of saturated sugar solution (SPG: 1.3) in the floatation step, it was replaced by a saturated salt solution (SPG: 1.2) to reduce the floated debris. Moreover, at the end of the flotation of the GIN eggs, the coverslip was washed with distilled water into a 15 ml Falcon tube and then, the mixture passed through 100 µm, 80 µm and 40 µm strainers to purify the eggs. In the end, the tube was centrifuged at 3000 ×g for 5 mins, the supernatant was discarded, and the remaining pellet was transferred to a 1.5 ml tube [98].

2.1.1.4.2. Protocol

The LDA was done in three replicates for all treated and control groups. A DMSO group and a control group were included in the assay. IVM (Sigma-Aldrich®, USA, CAS #70288-86-7) reconstituted in DMSO (Fisher®, USA, CAS #67-68-5) solutions at different concentrations were prepared and used at the following working dilution concentrations in each replicate: 0.1 ηM, 0.5 ηM, 1 ηM, 2 ηM, 4 ηM, 8 ηM, 16 ηM and 32 ηM. The nutritive media was composed of the one-part Phosphate-buffered saline (PBS) solution (10X) and nine parts of one gram yeast extract (Oxoid®, France, Cat #LP0021) dissolved in 90 ml autoclaved NaCl (Fisher®, Canada, CAS #7647-14-5) 0.85% solution [119]. The egg solution was adjusted to have about 100 eggs per 15 µl, and

Amphotericin B (Gibco®, Cat #15290-18) was added (5 µg/ml) to the egg solution to prevent secondary infection in the wells [119]. Milli-Q water, nutritive media and IVM dilutions were added to a tube for each replicate, mixed and subsequently added to the wells. The egg suspension was added individually to the wells in order to have almost equal numbers of eggs in each well (about 100 eggs). All left marginal empty wells on the plate were filled with milli-Q water, and the plates were covered with a lid before insertion inside the incubator to decrease the evaporation. Finally, the 96-well plates were incubated at 25°C for nine days [119]. Parasite development was monitored by microscopical examination; all the changes in each well regarding the hatching and growth of larvae were recorded daily until the end of the experiment. On day five of the experiment, 10 µl of milli-Q water was added to the wells to replace the evaporated water.

	Final volume in each well	Final volume in three replicates
Milli-Q H2O	114.666 µl	344 µl
Nutritive media	20 µl	60 µl
IVM solution	0.334 µl	1 µl
Egg suspension	15 µl	45 µl
Total	**150 µl**	**450 µl**

Table 6. – Composition of the different solutions in the wells for the LDA method [119].

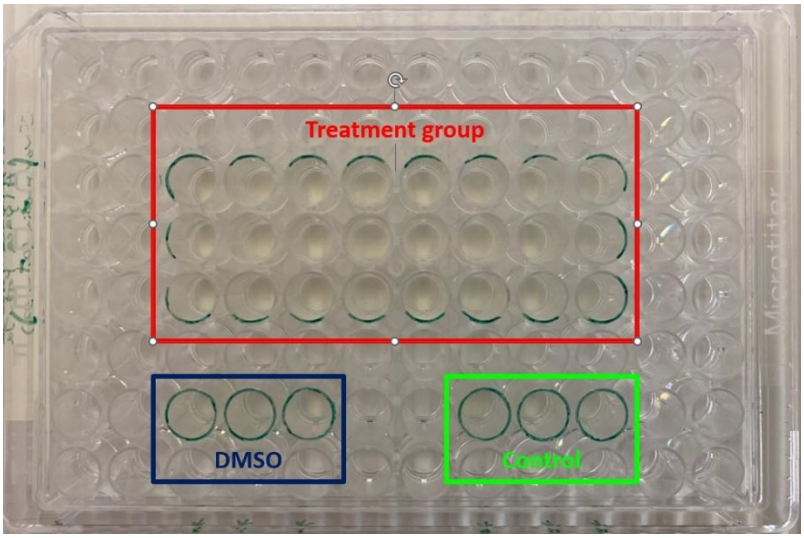


Figure 10. – LDA plate, including treatment, DMSO and control groups.

2.1.1.5. Peanut agglutinin (PNA) staining for *H. contortus* identification

GIN eggs recovered, as described in the LDA section, were transferred to a 1.5 ml tube and centrifuged at 5500×g for 5 mins. After removing the supernatant, 1 ml of fluorescein-labelled peanut agglutinin (5 mg/ml, lectin from *Arachis hypogaea*, FITC conjugate, Sigma-Aldrich®, product no. #L7381) was added to the pellet and resuspended by pipetting [98]. The tube was covered with an aluminum sheet and incubated at room temperature with consistent shaking. Later, the tube was centrifuged at 5500× g for 5 mins, followed by discarding the supernatant and adding 1 ml of PBS 1X solution. Furthermore, it was centrifuged at 5500× g for 5 mins, and the supernatant was removed. Finally, the pellet was resuspended in 250 µl of saturated salt solution (SPG 1.05), and 50 µl of this solution was mounted on a glass slide and covered by a coverslip. The specimen was analyzed by a UV microscope with FITC filters to identify the *H. contortus* eggs with a green-fluorescent outline (480–490 excitation/527/30 emission) [98].

2.1.2. Molecular identification of GIN species

2.1.2.1. GIN eggs recovery

GIN eggs recovery was performed as described in section 2.1.1.4.1; however, at the final step, the eggs passed only through 100µm and 20µm to decrease the risk of eggs loss during straining.

2.1.2.2. DNA extraction

Two hundred to one thousand eggs were used for DNA extraction. The samples with a lower number of eggs were incubated for two to three days at 24 °C to achieve L1s in order to increase the potential template [120]. DNA extraction was performed using two methods that are explained below.

2.1.2.2.1. Extraction with a commercial kit

Quick-DNA fecal/soil microbe miniprep kit (ZYMO RESEARCH®, USA, Cat #D6010) was used to extract DNA with the manufacturer's instructions. The lysis process of this kit is based on mechanical (with beads) and chemical methods.

2.1.2.2.2. Manual extraction

This method was obtained from the Nemabiome website (https://www.nemabiome.ca/) with slight changes to prepare the DNA lysates as described below. In this regard, a made-in-house lysis buffer was prepared with a recipe as shown below in Table 7. [120].

Volume (µl)	Stock reagent	Concentration in lysis buffer
2500	1M KCl (BioShop®, Canada, CAS #7447-40-7)	50mM KCl
500	1M Tris (pH: 8.3) (Fisher®, Canada, CAS #77-86-1)	10 mM Tris
125	1M MgCl2 (BioShop®, Canada, CAS #7791-18-6)	2.5 mM MgCl2
225	Nonidet p-40 (BioShop®, Canada, CAS #9016-45-9)	0.45% Nonidet p-40
225	Tween-20 (BioShop®, Canada, CAS #9005-64-5)	0.45% Tween-20
250	2% gelatin (BioShop®, Canada, CAS #9000-70-8)	0.01% gelatin

Table 7. – The recipe for made-in-house lysis buffer [120].

About 200-1000 GIN eggs (and/or larvae) were transferred to a 1.5 ml tube, and 1.3 ml of lysis buffer was added, mixed and incubated at room temperature for five mins. After, centrifugation was carried out at 13000× g for four mins; the supernatant was discarded without touching the pellet. Later the pellet was resuspended in 1 ml of lysis buffer, centrifuged at 13000× g for four mins and the supernatant discarded. The latter step was performed three times. At the end, about 100µl was left in the tube, and 50µl of new lysis buffer were added for pellet resuspension. Later, the tube was incubated at 95°C for 15 mins, and the tube was vortexed every one min. Afterwards, the pellet was kept at -80°C for two h and then defrosted on ice. Further, 6µl of proteinase K (Sigma-Aldrich®, USA, CAS #39450-01-6) (20 mg/ml) were added to the tube and incubated at 55°C for two h with vortexing at the maximum speed every one min. Finally, the tube was incubated at 95°C for twenty mins to denature the proteinase K. The final DNA lysate was diluted 1:10 to use as a template for further experiments [120].

2.1.2.5. Primers

The *ITS-2* primers used to identify *Haemonchus contortus*, *Teladorsagia circumcincta*, *Trichostrongylus axei*, *Trichostrongylus colubriformis*, *Trichostrongylus vitrinus*, *Chabertia ovina*,

Oesophagostomum venulosum, *Cooperia curticei*, *Camelostrongylus mentulatus* (GenBank accession number: KY930444.1) and *Marshallagia marshalli* (GenBank accession number: MT110920.1) in different small ruminants are shown in the table below.

Primer	Sequence (5' → 3')		Product size (bp)	Annealing temp (°C)
Generic [121]	FW	CACGAATTGCAGACGCTTAG	370-398	53
	RV	GCTAAATGATATGCTTAAGTTCAGC		
H. contortus 1 [121]	FW	CACGAATTGCAGACGCTTAG	170	53
	RV	CTTGAACTGAAATGGGAATTGTCT		
H. contortus 2 [122]	FW	GTTACAATTTCATAACATCACGT	321	55
	RV	TTTACAGTTTGCAGAACTTA		
T. circumcincta [122]	FW	ATACCGCATGGTGTGTACGG	421	58
	RV	CAGGAACGTTACGACGGTAAT		
T. axei [122]	FW	AGGGATATTAATGTCGTTCA	67	56
	RV	TGATAATTCCCATTTTAGTTT		
T. colubriformis [122]	FW	CCCGTTAGAGCTCTGTATA	165	59
	RV	TGCGTACTCAACCACCACTAT		
T. vitrinus [122]	FW	AGGAACATTAATGTCGTTACA	100	54
	RV	CTGTTTGTCGAATGGTTATTA		
Ch. Ovinia [122]	FW	CATGTGTGATCCTCGTACTAGATAAGA	158	54
	RV	ATGAACCGTACACCGTTGTCA		
O. venulosum [122]	FW	TGTTTACTACAGTGTGGCTTG	280	54
	RV	CGGTTGTCTCATTTCACAGGC		
C. curticei [122]	FW	TATACTACAGTGTGGCTAGCG	143	54
	RV	TCATACCATTCAGAAATGTTC		
C. mentulatus	FW	CTTCGGCACGTCTGGTTCAG	278	55
	RV	TGAGCTCAGGTTGCAATACAAA		
M. marshalli	FW	TCATGAATGACACATGCAACA	188	53
	RV	TAAGTTCAGCGGGTAATCACG		

Table 8. – The *ITS-2* primers and annealing temperatures.

Furthermore, mitochondrial cytochrome c oxidase subunit 1 (*COX-1*) primers were used to identify *Lamanema chavezi* (GenBank accession number: MG598421.1) in camelid farms. (Table 9). As an alternative to *ITS-2* for *L. chavezi*, the reference gene for mitochondrial *COX-1* has been used to accurately identify species [123] and was included in this study.

Primer	Sequence (5' → 3')		Product size (bp)	Annealing temp (°C)
L. chavezi	FW	TTTGGGCATCCTGAGGTTTA	157	53
	RV	GAGCTCAAACCACACAACCA		

Table 9. – The *COX-1* primers and annealing temperatures.

2.1.2.6. Polymerase chain reaction (PCR)

Thermocycling (Bio-Rad T100™) conditions were 95°C for three mins, followed by forty cycles of the second denaturation at 98°C for 30 seconds, annealing for 20 seconds, extension at 72°C for 1 min with the final extension of 10 mins at 72°C. Further, PCR amplicons were analyzed on agarose gels and verified for their respective expected sizes.

2.2. Gene expression analysis on ABC transporters from *H. contortus* field isolates

2.2.1. RNA extraction

All the parasitic materials, including eggs, L1, L2 and L3s of treatment groups of LDA corresponding to each sample, were transferred to one 1.5 ml tube, centrifuged, and supernatant discarded. After adding 500 µl of TRIZOL® (Ambion®, USA, REF #15596026) [124], individual samples homogenized by using the high-speed homogenizer, adding another aliquot 500 µl of TRIZOL™ to the mixture. After, the extraction was performed based on the manufacturer's instructions. Further, the purified RNA were placed on ice and individually quantified by Nanodrop. The extracted RNA were stored at -80°C for further experiments.

2.2.2. Reverse transcription

Complementary DNA (cDNA) was synthesized from each sample by utilizing the high-capacity cDNA reverse transcription commercial kit (applied biosystems™ by Thermo Fisher Scientific®, Lithuania, REF #4368814) according to manufacturer's instructions. The thermocycler (Bio-Rad T100™) conditions were 25°C for ten mins, 37°C for 120 mins, and 85°C for five mins.

2.2.3. Primers

The list of *H. contortus* ABC transporters genes primers used for relative gene expression analysis is shown in the table below.

Primer	Sequence (5' → 3')		Product size (bp)	Annealing temp (°C)
GAPDH [20]	FW	TGGGTGTGAACCACGAGAC	213	55
	RV	GCAGCACCACGTCCATCA		
Actin [20]	FW	GAGTCATGGTTGGTATGGGAC	140	52
	RV	GGAGCTTCGGTCAAAAGTACG		
pgp-2 [20]	FW	GGACAAAAGCAGCGAATTGCC	169	55
	RV	ACAGACGATGCGCTACAATGAC		
pgp-3 [20]	FW	CCGGCAACTTGTACTTCAAGGC	94	52
	RV	TCACTGTGCTCTTTCCGCAAC		
mrp-5 [20]	FW	TGTCGGTAGAACGGGAAGTG	125	52
	RV	GCAGGGTATGCAAAGGAATAGA		
abcf-2 [20]	FW	ACGTGTAGCCTTGGTTGGTC	157	52
	RV	TTCAAGTGGGAGCTCTTCG		

Table 10. – *H. contortus* ABC transporter and housekeeping genes primers used in qPCR.

2-2-4- Real time PCR (qPCR)

The thermocycler (Bio-Rad CFX Opus 96™) protocol used for qPCR was 98°C for 30 seconds, followed by 40 cycles of 95°C for 10 seconds, annealing for 30 seconds (plate read). *Glyceraldehyde 3-phophate dehydrogenase* (*GAPDH*) and *Actin* were used as a housekeeping

genes [20], and the delta-delta Ct method [125] was used for relative quantitation of the expression levels for each gene of interest, expressed as relative fold change [20].

2.3. Lipidomics analysis on the ESP profile of *H. contortus* and the involvement of ABC transporters in their extrusion

2.3.1. *H. contortus* adult worm recovery

Two Dorset lambs (approved animal ethics protocol # 19-Rech-2031) were infected with approximately 4,000 L3s of *H. contortus* PF strain (susceptible strain [126]) and monitored over 45 days post-infection for egg output and clinical follow-up on experimental infection. After verifying a high number of eggs (>500 EPG) indicative of *H. contortus* adult worms, sheep were humanely euthanized, and the abomasum was recovered.

2.3.2- *Ex-vivo* incubation of *H. contortus* adult worms

H. contortus adult worms were recovered carefully from the abomasum and washed several times in 1X PBS (Phosphate Buffer Saline). Then male and female adult worms were transferred to RPMI medium containing penicillin/streptomycin and incubated at 37^{o}C with 5% CO_2 [127]. Treatment groups (20 adult worms per group) with ABC transporter inhibitors, including fumitremorgin C, ketoconazole and MK-571 at three different concentrations 0.5 µM, 1 µM and 5 µM. Also, a blank RPMI group as a control group (20 adult worm) was included. At three time points (2h, 4h & 8h post-incubation) medium was collected from culture media and subsequently replaced. Culture media samples were stored at -80°C until further analysis.

2.3.3. Global lipidomic analysis on *H. contortus* culture medium

Samples were sent for lipidomic analysis at The Metabolomics Innovation Centre (TMIC) at University of Alberta, Edmonton, AB, Canada.

2.3.3.1. Samples preparation and lipid extraction

Modified Folch liquid-liquid extraction protocol was used for samples preparation [128, 129]. Samples were dried with a nitrogen blowdown evaporator at 24°C. After resuspending the residue

in 40µl of water, 1.34µL of internal standard solution and 274µL of methanol were added and vortexed. Later, extraction was performed with 551µl of dichloromethane, followed by clean-up with 166.6µl water [130]. Further, samples were incubated at room temperature (RT) for 10 mins and centrifuged at 16000×g for 10 min at 4°C. Additionally, the organic layer was dried with a nitrogen blowdown evaporator [130]. Finally, 4µl of Nova^{MT} MixB was added to the residue, and after resuspending by vortex for 1 min, 36µL of Nova MT Mix A added. Also, for quality control purposes a pooled mixture of all sample extracts was prepared.

2.3.3.2. Liquid Chromatography-Mass Spectrometry (LC-MS) Analysis condition

The LC-MS analyses on samples were performed in a Thermo Fisher Doinex UltiMate 3000 UHPLC (Thermo Fisher Scientific, Waltham, MA, USA) linked to Bruker Maxis II QTOF Mass Spectrometer (Bruker Corporation, Billerica, USA), in both positive and negative ion mode [131]. Sample and quality control injections were performed in positive and negative ionization for MS/MS identification spectra. Samples were transferred into Waters Acquity CSH C18, 1.7µm columns with injection duplicates (i.e., two runs per sample extraction in each polarity). Acquisition analysis was performed in two mobile phases, NovaMT Mix A and NovaMT Mix B, with 26-min-gradient and a flow rate was 250µL/min at 42°C. Furthermore, the injection volume for the LC-MS analysis was 5µL and 12.5µL for positive and negative ionization, respectively. Also, the mass range (m/z) was from 150-1500 and the MS/MS collision energies were 10-90 eV (electron-Volts). Finally, LC-MS data from sample and quality controls (QC) were extracted and aligned for lipid features identification through the NovaMT Lipid screener (Edmonton, AB, Canada).

2.3.3.3. Lipid Identification

Lipids identification was carried out with a three-tier ID approach based on the MS/MS identification and MS match [129, 130] with the following parameters:

Tier 1 (MS/MS identification): match score ≥ 500, precursor m/z error ≤ 5mDa

Tier 2 (MS/MS identification): match score ≥ 100, precursor m/z error ≤ 5mDa

Tier 3 (MS match): Mass match with m/z error ≤ 5mDa

NovaMT LipidScreener was used for Six-tier filtering and scoring analysis in Tier 1 and Tier 2 to put a limit on the number of isomers and also determine the best matches for lipid subclasses [129, 130, 132]. Moreover, LipidLynxX (available at the database from LIPID MAPS (https://www.lipidmaps.org/) was used to run an MS match to identify lipids in Tier 3 [133, 134].

2.3.3.4. Data normalization

Fourteen internal standards from different lipid classes were used for data normalization. In accordance with lipid class similarities and expected retention time range, the identified lipids matched one of these fourteen internal standards [129]. The intensity of each lipid is divided by intensity of the matched internal standard in order to calculate normalized intensity ratios.

2.3.3.5. Statistical Analysis

Lipid features were uploaded on the MetaboAnalyst 4.0 (https://www.metaboanalyst.ca) software (McGill University, QC, Canada) for statistical analysis. Later, the data set was normalized by autoscaling and median.

2.3.4. Gene expression analysis on *H. contortus* ABC transporters

2.3.4.1. RNA extraction

After eight hours of parasites culture including control (non-treated) and treated groups with ABC transporters' inhibitors, adult worms were collected into cryovials and stored at -80°C. Later the adult worms from different conditions corresponding to each sample were transferred to a plastic beaker, and 1 ml of TRIZOL® (Ambion®, USA, REF #15596026) was added [124]. By using the high-speed homogenizer, the adult worms were homogenized completely, and the prepared mixture was transferred to a 1.5 ml tube. After, the extraction continued as described previously in section 2.2.1.

2.3.4.3. Reverse transcription

Complementary DNA (cDNA) was synthesized using 1µg of extracted total RNA as described previously (section 2.2.2.).

2.3.4.4. Primers

The list of *H. contortus* ABC transporters genes primers used for relative gene expression analysis is shown in Table 11.

Primer	Sequence (5’ → 3’)		Product size (bp)	Annealing temp (°C)
GAPDH [20]	FW	TGGGTGTGAACCACGAGAC	213	56.3
	RV	GCAGCACCACGTCCATCA		
pgp-2 [20]	FW	GGACAAAAGCAGCGAATTGCC	169	55
	RV	ACAGACGATGCGCTACAATGAC		
p-gp-3 [20]	FW	CCGGCAACTTGTACTTCAAGGC	94	53.1
	RV	TCACTGTGCTCTTTCCGCAAC		
pgp-11 [20]	FW	ACCACGAAGCTGAACGAGAA	150	56.9
	RV	CACCAGAGTGATACGCCAGTC		
mrp-5 [20]	FW	TGTCGGTAGAACGGGAAGTG	125	55
	RV	GCAGGGTATGCAAAGGAATAGA		
haf-6 [20]	FW	CAATCAAACCCAGAGCGATAA	250	47.1
	RV	CAACAGCGAGCTTGAAACAG		

Table 11. – *H. contortus* ABC transporter and housekeeping genes used in qPCR.

2.3.4.5. qPCR

The thermocycling protocol and relative fold change calculations were used as described previously (section 2.2.4.). *GAPDH* was used as a housekeeping gene [20].

Chapter 3 – Results

3-1- IVM efficacy and gastrointestinal nematode (GIN) species identification

3-1-1- IVM efficacy on GINs in different ruminant farms

3-1-1-1- Fecal egg count (FEC)

FEC results for all samples are shown in Tables 12-15 as a mean EPG of three replicates. Moreover, the fecal samples were analyzed with Wisconsin method, except for farm 4 and 5 for which samples were submitted to the mini-Flotac method. Furthermore, due to the low amount of fecal material, the SHP 13a and SHP 33a samples were analyzed in one replicate.

3-1-1-1-1- FEC results of sheep samples

Farm	Sample	Type	Total GINs		*Nematodirus spp.*		*Trichuris spp.*	
			EPG mean	95% confidence	EPG mean	95% confidence	EPG mean	95% confidence
1	SHP 11a	Pre-tx	0.3	0.34	0	NA	0	NA
1	SHP 12a	Pre-tx	22.3	4.98	0.7	1.36	0	NA
1	SHP 13a	Pre-tx	0	NA	0	NA	0	NA
1	SHP 14a	Pre-tx	138.6	25.12	3.7	2.38	0	NA
1	SHP 15a	Pre-tx	61.7	39.04	0.3	0.34	0.1	0.23
1	SHP 15b	Post-tx	12.2	3.85	0	NA	0	NA
1	SHP 16a	Pre-tx	120.4	31.91	2.3	0.34	1.6	0.79
1	SHP 16b	Post-tx	5.4	1.58	0	NA	0	NA
1	SHP 17a	Pre-tx	31.9	16.75	0	NA	0	NA
1	SHP 17b	Post-tx	0.6	0.23	0	NA	0	NA
1	SHP 18a	Pre-tx	0	NA	0	NA	0	NA
1	SHP 18b	Post-tx	4.8	2.15	0.3	0.34	0	NA
1	SHP 19a	Pre-tx	35.2	24.89	0	NA	0	NA

1	SHP 19b	Post-tx	16.3	6.34	0	NA	0	NA
1	SHP 20a	Pre-tx	1013.2	158.08	0.3	0.34	0.4	0.57
1	SHP 20b	Post-tx	31.9	12.11	0	NA	0	NA
1	SHP 21a	Pre-tx	248	37.79	0	NA	0	NA
1	SHP 21b	Post-tx	1.8	1.24	0	NA	0	NA
1	SHP 22a	Pre-tx	239.4	270.45	0	NA	0	NA
1	SHP 22b	Post-tx	0.1	0.23	0	NA	0	NA
1	SHP 23a	Pre-tx	20.6	12.45	1.2	0.91	0	NA
1	SHP 23b	Post-tx	0	NA	0	NA	0	NA
1	SHP 24a	Pre-tx	0.7	1.02	0	NA	0	NA
1	SHP 25a	Pre-tx	594.1	198.93	3.1	0.45	0.6	0.23
1	SHP 26a	Pre-tx	1019.1	303.60	61.1	15.39	1.8	0.45
1	SHP 27a	Pre-tx	3.3	NA	0	NA	0	NA
1	SHP 28a	Pre-tx	337	114.18	7.1	2.83	0.3	NA
1	SHP 29a	Pre-tx	335.8	401.83	0.8	1.24	0	NA
2	SHP 30a	Pre-tx	714.2	161.14	0	NA	0	NA
2	SHP 30b	Post-tx	0	NA	0	NA	0	NA
3	SHP 31a	Pre-tx	196.7	53.52	0	NA	23.3	13.01
3	SHP 32a	Pre-tx	113.3	62.35	0	NA	0	NA
4	SHP 33a	Pre-tx	1158	NA	0	NA	0	NA

Table 12. – Mean EPG and 95% confidence intervals of GINs in sheep fecal samples.

NA: not applicable.

3-1-1-1-2- FEC result of goat sample

Farm	Sample	Type	Total GINs		*Nematodirus spp.*		*Trichuris spp.*	
			EPG mean	95% confidence	EPG mean	95% confidence	EPG mean	95% confidence
3	GOT 11	Pre-tx	416	39.72	0.0	NA	0.0	NA

Table 13. – Mean EPG and 95% confidence intervals of GINs in goat fecal sample.

NA: not applicable.

3-1-1-1-3- FEC results of llama samples

Farm	Sample	Type	Total GINs		*Nematodirus spp.*		*Trichuris spp.*	
			EPG mean	95% confidence	EPG mean	95% confidence	EPG mean	95% confidence
1	LAM 11a	Pre-tx	76.4	30.55	2.0	1.13	0.0	NA
1	LAM 12a	Pre-tx	526.6	393.11	1.0	1.36	0.1	0.23
1	LAM 12b	Post -tx	185.2	124.25	0.0	NA	0.0	NA
1	LAM 13a	Pre-tx	21.3	13.47	1.8	1.36	0.1	0.23

Table 14. – Mean EPG and 95% confidence intervals of GINs in llama fecal samples.

NA: not applicable.

3-1-1-1-4- FEC results of alpaca samples

Farm	Sample	Type	Total GINs		*Nematodirus spp.*		*Trichuris spp.*	
			EPG mean	95% confidence	EPG mean	95% confidence	EPG mean	95% confidence
5	ALP 11a	Pre-tx	41.4	31.46	11.6	16.18	0.4	1.13
5	ALP 11b	Post-tx	105.0	151.97	10	13.92	2.6	3.85
5	ALP 12a	Pre-tx	11.6	11.88	3.0	4.53	1.3	2.72
5	ALP 12b	Post-tx	26.8	26.71	2.9	4.07	5.0	7.58
5	ALP 13a	Pre-tx	46.1	57.03	5.4	7.36	7.9	9.73
5	ALP 13b	Post-tx	38	57.71	12.0	14.94	5.0	4.30
5	ALP 14a	Pre-tx	17.0	22.18	5.3	14.60	3.9	7.24
5	ALP 14b	Post-tx	10.0	12.45	2.0	3.96	1.0	1.58
6	ALP 15a	Pre-tx	11.9	7.69	0.1	0.23	0.1	0.23
6	ALP 16a	Pre-tx	1.6	1.70	0	NA	1.3	1.47
6	ALP 17a	Pre-tx	4.1	2.26	0.1	0.23	3	1.36
6	ALP 18a	Pre-tx	8.0	0.34	0	NA	0.3	0.34
7	ALP 19a	Pre-tx	137.9	56.24	0	NA	0	NA
8	ALP 20a	Pre-tx	35.7	7.24	2.2	1.47	0	NA
8	ALP 21a	Pre-tx	2.8	1.92	0.6	0.79	0	NA

8	ALP 21a	Pre-tx	1.3	0.34	0.8	0.45	0	NA
8	ALP 22a	Pre-tx	1.1	0.45	0.6	0.79	0	NA
8	ALP 23a	Pre-tx	15.3	5.32	10	4.98	0	NA
8	ALP 24a	Pre-tx	8.3	2.15	1.8	0.91	0	NA
8	ALP 25a	Pre-tx	5.7	2.38	5.1	2.15	0	NA

Table 15. – Mean EPG and 95% confidence intervals of GINs in alpaca fecal samples.

NA: not applicable.

3-1-1-2- Fecal egg count reduction test (FECRT)

FECRT was applied only in fifteen groups that were treated with IVM. Individual animals or groups with low EPG count were not treated by the veterinarian based on the FEC results. The FECRT was calculated with the formula mentioned in chapter 2, and the results are shown in Table 16.

Farm	Sample	Total EPG pre-tx	Total EPG post-tx	FECRT (%)
Farm 1	SHP 15	61.7 ± 34.5	12.2 ± 3.4	80.2
Farm 1	SHP 16	120.4 ± 28.2	5.4 ± 1.4	95.5
Farm 1	SHP 17	31.9 ± 14.8	0.6 ± 0.2	98.1
Farm 1	SHP 18	0.0	4.8 ± 1.9	0.0
Farm 1	SHP 19	35.2 ± 22.0	16.3 ± 5.6	53.7
Farm 1	SHP 20	1013.2 ± 139.7	31.9 ± 10.7	96.9
Farm 1	SHP 21	248.0 ± 33.4	1.8 ± 1.1	99.3
Farm 1	SHP 22	239.4 ± 239	0.1 ± 0.2	100.0
Farm 1	SHP 23	20.6 ± 11.0	0.0	100.0
Farm 2	SHP 30	714.2 ± 142.4	0.0	100.0
Farm 1	LAM 12	526.6 ± 347.4	185.2 ± 109.8	64.8
Farm 5	ALP 11	41.4 ± 27.8	105.0 ± 134.3	0.0
Farm 5	ALP 12	11.6 ± 10.5	26.8 ± 23.6	0.0
Farm 5	ALP 13	46.1 ± 50.4	38.0 ± 51.0	17.6
Farm 5	ALP 14	17.0 ± 19.6	10.0 ± 11.0	41.2

Table 16. – FECRT (%), GINs EPG for pre-tx and post-tx samples of different ruminant farms.

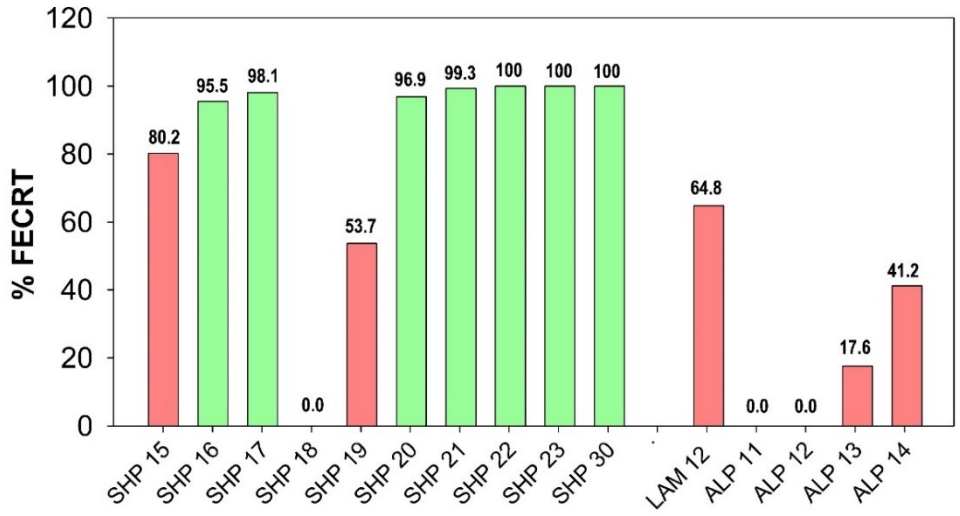


Figure 11. – FECRT (%) of different ruminant farms treated with IVM.

optimal (<95%), inefficient or indicative of resistance (<90%).

3-1-1-3- Microscopic identification of *Nematodirus* and *Trichuris* species

During coprology analysis, *Nematodirus* and *Trichuris* species were identified based on their specific size and shape as described in chapter 2 and the results are shown below.

	Farm 1		Farm 2	Farm 3		Farm 4	Farm 5	Farm 6	Farm 7	Farm 8
	Sheep	Llama	Sheep	Sheep	Goat	Sheep	Alpaca	Alpaca	Alpaca	Alpaca
Nematodirus spp.	pos	pos	neg	neg	neg	neg	pos	pos	neg	pos
Trichuris spp.	pos	pos	neg	pos	neg	neg	pos	pos	neg	neg

Table 17. – *Nematodirus* and *Trichuris* species identified in different ruminant farms.

spp.: species; pos= positive; neg=negative.

3-1-1-4- Larval Development assay (LDA)

LDA was performed with alpaca samples (farm #5) and sheep samples (farm #3). For farm #5 the LDA was run in 3 replicates, whereas for farm #3 it was only run with 2 replicates due to the low amount of fecal material. As shown in Figure 12, in alpaca farm, third stage larvae developed even in 16ηM concentration of IVM; however, in sheep samples (farm #3) L3s are present until 8ηM concentration of IVM (Figure 13).

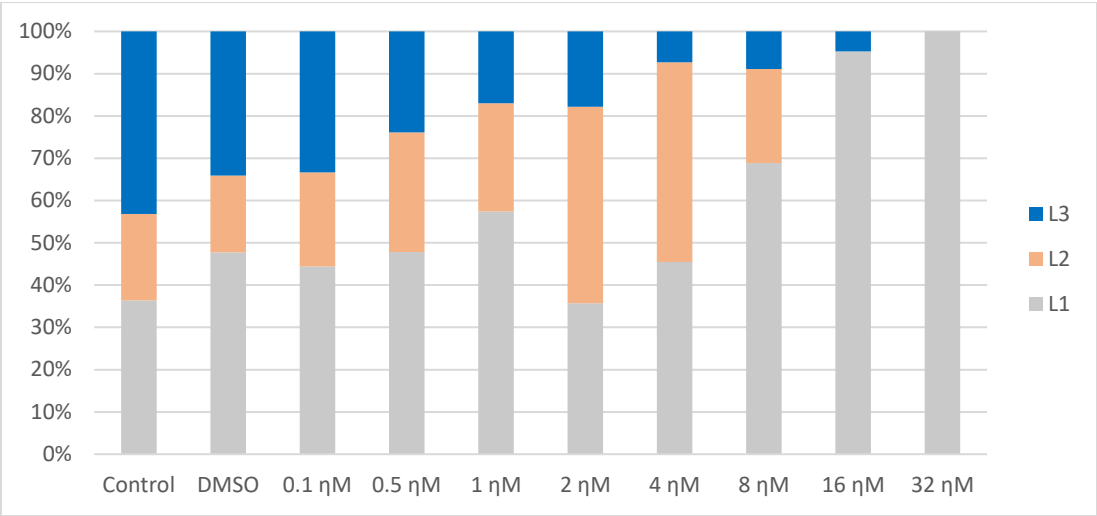


Figure 12. – LDA results for farm #5 with mean percentage of larval stages in control, DMSO and IVM treatment groups.

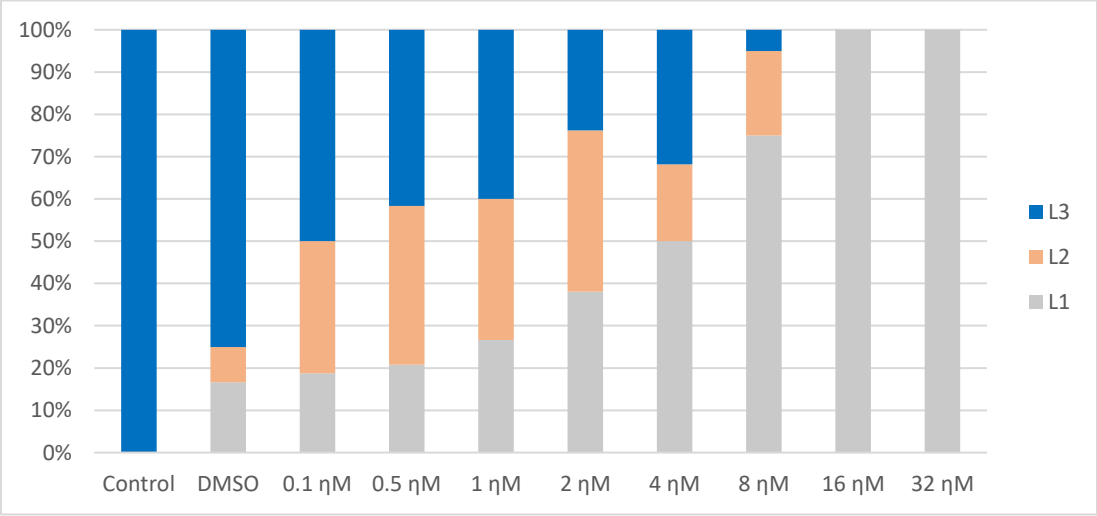


Figure 13. – LDA results for farm #3 (sheep) with mean percentage of larval stages in control, DMSO and IVM treatment groups.

3-1-1-5- Peanut agglutinin (PNA) staining for *H. contortus'* eggs identification

In order to identify *H. contortus* eggs among other GIN eggs, PNA staining was performed. After microscopy examination, *H. contortus* eggs were identified in farm 1 (sheep and llama samples) (Figure 14) and farm 2 (sheep sample) (Figure 15) by using this method. However, due to the presence of high debris in the final specimen and also the decrease of fluorescence signal by

passing time, it became complicated to identify *H. contortus* eggs; as a result, molecular identification replaced this method.

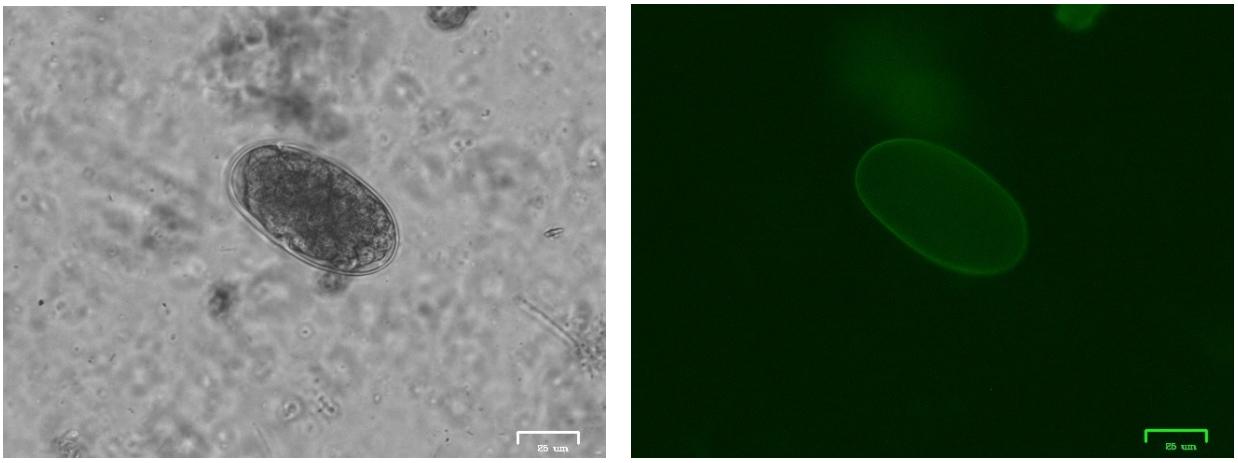


Figure 14. – *H. contortus* egg in sample farm #1 with PNA staining.

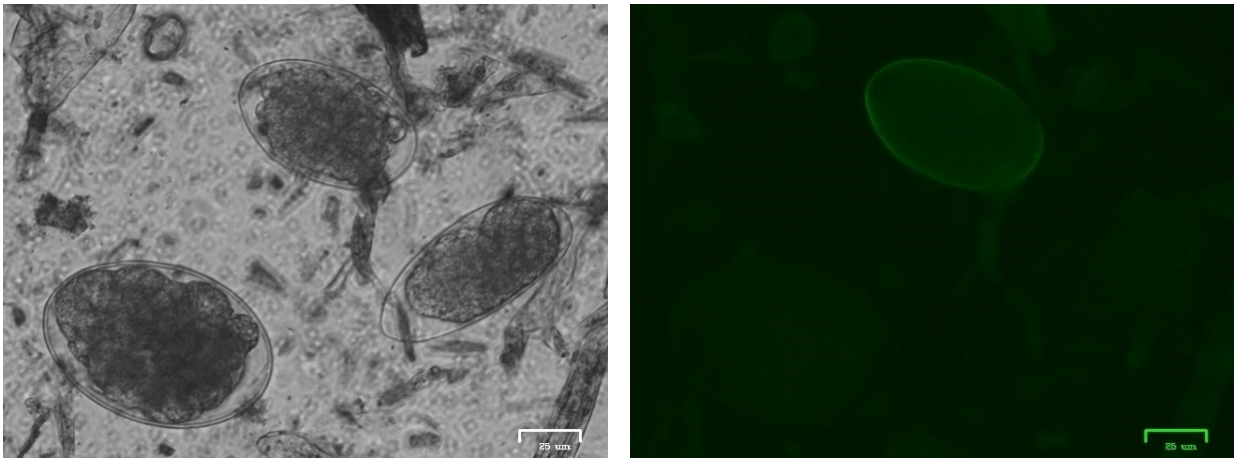


Figure 15. – *H. contortus* egg among other GIN eggs in farm #2 with PNA staining.

3-1-2- Molecular identification of GIN species

GIN species were identified in different ruminant farms, as shown in Table 18. Furthermore, species identification was not applicable to farm #8 (alpaca samples) due to unsuccessful DNA extraction.

	Farm 1		Farm 2	Farm 3		Farm 4	Farm 5	Farm 6	Farm 7	Farm 8
	Sheep	Llama	Sheep	Sheep	Goat	Sheep	Alpaca	Alpaca	Alpaca	Alpaca
H. contortus *	pos	pos	pos	pos	pos	pos	pos	pos	pos	NA
T. circumcincta *	pos	pos	pos	pos	neg	pos	neg	neg	neg	NA
t. axei *	pos	pos	neg	pos	neg	neg	neg	neg	neg	NA
*T. colubriformis**	neg	neg	pos	neg	neg	neg	neg	neg	neg	NA
T. vitrinus *	pos	pos	pos	pos	pos	pos	pos	pos	neg	NA
O. venulosum *	pos	neg	neg	neg	neg	neg	neg	pos	neg	NA
C. curticei *	pos	neg	neg	neg	neg	neg	neg	neg	neg	NA
Ch. ovina *	pos	pos	neg	pos	neg	neg	neg	neg	neg	NA
C. mentulatus *	NA	neg	NA	NA	NA	NA	pos	pos	pos	NA
L. chavezi **	NA	neg	NA	NA	NA	NA	pos	neg	neg	NA
M. marshalli *	NA	neg	NA	NA	NA	NA	pos	neg	neg	NA

Table 18. – The list of identified GIN species in different ruminant farms.

pos=positive; neg=negative; NA=not applicable; *identified by ITS-2 gene; ** identified by COX-1 gene.

3-2- Gene expression analysis on ABC transporters from *H. contortus* field isolates

Melting curves for all *H. contortus* ABC transporter genes in qPCR analysis are shown below in Figures 16 and 17, which reveal the specific peak for each set of primers.

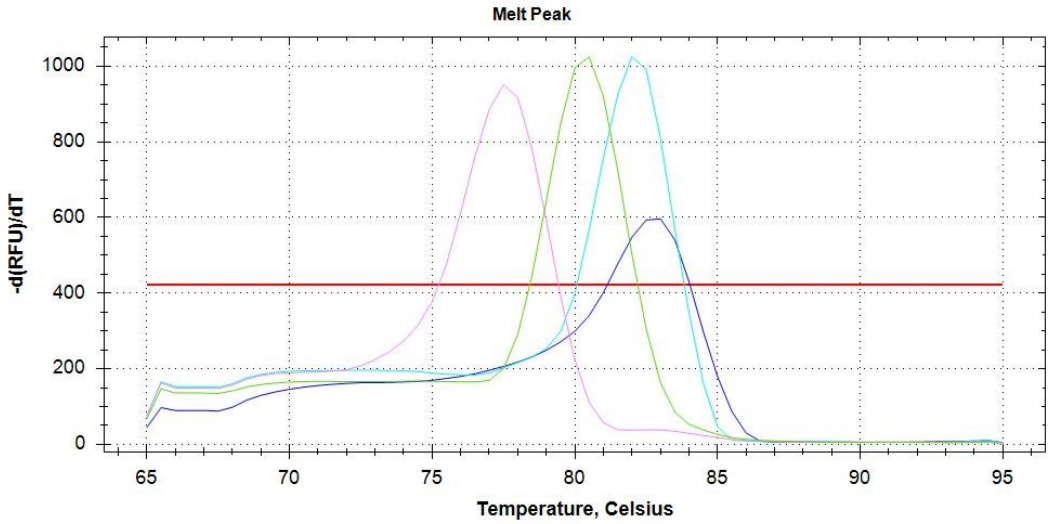


Figure 16. – Melt peaks from qPCR of housekeeping and ABC transporter genes.

actin, *mrp-5*, *abcf-2*, *pgp-3*.

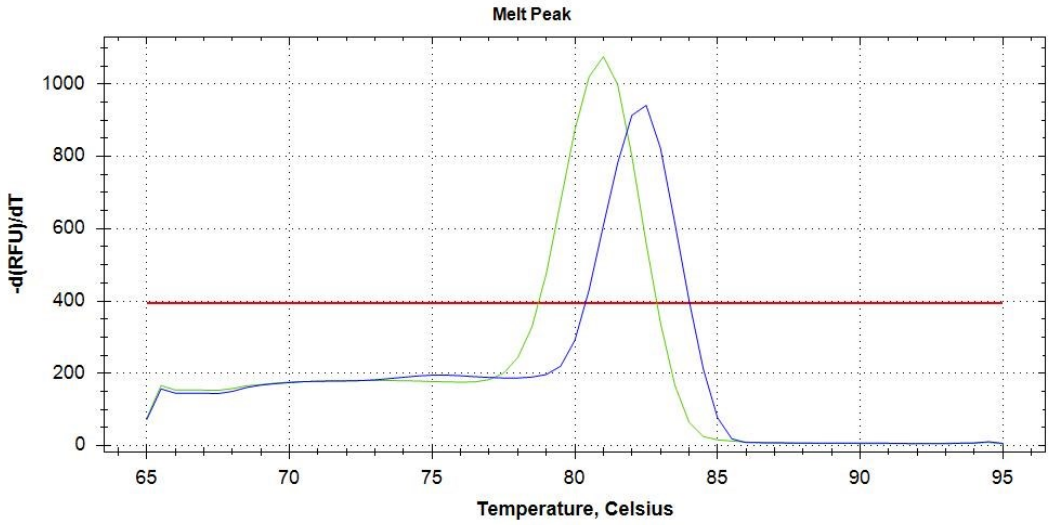


Figure 17. – Melt peaks from qPCR of housekeeping and *pgp-2* genes.

GAPDH, *pgp-2.*

As shown in Figure 18, *pgp-2* and *pgp-3* genes are overexpressed (13.11 and 2.85 by fold change, respectively) significantly compared with PF control (susceptible); however, *mrp-5* and *abcf-2* genes are significantly downregulated (10.39 and 2.24 by fold change respectively) significantly in comparing with susceptible strain.

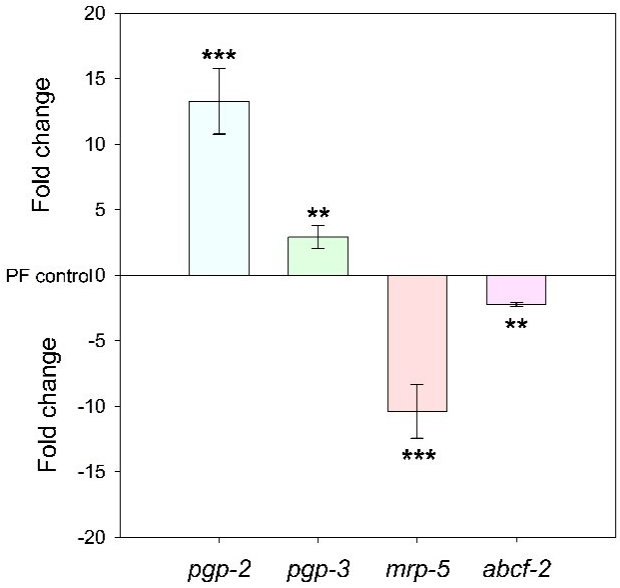


Figure 18. – Fold changes of relative ABC transporter genes expression in farm #5 (alpaca) LDA treated groups (IVM) compared with PF control.

**P<0.01; ***P<0.001.

3-3- Lipidomic Excretory/secretory products (ESP) profile of *H. contortus* and the involvement of ABC transporters in their extrusion

3-3-1- Lipidomic ESP profile of *H. contortus* non-treated control group

In total, 2562 lipid features were positively and putatively identified from media (*H. contortus* incubated) collected at time 0 (RPMI) 2h, 4h and 8h samples, as shown in Table 19. As a result, 262 lipids were identified positively with tandem mass spectrometry (MS/MS) in tier 1 and 2. Moreover, 2300 lipid features were identified putatively in tier 3 by matching with LIPID MAPS data base (http://www.lipidmaps.org/databases). The number of identified lipid features belonging to each sample and common features among samples are shown in Figure 19.

Lipids identification method	No. of lipid features
Tier 1: MS/MS match score ≥ 500; precursor m/z error ≤ 5mDa	178
Tier 2: MS/MS match score ≥ 100; precursor m/z error ≤ 5mDa	84
Tier 3 (MS match): Mass match with m/z error ≤ 5mDa; (LipidMaps)	2300
Total number of identified lipid features	2562

Table 19. – Number of identified lipid features by three tier approach.

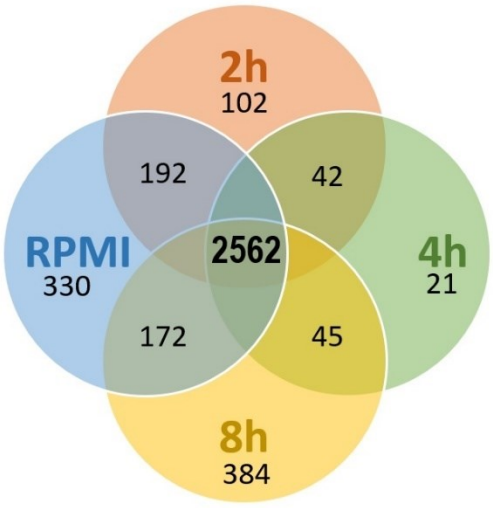


Figure 19. – Venn diagram showing the number of identified lipid features in four samples (RPMI, 2h, 4h & 8h).

After normalizing identified lipid features data with RPMI (time 0), 1057 *H. contortus* ESP (Hc-ESP) lipids were identified among three tiers, which consist of lipids from different classes as shown in Figure 20. In total, 171 positive and 886 putative unique lipid features were identified in Hc-ESP. The most predominant lipids of Hc-ESP are Phosphatidylcholines (PC), Fatty Acyls (FA), Triacylglycerols (TG), Monoacylglycerols (MG) and Sterols (ST).

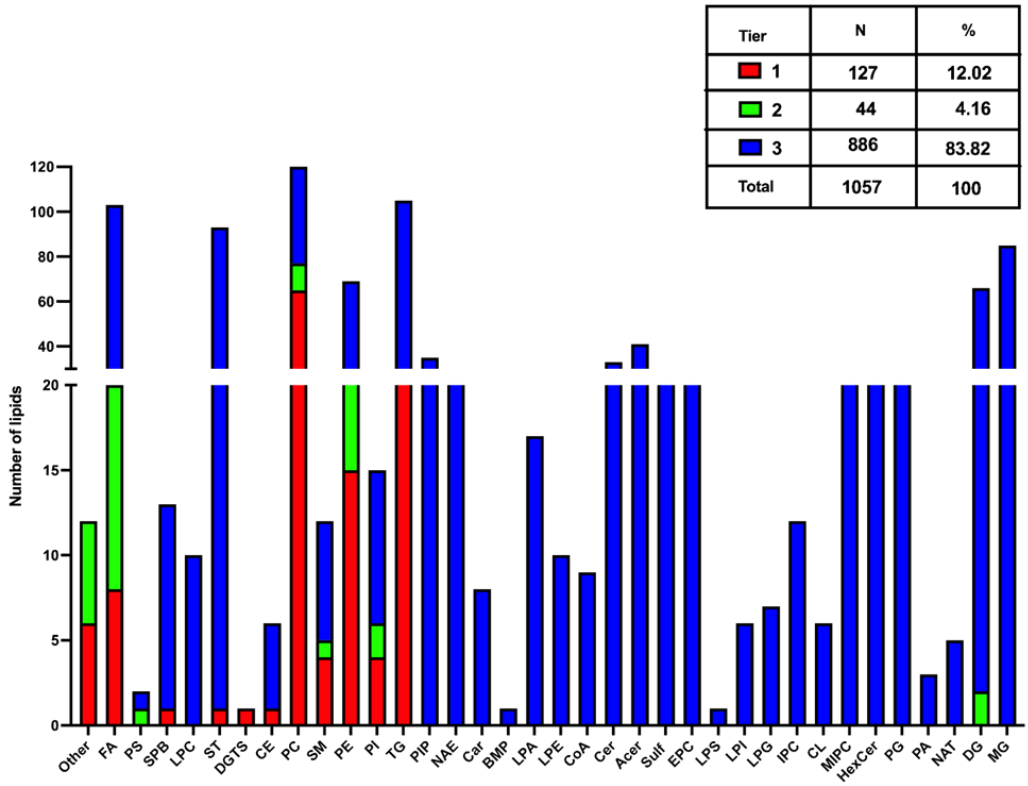


Figure 20. – Identified Hc-ESP lipids in different subclasses among three tiers.

Fatty acids and conjugates (FA); Phosphatidylserines (PS); Sphingoid bases or sphingoid base-phosphates (SPB); Lysophosphatidylcholine (LPC); Sterols (ST); Diacylglyceryltrimethylhomoserine (DGTS); Cholesteryl esters (CE); Phosphatidylcholines (PC); Sphingomyelins (SM); Phosphatidyl ethanolamines (PE); Phosphatidylinositols (PI); Triacylglycerols (TG); Phosphatidylinositol-phosphates (PIP); N-acyl ethanolamines (endocannabinoids) (NAE); Fatty acyl carnitines (Car); Bio(monoacylglycerol)phosphates (BMP); Lysophosphatidic acid (LPA); Lysophosphatidyl-ethanolamine (LPE); CoEnzyme A's (CoA); Ceramides or ceramide phosphates (Cer); Acylceramides (Acer); Sulfoglycosphingolipids (sulfatides) (Sulf); Ceramide phosphoethanolamines (EPC); Lysophosphatidylserine (LPS); Lysophosphatidylinositol (LPI); Lysophosphatidylgylcerols (LPG); Ceramide phosphoinositols (IPC); Cardiolipins (CL); Ceramide phosphoinositols (MIPC);

Hexosylceramides (HexCer); Phosphatidylglycerol (PG); Phosphatidic acid (PA); N-acyl amines or taurines (NAT); Diacylglycerols (DG); Monoacylglycerols (MG).

Furthermore, we applied Principal Component Analysis 2-dimensional (PCA 2D) and Partial least squares discriminant analysis (PLS-DA) at three time point and RPMI (time 0). As shown in Figure 21, the identified lipids clustered in each sample which shows the identified lipids at three time points post incubation (2h, 4h & 8h) are different from the identified lipids at RPMI (time 0).

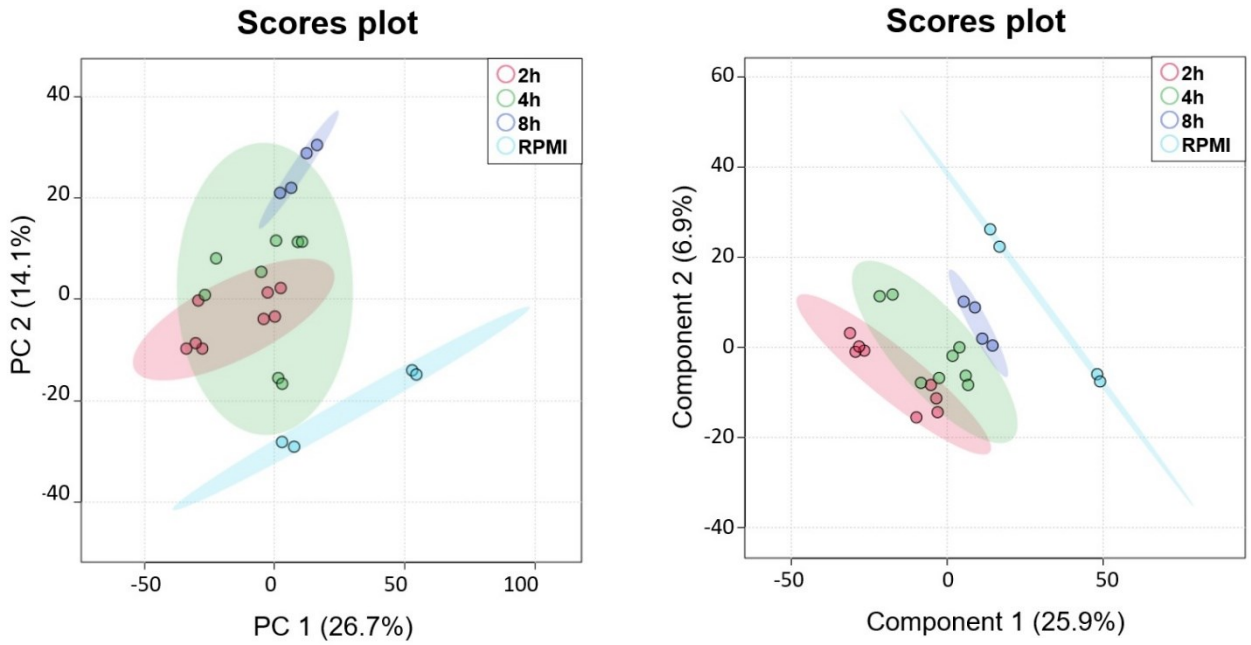


Figure 21. – PCA 2D scores plot (left) and PLS-DA scores plot (right).

Furthermore, we analyzed the significantly altered lipids concerning the lipid subclasses (Figure 22) and main lipid categories (Figure 23). As a result, at 2h post incubation, totally 258 lipids significantly altered comparing with time 0 (RPMI). Phosphatidylcholines (N=57), Fatty acids and conjugates (N=31), Phosphatidyl ethanolamines (N=30), sterols (N=19), N-acyl ethanolamines (endocannabinoids) (N=17), Diacylglycerols (N=15) and Sulfatides (N=10) were the most altered lipids at 2h comparing with RPMI. In comparing identified lipids at 4h with 2h, only eight lipids altered significantly: belonging to Fatty acyl carnitines (N=1), N-acyl ethanolamines (endocannabinoids) (N=2), Diacylglycerols (N=1), Lysophosphatidylcholine (N=1), Lysophosphatidyl-ethanolamine (N=1) and Ceramides or ceramide phosphates (N=1). Finally, at 8h in total 126 lipids were altered significantly comparing with 4h and the highest number of

identified lipids were Phosphatidylcholines (N=25), Phosphatidyl ethanolamines (N=20), Diacylglycerols (N=14), and sterols (N=11).

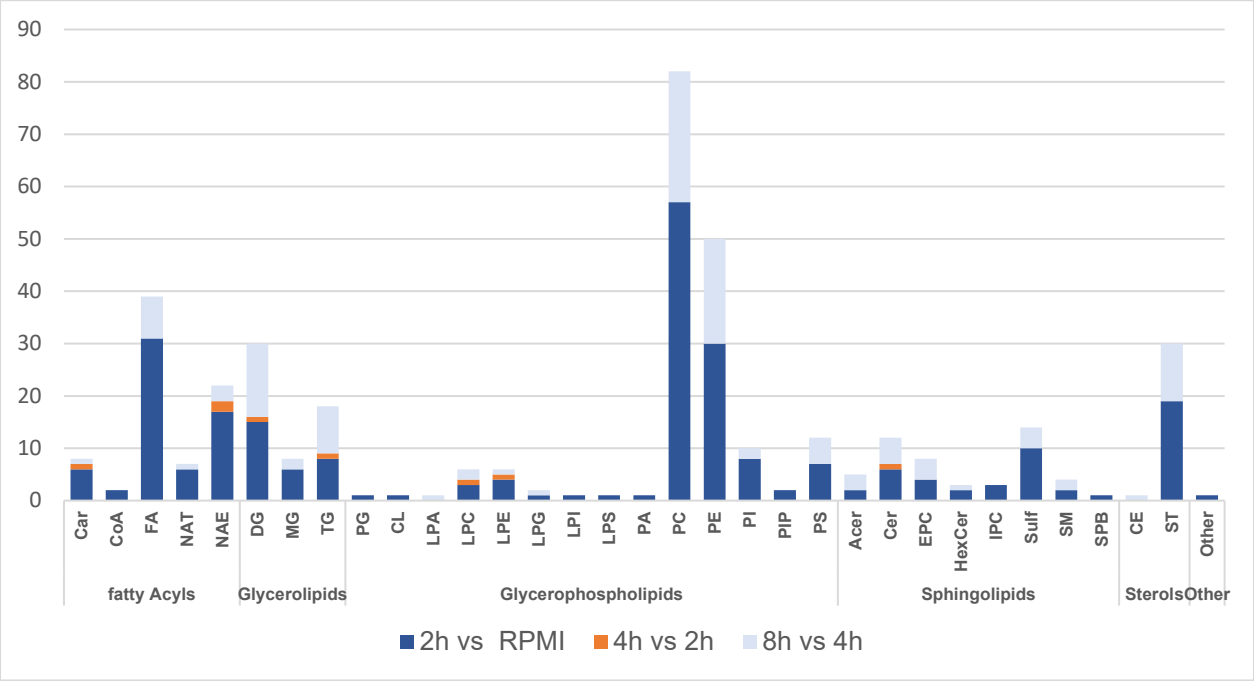


Figure 22. – Significantly altered lipids among different time points in each subclass.

Fatty acyl carnitines (Car); CoEnzyme A's (CoA); Fatty acids and conjugates (FA); N-acyl amines or taurines (NAT); N-acyl ethanolamines (endocannabinoids) (NAE); Diacylglycerols (DG); Monoacylglycerols (MG); Triacylglycerols (TG); Phosphatidylglycerol (PG); Cardiolipins (CL); Lysophosphatidic acid (LPA); Lysophosphatidylcholine (LPC); Lysophosphatidyl-ethanolamine (LPE); Lysophosphatidylgylcerols (LPG); Lysophosphatidylinositol (LPI); Lysophosphatidylserine (LPS); Phosphatidic acid (PA); Phosphatidylcholines (PC); Phosphatidyl ethanolamines (PE); Phosphatidylinositols (PI); Phosphatidylinositol-phosphates (PIP); Phosphatidylserines (PS); Acylceramides (Acer); Ceramides or ceramide phosphates (Cer); Ceramide phosphoethanolamines (EPC); Hexosylceramides (HexCer); Ceramide phosphoinositols (IPC); Sphingomyelins (SM); Sulfoglycosphingolipids or sulfatides (Sulf); Sphingoid bases or sphingoid base-phosphates (SPB); Cholesteryl esters (CE); Sterols (ST);

In the meantime, we compared the main lipid categories that are altered significantly in different timepoints (figure 23). The highest fold changes (FC) belong to 2h comparing with RPMI time (0) in which sphingolipids has the highest fold change and then Glycerolipids and

Glycerophospholipids are the next most altered lipid categories in this time point. We observe that sphingolipid at 8h vs 4h sample have high fold changes.

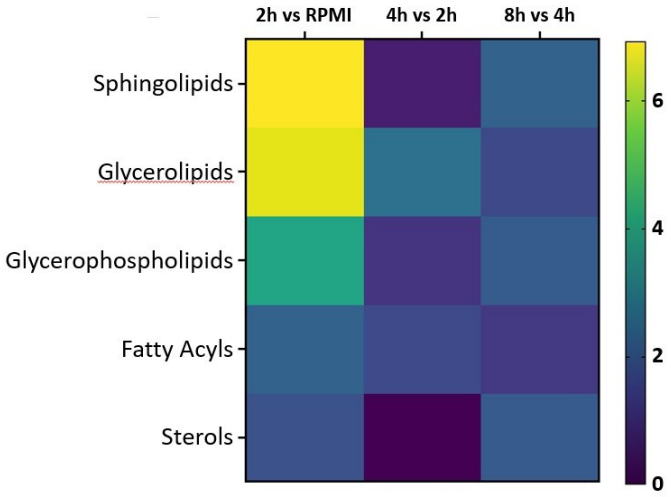


Figure 23. – Significantly altered lipids (categories) among different time points.

Finally, we identified the 15 most important lipids based on the VIP scores in PLS-DA among different time points (Figure 24).

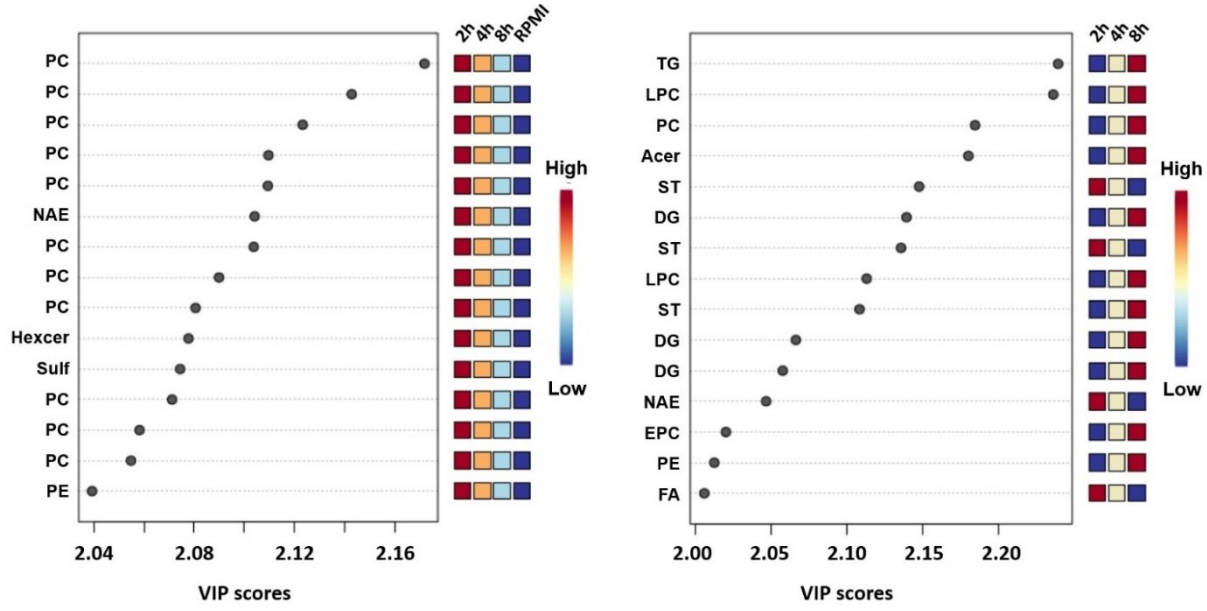


Figure 24. – Top 15 lipids with the highest VIP scores from the PLS-DA at different time points; without RPMI-blank (right); with RPMI-blank (left).

Fatty acids and conjugates (FA); Hexosylceramides (HexCer); Lysophosphatidylcholine (LPC); Sterols (ST); Phosphatidylcholines (PC); Phosphatidyl ethanolamines (PE); Triacylglycerols (TG); N-acyl ethanolamines (endocannabinoids) (NAE); Fatty acyl carnitines (Car); Lysophosphatidic acid (LPA); Acylceramides (Acer); Sulfoglycosphingolipids (sulfatides) (Sulf); Ceramide phosphoethanolamines (EPC).

The panel of lipids includes the RMPI (time 0) time-point groups consist mainly of phosphatidylcholines (N=11), N-acyl ethanolamine (N=1), Hexasocylceramide (N=1), Sulfatide (N=1), and Phosphatydylethanolamine (N=1). All the fifteen identified lipids have the same pattern in which they have the highest score at 2h. All the fifteen identified lipids do not show a significant different at 4h timepoint. However, at 2h timepoint, two sterols (ST), one Fatty acids and conjugates (FA) and one N-acyl ethanolamines (NAE) show the highest score at 2h and at the end of the incubation (8h) have the lowest scores. In contrast the remaining eleven lipids have the low score at 2h and by passing the time at 8h have the highest scores. Briefly, significant altered lipids abundance varies in time manner.

3-3-2- Lipidomic ESP profile of *H. contortus* PF strain treated with ABC transporter inhibitors

As mentioned before, in order to identify the role of ABC transporters in translocating lipids as ESP, *H. contortus* PF strain adult worms were treated with ABC inhibitors. As shown in Figure 25, the one hundred most important altered lipids have been upregulated and downregulated in the different treated groups. It is obvious that some lipids are downregulated in some groups, and even some of them are downregulated in all groups. Moreover, different ABC transporters inhibitors groups in three concentrations altered lipids distinctly which might be due to variable inhibitory effects of inhibitors on different ABC transporters and their role in extrusion of different groups of lipids. In order to better differentiate, the 20 most important lipids heatmap is also included which shows the alterations in different treated groups (Figure 26). Sterols are the most affected lipids (10 out of 20) showing down regulation among the treated groups. Polyketides are second lipid group with highest numbers (7 out of 10). One lipid feature corresponding to lysophosphatidic acid is downregulated in all treated groups. Also, there is one lipid of fatty acyls group and another one from FA carnitine class.

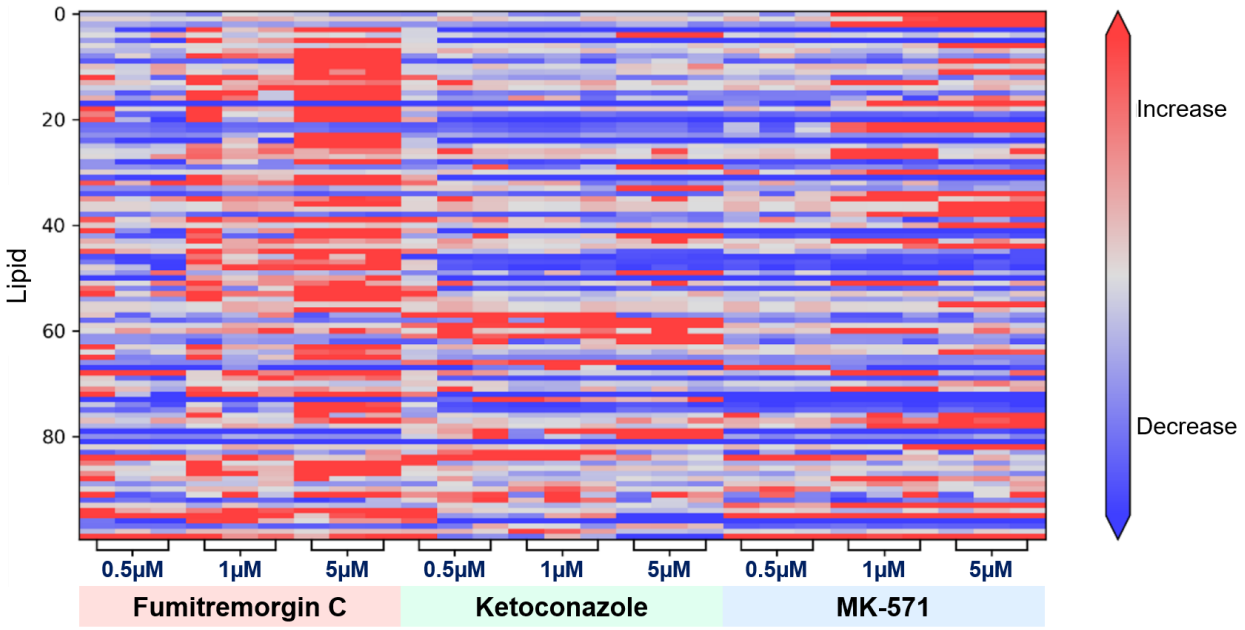


Figure 25. – Heatmap of top 100 altered lipids among nine treated groups.

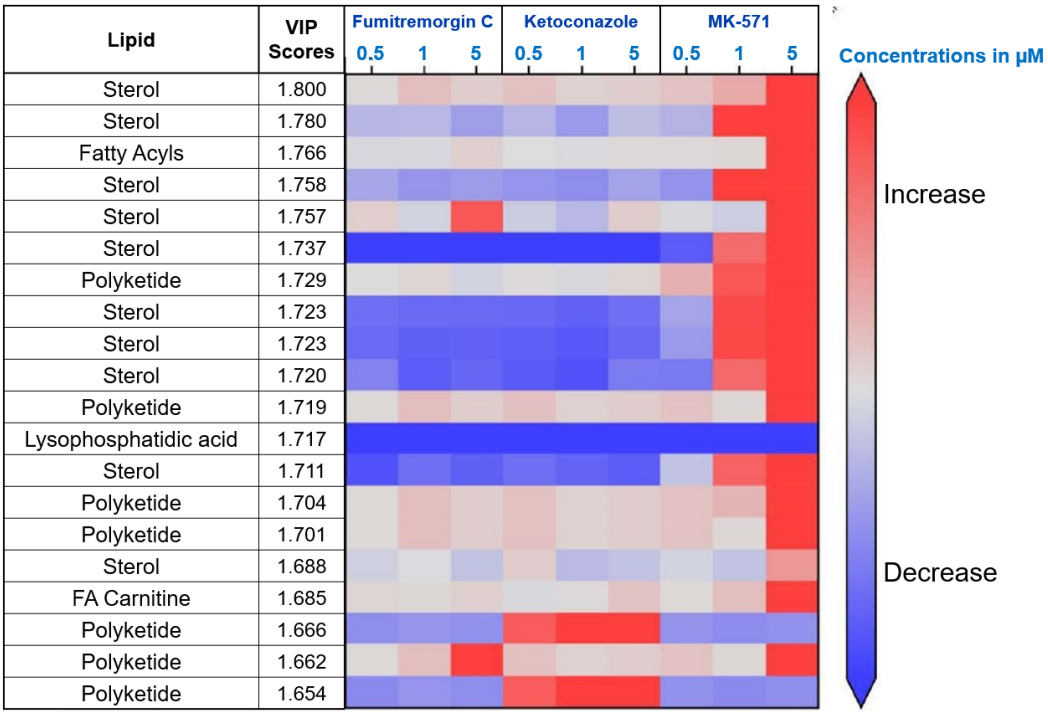


Figure 26. – Twenty most important altered lipids among nine treated groups.

Comparing the different ABC inhibitor groups at different time points (Figure 27), there are still alterations among the one hundred most important lipids at different time points. This variability

in timepoints shows the potential planned and purposeful secretion of these lipids by *H. contortus*. Deeper analysis selects the top twenty most important lipids altered among time points, there are diverse lipid species affected differently in time manner (Figure 28). Almost all the identified lipids are downregulated in Ketoconazole and MK-571 groups; in which some are completely down regulated such as three sterols, two polyketides, one prenol, one lysophosphatidylinositol and a fatty acyls' member. However, in Fumitremorgin C group, all of the lipids are upregulated or neutral. Moreover, almost all the identified lipids are down regulated by passing the time in Fumitremorgin C treated group. One of the identified lysophosphatidylinositols is upregulated in all groups except in 8h timepoint in Mk-571 treated group.

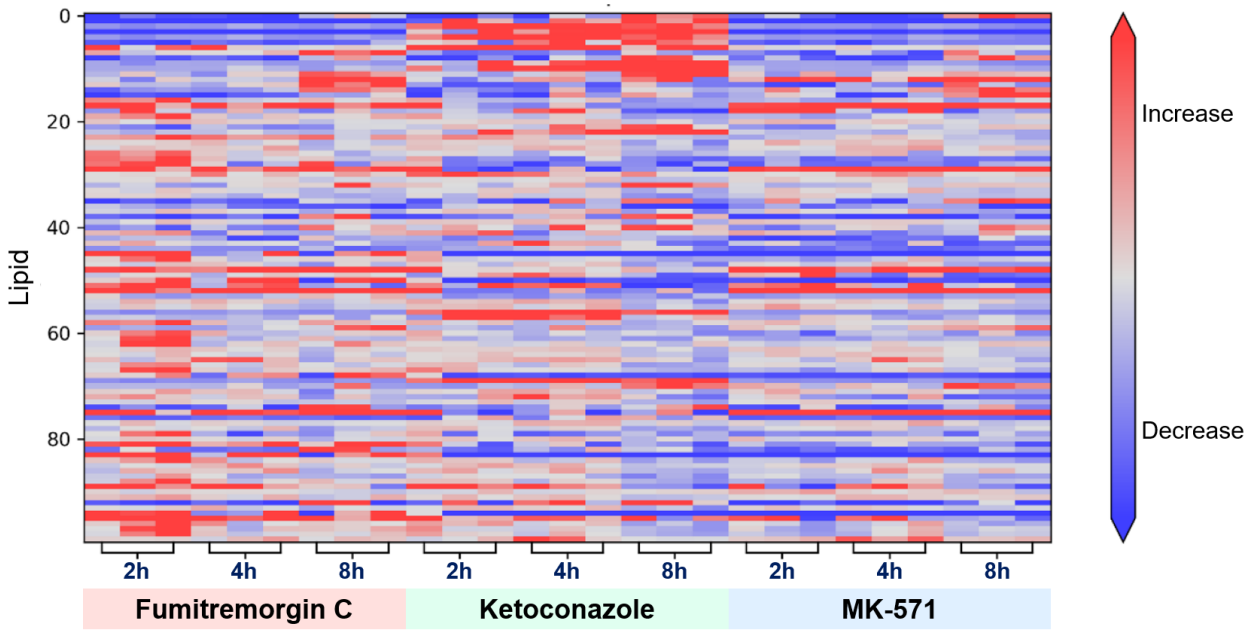


Figure 27. – Heatmap of top 100 altered lipids among 2h, 4h and 8h in treated groups.

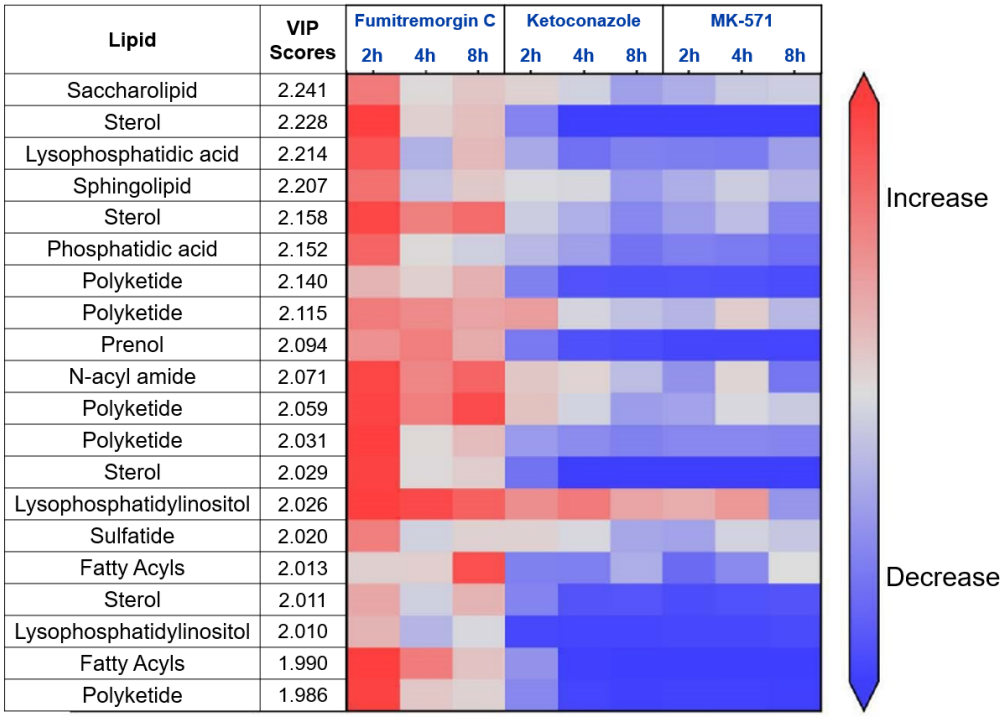


Figure 28. – Twenty most important altered lipids among nine treated groups.

3-3-3- Relative ABC transporters' gene expression analysis

Melting curves for all *H. contortus* ABC transporter genes in qPCR analysis are shown below in Figures 29 and 30, which reveal the specific peak for each set of primers.

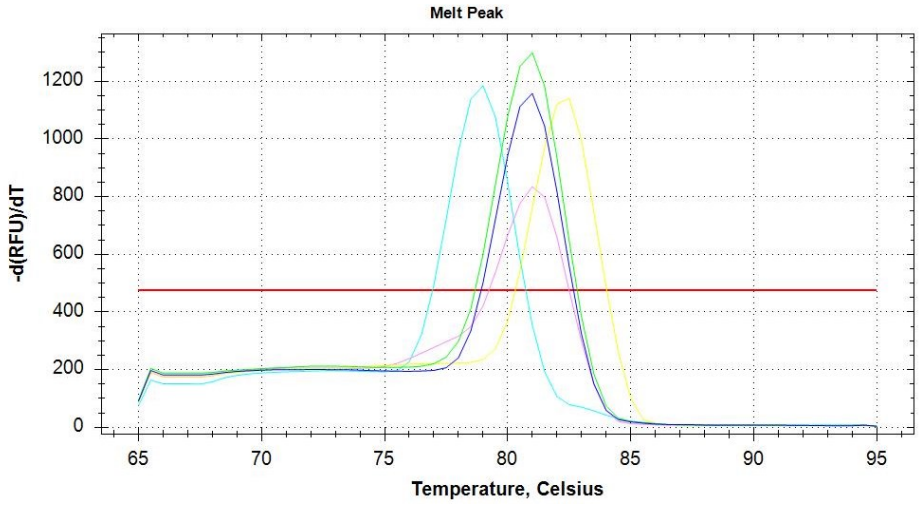


Figure 29. – Melt peaks from qPCR of Housekeeping and ABC transporter genes.

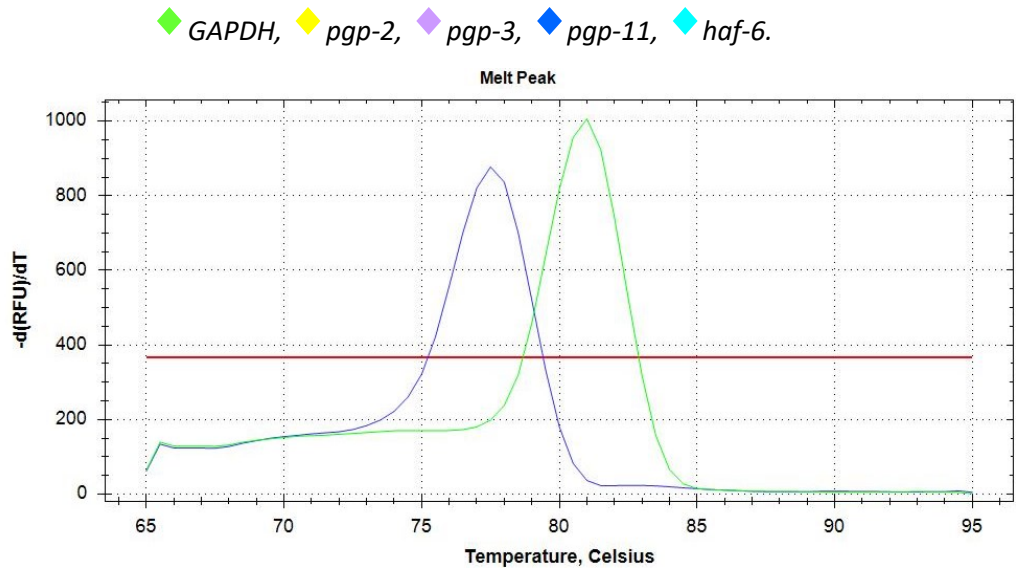
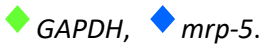


Figure 30. – Melt peaks of Housekeeping and ABC transporter genes primers in qPCR.



At the end of the incubation (8h) of control and treatment groups, the relative gene expression of some important ABC transporters was analyzed, and the results are shown in Figure 31. *pgp-2* upregulated significantly in all treated groups except in Ketoconazole 5µM group, which shows downregulation. Moreover, *pgp-3*, *haf-6* and *mrp-5* show upregulation in all treated groups compared with the control group, which is significant ($p<0.05$) except for Fumitromorgin C 0.5µM and 5µM groups for *pgp-3* and *haf-6*, respectively. In the meantime, *haf-6* in ketoconazole 0.5µM has the greatest fold change. In addition, *pgp-11* was downregulated significantly ($p<0.05$) in all treatment groups in comparison with control group.

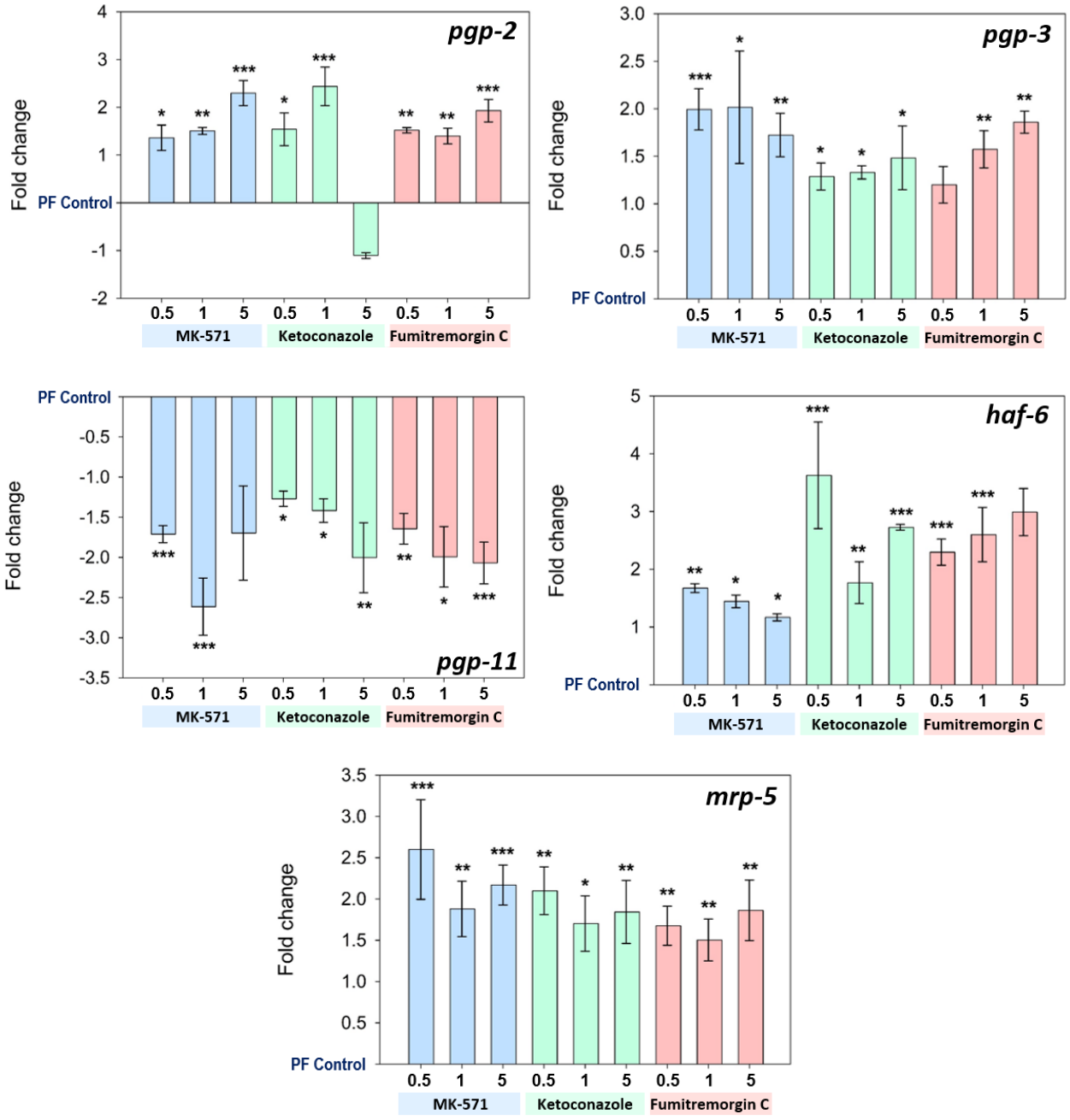


Figure 31. – Fold changes of ABC transporter genes in treatment groups compared with the non-treated control group.

Chapter 4 – Discussions

4-1- Diverse GIN species identification and varied IVM efficacy in different ruminant farms

As anthelmintic resistance (AR) is a general problem in the control of GINs in several livestock species worldwide, one of our objectives addressed the efficacy of IVM, the most common anthelmintic compound used in animal health, against GINs, including *H. contortus*. Moreover, there is no officially published molecular study regarding the GINs prevalence, diversity, infection intensity and IVM resistance in small ruminants and, particularly, in camelid farms in Quebec, Canada. However, there are published data in other provinces of Canada, including Alberta, British Columbia, Manitoba, Ontario Saskatchewan, regarding the IVM resistance and prevalence of GIN species in sheep farms [99, 135]. Also, two old and small studies were done on a limited number of individual sheep by necropsy in Saskatchewan and Alberta provinces in 1979 and 2002, respectively [136, 137]. Additionally, Mederos et al. (2010) cultured fecal eggs to L3s in order to identify different GIN species in sheep farms of Ontario and Quebec [138].

Among various groups of animals in different farms recruited in this study, FEC results varied between 0.0 and 1158.0 EPG, involving thirteen different GIN species. In total, ten and eleven GIN species were identified in small ruminant and camelid farms, respectively. *H. contortus* was the most prevalent species in all of the small ruminant and camelid farms. The high prevalence of *H. contortus* is in agreement with the findings from recent work by Queiroz et al. (2020) in 92 sheep farms in western Canada, including four provinces, where they reported 100% prevalence and 64% intensity for *H. contortus [99]*. Additionally, according to Mederos et al. (2010), *H. contortus* was the most prevalent GIN species (by the cultivation of larvae from fecal samples) in Quebec sheep farms [138]. However, in another study in the Quebec, *H. contortus* prevalence was reported at 73% in 11 sheep farms [98]. In addition, Moteane et al. (1979) reported 34% *H. contortus* prevalence among fifty necropsied sheep in Saskatchewan [137]. The increase in *H. contortus* prevalence during these years in sheep farms, despite the cold weather conditions in the Canadian provinces and the introduction of newer anthelmintics in recent years, reveals the

high survival capabilities inside and outside the host body [97, 99, 136, 137]. In this regard, climate change and anthelmintic resistance would be the most probable factors, and more studies are needed to determine their exact role [99].

T. circumcincta was identified in 80% and 25% of small ruminants and camelid farms, respectively; however, it has been reported in all sheep farms in western Canada [99]. Earlier, Mederos et al. (2010) reported *T. circumcincta* as the most prevalent GIN species detected in the cultivated fecal samples from sheep farms and necropsied lambs in Ontario [138]. Additionally, in a small-scale study on slaughtered lambs in Alberta, Colwell et al. (2002) reported 8-100% prevalence for *T. circumcincta* at different times of the year [136].

T. vitrinus was the second most prevalent GIN species identified in all small ruminant farms and in 75% of camelid farms. In contrast, Queiroz et al. (2020) reported a 50% prevalence in western Canada sheep farms. We identified *T. colubriformis* in only one sheep farm among the studied farms; however, in a recent study in four provinces, *T. colubriformis* was the third most abundant species (69% of the farms) [99]. *T. axei* was present in 60% of small ruminant farms but not detected in camelid farms. On the other side, Queiroz et al. (2020) reported 29% for *T. axei* prevalence in sheep farms in western Canada. In addition, *T. axei*, *T. colubriformis* and *T. vitrinus* worms were identified in 93%, 100% and 7% of necropsied lambs in Ontario, respectively [138]. Also, Moteane et al. (1979) reported the presence of *Trichostrongylus spp.* worms only in the small intestine of 38% of necropsied sheep. It should be noted that *T. axei* resides in abomasum, while the other *Trichostrongylus* species are found in the small intestine [1].

Ch. ovina prevalence was 40% and 25% in small ruminant and camelid farms, respectively, similar to the results on the western Canada study which reported a 35% prevalence in sheep farms [99]. However, in the older study conducted in Saskatchewan, the prevalence of *Ch. ovinia* was reported as 12% [137]. Moreover, the prevalence of *Ch. Ovinia* was reported to have a low proportion among cultivated GIN larvae from fecal samples collected in Ontario and Quebec sheep farms [138].

Prevalence of *O. venulusom* in small ruminant and camelid farms were 20% and 25%, respectively. This finding is in agreement with the earlier study conducted in western Canada in which they reported 21% of *O. venulosum* prevalence in sheep farms [99].

C. curticei was identified only in 20% of small ruminants and did not identify in camelid farms. In contrast, in a necropsy-based study Moteane et al. (1979) reported *Cooperia* worms in only 6% of sheep. In the epidemiologic study in western Canada, the authors did not report *C. curticei*, but they reported *C. oncophora* in a very low percentage (< 5%) of sheep farms [99].

In addition, *Nematodirus* and *Trichuris* species were identified in FEC under a conventional microscope. Therefore *Nematodirus* spp. was identified in 20% of small ruminant and 80% of camelid farms. Also, we identified *Trichuris* spp. in 40% and 60% of small ruminant and camelid farms, respectively. Surprisingly, despite using a high-sensitive species identification method, Queiroz et al. (2020) did not report any data regarding the prevalence of *Nematodirus* and *Trichuris* species. Colwell et al. (2002) reported *Nematodirus* spp. varying between 0-80% and *Trichuris* 11-40% in slaughtered lambs of central Alberta at different times of the year. Moreover, in Saskatchewan, *Nematodirus* and *Trichuris* worms were identified in 50% and 10% of necropsied sheep, respectively [137].

Finally, in camelids, *C. mentulatus*, *L. chavezi* and *M. marshalli* were identified in 75%, 25% and 25% of evaluated farms proportionately. To the best of our knowledge, there are no other reports on GINs prevalence in camelid farms in Canada. This is the first study addressing the identification of GIN species for this kind of ruminant productions. In an epidemiologic study of prevalence of GINs of alpacas in Australia, *C. mentulatus* was found in 69% of 89 evaluated farms; however, the authors did not report any data regarding the prevalence of *L. chavezi* and *M. marshalli* [27]. In camelids, *L. chavezi* was reported in 13-64% of different alpaca and llama breeds in South America [139]. As outlined above, in the first chapter, GINs prevalence could be directly affected by various factors such as the environmental (temperature, humidity), animal and herd health conditions, nutrition, management, etc.

In order to control various mentioned GINs in different ruminant farms, IVM is one of the most important used anthelmintics. However, there is a small data regarding IVM efficacy in Canada

and no published data in Quebec. It should be noted data are majorly done in sheep farms and camelids have not been studied in Canada. Therefore, we determined the IVM efficacy in different ruminant farms utilizing the FECRT, as recommended by the WAAVP (World Association for the Advancement of Veterinary Parasitology) guidelines [33, 115]. In total, the IVM efficacy varied between 0.0% and 100%, with an average of 63.2% among 15 groups of animals. IVM efficacy was optimal in 70% of sheep groups, and the remaining 30% had low efficacy. However, all the IVM-treated groups of camelids had low efficacy, with an average of 24.7% FECRT.

In another study in Canada, Falzon et al. (2013) reported 97% of sheep farms treated with IVM had FECRT below the optimal range (<95%), suggesting the presence of resistant GIN populations. Also, in the epidemiological study of western Canada, among 31 sheep farms that were treated with IVM, 30 farms (97%) showed the FECRT below the optimal range [99].

In order to prove the IVM resistance in GINs, including *H. contortus* more precisely, the larval development assay (LDA) is the further step to confirm the results of FERCT; however, there are many limitations regarding this assay, including the need for very fresh parasitic material, a high number of EPG, and is only readable to confirm ML resistance, for a couple of GIN species including *H. contortus* [140]. We tried to perform a LDA with one alpaca farm and all sheep and goat farms with high EPG, but unfortunately, we were only able to perform it with one alpaca farm (farm #5) with a very low IVM efficacy. Therefore, LDA was performed unsuccessfully on farms #2, #3 and #4, because the GIN eggs were not fresh, and the eggs did not hatch at the acceptable range. Furthermore, lack of sufficient amount of fecal material was another issue in farm #3. Nevertheless, we needed a *H. contortus* IVM-resistant strain (which was not available in our laboratory) as positive control to validate our initial LDA results and establish more clear conclusions.

It should be noted that we recruited an important number of farms for this project, however, only a limited number of farms fully collaborated with us. On the other hand, some fecal samples were collected individually instead of the pool samples, therefore, we made pool samples from the individual ones (in each group) to have an equal amount of fecal material from each individual to decrease the impact of sampling on the final FEC results. Moreover, the low amount of fecal

materials from some farms made challenging to complete three replicates of FEC and for GIN species identification. In addition, there is an unequal share of different GIN species among total FEC numbers in a sample, which made difficult to recover eggs, DNA extraction and molecular identification of GIN species, especially for those with very low abundance. Despite all these limitations, we identified a quite diverse GIN species not reported previously in Canada, using a more precise molecular method such as sequencing.

The diversity of identified GINs in different ruminant farms can give a more comprehensive view to veterinarians in Quebec in the face of gastrointestinal parasitic disease. This latter is much more important when dealing with alpacas, in which there is a very limited information on the GINs infection, especially when alpacas are pastured with other ruminants such as sheep and goats. On the other hand, ineffective IVM treatments, especially at very low percentages (0%) in some farms of Quebec, are critical signs of IVM resistance, which may result in a higher number of farms with resistant GIN populations in the near future. Therefore, the need for designing stricter guidelines for small ruminant anthelmintic therapy and the use of alternatives in order to reduce resistance GIN populations in Quebec province seems more crucial than before.

4-2- Assessment of the transcriptional level on ABC transporter genes from *H. contortus* field isolates

Previously, an overexpression of ABC transporter genes, most notably some p-glycoproteins (*pgps*) has been linked with a ML resistant phenotype in isolates from different GIN species [19, 92-94, 141]. Furthermore, *in vitro* experiments including *H. contortus* ML-resistant strains in presence of ABC transporter inhibitors has shown an increase in the susceptibility to IVM, consistent with the evidences that nematode ABC transporters may participate on the mechanisms of ML resistance in parasitic nematodes [142].

Most of the studies on gene expression levels of ABC transporters have been performed in *pgps* genes and mainly in adult stage of the parasite. However, in the present study, we measured the transcription level of *Hco-pgp-2*, *Hco-pgp-3*, *Hco-mrp-5* and *Hco-abcf-2* (half ABC transporter) genes in *H. contortus* larval stage (L3s). *Hco-pgps* are one of the first ABC transporters groups

known to be associated with ML resistance [91]. However, multidrug resistance associated proteins (MRPs) are also known to be involved in IVM detoxification in mammalian models and in *C. elegans* [91]. We extended the study of gene expression assessment on *H. contortus* ABC transporters including *Hco-abcf-2*, as some evidences in parasitic nematodes such as *Cooperia oncophora* and the human filarial *Onchocerca volvulus* have established a genetic association of half transporters with IVM resistance [94, 143].

Considering all these antecedents, we compared relative ABCs gene expression levels from larvae of *H. contortus* isolates (farm #5) that were suspected of IVM resistance with *H. contortus* susceptible (PF) strain [126].

We found that *Hco-pgp-2* was upregulated (FC=13.27) very significantly ($p<0.001$) in suspected IVM-resistant isolates compared with PF strain. Similarly, Prichard & Roulet (2007) and Reza et al. (2016) reported that in IVM-resistant *H. contortus*, in ML-resistant adult worm isolates and in L3s after selection with IVM, the transcriptional level of *Hco-pgp-2* was significantly upregulated.

In the present study we found that *Hco-pgp-3* was significantly upregulated ($p<0.01$) in suspected IVM-resistant isolates compared with PF susceptible strain. A previous report from Prichard and Roulet (2007) also described an over-expression of *Hco-pgp-C* (currently renamed as *Hco-pgp-3* from its common ancestor in *C. elegans Ce-pgp-3*) in *H. contortus* ML-resistant isolates [144]. In this free-living nematode, Broeks et al. (1995) reported that *Ce-pgp-3* participates actively in extruding natural toxins in *C. elegans* [111], suggesting that P-glycoproteins may have a wider activity in detoxification, including further transport of anthelmintic drugs such as the MLs. In addition, *Hco-pgp-3* is involved in host-parasite interactions in order to probably participate in the detoxification of host immune cell products [145], implying that nematode ABC transporters could represent a multipurpose extrusion system with implications in drug resistance and in the host-parasite interplay.

Furthermore, we observed relatively low gene expression levels of *mrp-5* and *abcf-2* in *H. contortus* field isolates in comparison with PF strain. Reza et al. (2016) did not report any significant alterations in expression levels of *mrp-5* and *abcf-2,* neither in resistant nor susceptible *H. contortus* L3 isolates after their exposure to IVM. However, experimental selection with IVM

on *C. elegans* and the human parasitic nematode has shown an up-regulation of *Ce-mrp-5* [91], and different ABC transporter genes in the human parasitic nematode *Brugia malayi* (agent of Lymphatic Filariasis) including P-glycoprotein, half transporters and MRPs [146].

Collectively, our results are in line with the literature regarding the involvement of P-glycoproteins in ML resistance, mainly to IVM, in *H. contortus* and other parasitic nematodes [19, 66, 92, 94, 143, 144, 146].

Debatable with the body of publication and research efforts throughout the last three decades with not concluding on reliable genetic markers for ML resistance in GINs [66, 147], we found some evidences from a *H. contortus* population that has been under selection with IVM, that link *Hco-pgp-2* and *Hco-pgp-3* with a relative up-regulation of their transcripts in this *H. contortus* isolate in Canada. Firstly, and differently from other countries where several anthelmintic drugs are used by veterinarians and animal producers for the control of GINs, we should contextualize the use of IVM in some alpaca farms as off-label with the purpose to control the meningeal parasitic nematode *Paralestrongylus tenuis* [148]. Secondly, IVM is the only anthelmintic used in several camelid farms across eastern North America (in the US and Canada) where the control of parasitic nematodes, including *P. tenuis* and *H. contortus*, has been extended for several years. We then can conclude that under exposure to IVM solely for long period, it could be possible to see a selection on *H. contortus* population, reflected on the lower efficacy outcome from the FECRT, and some genetic signatures from this selection with IVM, correspond to the up-regulation of *Hco-pgp-2* and *Hco-pgp-3*. If the transcript level from these genes corresponds positively with the protein level level, or whether the up-regulation of these *Hco-pgp* genes is held in adult worms, are interrogations that will need investigation with further research.

RNA extraction was the most challenging step in this part of the project, first because of the low number of larvae (raised from a low amount of received fecal material) and second due to the hard cuticular layer of the external surface of larvae. As a result, we had to target a limited number of ABC transporter genes instead of all of them. On the other hand, performing it on the other farms was not applicable due to the minimal amount of extracted RNA from other samples.

4-3- Lipidomic ESP profile of *H. contortus* and the involvement of ABC transporters in their extrusion

Helminths need to interact with their external environment and hosts during different stages of their lifecycle and, finally, in successful long-term infection in the host [149]. In this regard, helminths release a wide range of biomolecules, including peptides, amino acids, proteins, small molecules, extracellular vesicles, secondary metabolites and lipids, to the outside of their body as excretory/secretory products in order to communicate with environment, other parasites and final host [101, 149, 150]. Most of the molecules mentioned as Hc-ESPs are well studied, but there is no data regarding the ESP lipidomics of *H. contortus*. It has been reported that regulating lipids by *H. contortus* is a strategy to adapt to the host environment [103]. As in other eukaryote organisms, lipids play an essential role in nematodes biology including their constitution in cellular membranes, as energy storage and as signaling molecules [103, 150]. In this study, we identified the ESP lipidomics profile of the adult *H. contortus* by LC-MS/MS and evaluated their extrusion with different ABC transporters by blocking ABCs. Furthermore, we measured the relative gene expression of different ABC transporters (*Hco-pgp-2*, *Hco-pgp-3*, *Hco-pgp-11*, *Hco-mrp-5* and *Hco-haf-6*) of adult *H. contortus* PF strain in the presence of ABC inhibitors.

We identified 1057 lipids belonging to more than 36 classes present in the Hc-ESp lipidomic profile. The most abundant lipids were Phosphatidylcholines (PC), Triacylglycerols (TG), Fatty acids (FA) and Sterols (ST). PC belong to the glycerolipids category, representing one of the major phospholipids composing cellular membranes. Also, in *C. elegans*, PC have been described as an antioxidant, producing the expansion of the nematode's lifespan [151]. Also, PC are involved in the larval development and fat storage in *H. contortus* [152]. In addition, PC were identified in secreted lipidomics of exsheated L3s of *H. contortus* [153]. Wang et al. (2018) also identified PC as one of the most abundant lipids in the structure of different stages of *H. contortus*. Among other identified glycerolipids, some identified phospholipids in previous studies also participate in nematodes' biology. For instance, phosphatidylinositols (PI) and lysophosphatidylcholines (LPC) are proven to have a key role in the development process and also as signaling molecules in *H. contortus* [26].

TG, the second most abundant Hco-ESP lipidomics, is known as the major form of energy storage in eukaryotes, including nematodes [154]. Further, TG have been the most abundant lipid in the structure of *H. contortus,* in different life stages [103, 109]. In several studies, it has been reported that infection with nematodes, including haemonchosis, results in a decrease in serum TG levels in calves [155]. The consumption of host TG by *H. contortus* and TG identification as a predominant lipid, either in the structure or ESP, shows the crucial role of TG in nematode's biology.

FA are also the major lipids that participate in membrane formation and the structure of storage lipids [156]. In a recent study FA were identified in secretions of exsheated L3s of *H. contortus* [153]. FA also participates in the conformation of prostaglandin E2 (PGE2) synthesis which has been detected in various nematodes and is known to affect the host metabolism and immune response [150, 157]. Prostaglandin is known as an important promotor of pro-inflammatory or anti-inflammatory effects in various biological systems [158]. In addition, prostaglandins help *S. mansoni* (cercaria) to limit the host capability to induce protective immune response [159]. Moreover, it has been suggested that the nematodes' strategy to adapt to environmental temperature alterations is to regulate membrane FA saturation levels. An example of this is in nematodes such as *C. elegans* where at high temperatures, membrane-saturated FA increase [103, 160].

Sterols were one of the most abundant lipids among the twenty most altered lipids in this study. Sterols are one of the essential precursors of steroidal hormones, such as dafachronic acid, which is known to have an essential role in the larval development and energy storage of various nematodes, including *H. contortus* [152, 161, 162]. In addition, it has been proven that steroidal hormones participate in reproductive regulation and increase the life span of *C. elegans* [161, 162].

In addition, we identified one LPS lipid as Hco-ESP, which are known as important lipids with immune polarization effects in *S. mansoni* [163]. We may assume a similar function the repertoire of lipids secreted by *H. contortus*.

Further, we identified lipids in different classes of sphingolipids category. Sphingolipids are a category of complex lipids present in cell membranes, and more importantly, they are known to be involved in cell recognition and interactions between cells [164].

Considering all the information about the identified lipid molecules as Hc-ESP and their crucial role in host-parasite interactions, the translocation pathway of Hc-ESP lipidomics is unclear. ABC transporters in mammalians have central role in transporting lipids and also the pathogenesis of different disease related to impaired translocation of lipids [110]. Therefore, identification of the role of ABC transporters as an important candidate in extrusion of Hc-ESP lipid molecules, which some might be included in pathogenesis process, is of great importance.

In order to determine the potential link between the extrusion of identified lipids and ABC transporters' activity in adult *H. contortus*, we used three different ABC inhibitors including Fumitremorgin C, ketoconazole and MK-571, to block different groups of ABCs. Fumitremorgin C is a mycotoxin that blocks the half ABC transporters in human cells [165, 166], and ketoconazole, an antifungal compound, known to block P-gps [166], while MK-571 has typical inhibitory effects on *mrps* [167]. Moreover, ketoconazole and MK-571 have shown reversal on larval stages of *H. contortus* [142] and *C. elegans* [91] IVM-selected strains, respectively. Since there was no information about the effective and non-toxic doses of the mentioned ABC inhibitors in adult *H. contortus*, we incubated the worms at three concentrations, (0.5 µM, 1µM and 5µM) for eight hours. As we used different ABC transporter inhibitors, we measured the relative gene expression of *Hco-pgp-2*, *Hco-pgp-3*, *Hco-pgp-11*, *Hco-haf-6* and *Hco-mrp-5* in treated and control groups. As a result, the expression of *Hco-pgp-3*, *Hco-haf-6* and *Hco-mrp-5* was upregulated in all treated groups. Also, *Hco-pgp-2* was upregulated in all treated groups except in the ketoconazole 5µM group, in which it was downregulated ($P>0.05$). We hypothesized that the upregulation of these ABC transporters-encoding genes is probably a response to the blocking on the nematode transporters in adult *H. contortus*, in order to generate more protein and keep their transport function in the organism.

Surprisingly, *pgp-11* was downregulated in all treated groups in comparison with control group. Although there is no data regarding the partial or total blocking effect of used ABC inhibitors on

Hco-pgp-11; however, even by considering the actual inhibition of *pgp-11*, this downregulation could be explained at the epigenetic level. In such scenario, we may speculate about the promoter region upstream the *Hco-pgp-11* sequence, might have a distinctive sequence from the other *Hco*-ABC transporter genes, and may not be activated by the same transcription factor. Although evidences in *C. elegans* indicate that *Ce-pgp-11* gene among other paralogue *pgps*, are governed by the transcription factor termed Nuclear Hormone-Receptor, NHR-8 [168, 169], we may not extrapolate the same in *H. contortus*, as the study of transcription factors in parasitic nematodes is vastly unknown. Conversely, we may explain the downregulation of *Hco-pgp-11* in *H. contortus* by the ABC transporter inhibitors, as a model where the gene and its transcript regulation are dependent on a substrate-stimulus that can induce its gene expression. In fact, the knowledge on *Hco-pgp-11* from *H. contortus*, indicates that it is one of the genes that was not able to increase significantly its transcript when adult worms where incubated in presence of eosinophilic granules, compared with the vast majority of *Hco-pgp* genes that after the exposure to this stimulus, were markedly up-regulated in a concentration-dependent manner [145]. As these authors suggest, some *Hco-pgp* genes such as *Hco-pgp-11*, increase their transcript level at the adult stage once they have been exposed to other stimuli from the host or exogenous substrate, i.e., an anthelmintic, that can induce their expression and detoxification activity. In fact, the nematode transporter orthologues in *P. equorum*, *Pe-pgp-11* [170], and in *C. oncophora*, *Co-pgp-11* [94], have shown an increase in IVM-resistant isolates. Although *Hco-pgp-11* is not reported previously to be involved in adult *H. contortus* IVM resistance, this may not exclude its contribution to IVM resistance in *H. contortus* and IVM might correspond to one of those stimuli-substrates that induce its gene expression.

We further investigated the contribution of ABC transporters in the translocation of lipids. ABC transporters are not substrates-specific, having a wide range of molecules, including various lipids for efflux activity [87]. However, there are some structural characteristics from substrates that confer them coupling and their extrusion by the ABC transporters, such as the presence of hydrogen bonding acceptor radicals (or hydroxyl groups) and planar aromatic moieties [87]. Moreover, previous studies have shown that many ABC transporters are able to extrude various drugs in eukaryotes and prokaryotes [86, 87]. On the other hand, lipids metabolism and their

complex role in different nematode biological processes are poorly understood [150]. Research in human cells has established that disruption on lipid transport by ABC pumps is involved in the pathogenesis of several diseases [110]. As an example, in humans, it has been proven that cholesterol, as an essential biomolecule, has a critical role in the activation of specific efflux ABC transporters and also in the release of interleukins [171]; The membrane lipid phosphatidylinositol also acts as a link between ABCs (for cholesterol efflux), cellular cholesterol level and inflammation induction [171]. Similarly, it has been reported that cholesterol in *H. contortus* can modulate the activity of P-gps and consequently alter the level of resistance to thiabendazole [172].

Localization of ABC transporters along body of nematodes including *H. contortus* [111, 112, 124, 173] and their critical role in transporting different lipid molecules, that some are known as important players in host-parasite interactions [145, 151, 158, 159, 161], reveals their important role in nematodes biological processes. Since adult *H. contortus* reside, feed, mate and produce offspring in the host abomasum [1], interacting with the host is undeniable to survive. Therefore, the identified wide range of Hc-ESP lipids and their extrusion with ABCs indicate the specific role of lipids along with their exporters in the nematode biological cycle and their potential role in *H. contortus* interaction with outside of body.

Chapter 5 – Conclusions

The efficacy of IVM against diverse GINs in ruminant farms has variable outcomes including evidence of resistance to IVM; and *H. contortus* was the most common GIN species found in sampled farms.

Hco-pgp-2 is overexpressed in *H. contortus* IVM field isolates, possibly involved in the mechanism of ML resistance.

The lipid profile released by *H. contortus* contains several classes of lipids including novel features and varies in a time-dependent manner. The lipid profile secreted by *H. contortus* changed in the presence of ABCs inhibitors which indicates their extrusion most probably via ABC transporters. Therefore, some ABC transporters in *H. contortus* may be involved in IVM detoxification and lipid extrusion, representing a significant mechanism in IVM resistance and in the host-parasite interactions.

Chapter 6 – Contribution and perspectives

This study provides information about the current status of ML efficacy against *H. contortus* and its potential development of anthelmintic resistance. The transfer of this knowledge to veterinarians and animal producers, would help to elaborate an integrated management plan for small ruminant and camelid farms in Quebec.

Further detail experiments such as functional genomics will allow to validate the transport of MLs and lipids by nematode ABC transporters.

Future lipidomic analyses are necessary to identify potential lipid biomarkers for ML resistance in *H. contortus*.

Novel lipidomic studies will allow Identification of potential lipids as therapeutic and diagnostic targets for controlling *H. contortus.*

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