The anthelmintic effect of *Bacillus thuringiensis* Cry5B on *Haemonchus contortus* in sheep

John P. Sanders

Thesis submitted to the faculty of the Virginia Polytechnic Institute and State University
in partial fulfillment of the requirements for the degree of

Master of Science
In
Biomedical and Veterinary Science

Anne M. Zajac, Committee Chair
David S. Lindsay
Clayton C. Caswell

May 28, 2020
Blacksburg, VA

Keywords: *Haemonchus contortus*, *Bacillus thuringiensis*, Cry5B, sheep, internal parasite, control

The anthelmintic effect of *Bacillus thuringiensis* Cry5B on *Haemonchus contortus* in sheep

John P. Sanders

ABSTRACT

Widespread anthelmintic resistance in trichostrongyle nematodes of ruminants has created an urgent need for alternatives to commercial anthelmintics. The bacterium *Bacillus thuringiensis* (*Bt*) can produce crystal proteins during sporulation, which can be lethal to insects in multiple orders when ingested. One protein, Cry5B, has demonstrated effectiveness against multiple parasitic nematodes. We hypothesized that Cry5B would be effective against *Haemonchus contortus,* a highly pathogenic parasite, in sheep.

Two experiments tested efficacy of Cry5B in sheep experimentally infected with *H. contortus*. In the first, a live genetically modified, asporogenous strain of *B. thuringiensis* expressing cytosolic Cry5B protein (BaCC) was administered orally daily for four days (~40mg/kg Cry5B/day). The mean fecal egg count (FEC) of treated animals was reduced by 94% three days after treatment, and at necropsy the female worm burden was significantly reduced by 98%. In the second experiment inactivated, asporogenous *Bt* expressing cytosolic Cry5B (IBaCC) was used. Treated animals received 60mg/kg Cry5B, administered daily for three days. By 72 hours after the first treatment FEC was reduced by 91%. Mean total worm burden of treated sheep at necropsy was significantly reduced, with female worms reduced by 95%. A third study tested the effect of BaCC and IBaCC on development of eggs to infective larvae in feces under laboratory and outdoor environmental conditions. Cry5B (15mg) added to feces (10g) reduced numbers of infective larvae by 99% in both environments within 12 days. Cry5B appears to have

potential for controlling *H. contortus* in sheep. All protocols approved by VT IACUC and IBC.

The anthelmintic effect of *Bacillus thuringiensis* Cry5B on *Haemonchus contortus* in sheep

John P. Sanders

GENERAL AUDIENCE ABSTRACT

Many animals and humans can be infected with roundworm, also called nematode, parasites. Infection of animals and humans by parasitic nematodes can result in disease. Some animals like ruminants (cows, sheep, and goats) can be infected with multiple species at once with few effects on the host. However, certain species can cause major disease, and even kill their ruminant host. Younger animals like lambs can easily become overwhelmed by these parasites. Anthelmintics are the type of drug used to treat those infected with these parasitic worms. However, just like bacteria are becoming resistant to antibiotics, these worms are also becoming resistant to anthelmintics. Because of this, researchers are looking for new compounds and materials that are lethal to the parasite and can be used to treat infected animals.

One species of bacterium, *Bacillus thuringiensis*, is usually found in the soil. This bacterium can produce a large crystal structure that is made up of proteins. These crystal (Cry) proteins can be lethal to pest insects like beetles, caterpillars, and mosquitos. When the insect eats the protein, it binds to cells in the insect intestine, creating holes in the insect gut. These proteins can be lethal to nematodes as well when they are eaten by the worms. Because of this, these proteins are being investigated as potential alternative treatments for parasitic nematodes. One type of protein, Cry5B, has been tested in hamsters, mice, and pigs. We hypothesized that Cry5B would also be effective against a sheep stomach worm called *Haemonchus contortus*. We tested the Cry5B in two different

formulations and found that the protein was effective against both the adult worm in the stomach, and the young worms in the feces of the host. This protein could potentially be used to treat parasitic nematodes that have become resistant to anthelmintics.

DEDICATION

This thesis is dedicated to my family and friends. I would never have made it without their support and guidance.

ACKNOWLEDGEMENTS

I would like to thank Dr. Anne Zajac for allowing me to work in her lab, and offering her constant support, insight, and guidance which were critical to my time at Virginia Tech. I would also like to thank the rest of my committee, Dr. David Lindsay and Dr. Clay Caswell, for generously offering their time and advice whenever I asked. I am incredibly grateful for the funding provided by the USDA National Institute of Food and Agriculture, without which I would not be here today. I would next like to thank the members of my lab. Emily Suess and Kate Pouliot guided me when I started and helped me find my footing. If I ever had a problem, they would be there to help. Caroline Anderson, Brittany Weicht, Olivia Stover, and Rebecca Wilson were invaluable to my research, always happy to assist in the lab or with the animals. Finally, I would like to thank my family and friends for their enduring support over the course of the past two years.

TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

CHAPTER 1: Literature Review

1. Gastrointestinal nematodes of small ruminants

Small ruminants (sheep and goats) are affected by many parasites, both internal and external. Ticks, mites, lice, and flies are all ectoparasites of sheep and goats. Internal parasites include nematodes, tapeworms, flukes, and protozoan parasites (Sykes, 1994). Nematodes are the most diverse group of small ruminant parasites and can affect multiple organ systems. The most important, and costly, group of parasitic nematodes are the gastrointestinal nematodes. All major gastrointestinal nematode genera belong to the superfamily Trichostrongyloidea, within the order Strongylida (Sutherland and Scott, 2010). The Trichostrongylid genera can be grouped by the organ they affect. The genera *Haemonchus*, *Teladorsagia*, and *Trichostrongylus* can be found in the abomasum of sheep and goats. Species of *Trichostrongylus* can also be found in the small intestine, along with *Nematodirus* and *Cooperia* (Sutherland and Scott, 2010). The species most commonly associated with disease and production loss are *Haemonchus contortus*, *Teladorsagia* (*Ostertagia*) *circumcincta*, *Trichostrongylus axei*, and *Trichostrongylus colubriformis* (Roebor et al., 2013; Vlassoff et al., 2001).

Haemonchus contortus, also called the barber pole worm, is a common and problematic nematode for producers and veterinarians across the globe (Kaplan and Vidyashankar, 2012). It is arguably the most important of all gastrointestinal nematodes due to its high pathogenicity, resulting in major economic and production losses (Emery et al., 2016). *H. contortus* is the largest common abomasal parasite (up to 2.5 cm) and is more pathogenic than most other gastrointestinal species of sheep and goats due to it being a blood-feeder (Sutherland and Scott, 2010). The worm flourishes in warm, humid climates and can complete its life cycle rapidly in favorable conditions (Emery et al., 2016). Rapid development of larvae paired with the high

fecundity of the adult females allows for pastures to become contaminated quickly, leading to the rapid buildup of infections.

The life cycle of *H. contortus* is direct, which means the only host required to complete the life cycle is the sheep or goat definitive host. Within the abomasum of the animal, adult worms breed, and the females release 5,000 to 15,000 eggs a day (Emery et al., 2016). These eggs are passed out of the animal in the feces. The eggs develop on the pasture, and the larva emerges from the egg. The newly hatched worm is a first stage larva (L1). The larva must reach the third larval stage (L3) before it is ready to infect a host. Water and oxygen are important requirements for larval development, as the eggs and L1/L2 require adequate moisture levels to prevent desiccation. The feces retain moisture, providing a protective environment against water loss (Sutherland and Scott, 2010). As an L1, the larva feeds on bacteria within the feces and molts its cuticle, becoming an L2. The L2 feeds on bacteria, grows, but does not shed the old cuticle when it molts. Instead, the L3 is ensheathed in the L2 cuticle, which protects the larva from desiccation as it waits to find an appropriate host. While it is ensheathed, the L3 is unable to feed due to the L2 cuticle covering. The larva survives on stored energy, and its survival time is dependent on environmental conditions (Craig, 1986). If the environment is warm, the energy stores are used up faster than they would be in cold environments.

The L3 are ingested by the host as it feeds on the pasture. A moist environment is required for the L3 to move away from the feces, and larvae use dew to travel up the stems of the grass or forage. This facilitates the ingestion of the larvae by the host as it grazes, which is required for the life cycle to continue. Once ingested by the host, *H. contortus* L3 larvae will exsheathe in the rumen, likely prompted by CO_2 levels (Sutherland and Scott, 2010). The L3 then travel to the abomasum, where they burrow into the wall. They feed, molt once more, and

become fourth stage larvae (L4). The L4 emerge, and fully develop into adult worms feeding on blood from the abomasal lumen. The L4 and adults possess a buccal lancet, a cuticular tooth like structure that protrudes from the buccal cavity. The lancet is used to create lesions on the abomasal wall, allowing the parasite to feed on the blood of the host (Qamar, 2009; Weise, 1977). The time from establishing infection to adult takes a minimum of two to three weeks (Laing et al., 2013). The larvae entering the wall of the abomasum may also enter a state of arrested development called hypobiosis instead of emerging. For *H. contortus*, the L4 arrest when conditions outside of the host are not favorable to survival, such as during winter in cold climates, or during the dry season in arid ones (Besier et al., 2016). They can also arrest if conditions inside the host are not favorable, as hypobiosis can help protect them from the immune response. Once conditions are more favorable, they can resume development, leading to large spikes in egg counts and pasture contamination.

Haemonchosis is the disease caused by a heavy *Haemonchus* infection (Qamar, 2009). The main clinical sign of haemonchosis is anemia, with other signs relating to the varying levels of blood loss. Infected sheep and goats can present with lethargy, ill-thrift, weight loss, and bottle jaw (submandibular edema due to hypoproteinemia). When the infection is heavy enough, or when the number of worms increase rapidly, the animals can die from blood loss (Sutherland and Scott, 2010). In acute disease this can occur in animals during the prepatent period before they are able to shed any eggs or show any signs of infection. A single adult *H. contortus* can consume 30 µL of blood a day. The number of worms needed to kill a sheep or goat is dependent on factors such as the age, nutrition status, and immune response of the host, but over 500 adult worms can cause death (Emery et al., 2016).

A few weeks before lambing begins, and for six to eight weeks after the lambs are born, a

phenomenon called the periparturient egg rise can be seen (Abbott et al., 2012). During this time, ewes are immunosuppressed, and less able to prevent larval establishment and control current infections (O'Sullivan and Donald, 1973; Thomas and Ali, 1983). Egg counts sharply rise as larvae that were in hypobiosis emerge and continue their development, which can lead to pastures becoming highly contaminated (Gibbs, 1986). Lambs are more susceptible to parasitic infection than adults, due to their immature immune systems (Colditz et al., 1996). The foraging lambs can become quickly overwhelmed as they are more susceptible to establishment, and many fewer worms are needed to kill a lamb from acute blood loss than an adult (Gibbs, 1986).

2. Anthelmintics and anthelmintic resistance

Anthelmintics are a group of drugs used to treat parasitic worms. The first broad-spectrum anthelmintic, thiabendazole, was discovered in 1961 (Brown et al., 1961). Since then, many new drugs have been approved for treating parasitic helminths in both animals and humans. There are three main classes of anthelmintics used to treat ruminant parasitic worms which have been approved for commercial use across the globe.

The first class is the benzimidazoles. Examples of drugs within this group are thiabendazole, albendazole, fenbendazole, and oxfendazole (Bogan and Armour, 1987). This class of drug acts by inhibiting β-tubulin binding and subsequently microtubule formation. Microtubules are crucial cellular structures that make up the cytoskeleton of the cell, are required for mitosis, and are needed for transport of materials inside and outside of the cell (Kohler, 2001). Once microtubules are unable to form, cell and organism death follow. The second main class of anthelmintics are nicotinic agonists. Examples of this class are levamisole, butamisole, pyrantel, and morantel (Martin, 1997). These drugs act as agonists at the synaptic and extra synaptic nicotinic-acetylcholine receptors found on the somatic muscle cells. This leads to

depolarization and spastic paralysis of the worms, which will cause the worms to die by making them unable to feed, or unable to stay inside the host (Martin, 1997; Martin et al., 1997). The third main class is the macrocyclic lactones. This class contains ivermectin, abamectin, doramectin, and moxidectin (Kohler, 2001). These drugs have a high affinity to glutamate gated chloride channels in muscle and nerve cells. They increase chloride permeability, which hyperpolarizes the cell leading to paralysis. This paralysis prevents feeding and results in worm death (Kohler, 2001; Martin, 1997; Martin et al., 1997).

Modern anthelmintics aim for an efficacy of ~99% (Jabbar, 2006). When an animal is treated with anthelmintics, there is a chance that a portion of the population will survive the treatment. Use of anthelmintics provides a selection pressure on the worms in a host. Parasitic nematodes have high gene flow and large diversity within their populations (Blouin et al., 1995). This, along with the high fecundity of the adults (Emery et al., 2016) allows for parasitic nematodes to have high mutation rates, and for new alleles to arise within a relatively short amount of time. These factors contribute to the development of resistant individuals and populations.

Anthelmintic resistance has been defined as the declining efficacy of an anthelmintic drug on a parasite population that was previously susceptible to the drug (Sangster and Gill, 1999). There are three population factors that determine how fast anthelmintic resistance will develop (Coles, 2005). The first is the number of worms in the refugia, the portion of the population that was not exposed to the anthelmintic. The larger the refugia, the higher the chance that some susceptible worms will be present in the population. The second factor is how frequently the genes for resistance appear in the population, and whether these genes are dominant or recessive. The third main factor is the biological fitness of the resistant worms when

compared to those lacking the resistant genes. The resistant worms may possess the ability to survive anthelmintic treatment, but if they are less able to pass on those genes than the non-resistant worms, the anthelmintic resistance in the population will not grow as quickly.

The use of anthelmintics selects for those members of the population that have resistance to the drugs. There are also factors that speed up the development of anthelmintic resistance (Jabbar, 2006). One important factor that contributes is under dosing (Edwards et al., 1986). When animals are giving lower doses of anthelmintics than are recommended, this increases the chance that less resistant or tolerant worms may survive. These worms may not have been able to survive exposure to a full dose, but now they are able to pass on their genes and decrease the effectiveness of the anthelmintic in the future. Another factor is frequent treatment (Martin et al., 1982). By treating a population too frequently the worms that are not resistant are continually killed, leaving the most resistant individuals behind to contribute their offspring to the next generation. A third factor is the use of only one type of anthelmintic (Van Wyk et al., 1989). When only one drug, or one class of drug, is used repeatedly without alternating to another class, anthelmintic resistance is hastened. The worms that survived the treatment were either resistant, or not exposed to the drug. These worms produce the next generation that is then exposed to the same treatment. Each time, the portion of the population that is resistant to the drug increases.

Since 1961, with the introduction of the benzimidazole class of drugs, anthelmintic resistance has appeared for each new class within the decade that the class was implemented (James et al., 2009; Kaplan, 2004). Thiabendazole was introduced in 1961, and in 1964 resistance was reported. Levamisole was introduced in 1970, with resistance reported in 1979, and ivermectin which was introduced in 1981 had resistance reported in 1988. Today, anthelmintic resistance is seen in every continent where small ruminants are raised. Studies have

shown anthelmintic resistance in Ontario (Barrere et al., 2013), Brazil (Oliveira et al., 2017), Europe (Papadopoulos et al., 2012), Africa (Van Wyk et al., 1989), India (Chaudhry et al., 2015), Australia (Besier and Love, 2003), and New Zealand (Pomroy, 2006). Not only are worms becoming resistant to an entire class of anthelmintics, some studies have shown that certain parasitic populations have become resistant to all classes currently available (Chandrawathani et al., 2004; Kaplan and Vidyashankar, 2012).

The development of anthelmintic resistance is an inevitability. These drugs place selection pressure on the worms that are treated, and naturally resistant members of the population will survive to pass on their resistant genes. This process is being sped along by unsustainable treatment practices, and already conventional anthelmintics are becoming unable to effectively manage the parasites they used to control. The rapid and widespread rise of anthelmintic resistance has led to the need for more sustainable farming practices that will slow the growth of resistance and offer alternative control and treatment options.

3. Control methods

3.1. Refugia and targeted treatment

One of the most important factors to consider when trying to slow the progression of anthelmintic resistance in parasitic nematodes is the concept of refugia. Van Wyk (2001) defines refugia as: “the proportion of the parasite population that is not exposed to a particular given control measure, thus escaping selection for resistance”. Refugia offers the potential for a mix of resistant and susceptible individuals, which allows there to be a population of worms with varied levels of resistance for new infections. Instead of just the resistant worms surviving and creating the next generation, maintaining refugia increases the chance that there will be some susceptibility within the next generation. It is crucial to dilute the impact of the resistant worm

eggs by increasing the contribution of refugia to the future generations of worms.

Martin et al. (1981) performed an experiment to illustrate how large an impact refugia can have on slowing the progression of anthelmintic resistance using thiabendazole resistant *Haemonchus contortus*. The results showed that when the refugium was higher, the rate of growth for anthelmintic resistance was slowed, compared to low refugia and a higher number of resistant individuals. This is due to the dilution effect offered by refugia. An experiment by Waghorn et al. (2008) also tested this concept, this time by leaving some lambs untreated, thereby creating a larger refugia than the groups where all lambs were treated. The treatment groups that had their 10% and 20% of their lambs untreated showed a slower development of anthelmintic resistance than the group that had all lambs treated. However, those groups had higher levels of parasitism, and pastures that were more heavily contaminated, as more worms survived compared to groups that received full treatment. This highlights the fact that the concept of refugia should be used with the knowledge that it can cause higher levels of pasture contamination and parasitism, which may affect production.

The FAMACHA© system was developed in South Africa for use in determining anemia in sheep to assist with targeted anthelmintic treatment (Papadopoulos, 2008). The system, named after Dr. Faffa Malan (FAffa MAlan CHArt), uses a chart with five colors that depict a range from non-anemic to severe anemia and is held up to the mucous membranes of the eye of a sheep or goat (Van Wyk and Bath, 2002; Vatta et al., 2001). The chart scores the level of anemia by giving a corresponding score to a color. Severely anemic is white, and the animal would be given a score of 5. Bright red is healthy, non-anemic and the animal would be scored at 1. Animals with a score of 1 or 2 are considered to not require treatment. Those that score 4 or 5 require de-worming, and those that score 3 could use treatment depending on the animal. The goal of this

system is to identify the animals that are hit the hardest by their *H. contortus* infection and to treat them, rather than the entire herd. The implementation of the FAMACHA© system allows for targeted treatment of only the most heavily affected animals, rather than treating the entire herd (Torres-Acosta and Hoste, 2008). Parasite distribution in a host population follows an aggregated pattern (Barger, 1985; Hoste et al., 2002; Sréter et al., 1994). This means that a minority of the sheep or goats infected in a herd have the majority of the worm burden. If there is a large parasite population on a pasture, the goal at that point would be to ensure that refugia is high and the resistant population is low. Treating animals that do not have a heavy or damaging parasite load would only serve to increase the resistant population, as every use of an anthelmintic is another selection pressure that favors the resistant individuals. Therefore, anthelmintics should be viewed as the limited resource that they are and used only for those animals that truly need them.

3.2. Grazing management

The goal of grazing management is to provide a pasture that has a low level of infective parasite larvae for susceptible individuals (Barger, 1999). A safe pasture is one that may not be completely free of infective larvae, but the number is low enough to not pose any real threat in that grazing season (Brunsdon, 1980). There are multiple methods to provide these safe pastures. One method is to keep animals off a pasture and to cut short any grass or weeds (Fleming et al., 2006). The infective larval stage can survive for long periods of time, depending on the weather and climate. In warm wet conditions, the larvae will use up their energy stores quicker and die off faster than if they are in cold, dry environments (Barger, 1999). The time of year will also play a role in determining how long the larvae can survive. Pastures that have lots of growth provide protection for the larvae from ultraviolet light, which can kill off the larvae (Fleming et

al., 2006). A pasture can also be divided into sections, and the animals rotated through them, depending on the size of the herd and the land available. By dividing up a pasture, animals can graze on one section, and then move to the next. Once they have been moved off the first section, no new parasite eggs will be added to the pasture, and the larvae will begin to die off, leading to a safe pasture.

Another method is mixed grazing, where different species are rotated through a pasture. The theory behind this method is that small ruminants, cattle, and horses are affected by different species, with only *Trichostrongylus axei* as a common parasite (Fleming et al., 2006). By grazing sheep and cattle together in one pasture, or by rotating cattle into a pasture that had been grazed by sheep, the parasitic larvae will be ingested by other livestock that they are unable to infect and be killed. *H. contortus* parasitizes sheep and goats but is unable to infect mature cattle or horses. If pastured together, the cattle would consume a portion of the infective *H. contortus* larvae population and lower the worm burden in sheep when compared to sheep that graze alone. If the cattle followed the sheep into the pasture, they could help clean up the pasture. A three-year study performed by Jordan et al. (1988) looked at the effect of mixed grazing with sheep and cattle. Three groups were formed with the first being lambs and ewes only. The second was calves and cows only, and the third mixed all animals together. The results were that the lambs that grazed with cattle and the ewes had lower worm burdens and gained more weight each year than lambs that grazed with just the ewes.

3.3. Selective breeding

The use of genetic selection for parasite control offers multiple advantages for producers. First, selective breeding will decrease the need for anthelmintics, slowing the development of anthelmintic resistance. Secondly, the improvements in host growth by selecting for resistance

are life long and can be passed on to the next generation of host. Finally, improved resistance will decrease the overall pasture contamination and larval buildup, which will provide a benefit for all individuals, including those that are more susceptible to disease (Bishop, 2012; Bishop and Morris, 2007).

Two traits, resistance and resilience, need to be considered for breeding programs. Resistance is the measure of how well the host can prevent the establishment of infection, while resilience is the ability of the host to resist the impact of their infection (Bisset et al., 2002). Resilience is considered as less heritable than resistance, and more heavily impacted by factors such as nutrition and severity of the larval challenge (Sutherland and Scott, 2010). Therefore, breeding programs focus on the selection of the most resistant individuals (Bishop, 2012). Studies in both sheep (Kemper et al., 2010) and goats (Vagenas et al., 2002) have shown that fecal egg counts can be used as an indicator of resistance. Programs can focus on choosing to only use breeds that have demonstrated resistance such as the Scottish Blackface or Red Maasai, or selecting the most resistant animals within a breed (Fleming et al., 2006).

As resistance and resilience are two separate measures, not all animals with low fecal egg counts will also be resilient to infection. Due to this fact, animals with low fecal egg counts that also show the highest productivity under the parasite challenge should be the ones chosen for breeding purposes (Bisset et al., 2002). By selecting the most resistant individuals, the overall pasture contamination will decrease, as illustrated by Williams et al. (2010). The study took two lines of merino ewes, one control and one resistant line, and determined fecal egg counts starting five weeks before lambing and three weeks after. They found the resistant line had significantly lower fecal egg counts than the control line one week before lambing through two weeks after. The rise in fecal egg counts of the resistant line was less steep than the control, with control ewes

displaying the rapid increase expected during the periparturient period. There were also significantly fewer larvae in the resistant ewe pasture both pre and post lambing. The results show that lambs of the resistant ewes would face a reduced larval challenge, and if a mixed population of sheep were on the same pasture, the other animals would also be better protected. This study shows how breeding for resistance is a critical tool for reducing larval contamination while decreasing the reliance on anthelmintics. Anthelmintics would still be needed, as less resilient individuals could still be overwhelmed by the parasite challenge, but the overall need should decrease with each generation.

4. Alternative anthelmintic compounds

4.1. Copper oxide wire particles

Increasing the number of alternative treatments and control methods will preserve the efficacy of anthelmintics and slow the further development of resistance. One alternative control method that shows some promise is the use of copper oxide wire particles. Copper oxide particles were originally used as dietary supplements for livestock with copper deficiencies (Torres-Acosta and Hoste, 2008). These particles have been used in cattle, goats, and sheep, but are used less commonly in sheep due to the risk of toxicity (Cavanagh and Judson, 1995; Fleming et al., 2006). The copper wire particles are ingested by the animal, and travel to the abomasum. The acidic environment causes copper to be released, increasing copper levels in the abomasum and its contents, but also in the liver (Terrill et al., 2012). The risk for copper toxicity increases as more copper builds up in the liver of the animal (Cavanagh and Judson, 1995). The observation was made that animals treated with copper particles had lower levels of parasitism.

A study by Bang et al. (1990a) was performed to determine the effect of copper oxide wire particles on the establishment of *Haemonchus contortus, Trichostrongylus colubriformis,*

and *Teladorsagia circumcincta* larvae in lambs. The study found that copper oxide wire particles caused a 96% decrease in worm burdens in lambs infected with *H. contortus*, a 56% decrease in *T. circumcincta* levels and no significant difference in *T. colubriformis*. These results have been confirmed by other studies with both sheep and goats (Chartier et al., 2000; Knox, 2002; Vatta et al., 2009). These studies have shown that the copper oxide wire particles can lower the establishment of larvae, decrease the worm burden, and reduce the FEC of treated animals (Chartier et al., 2000; Knox, 2002). As copper levels in the abomasum decrease over time the effect on larval establishment is reduced, with the levels beginning to drop after four days (Knox, 2002; Bang et al., 1990b). However, there are factors that limit the effective use of this treatment. Both sheep and goats are susceptible to copper toxicity if given too high a dose or if treated too often, with sheep even more sensitive than goats to toxicity. While the initial administration of the copper wire oxide particles will reduce the burden of the worms inside the animal and the success of new infections being established, this effect only lasts while the abomasal copper levels are high enough. The strengths of copper wire oxide particles in treating and preventing *H. contortus* infection suggest that this could be one more tool used for the management of these worms in goats, and to a lesser extent sheep.

4.2. Plant secondary compounds

Another control method that shows promise for controlling gastrointestinal nematodes are tannin-rich plants and feeds. Many forages contain tannins, which can produce beneficial effects in ruminants when ingested, such as reducing bloat and gas production (Min et al., 2006). Studies have also begun to investigate the potential anthelmintic qualities of tannin-rich plants. Many plants have been tested to determine nematocidal effectiveness *in vitro*. One plant in the legume family, *Lespedeza cuneata*, commonly known as sericea lespedeza, has received

attention for its effects in small ruminants when fed as both forage and hay (Fleming et al., 2006). In multiple tests conducted with sheep and goats, sericea lespedeza has demonstrated a significant ability to lower fecal egg counts and may act by damaging the cuticle of the adult worms (Joshi et al., 2011; Kommuru et al., 2015; Lange et al., 2006; Terrill et al., 2012). In the study by Kommuru et al. (2015), sericea lespedeza was fed to goats as leaf meal pellets. The goats fed the sericea lespedeza pellets showed significantly lower fecal egg counts than the control goats. Adult female *H. contortus* worms were removed from the abomasum after slaughter and examined by scanning electron microscopy. The images showed damage to the cuticle in worms exposed to sericea lespedeza, with longer exposure leading to more damage.

4.3. Nematode trapping fungi

Another potential treatment for preventing gastrointestinal nematode infection is the use of nematode-trapping fungi. These fungi feed naturally on free-living soil nematodes by trapping them with their hyphae and consuming the nematodes (Fleming et al., 2006). When the fungal spores are ingested by sheep or goats, they pass through the gastrointestinal tract and are shed in the feces, along with the eggs of the parasitic nematodes. Once the feces are on the pasture, the eggs and fungal spores develop together, and the fungi trap the larvae with their mycelia (Fontenot et al., 2003; Terrill et al., 2012). One species of fungus, *Duddingtonia flagrans*, has a chlamydospore with a particularly thick wall which gives it a better chance of survival than other fungi when passing through the gastrointestinal tract of small ruminants (Larsen et al., 1997). Because of this, multiple studies have looked at the effect of *D. flagrans* on gastrointestinal nematodes when fed to sheep and goats (Jackson and Miller, 2006).

In a study by Waghorn et al. (2003) *D. flagrans* had a significant effect on limiting the development of *H. contortus*, *T. colubriformis*, and *T. circumcincta* larvae when ingested by

sheep and goats. *Duddingtonia flagrans* in lambs demonstrated 84.8% to 93.4% efficacy against *H. contortus* L3, depending on dose. In the study by Peña et al. (2002), when *D. flagrans* was fed to infected lambs, the fungus caused an 82.8% to 99.7% reduction in the number of *H. contortus* larvae that developed when compared to control lambs. Once the spores were no longer fed to the lambs, these percentages fell to 76.1% and 66.1% for the last two days of the study. In the study by Fontenot et al. (2003), ewes were fed the fungus, decreasing larval numbers and when tracer lambs free of infection were placed on the control and treatment pastures, a 96.8% reduction in worm burdens was seen in lambs fed on the treated ewe pasture. These studies show that *D. flagrans* is highly effective at preventing the development of gastrointestinal nematode larvae, especially *H. contortus*. *Duddingtonia flagrans* could be used to significantly lower the population of *H. contortus* larvae on a pasture and slow the contamination of pastures.

5. *Bacillus thuringiensis* Cry proteins and Cry5B

Bacillus thuringiensis (*Bt*) is a gram-positive soil dwelling bacterium that produces an endospore, first isolated in 1901 from infected silkworms. The bacterium is ubiquitous, and has been found in soil, feces, sewage, stored grains, and forests (El-kersh et al., 2016). The bacterium was first used for insect control in the late 1920's against the European corn borer, *Ostrinia nubilalis*, and the gypsy moth, *Lymantria dispar* (Sanchis, 2011). The first commercial insecticide using the bacteria was made in 1938, and it was used mainly to control flour moths. Beginning in 1996 with cotton, transgenic crops that incorporate *B. thuringiensis* genes have been used worldwide (Ibrahim et al., 2010). The reason that *B. thuringiensis* is so widely used as a biopesticide is due to the parasporal crystals that can be produced during the sporulation phase of the bacterium. Parasporal crystals are produced next to the spore, with one cell being capable of producing multiple of these crystals (Ibrahim et al., 2010). The large parasporal crystal is

made up of crystal, or Cry, proteins (Bravo et al., 2007). While the production of these crystals is a distinguishing characteristic of *B. thuringiensis*, some strains have been described that do not produce these parasporal bodies (Roh et al., 2007).

There are many Cry proteins, with an individual bacterium capable of producing one or multiple proteins, depending on the strain. Different strains are also capable of producing different numbers of parasporal crystals. Most of the genes responsible for producing the Cry proteins belong to the *cry* gene family, which are mainly found on plasmids (Ibrahim et al., 2010). Many of the Cry proteins are protoxins which, when proteolytically cleaved, create pore forming toxins called Cry toxins (Bravo et al., 2007). These Cry proteins are highly effective bio-pesticides. They can affect a range of insect orders, including Coleoptera, Diptera, Hymenoptera, and Lepidoptera. Usually, one Cry protein has a narrow spectrum of effectiveness, affecting a few species within one order. However, other proteins have been shown to affect species in multiple orders (Roh et al., 2007).

The mode of action for the Cry1 group of proteins against insects in the order Lepidoptera has been thoroughly described. The primary mode of action for these proteins is to form pores in the insect gut, with death following due to cell lysis or sepsis (Bravo et al., 2007). When consumed, the Cry proteins are solubilized and broken down in the alkaline conditions of the insect midgut. The proteins require a high pH (10+) environment to become solubilized (Betz et al., 2000). The activated Cry toxin then binds to receptors on the epithelial cells of the midgut, and insert into the membrane, forming pores (Bravo et al., 2007). This causes a disruption of the K^+ and pH gradients of the epithelial cells leading to osmotic lysis. The lysis of the cells provides a medium that allows any ingested spores to germinate, and bacteria present in the midgut can exit and invade the body cavity. Death can occur from either cell lysis, sepsis, or a combination

of the two (De Lara et al., 2016; Kotze et al., 2005). The mode of action in other insect orders, such as the effect of the proteins Cry 11Aa, Cry 4A, and Cry4B on mosquitos is very likely to be the same (Bravo et al., 2007). The formation of the spore and parasporal inclusion body are linked, and it is likely the Cry proteins help the bacteria survive. Like other spore forming bacteria, *Bt* undergoes sporulation when conditions are unfavorable. If both the spore and the protein are ingested by the insect, the subsequent cell lysis provides a culture allowing the spores to germinate (Aronson, 2002).

Because of the effect of Cry proteins on pest insects, *Bt* is the one of the most commonly used biopesticides, making up 95% of all commercial bio-insecticides (El-kersh et al., 2016). *Bacillus thuringiensis* spore and protein mixtures are implemented as sprays used for Lepidopteran pests. Mosquitos and black flies are also targeted using these proteins (Bravo et al., 2007). As mentioned above, crops around the world have been genetically modified to express Cry proteins (Betz et al., 2000). In the U.S., 83% of corn acreage is filled with *Bt* modified crops, and 92% of cotton has been modified (USDA, 2019). Most of the *cry* genes are found on plasmids, and these genes are inserted into the plant cells along with the required promoter to allow the foreign genes to be expressed. There are two methods by which the genes can be transferred. The first is through the bacterium, *Agrobacterium tumefaciens*, which can inject its genes into a plant and hijack the plant machinery. The second method is using a gene gun, which fires particles containing the desired DNA into the cells (Abbas, 2018). Once the desired genes are introduced, the plant will then begin to produce the Cry proteins, allowing them to resist the insect pests. A major benefit this provides is lowering the amount of chemical insecticides needed for pest control (Bravo et al., 2007).

The article by Clark et al. (2005) describes the results of multiple studies looking at the

effect of transgenic plant Cry proteins on organisms besides the target insects. One study found that *Chrysoperla carnea* (green lacewing) larvae, when fed insects with a diet of corn leaves expressing Cry protein, had a statistically higher mortality rate than control larvae. It was not clear whether the higher mortality rate was due to the Cry protein, or the diet of sick and dying prey insects (Hilbeck et al., 1998). Another study by Romeis et al. (2004) fed Cry protein directly to the *C. carnea* larvae and found no negative effects. The doses tested in this study were much higher than the larvae would have been exposed to in the study by Hilbeck et al. (1998), which supported the theory that the higher mortality in that study was due to the poor diet provided by the dying prey insects. Clark et al. (2005) also describes the results of multiple studies that were performed looking at the effect of these proteins on nontarget Lepidoptera, which found that species like *Danaus plexippus* (monarch butterfly) were susceptible. However, the studies performed in the field showed higher amounts of protein required for toxicity than in studies performed in the lab, suggesting that multiple factors determine both exposure and severity of Cry proteins to nontarget insects.

Other studies have investigated the safety of these proteins in vertebrates which found that vertebrate systems lacked the conditions required for the toxin to activate as well as the receptors to which the toxin binds. The Cry1 group of protein may be the most well studied, with the mode of action of these proteins thoroughly described. The Cry1 group requires an alkaline environment for the protein to first be solubilized, before the protoxin can be cleaved and activated. The acidic environment of the mammalian stomach would not provide this opportunity, instead degrading the protein before it left, and the receptors needed for the toxin to bind and create pores are lacking in vertebrates (Betz et al., 2000; Hu and Aroian, 2012). Kotze et al. (2005) demonstrated that Cry proteins rapidly degrade when exposed to acidic pH

environments, with the Cry proteins tested being significantly deactivated after exposure to environments with a pH of 3 or less for 15 minutes. Hu et al. (2010) documented that Cry5B in simulated gastric fluid almost completely broke down in just four minutes. In the paper by Betz et al. (2000), there is a table listing Cry proteins and the time it took to digest the proteins in simulated gastric fluid; the longest lasting protein, Cry1Ab, took one minute. Hu and Aroian (2012) acknowledged that a very small amount of Cry protein probably survives the stomach to reach the small intestine, yet there were still significant anthelmintic affects seen against *Heligmosomoides bakeri* in Hu et al. (2010). It is likely that only a small amount of the original dose survives the stomach, but that amount is enough for significant results.

Studies have been performed to determine whether the bacteria or the Cry proteins are toxic when ingested by mammals, with none showing significant impact on the mammal wellbeing (Guerrero et al., 2007; Koch et al., 2015; Mesnage et al., 2012; Schrøder et al., 2007; Shimada et al., 2006). One study by Santos-Vigil et al. (2018) found that activated Cry1Ac toxin administered intragastrically produced a moderate allergic response in mice. However, this study used the activated toxin, rather than the protein to illicit his response. Feed studies using Cry proteins have found no such allergic response. The study by Hadley et al. (1987) showed that there was no significant impact of feeding 500 mg of *B. thuringiensis* spores/kg body weight to sheep daily during a five-month study. The review by Rubio-Infante and Moreno-Fierros (2016) lists multiple mammal toxicity studies performed, with no signs of toxicity from either *Bt* based insecticide or purified toxins.

The extensive use of *Bt* Cry proteins as biopesticides and the modification of crops to express the proteins has led to the development of resistance in the insect pests that these proteins were originally used against. As seen with anthelmintics and antibiotics, resistance to

Cry proteins will develop in the organisms that are exposed. The first report of insect resistance to *Bacillus thuringiensis* was reported in *Plodia interpunctella* (Indianmeal moth) in 1985 (Singh et al., 2018). As the area covered by transgenic crops increased exposure by pest insects increased, and subsequently so did the number of cases of resistance (Tabashnik et al., 2013). Similar strategies as those used to deal with anthelmintic resistance are being implemented to slow the development of resistance against *Bt* crops in pest insects. The main method to slow resistance developing is to ensure the presence of refuges of non *Bt* crops planted near to *Bt* crops, which allows resistant individuals to breed with susceptible ones (Bravo and Soberón, 2008).

There are multiple mechanisms of resistance to Cry proteins that have been demonstrated by insects. One is seen in *Plodia interpunctella*, where resistant individuals lack a protease in the gut which is needed to activate the protoxin (Oppert et al., 1997). A second method of resistance is related to the binding site of the Cry toxin. In resistant strains of *Helicoverpa armigera* (cotton bollworm), resistance was related to mutations in the genes that produce cadherin, the primary receptor site for the Cry proteins (Xu et al., 2005). A third method is related to the immune response of the insect. In the study by Ma et al. (2005), a resistant strain of *Helicoverpa armigera* was shown to have an elevated immune response. A protein that promotes the coagulation of toxins bound to the protein, forming an insoluble aggregate. The mode of action of Cry proteins provide multiple points at which the protein or toxin could be inhibited or inactivated, as well as the binding site altered. These methods of resistance need to be considered if Cry proteins are used against other organisms, such as nematodes.

Studies have found that *B. thuringiensis* Cry proteins are not only toxic to insects, but also to nematodes, likely with the same mode of action (Hu and Aroian, 2012). There are many

strains of Cry proteins that have shown potential anthelmintic properties including Cry5A, Cry5B, Cry6A, Cry11Aa, Cry13, Cry14A, and Cry21A (De Lara et al., 2016; Hu and Aroian, 2012; Kotze et al., 2005). These Cry proteins have been demonstrated to be toxic to many nematodes, including free-living species like *Caenorhabditis elegans* (Wei et al., 2003), as well as parasitic species such as *Ancylostoma ceylanicum* (Cappello et al., 2006), *Ascaris suum* (Urban et al., 2013), and *H. contortus, T. colubriformis, and T. circumcincta* (Kotze et al., 2005).

In the study by Kotze et al. (2005), *H. contortus*, *T. circumcincta*, and *T. colubriformis* larvae and adults were exposed to multiple *B. thuringiensis* strains. The impact of the *Bt* was measured using a larval development assay and a motility assay for the adults. Two strains were seen to significantly inhibit the larval development of all three species and were toxic to the adults. For the larval development assays, all three species experienced complete larval development inhibition at the highest dose. For the adult motility assays, at the highest dose, the adult *H. contortus* and *T. circumcincta* displayed complete cessation of movement at 2 and 4 days, while the *T. colubriformis* was less affected, but displayed reduced mobility at day 2. PCR revealed these two strains possessed the genes needed to produce Cry13, Cry5A, and Cry5B. Sinott et al. (2012) took multiple species of *Bacillus* and tested their ability to inhibit larval development of *H. contortus* in sheep feces. The study found that *B. thuringiensis* var. *oswaldocruzi* had an 81.06% reduction, *B. thuringiensis* var. *kurstaki* produced a 79.9% reduction, *B. thuringiensis* var. *israelensis* had a 76.95% reduction, and *B. circulans* caused an 80.9% reduction in larvae.

In a recent study, De Lara et al. (2016) looked at the effect of *B. thuringiensis* var. *israelensis* Cry11Aa toxin on *H. contortus* larvae. Two suspensions were made and added to sheep fecal cultures to determine the larval inhibition of the Cry11Aa protein. One suspension

was composed of *B. thuringiensis* var. *israelensis* spores with Cry11Aa protein, and the other was composed of recombinant *Escherichia coli* bacteria that expressed the Cry11Aa. The two suspensions produced a 62% and 81% reduction, respectively, in larvae compared to controls of water and non-recombinant *E. coli*. Lambs naturally infected with *H. contortus* were administered the two suspensions and two controls, and fecal samples were collected 12 hours following administration. The suspensions of *B. thuringiensis* var. *israelensis* and recombinant *E. coli* caused a reduction in larvae by 79% and 90% when compared to control samples.

Of the Cry proteins mentioned above, one in particular has received much attention for its broad-spectrum effect on nematodes. Cry5B has been demonstrated to be toxic to many nematode parasites not only *in vitro,* but also *in vivo*. Mice infected with *Heligmosomoides bakeri* treated with a single dose of 90-100 mg/kg Cry5B experienced greater than 90% reduction in fecal egg counts and approximately 70% reduction in total worm burden (Hu et al., 2010). In a study by Cappello et al. (2006), hamsters infected with *Ancylostoma ceylanicum*, a hookworm that can also affect humans, were given 1 mg Cry5B repeated over 3 days resulting in a 75% reduction in fecal egg counts, as well as almost a 90% reduction in worm burden. A conventional anthelmintic used to treat *A. ceylanicum*, mebendazole, was also administered as a control and produced similar results. In a study by Urban et al. (2013), Cry5B was once again tested *in vivo*, this time in pigs infected with *Ascaris suum*. Pigs were administered two doses of 20 mg/kg Cry5B and slaughtered 15 days post infection when *A. suum* L4 were in the intestine. The pigs dosed with Cry5B demonstrated a 97% reduction in worm burden, and those that remained were significantly shorter than the worms found in the control pigs. While studies have been performed to measure the efficacy of *B. thuringiensis* against ruminant parasitic nematodes, none have been performed to determine the effect of Cry5B specifically.

The gastrointestinal system of the ruminant is more complex than the rodent or porcine system. Ruminants are foregut fermenters, with a multi-chambered stomach. The rumen and reticulum act as a fermentation chamber. From there the food moves to the omasum, and then into the abomasum. The abomasum is the "true stomach" where enzymatic digestion occurs. The abomasum empties into the small intestine through the pyloric sphincter (Dehority, 2002). This complex, multi-chambered stomach presents a challenge when considering the treatment of gastrointestinal nematodes with Cry protein. The time food takes to move out of the rumen and reticulum vary by animal. In sheep, this time can take 0.8-2.2 days, depending on the size of the food particles (Dehority, 2002). Urban et al. (2013) demonstrated that Cry5B ingested by pigs was able to reach the small intestine and have a significant impact on the *A. suum* L4. These results are promising, as the Cry protein had to survive passage through the acidic environment of the stomach, where there is high potential for the proteins to degrade beyond effectiveness, and reach the small intestine to be consumed by the target parasite. If Cry5B were to be used to treat ruminant nematodes, it would need to survive passage through the rumen and at least reach the abomasum without degrading.

References

Abbas, M.S.T., 2018. Genetically engineered (modified) crops (*Bacillus thuringiensis* crops) and the world controversy about their safety. Egypt J. Biol. Pest Control. 28, 52.

Abbott, K.A., Taylor, M.A., Stubbings, L.A., 2012. Sustainable worm control strategies for sheep, 4th edition. SCOPS Publishing.

Aronson, A., 2002. Sporulation and δ-endotoxin synthesis by *Bacillus thuringiensis*. Cell. Mol. Life Sci. 59, 417-425.

Bang, K.S., Familton, A.S., Sykes, A.R., 1990a. Effect of copper oxide wire particle treatment on establishment of major gastrointestinal nematodes in lambs. Res. Vet. Sci. 49, 132-137.

Bang, K.S., Familton, A.S., Sykes, A.R., 1990b. Effect of ostertagiasis on copper status in sheep: a study involving the use of copper oxide wire particles. Res. Vet. Sci. 49, 306-314.

Barger, I.A., 1985. The statistical distribution of trichostrongylid nematodes in grazing lambs. Int. J. Parasitol. 15, 645-649.

Barger, I.A., 1999. The role of epidemiological knowledge and grazing management for helminth control in small ruminants. Int. J. Parasitol. 29, 41-47.

Barrere, V., Falzon, L.C., Shakya, K.P., Menzies, P.I., Peregrine, A.S., Prichard, R.K., 2013. Assessment of benzimidazole resistance in *Haemonchus contortus* in sheep flocks in Ontario, Canada: comparison of detection methods for drug resistance. Vet. Parasitol. 198, 159-165.

Besier, R.B., Kahn, L.P., Sargison, N.D., Van Wyk, J.A., 2016. The pathophysiology, ecology, and epidemiology of *Haemonchus contortus* infection in small ruminants. Adv. Parasitol. 93, 95-143.

Besier, R.B., Love, S.C.J., 2003. Anthelmintic resistance in sheep nematodes in Australia: the need for new approaches. Aust. J. Exp. Agric. 43, 1383-1391.

Betz, F.S., Hammond, B.G., Fuchs, R.L., 2000. Safety and advantages of *Bacillus thuringiensis*-protected plants to control insect pests. Regul. Toxicol. Pharmacol. 32, 156-173.

Bishop, S.C., 2012. Possibilities to breed for resistance to nematode parasite infections in small ruminants in tropical production systems. Animal. 6, 741-747.

Bishop, S.C., Morris, C.A., 2007. Genetics of disease resistance in sheep and goats. Small Rumin. Res. 70, 48-59.

Bisset, S.A., Morris, C.A., McEwan, J.C., Vlassof, A., 2002. Breeding sheep in New Zealand that are less reliant on anthelmintics to maintain health and productivity. N. Z. Vet. J. 49, 236-246.

Blouin, M.S., Yowell, C.A., Courtney, C.H., Dame, J.B., 1995. Host movement and the genetic structure of populations of parasitic nematodes. Genetics. 141, 1007-1014.

Bogan, J., Armour, J., 1987. Anthelmintics for ruminants. Int. J. Parasitol. 17, 483-491.

Bravo, A., Gill, S.S., Soberón, M., 2007. Mode of action of *Bacillus thuringiensis* Cry and Cyt toxins and their potential for insect control. Toxicon. 49, 423-435.

Brown, H.D., Matzuk, A.R., Ilves, I.R., Peterson, L.H., Harris, S.A., Sarett, L.H., Egerton, J.R., Yakstis, J.J., Campbell, W.C., Cuckler, A.C., 1961. Antiparasitic drugs. IV. 2-(4'-Thiazolyl)-Benzimidazole, a new anthelmintic. J. Am. Chem. Soc. 83, 1764-1765.

Bravo, A., Soberón, M., 2008. How to cope with insect resistance to Bt toxins? Trends Biotechnol. 26, 573-579.

Brunsdon, R.V., 1980. Principles of helminth control. Vet. Parasitol. 6, 185-215.

Cappello, M., Bungiro, R.D., Harrison, L.M., Bischoff, L.J., Griffitts, J.S., Barrows, B.D., Aroian, R.V., 2006. A purified *Bacillus thuringiensis* crystal protein with therapeutic activity against the hookworm parasite *Ancylostoma ceylanicum*. Proc. Natl. Acad. Sci. U.S.A. 103, 15154-15159.

Cavanagh, N.A., Judson, G.J., 1995. Copper oxide powder as a copper supplement for sheep. J. Trace Elem. Electrolytes Health Dis. 8, 183-188.

Chandrawathani, P., Yusoff, N., Wan, L.C., Ham, A., Waller, P.J., 2004. Total anthelmintic failure to control nematode parasites of small ruminants on government breeding farms in Sabah, East Malaysia. Vet. Res. Commun. 28, 479-489.

Chartier, C., Etter, E., Hoste, H., Pors, I., Koch, C., Dellac, B., 2000. Efficacy of copper oxide needles for the control of nematode parasites in dairy goats. Vet. Res. Commun. 24, 389-399.

Chaudhry, U., Redman, E.M., Raman, M., Gilleard, J.S., 2015. Genetic evidence for the spread of benzimidazole resistance mutation across southern India from a single origin in the parasitic nematode *Haemonchus contortus*. Int. J. Parasitol. 45, 721-728.

Clark, B.W., Phillips, T.A., Coats, J.R., 2005. Environmental fate and effects of Bacillus thuringiensis (Bt) proteins from transgenic crops: a review. J. Agric. Food Chem. 53, 4643-4653.

Colditz, I.G., Watson, D.L., Gray, G.D., Eady, S.J., 1996. Some relationships between age, immune responsiveness and resistance to parasites in ruminants. Int. J. Parasitol. 26, 869-877.

Coles, G.C., 2005. Anthelmintic resistance – looking to the future: a UK perspective. Res. Vet. Sci. 78, 99-108.

Craig, T.M., 1986. Epidemiology and control of gastrointestinal nematodes and cestodes in small ruminants. Vet. Clin. North Am. Food Anim. Pract. 2, 367-372.

Dehority, B.A., 2002. Gastrointestinal tracts of herbivores, particularly the ruminant: anatomy, physiology and microbial digestion of plants. J. Appl. Anim. Res. 21, 145-160.

De Lara, A.P., Lorenzon, L.B., Vianna, A.M., Santos, F.D.S., Pinto, L.S., Berne, M.E.A., Leite, F.P.L., 2016. Larvicidal activity of *Bacillus thuringiensis* var. *israelensis* Cry 11Aa toxin against *Haemonchus contortus*. Parasitology. 1, 1-7.

Edwards, J.R., Wroth, R., De Chaneet, G.C., Besier, R.B., Karlsson, J., Morcombe, P.W., Dalton-Morgan, G., Roberts, D., 1986. Survey of anthelmintic resistance in Western Australian sheep flocks. 2. Relationship with sheep management and parasite control practices. Aust. Vet. J. 63, 139-144.

El-kersh, T.A., Ahmed, A.M., Al-sheikh, Y.A., Tripet, F., Ibrahim, M.S., Metwalli, A.A.M., 2016. Isolation and characterization of native *Bacillus thuringiensis* strains from Saudi Arabia with enhanced larvicidal toxicity against the mosquito vector *Anopheles gambiae* (s.l.). Parasit. Vectors. 9, 647.

Emery, D.L., Hunt, P.W., Le Jambre, L.F., 2016. *Haemonchus contortus*: the then and now, and where to from here? Int. J. Parasitol. 46, 755-769.

Fleming, S.A., Craig, T., Kaplan, R.M., Miller, J.E., Navarre, C., Rings, M., 2006. Anthelmintic resistance of gastrointestinal parasites in small ruminants. J. Vet. Intern. Med. 20, 435-444.

Fontenot, M.E., Miller, J.E., Peña, M.T., Larsen, M., Gillespie, A., 2003. Efficiency of feeding *Duddingtonia flagrans* chlamydospores to grazing ewes on reducing availability of parasitic nematode larvae on pasture. Vet. Parasitol. 118, 203-213.

Gibbs, H.C., 1986. Hypobiosis and the periparturient rise in sheep. Vet. Clin. North Am. Food Anim. Pract. 2, 345-353.

Guerrero, G.G., Russell, W.M., Moreno-Fierros, L., 2007. Analysis of the cellular immune response induced by *Bacillus thuringiensis* Cry1A toxins in mice: effect of the hydrophobic motif from diphtheria toxin. Mol. Immunol. 44, 1209-1217.

Hadley, W.M., Burchiel, S.W., McDowell, T.D., Thilsted, J.P., Hibbs, C.M., Whorton, J.A., Day, P.W., Friedman, M.B., Stoll, R.E., 1987. Five-month oral (diet) toxicity/infectivity study of *Bacillus thuringiensis* insecticides in sheep. Fundam. Appl. Toxicol. 8, 236-242.

Hilbeck, A., Baumgartner, M., Fried, P.M., Bigler, F., 1998. Effects of transgenic Bacillus thuringiensis corn-fed prey on mortality and development time of immature *Chrysoperla carnea* (Neuroptera: Chrysopidae). Environ. Entomol. 27, 1255-1263.

Hoste, H., Chartier, C., Le Frileux, Y., 2002. Control of gastrointestinal parasitism with nematodes in dairy goats by treating the host category at risk. Vet. Res. 33, 531-545.

Hu, Y., Georghiou, S.B., Kelleher, A.J., Aroian, R.V., 2010. *Bacillus thuringiensis* Cry5B protein is highly efficacious as a single-dose therapy against an intestinal roundworm infection in mice. PLoS Negl. Trop. Dis. 4, e614.

Hu, Y., Aroian, R.V., 2012. Promise of *Bacillus thuringiensis* crystal proteins as anthelmintics. In: Selzer, P.M., Caffrey, C.R. (Eds.), Parasitic Helminths. Wiley-Blackwell, Hoboken, NJ, pp. 267-281.

Ibrahim, M.A., Griko, N., Junker, M., Bulla, L.A., 2010. *Bacillus thuringiensis*: a genomics and proteomics perspective. Bioeng. Bugs. 1, 31-50.

Jabbar, A., Iqbal, Z., Kerboeuf, D., Muhammad, G., Khan, M.N., Afaq, M., 2006. Anthelmintic resistance: the state of play revisited. Life Sci. 79, 2413-2431.

Jackson, F., Miller, J., 2006. Alternative approaches to control-Quo vadit? Vet. Parasitol. 139, 371-384.

James, C.E., Hudson, A.L., Davey, M.W., 2009. Drug resistance mechanisms in helminths: is it survival of the fittest? Trends Parasitol. 25, 328-335.

Jordan, H.E., Phillips, W.A., Morrison, R.D., Doyle, J.J., McKenzie, K., 1988. A 3-year study of continuous mixed grazing of cattle and sheep: parasitism of offspring. Int. J. Parasitol. 18, 779-784.

Joshi, B.R., Kommuru, D.S., Terrill, T.H., Mosjidis, J.A., Burke, J.M., Shakya, K.P., Miller, J.E., 2011. Effect of feeding sericea lespedeza leaf meal in goats experimentally infected with *Haemonchus contortus*. Vet. Parasitol. 178, 192-197.

Kaplan, R.M., 2004. Drug resistance in nematodes of veterinary importance: a status report. Trends Parasitol. 20, 477-481.

Kaplan, R.M., Vidyashankar, A.N., 2012. An inconvenient truth: global warming and anthelmintic resistance. Vet. Parasitol. 186, 70-78.

Kemper, K.E., Palmer, D.G., Liu, S.M., Greeff, J.C., Bishop, S.C., Karlsson, L.J.E., 2010. Reduction of faecal worm egg count, worm numbers and worm fecundity in sheep selected for worm resistance following artificial infection with *Teladorsagia circumcincta* and *Trichostrongylus colubriformis*. Vet. Parasitol. 171, 238-246.

Knox, M.R., 2002. Effectiveness of copper oxide wire particles for *Haemonchus contortus* control in sheep. Aust. Vet. J. 80, 224-227.

Koch, M.S., Ward, J.M., Levine, S.L., Baum, J.A., Vicini, J.L., Hammond, B.G., 2015. The food and environmental safety of *Bt* crops. Front. Plant Sci. 6, 283.

Kohler, P., 2001. The biochemical basis of anthelmintic action and resistance. Int. J. Parasitol. 31, 336-345.

Kommuru, D.S., Whitley, N.C., Miller, J.E., Mosjidis, J.A., Burke, J.M., Gujja, S., Mechineni, A., Terrill, T.H., 2015. Effect of sericea lespedeza leaf meal pellets on adult female *Haemonchus contortus* in goats. Vet. Parasitol. 207, 170-175.

Kotze, A.C., O'Grady, J., Gough, J.M., Pearson, R., Bagnall, N.H., Kemp, D.H., Akhurst, R.J., 2005. Toxicity of *Bacillus thuringiensis* to parasitic and free-living life-stages of nematode parasites of livestock. Int. J. Parasitol. 35, 1013-1022.

Laing, R., Kikuchi, T., Martinelli, A., Tsai, I.J., Beech, R.N., Redman, E., Holroyd, N., Bartley, D.J., Beasley, H., Britton, C., Curran, D., Devaney, E., Gilabert, A., Hunt, M., Jackson, F., Johnston, S.L., Kryukov, I., Li, K., Morrison, A.A., Reid, A.J., Sargison, N., Saunders, G.I., Wasmuth, J.D., Wolstenholme, A., Berriman, M., Gilleard, J.S., Cotton, J.A., 2013. The genome and transcriptome of *Haemonchus contortus,* a key model parasite for drug and vaccine discovery. Genome Biol. 14, R88.

Lange, K.C., Olcott, D.D., Miller, J.E., Mosjidis, J.A., Terrill, T.H., Burke, J.M., Kearney, M.T., 2006. Effect of sericea lespedeza (*Lespedeza cuneata*) fed as hay, on natural and experimental *Haemonchus contortus* infections in lambs. Vet. Parasitol. 141, 273-278.

Larsen, M., Nansen, P., Grønvold, J., Wolstrup, J., Henriksen, S.A., 1997. Biological control of gastro-intestinal nematodes- facts, future, or fiction? Vet. Parasitol. 72, 479-492.

Ma, G., Roberts, H., Sarjan, M., Featherstone, N., Lahnstein, J., Akhurst, R., Schmidt, O., 2005. Is the mature endotoxin Cry1Ac from *Bacillus thuringiensis* inactivated by a coagulation reaction in the gut lumen of resistant *Helicoverpa armigera* larvae? Insect Biochem. Mol. Biol. 35, 729-739.

Martin, P.J., Le Jambre, L.F., Claxton, J.H., 1981. The impact of refugia on the development of Thiabendazole resistance in *Haemonchus contortus*. Int. J. Parasitol. 11, 35-41.

Martin, P.J., Anderson, N., Jarrett, R.G., Brown, T.H., Ford, G.E., 1982. Effects of a preventative and suppressive control scheme on the development of thiabendazole-resistance in *Ostertagia* spp. Aust. Vet. J. 58, 185-190.

Martin, R.J., 1997. Modes of action of anthelmintic drugs. Vet. J. 154, 11-34.

Martin, R.J., Robertson, A.P., Bjorn, H., 1997. Target sites of anthelmintics. Parasitology. 114.

Mesnage, R., Clair, E., Gress, S., Then, C., Székács, A., Séralini, G.E., 2012. Cytotoxicity on human cells of Cry1Ab and Cry1Ac *Bt* insecticidal toxins alone or with a glyphosate-based herbicide. J. Appl. Toxicol. 33, 695-699.

Min, B.R., Pinchak, W.E., Anderson, R.C., Fulford, J.D., Puchala, R., 2006. Effects of condensed tannins supplementation on weight gain and *in vitro* and *in vivo* bloat precursors in steers grazing winter wheat. J. Anim. Sci. 84, 2546–2554.

O'Sullivan, B.M., Donald, A.D., 1973. Responses to infection with *Haemonchus contortus* and *Trichostrongylus colubriformis* in ewes of different reproductive status. Int. J. Parasitol. 3, 521-530.

Oliveira, P.A., Riet-Correa, B., Estima-Silva, P., Coelho, A.C.B., Santos, B.L., Costa, M.A.P., Ruas, J.L., Schild, A.L., 2017. Multiple anthelmintic resistance in Southern Brazil sheep flocks. Rev. Bras. Parasitol. Vet. 26, 427-432.

Oppert, B., Kramer, K.J., Beeman, R.W., Johnson, D., McGaughey, W.H., 1997. Proteinase-mediated insect resistance to *Bacillus thuringiensis* toxins. J. Biol. Chem. 272, 23473-23476.

Papadopoulos, E., 2008. Anthelmintic resistance in sheep nematodes. Small Rumin. Res. 76, 99-103.

Papadopoulos, E., Gallidis, E., Ptochos, S., 2012. Anthelmintic resistance in sheep in Europe, a selected review. Vet. Parasitol. 189, 85-88.

Peña, M.T., Miller, J.E., Fontenot, M.E., Gillespie, A., Larsen, M., 2002. Evaluation of *Duddingtonia flagrans* in reducing infective larvae of *Haemonchus contortus* in feces of sheep. Vet. Parasitol. 103, 259-265.

Pomroy, W.E., 2006. Anthelmintic resistance in New Zealand: a perspective on recent findings and options for the future. N. Z. Vet. J. 54, 265-270.

Qamar, M.F., Maqbool, A., Khan, M.S., Ahmad, N., Muneer, M.A., 2009. Epidemiology of *Haemonchosis* in sheep and goats under different management conditions. Vet. World. 2, 413-417.

Roebor, F., Jex, A.R., Gasser, R.B., 2013. Impact of gastrointestinal parasitic nematodes of sheep, and the role of advanced molecular tools for exploring epidemiology and drug resistance - an Australian perspective. Parasit. Vectors. 6, 153.

Roh, J.Y., Choi, J.Y., Li, M.S., Jin, B.R., Je, Y.H., 2007. *Bacillus thuringiensis* as a specific, safe, and effective tool for insect pest control. J. Microbiol Biotechnol. 17, 547-559.

Romeis, J., Dutton, A., Bigler, F., 2004. *Bacillus thuringiensis* toxin has no direct effect on larvae of the green lacewing *Chrysoperla carnea* (Stephens) (Neuroptera: Chrysopidae). J. Insect Physiol. 50, 175-183.

Rubio-Infante, N., Moreno-Fierros, L., 2016. An overview of the safety and biological effects of *Bacillus thuringiensis* Cry toxins in mammals. J. Appl. Toxicol. 36, 630-648.

Sanchis, V., 2011. From microbial sprays to insect-resistant transgenic plants: history of the biospesticide *Bacillus thuringiensis*. A review. Agron. Sustain. Dev. 31, 217-231.

Sangster, N.C., Gill, J., 1999. Pharmacology of anthelmintic resistance. Parasitol. Today. 15, 141-146.

Santos-Vigil, K.I., I Ilhuicatzi-Alvarado, D., García-Hernández, A.L., Herrera-García, J.S., Moreno-Fierros, L., 2018. Study of the allergenic potential of *Bacillus thuringiensis* Cry1Ac toxin following intra-gastric administration in a murine model of food-allergy. Int. Immunopharmacol. 61, 185-196.

Schrøder, M., Poulsen, M., Wilcks, A., Kroghsbo, S., Miller, A., Frenzel, T., Danier, J., Rychlik, M., Emami, K., Gatehouse, A., Shu, Q., Engel, K.H., Altosaar, I., Knudsen, I., 2007. A 90-day safety study of genetically modified rice expressing Cry1Ab protein (*Bacillus thuringiensis* toxin) in Wistar rats. Food Chem. Toxicol. 45, 339-349.

Shimada, N., Miyamoto, K., Kanda, K., Murata, H., 2006. *Bacillus thuringiensis* insecticidal Cry1AB toxin does not affect the membrane integrity of the mammalian intestinal epithelial cells: an *in vitro* study. In Vitro Cell. Dev. Biol. Anim. 42, 45-49.

Singh, G., Dwivedi, S.K., Nath, R., 2018. Mechanism of resistance for field insects in *Bt* crops and its management. Ann. Biol. 34, 152-154.

Sinott, M.C., Cunha-Filho, N.A., Castro, L.L.D., Lorenzon, L.B., Pinto, N.B., Capella, G.A., Leite, F.P.L., 2012. *Bacillus* spp. toxicity against *Haemonchus contortus* larvae in sheep fecal cultures. Exp. Parasitol. 132, 103-108.

Sréter, T., Molnár, V., Kassai, T., 1994. The distribution of nematode egg counts and larval counts in grazing sheep and their implications for parasite control. Int. J. Parasitol. 24, 103-108.

Sutherland, I., Scott, I., 2010. Gastrointestinal nematodes of sheep and cattle. West Sussex (UK): Wiley-Blackwell, 242 pp.

Sykes, A., 1994. Parasitism and production in farm animals. Anim. Sci. 59, 155-172.

Tabashnik, B., Brévault, T., Carriere, Y., 2013. Insect resistance to *Bt* crops: lessons from the first billion acres. Nat. Biotechnol. 31, 510-521.

Terrill, T.H., Miller, J.E., Burke, J.M., Mosjidis, J.A., Kaplan, R.M., 2012. Experiences with integrated concepts for the control of *Haemonchus contortus* in sheep and goats in the United States. Vet. Parasitol. 186, 28-37.

Thomas, R.J., Ali, D.A., 1983. The effect of *Haemonchus contortus* infection on the pregnant and lactating ewe. Int. J. Parasitol. 13, 393-398.

Torres-Acosta, J.F.J., Hoste, H., 2008. Alternative or improved methods to limit gastro-intestinal parasitism in grazing sheep and goats. Small Rumin. Res. 77, 159-173.

Urban, J.F. Jr., Hu, Y., Miller, M.M., Scheib, U., You, Y.Y., Aroian, R.V., 2013. *Bacillus thuringiensis*-derived Cry 5B has potent anthelmintic activity against *Ascaris suum*. PLoS Negl. Trop. Dis. 7, e2263.

USDA, 2019. Recent trends in GE adoption. Available at: https://www.ers.usda.gov/data-products/adoption-of-genetically-engineered-crops-in-the-us/recent-trends-in-ge-adoption.aspx

Vagenas, D., Jackson, F., Russel, A.J.F., Merchant, M., Wright, I.A., Bishop, S.C., 2002. Genetic control of resistance to gastro-intestinal parasites in crossbred cashmere-producing goats: responses to selection, genetic parameters and relationships with production traits. Anim. Sci. 74, 199-208.

Van Wyk, J.A., Malan, F.S., Gerber, H.M., Alves, R.M.R., 1989. The problem of escalating resistance of *Haemonchus contortus* to the modern anthelmintics in South Africa. Onderstepoort J. Vet. Res. 56, 41-49.

Van Wyk, J.A., 2001. Refugia-overlooked as perhaps the most potent factor concerning the development of anthelmintic resistance. Onderstepoort J. Vet. Res. 68, 55-67.

Van Wyk, J.A., Bath, G.F., 2002. The FAMACHA© system for managing haemonchosis in sheep and goats by clinically identifying individual animals for treatment. J. Vet. Res. 33, 509-529.

Vatta, A.F., Letty, B.A., Van der Linde, M.J., Van Wijk, E.F., Hansen, J.W., Krecek, R.C., 2001. Testing for clinical anaemia caused by *Haemonchus* spp. in goats farmed under resource-poor conditions in South Africa using an eye colour chart developed for sheep. Vet. Parasitol. 99, 1-14.

Vatta, A.F., Waller, P.J., Githiori, J.B., Medley, G.F., 2009. The potential to control *Haemonchus contortus* in indigenous South African goats with copper oxide wire particles. Vet. Parasitol. 162, 306-313.

Vlassoff, A., Leathwick, D.M., Heath, A.C.G., 2001. The epidemiology of nematode infections of sheep. N. Z. Vet. J. 49, 213-22.

Waghorn, T.S., Leathwick, D.M., Chen, L.Y., Skipp, R.A., 2003. Efficacy of the nematode-trapping fungus *Duddingtonia flagrans* against three species of gastro-intestinal nematodes in laboratory faecal cultures from sheep and goats. Vet. Parasitol. 118, 227-234.

Waghorn, T.S., Leathwick, D.M., Miller, C.M., Atkinson, D.S., 2008. Brave or gullible: testing the concept that leaving susceptible parasites in refugia will slow the development of anthelmintic resistance. N. Z. Vet. J. 56, 158-163.

Wei, J.Z., Hale, K., Carta, L., Platzer, E., Wong, C., Fang, S.C., Aroian, R.V., 2003. *Bacillus thuringiensis* crystal toxins that target nematodes. Proc. Natl. Acad. Sci. U.S.A. 100, 2760-2765.

Weise, R.W., 1977. A light and electron microscopic study of the dorsal buccal lancet of *Haemonchus contortus*. J. Parasitol. 63, 854-857.

Williams, A.R., Greeff, J.C., Vercoe, P.E., Dobson, R.J., Karlsson, L.J.E., 2010. Merino ewes bred for parasite resistance reduce larval contamination onto pasture during the peri-parturient period. Animal. 4, 122-127.

Xu, X., Yu, L., Wu, Y., 2005. Disruption of a cadherin gene associated with resistance to Cry1Ac-endotoxin of *Bacillus thuringiensis* in *Helicoverpa armigera*. Appl. Environ. Microbiol. 71, 948-954.

CHAPTER 2: Effect of oral administration of live *Bacillus thuringiensis* containing Cry5B protein on experimental *Haemonchus contortus* infection in sheep

ABSTRACT

Trichostrongyle nematodes of small ruminants pose a danger to the health and productivity of their hosts. These parasites are mainly treated with anthelmintics, to which many species have become resistant. The ever-developing anthelmintic resistance seen across the globe has created a pressing need for new compounds that can be used to slow the development of resistance. Previous research has shown the considerable anthelmintic impact both *in vitro* and *in vivo* of the bacterium, *Bacillus thuringiensis*, Cry5B crystal protein against multiple intestinal nematode parasites of dogs, pigs, and rodents. To investigate the effect of the protein *in vivo* against the small ruminant parasite, *Haemonchus contortus*, five female and five castrated male lambs aged approximately 7-8 months were removed from pasture and housed in a barn on straw bedding for the duration of the study. All were dewormed to remove existing trichostrongyle infection. Sheep were subsequently each orally administered 10,000 third stage larvae of *H. contortus*. Following patency of infection, sheep were divided into two groups balanced for sex and fecal egg counts (FEC). Treatment group sheep were orally administered 200 mL daily for four days of a mixture of live genetically modified, asporogenous *Bacillus thuringiensis* producing Cry5B (~40 mg/kg). Control group sheep received an equal volume of water. FEC were determined daily for 7 days post treatment (PT) and thereafter every two days until necropsy at 14 days PT. Twenty-four hours after the first treatment, the mean FEC of the treated animals was reduced by 78% compared to controls, and by 94% after 72 hours. There was no significant difference in the mean total worm burden between the experimental groups, but the number of female worms in treated animals was reduced by 98%. Of the few eggs produced by

treated animals, only 27% developed to the infective larval stage compared to >95% of eggs from control lambs. These results suggest that *B. thuringiensis* Cry5B protein shows promise as a potential treatment for *H. contortus* in sheep.

1. Introduction

Haemonchus contortus is one of many species of gastrointestinal nematodes that infect small ruminants. This species is more pathogenic than most due to it being a blood feeding parasite. The adults are found in the abomasum, and infections can cause production losses for small ruminant producers. Signs of heavy infection include weight loss, lethargy, submandibular edema, anemia, and death (Sutherland and Scott, 2010). The nematode can quickly progress from the egg to the infective larva in warm and wet conditions, allowing pastures to become rapidly contaminated and susceptible individuals like lambs to develop heavy infections (Getachew et al., 2007). Due to the potential for production loss, small ruminant producers and veterinarians have used frequent anthelmintic treatments to control *H. contortus* infections. Practices such as underdosing or non-targeted treatment have contributed to the development of anthelmintic resistance by creating selection pressure for individual worms resistant to anthelmintics. Their offspring would constitute greater percentages of the total worm population as time went on if the resisted drug continued to be used (Kaplan, 2004). Resistance in small ruminant gastrointestinal nematodes has been reported to all widely available anthelmintics. *Haemonchus contortus* especially has shown the ability to rapidly develop resistance to a wide range of anthelmintics (Gilleard, 2013). Due to the rising rate of anthelmintic resistance, the current reliance on anthelmintics as the main control method is a problem that will become increasingly worse if no new treatment options are made available.

One potential alternative treatment is the crystal proteins produced by the bacterium *Bacillus thuringiensis*, a soil dwelling bacterium. During sporulation, *B. thuringiensis* can create large crystals next to the spore called parasporal crystals. These parasporal crystals are composed of crystal (Cry) proteins. Crystal proteins are widely used biopesticides in many forms, ranging from spray applications to crops genetically modified to produce the protein directly (Bravo et al., 2007). Crystal proteins are lethal to members of the orders Coleoptera, Diptera, Lepidoptera, and Hymenoptera. There are many Cry proteins, with one protein usually lethal to just a few species, but some proteins are lethal to species in multiple orders (Roh et al., 2007). The mode of action is well described in Lepidopteran species. When ingested, the crystal protein is solubilized in the alkaline conditions of the insect midgut and, once proteolytically cleaved, is activated and becomes a pore-forming toxin that has a high affinity for the epithelial cells of the invertebrate intestine (Kotze et al., 2005; Bravo et al., 2007). The glycolipid receptors to which the toxin binds are lacking in vertebrates, so the toxin has no impact on them when ingested (Betz et al., 2000). Due to the effectiveness against insects, and the fact that the same receptor is present in the nematode intestine, studies have been performed looking at the impact of the Cry proteins on nematodes.

Some Cry proteins are toxic to multiple organisms, such as Cry11Aa, which displays nematocidal and mosquitocidal properties (Bravo et al., 2007). Studies have shown that multiple *B. thuringiensis* Cry proteins are effective against nematode species (Hu and Aroian 2012; Kotze et al., 2005), ranging from free-living nematodes like *Caenorhabditis elegans* (Wei et al., 2003) to parasitic species like *H. contortus* (De Lara et al., 2016). One protein, Cry5B, has been studied for its effect on multiple parasitic species *in vivo*. Hu et al. (2010) found mice infected with *Heligmosomoides bakeri* experienced a 70% reduction in worm burden after treatment with

Cry5B. Cappello et al. (2006) reported a 90% reduction in the burden of hamsters infected with *Ancylostoma* c*eylanicum*, and Urban et al. (2013) reported a 97% reduction in worm burden in pigs infected with *Ascaris suum* fourth stage larvae. While there are no published reports of Cry5B being tested against *H. contortus*, studies have shown that *H. contortus* is susceptible to other Cry proteins (De Lara et al., 2016; Kotze et al., 2005). Based on the vulnerability of *H. contortus* to Cry proteins, as well as the *in vivo* effectiveness of Cry5B against multiple parasitic species, we hypothesized that *B. thuringiensis* Cry5B protein would be an effective treatment for *H. contortus* infections in sheep.

2. Materials and methods

2.1. Animals

A group of Suffolk, Dorset, and Suffolk/Dorset cross lambs aged 7-8 months were selected for the experiment. Six castrated male and six female lambs were removed from pasture and housed during the study. All lambs were treated with levamisole (8.0 mg/kg), ivermectin (0.2 mg/kg), and albendazole (7.5 mg/kg) to remove any existing trichostrongylid infection. Anthelmintics were administered orally, one after another. The animals shared a single pen with straw bedding to minimize the chance of reinfection. When euthanized, sheep were given an intravenous injection of pentobarbital at a dose of 100 mg/kg. Death was confirmed by exsanguination once vital signs were no longer detected. All protocols followed in the study were approved by the VT Institutional Animal Care and Use Committee (Protocol #18-135, #18-141), and the Institutional Biosafety Committee (Protocol #17-006).

2.2. Haemonchus contortus

Third stage infective *H. contortus* larvae (L3) were obtained by culturing eggs present in

donor sheep feces. Feces from sheep infected with *H. contortus* were collected and cultured for 14 days to allow the eggs to develop to L3. Infective L3 were harvested using the modified Baermann technique (Zajac and Conboy, 2012) and stored in water at 4° C.

2.3. Cry5B

The *B. thuringiensis* and Cry5B protein used in the experiment was provided by the laboratory of Dr. Raffi Aroian at the University of Massachusetts Medical School (Hu et al., 2010). The treatment that was administered was live *B. thuringiensis* with cytosolic crystal protein (BaCC) and Cry5B in water. The *B. thuringiensis* bacteria were sporulation deficient and genetically modified to produce the Cry5B in the cytosol rather than during normal sporulation, encapsulating the protein in the vegetative bacterial cells. The mixture was stored at -80° C and thawed at the time of use at 4° C.

2.4. Haemonchus contortus infection

Haemonchus contortus L3 in water were diluted to a concentration of 1,000 L3/mL of water. Each animal was infected with 10,000 *H. contortus* L3 by oral administration using a syringe followed by an additional 10 mL of tap water. Following infection, FAMACHA© scores were determined weekly to monitor for anemia. Fecal samples were collected *per rectum* once weekly to determine fecal egg counts (FEC).

2.5. Fecal egg count determination and larval cultures

Rectal fecal samples were collected from sheep and fecal egg counts (FEC) were determined by modified McMaster's Test (Zajac and Conboy, 2012) with an egg detection limit of 50 eggs per gram of feces. For the larval cultures, composite fecal samples were prepared by thoroughly mixing 10-gram samples from each animal in each experimental group. Ten-gram

larval cultures were made for each group using the composite feces, and cultured for 14 days (Zajac and Conboy, 2012).

2.6. Worm count and measurement

To collect adult *H. contortus*, the abomasum was removed from each sheep, placed in a tray, cut along the greater curvature, and washed with warm water. Abomasal washings were collected, and a 10% aliquot was taken from the washings for each animal. An equal volume of 10% formalin was added to each aliquot for fixation of parasites. Half of the abomasum was placed in a tray, covered in warm saline, and incubated at 37° C overnight to allow any larvae to emerge from the abomasal wall. The other half of the abomasum was used in a separate study. The next day, the abomasa were removed from the incubator, and the same method was used to collect 10% aliquots of washings.

Adult worm numbers in each sample were determined in each sample using a dissecting microscope and a gridded petri dish. Lugol's iodine was added to stain the worms to assist with identification. These counts were multiplied by 10 to determine the total worm burden of each sheep. Worms collected during the counting process were measured using ImageJ software. Fifty male and 50 female worms were measured for each animal. For those animals with fewer than 50 worms of each sex, all worms were measured.

2.7. Larval cultures

Composite fecal samples for larval culture were created for each experimental group using 10 grams of feces per sheep. Three FEC determinations using the modified McMaster's test were performed on the composite samples to determine the number of eggs present in each culture. Ten-gram cultures were set up in triplicate for each of the two groups, with cultures being created from feces collected on the first day of treatment and on the final treatment day.

After two weeks, the cultures were harvested by a modification of the Baermann test (Zajac and Conboy, 2012). The collected liquid was placed in a 50 mL conical centrifuge tube and kept at 4°C for an hour to allow the L3 to sediment out. The supernatant was drawn off, and the L3 were transferred to a 15 mL tube, with the process repeated until the L3 were left in 2 mL of liquid. After mixing, the number of larvae in five 20-microliter aliquots was counted and the average used to calculate the total number of larvae in each culture.

2.8. Experimental protocol

The experimental timeline is displayed in Fig. 2.1. Following infection with 10,000 *H. contortus* L3 eight weeks prior to Day 0, FEC were determined weekly. The sheep were divided into control and treatment groups on Day 0, balanced for sex and individual FEC determined using the mean FEC over the previous three weeks. The male and the female sheep with the lowest FEC were removed from the study. The BaCC was orally administered to the treatment animals, with each animal receiving 200 mL of BaCC, containing approximately 40 mg/kg Cry5B. The control group received an equal volume of water. The same dose was repeated on Days 2-4 at the same time of day, before the animals were fed in the morning. Rectal fecal samples were collected from each sheep before treatment. FEC were determined on Days 1-8, 10, 12, and 14. Larval cultures were prepared on Days 1 and 4 using a composite sample from each group. On Day 15, the sheep were euthanized, and their abomasa removed to collect contents containing adult worms.

2.9. Statistics

The FEC and worm numbers were log [10] transformed to normalize the data. The geometric means of FEC and worm count data were used to analyze the worm count data. The FEC data was evaluated using a Mixed Model ANOVA, and the slice option was used to

compare the two groups on each day. A pooled T-test and a Mixed Model ANOVA were used on the worm count data. Total worm counts in the two groups were compared, as well as sex specific counts. A Mixed Model ANOVA was also used to determine if there was a significant difference in the lengths of treatment and control group worms. Statistical significance was established at $P \leq 0.05$ for all tests.

3. Results

3.1. Fecal egg counts

Arithmetic mean FEC for each experimental group over the course of the study are shown in Fig. 2.2. FEC were determined from fecal samples collected each morning and before treatment on Days 1-4. Day 0 mean FEC for control and treatment groups were 1940 (±473) and 2860 (±607) eggs per gram (±S.E), respectively. Day 14 mean FEC for control and treatment groups were 2900 (±937) and 67 (±17), respectively. For Days 1 and 2, there was no significant difference between the mean FEC of the two experimental groups. There was a significant difference ($P=0.0007$) starting on Day 3, after two treatment doses had been administered. This difference remained significant ($P<0.0001$) until the end of the study.

3.2. Worm counts

There was no significant difference ($P=0.01$) between the mean worm counts (±S.E.) of the control (1430 ±449) and treatment (416 ±201) groups, respectively (Fig. 2.3). The arithmetic mean worm count (±S.E.) of *H. contortus* based on worm sex and experimental group are displayed in Fig. 2.4. However, there was a significant difference ($P<0.0001$) between the mean number of female worms in the treated animals (16 ±2) versus the control animals (670 ±211). There was also a significant difference ($P<0.0001$) between the mean number of female worms

in the treated animals compared to the males (400 ±200). There was no significant difference (P=0.06) between the number of males in the treated animals compared to the controls (760 ±248). There was no significant difference when comparing the number of male and female worms retrieved from the control animal abomasa (P=0.6). Only adult *H. contortus* were seen in abomasal samples.

3.3. Worm lengths

The means for male and female worm length by treatment group are shown in Fig. 2.5. The mean lengths for control male and females (±S.E.) were 14.8 (±0.97) and 22.9 (±1.8), respectively. The mean lengths for treatment group males and females (±S.E.) were 14.7 (±1.1) and 22.4 (±2.6), respectively. Analysis showed no significant difference between the lengths of the control and treatment group males (P=0.9) or females (P=0.5).

3.4. Larval cultures

The results of the Day 1 and Day 4 larval cultures are displayed in Table 2.1. On Day 1, before any treatments were administered, over 95% of the estimated number of eggs in both the control and treatment group composite samples developed to L3. On Day 4, after three treatments, over 95% of the eggs in the control group composite samples developed to L3. However, in the treatment group composite samples, only 27% of the estimated eggs present in the feces reached the third larval stage.

4. Discussion

Results of this experiment provide support for our hypothesis by showing that Cry5B can significantly reduce FEC and numbers of female *H. contortus* in sheep. There was a dramatic impact on the female worms in the treated sheep. Their numbers were reduced by 98% compared

to the number of female worms in the control abomasa. A possible explanation for the difference in effect on males and females is based on the mode of action of the Cry5B. Female *H. contortus* are on average twice as long as males (21.44 mm vs. 10.7 mm), and able to produce 5,000-10,000 eggs a day (Kuchai et al., 2012; Emery et al., 2016), so it is possible that food intake of females is greater than in males. Since the Cry5B probably needs to be ingested for it to bind to the glycolipid receptors of the intestine, this could explain the greater impact on females. A potential experiment to test this theory *in vitro* would be to retrieve male and female adult *H. contortus* and measure their consumption rates using fluorescein isothiocyanate, like the methodology described in Geary et al. (1993), which measured pharyngeal function.

The Cry5B had a significant impact on the FEC, which stemmed from the impact seen on the treated females. On Day 2, after one treatment, there was a 78% reduction of treatment FEC when compared to the control group. This difference continued after two and three doses with an 85% and 94% reduction, respectively. The significant difference between the two groups remained until euthanasia. The Cry5B appears to offer potential for *H. contortus* management by killing the female worms inside the sheep, which has the added effect of massively reducing the number of eggs shed by infected hosts. Three treatments were able to reduce FEC in infected animals by over 90%, which would have a major effect on slowing the contamination of pastures by the eggs. This could be used with other treatments or compounds to not only kill adult worms, but to reduce egg and larval buildup on pasture. This would provide protection for lambs that can become rapidly infected in the spring due to factors like the periparturient rise, where ewes are less able to control their infections and larvae that were in hypobiosis emerge and continue their development, as well as the immature immune responses of the lambs (Gibbs, 1986).

The sheep treated with Cry5B showed no signs of toxicity. This was expected, as the

glycolipid receptor to which the toxin binds in the nematode gut is not present in vertebrates, and therefore the Cry5B would be unable to act on the hosts. The results of the experiment provide further proof that *B. thuringiensis* Cry proteins are safe for vertebrate consumption, with no measurable impact on the host (Betz et al., 2000). Previous studies show that Cry5B is effective against nematode parasites in multiple mammalian hosts, with no signs of toxicity in any of the hosts (Cappello et al., 2006; Hu et al., 2010). This is a crucial property any potential anthelmintic must possess.

Finally, the Cry5B may have inhibited the development of the *H. contortus* eggs produced by the worms in the treated animals either in the sheep or during development in the manure. The feces used for the Day 1 culture were collected before any treatment was administered, and in both the control and treatment feces, over 95% of the eggs developed to the L3 stage. In the Day 4 culture, after 3 doses were administered, over 95% of the eggs developed in the control feces, but only 27% of the eggs developed in the treatment feces. However, there were very few eggs (approximately 1166 eggs total) present in the treatment composite samples when compared to the control samples (38000 eggs total), which could affect the accuracy of the estimates of development. The fact that the Cry5B was encapsulated inside live bacterial cells may have allowed some to pass through the sheep in the feces with the parasite eggs. If this is the case, then it is possible L1 or L2 larvae consumed the BaCC, since the first two larval stages feed on bacteria in the feces. Another possibility is that BaCC acted on the worms inside the animal by reducing their ability to produce fertilized or viable eggs. The study by Cappello et al. (2006) found that adult female hookworms produced 95% fewer eggs *in vitro* when exposed to Cry5B at a dose of only 0.1 μg/mL.

Based on the FEC data and the impact on female worms, the experiment supported our

hypothesis that *B. thuringiensis* Cry5B protein has efficacy against *H. contortus* in sheep, as seen in the impact on adult females in treated animals. Future studies should use higher total doses of Cry5B to determine if male worms can also be removed. If, as suggested, females are feeding more heavily than males, then there needs to be a higher concentration of Cry5B available in the abomasum to increase the chance males are exposed. However, the protein is delicate, and will rapidly breakdown in the acidic environment of the abomasum (Kotze et al., 2005). Hu et al. (2010) showed that Cry5B exposed to simulated gastric fluid broke down in only four minutes. This makes it likely that even though a dose of 40 mg/kg is administered, there is much less fully functional Cry5B being consumed. Encapsulating the protein in the live bacteria may have delayed this breakdown.

Even if less than fully adulticidal, Cry5B showed a significant ability to reduce the number of eggs shed by infected sheep. The ability to reduce FEC, along with the potential larval development inhibition, suggests that Cry5B could one day be incorporated into parasite control programs to help slow the development of anthelmintic resistance. Killing the adults in an animal is just one step of parasite management. Cry5B could also be used to prevent pasture contamination or inhibit larval development of the eggs already on the pasture.

References

Betz, F.S., Hammond, B.G., Fuchs, R.L., 2000. Safety and advantages of *Bacillus thuringiensis*-protected plants to control insect pests. Regul. Toxicol. Pharmacol. 32, 156-173.

Bravo, A., Gill, S.S., Soberón, M., 2007. Mode of action of *Bacillus thuringiensis* Cry and Cyt toxins and their potential for insect control. Toxicon. 49, 423-435.

Cappello, M., Bungiro, R.D., Harrison, L.M., Bischoff, L.J., Griffitts, J.S., Barrows, B.D., Aroian, R.V., 2006. A purified *Bacillus thuringiensis* crystal protein with therapeutic activity against the hookworm parasite *Ancylostoma ceylanicum*. Proc. Natl. Acad. Sci. U.S.A. 103, 15154-15159.

De Lara, A.P., Lorenzon, L.B., Vianna, A.M., Santos, F.D.S., Pinto, L.S., Berne, M.E.A., Leite, F.P.L., 2016. Larvicidal activity of *Bacillus thuringiensis* var. *israelensis* Cry 11Aa toxin against *Haemonchus contortus*. Parasitology. 1, 1-7.

Emery, D.L., Hunt, P.W., Le Jambre, L.F., 2016. *Haemonchus contortus*: the then and now, and where to from here? Int. J. Parasitol. 46, 755-769.

Geary, T.G., Sims, S.M., Thomas, E.M., Vanover, L., Davis, J.P., Winterrowd, C.A., Klein, R.D., Ho, N.F.H., Thompson, D.P., 1993. *Haemonchus contortus*: Ivermectin-induced paralysis of the pharynx. Exp. Parasitol. 77, 88-96.

Getachew, T., Dorchies, P., Jacquiet, P., 2007. Trends and challenges in the effective and sustainable control of *Haemonchus contortus* infection in sheep. Review. Parasite. 14, 3-14.

Gibbs, H.C., 1986. Hypobiosis and the periparturient rise in sheep. Vet. Clin. North Am. Food Anim. Pract. 2, 345-353.

Gilleard, J.S., 2013. *Haemonchus contortus* as a paradigm and model to study anthelmintic drug resistance. Parasitology. 140, 1506-1522.

Hu, Y., Georghiou, S.B., Kelleher, A.J., Aroian, R.V., 2010. *Bacillus thuringiensis* Cry5B protein is highly efficacious as a single-dose therapy against an intestinal roundworm infection in mice. PLoS Negl. Trop. Dis. 4, e614.

Hu, Y., Aroian, R.V., 2012. Promise of *Bacillus thuringiensis* crystal proteins as anthelmintics. In: Selzer, P.M., Caffrey, C.R. (Eds.), Parasitic Helminths. Wiley-Blackwell, Hoboken, NJ, pp. 267-281.

Kaplan, R.M., 2004. Drug resistance in nematodes of veterinary importance: a status report. Trends Parasitol. 20, 477-481.

Kotze, A.C., O'Grady, J., Gough, J.M., Pearson, R., Bagnall, N.H., Kemp, D.H., Akhurst, R.J., 2005. Toxicity of *Bacillus thuringiensis* to parasitic and free-living life-stages of nematode parasites of livestock. Int. J. Parasitol. 35, 1013-1022.

Kuchai, J.A., Ahmad, F., Chishti, M.Z., Tak, H., Ahmad, J., Ahmad, S., Rasool, M., 2012. A study on morphology and morphometry of *Haemonchus contortus*. Pak. J. Zool. 44, 1737-1741.

Rasband, W.S., ImageJ, U. S. National Institutes of Health, Bethesda, Maryland, USA, https://imagej.nih.gov/ij/, 1997-2018.

Roh, J.Y., Choi, J.Y., Li, M.S., Jin, B.R., Je, Y.H., 2007. *Bacillus thuringiensis* as a specific, safe, and effective tool for insect pest control. J. Microbiol. Biotechnol. 17, 547-559.

Sutherland, I., Scott, I., 2010. Gastrointestinal nematodes of sheep and cattle. West Sussex (UK): Wiley-Blackwell, 242 pp.

Urban, J.F. Jr., Hu, Y., Miller, M.M., Scheib, U., Yiu, Y.Y., Aroian, R.V., 2013. *Bacillus thuringiensis*-derived Cry 5B has potent anthelmintic activity against *Ascaris suum*. PLoS Negl. Trop. Dis. 7, e2263.

Wei, J.Z., Hale, K., Carta, L., Platzer, E., Wong, C., Fang, S.C., Aroian, R.V., 2003. *Bacillus thuringiensis* crystal toxins that target nematodes. Proc. Natl. Acad. Sci. U.S.A. 100, 2760-2765.

Zajac, A., Conboy, G.A., 2012. Veterinary clinical parasitology. Chichester, West Sussex, UK: Wiley-Blackwell, 354 pp.

Tables and Figures

Table 2.1: Effect of *Bacillus thuringiensis* Cry5B protein on development of *H. contortus* eggs to third stage larvae based on experimental group. Day 1 cultures created before treatment administered, Day 4 cultures created after treatment concluded.

	Day 1 Cultures		Day 4 Cultures	
	Control	**Treatment**	**Control**	**Treatment**
Estimated Total Egg Number	26,333	29,166	38,000	1,166
Estimated L3 Harvested	25,130	29,358	46,680	315
% of Eggs Developed	**>95**	**>95**	**>95**	**27**

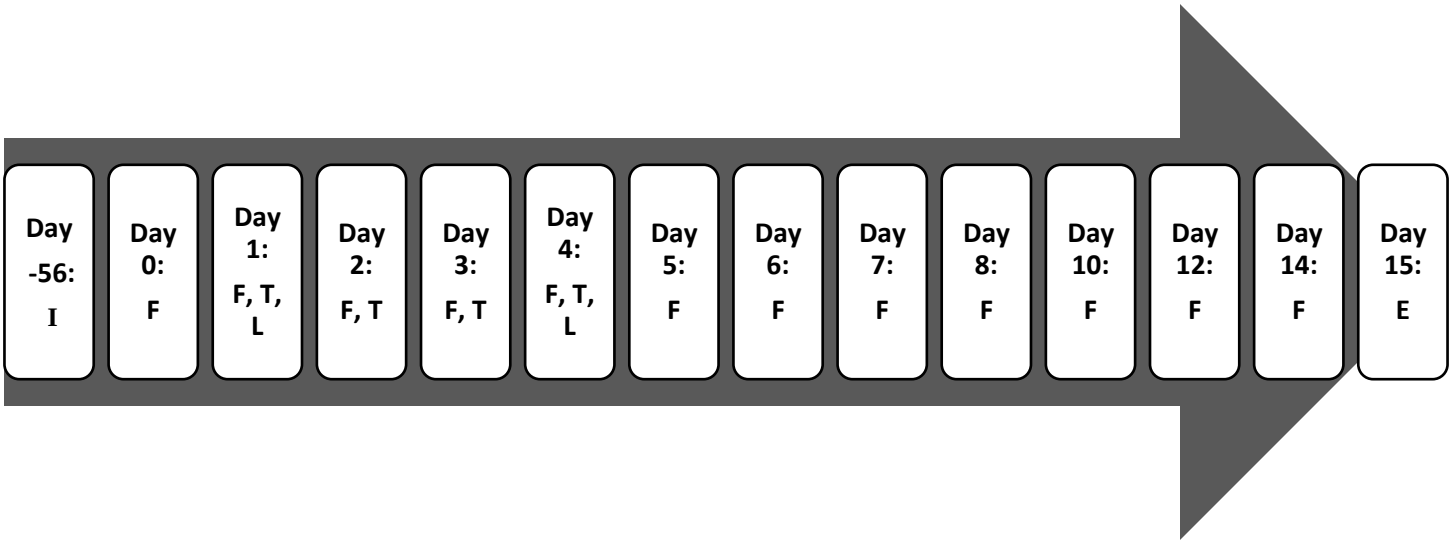

Fig. 2.1. Experimental timeline. **I**= each sheep infected with 10,000 *H. contortus* L3. **F**= FEC determined for each animal in the morning, prior to treatment. **T**= treatment administered, control sheep received 200 mL of water and treated sheep administer 40 mg/kg Cry5B. **L**= larval cultures set up using composite samples, three cultures per experimental group on Day 1 and 4. **E**= euthanasia and abomasa removal to determine worm burden.

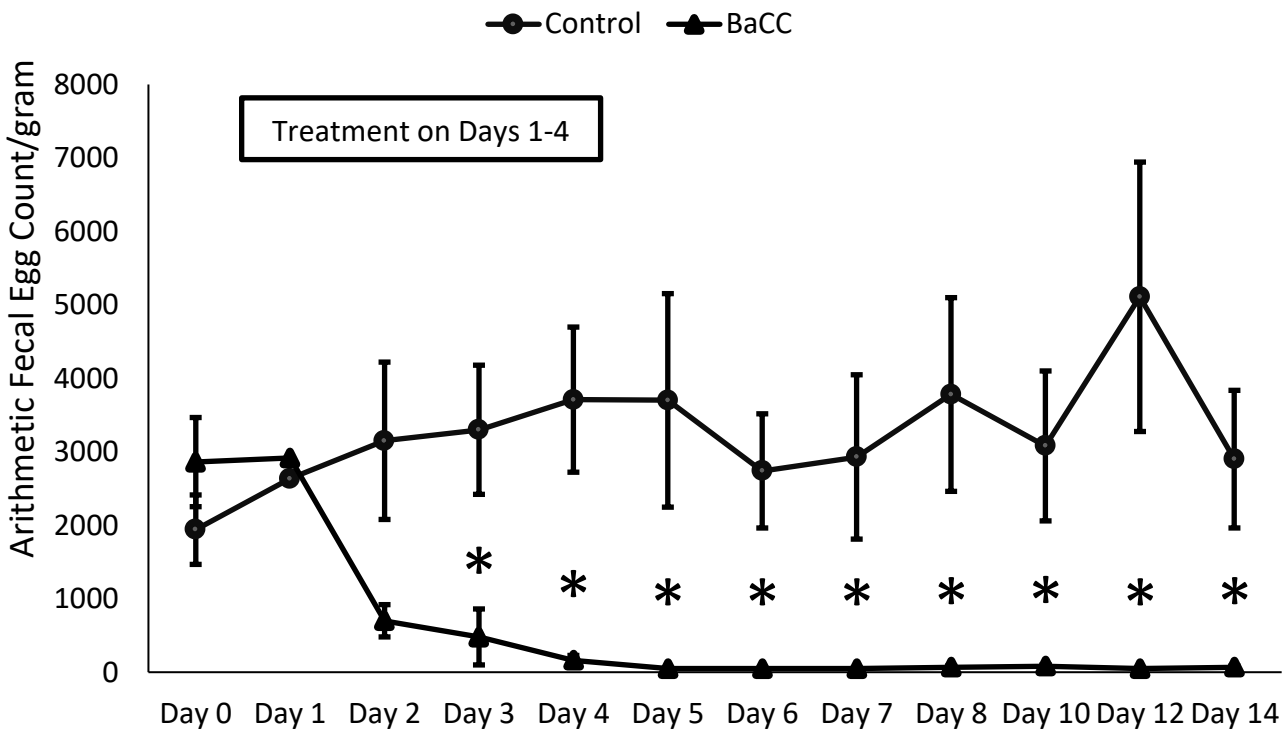

Fig. 2.2. Arithmetic mean FEC (±S.E.) of control sheep and sheep treated with 40 mg/kg Cry5B (BaCC) by day. Significant differences (P=0.0007, Day 3) and (P<0.0001, Days 4-14) denoted by asterisk.

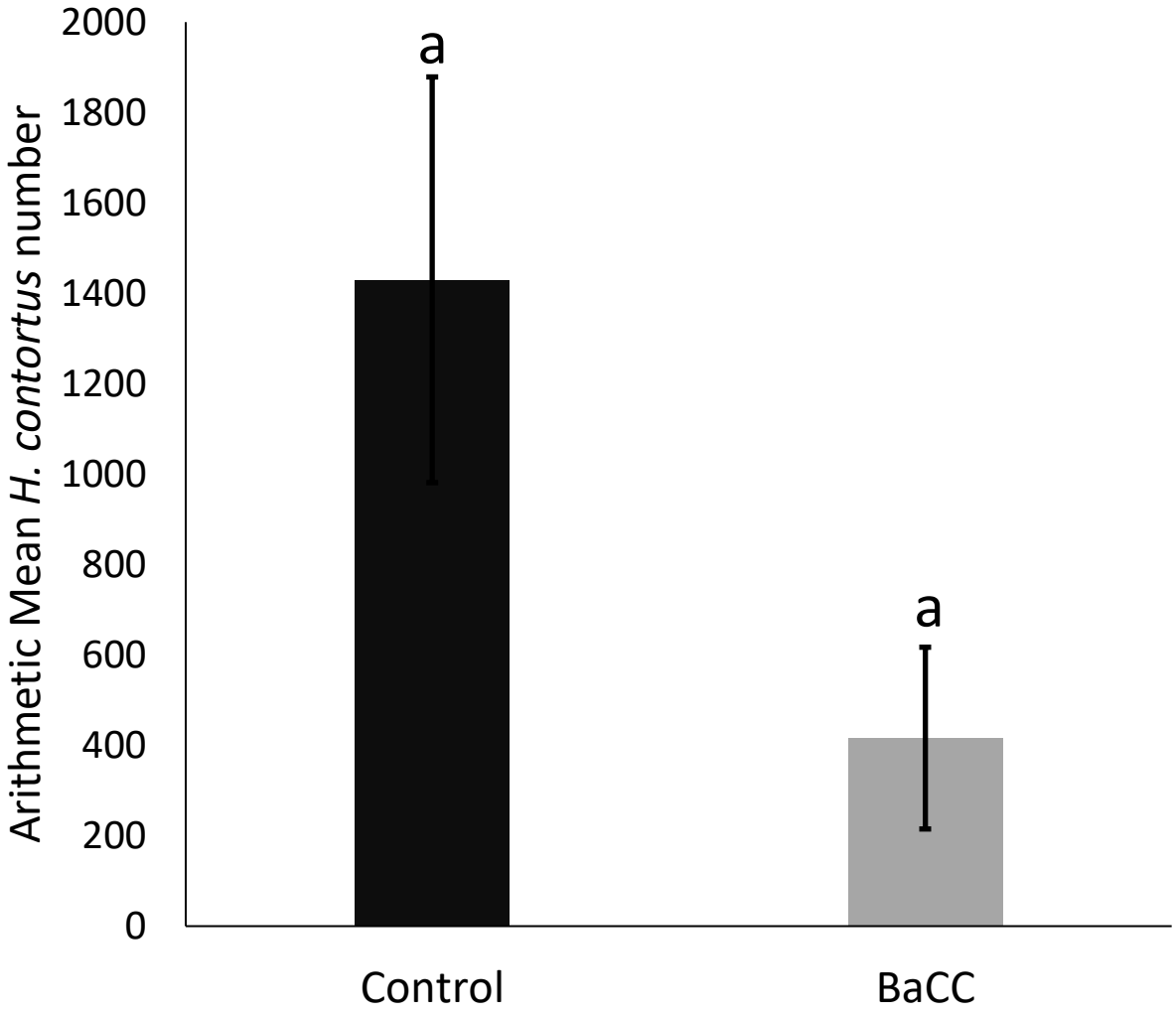

Fig. 2.3. Arithmetic mean adult worm burden (±S.E.) for control sheep and sheep treated with 40 mg/kg Cry5B (BaCC). Columns with the same letter are not significantly different (P=0.06).

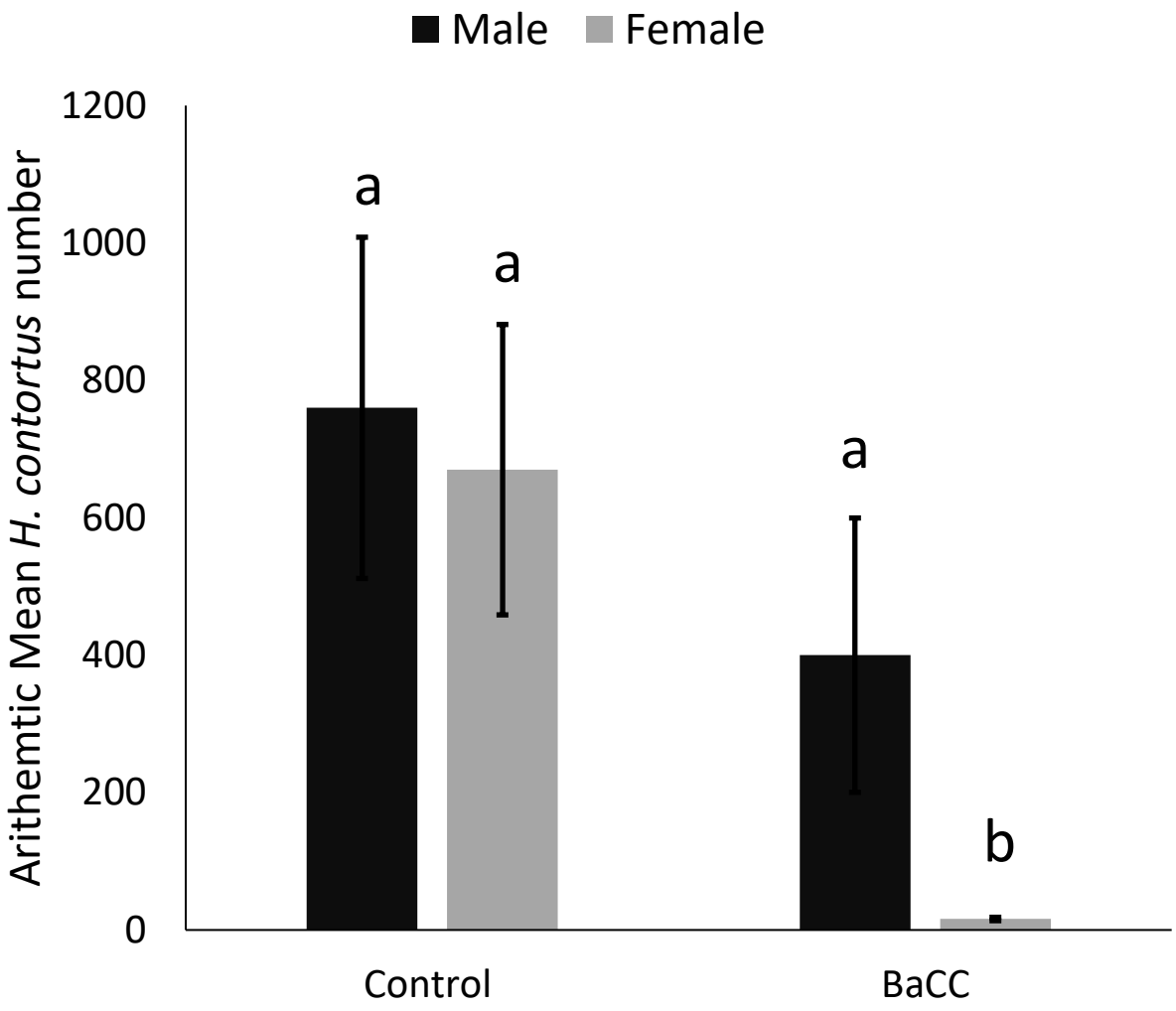

Fig. 2.4. Arithmetic mean male and female adult worm count (±S.E.) by experimental group for control sheep and sheep treated with 40 mg/kg Cry5B (BaCC). Columns with different letters are significantly different (P<0.0001).

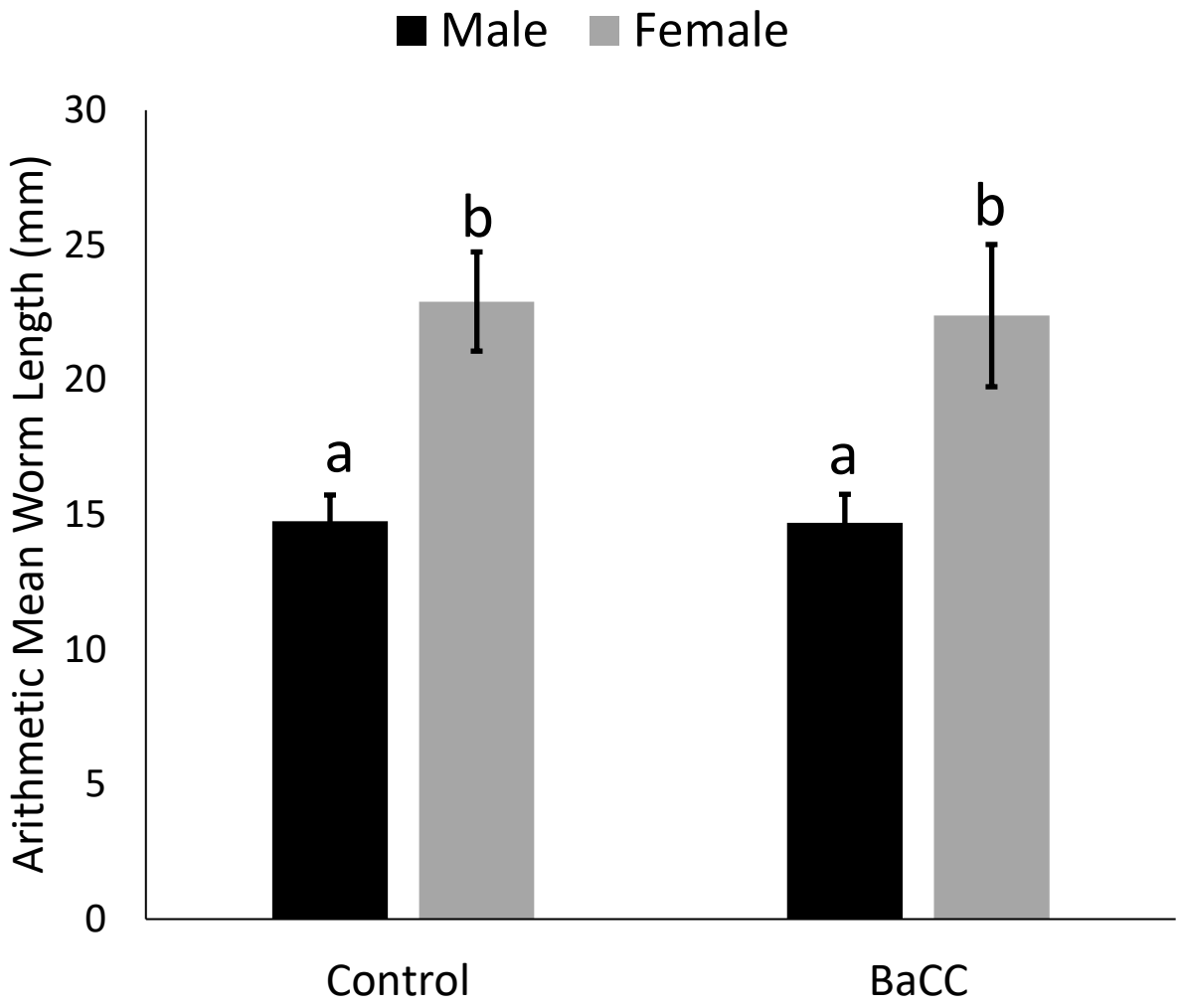

Fig. 2.5. Arithmetic mean male and female worm length (mm) (±S.E.) of worms retrieved from abomasa of control sheep and sheep treated with 40 mg/kg of Cry5B (BaCC). Columns with different letters are significantly different ($P<0.0001$).

CHAPTER 3: Effect of oral administration of inactivated *Bacillus thuringiensis* with cytosolic Cry5B protein on experimental *Haemonchus contortus* infection in sheep

ABSTRACT

Haemonchus contortus is a common abomasal nematode of small ruminants. This parasite is a major problem for its small ruminant hosts, as heavy infections can cause weight loss, anemia, and death. *H. contortus* is primarily controlled with anthelmintics, but the selection pressure imposed by these drugs has caused widespread resistance to emerge, creating an urgent need for new treatment methods. Previous studies have displayed the nematocidal properties of *Bacillus thuringiensis* Cry5B protein against multiple parasitic species. To investigate the effectiveness of this protein against *H. contortus* in sheep, six female and six castrated male lambs were removed from pasture and housed inside. All animals were dewormed and then infected orally with 10,000 *H. contortus* third stage larvae. After six weeks, the sheep were divided into control and treatment groups based on sex and mean fecal egg count. The treatment group received a preparation of genetically modified, non-sporulating, inactivated *B. thuringiensis* containing Cry5B protein (60 mg/kg orally once daily for three days). The control group received 200 mL of water for three days. FEC were determined daily for eight days post treatment, until the animals were euthanized to remove their abomasa and determine worm burden. There was a significant difference in the mean worm burden of the two groups, and females in the treated animals were reduced by 95% compared to the control animals. FEC were reduced by 91% after the three doses of Cry5B. These results support the anthelmintic potential of *B. thuringiensis* Cry5B protein as a treatment for *H. contortus*.

1. Introduction

There are many species of gastrointestinal nematodes that parasitize small ruminants. The main group of concern for producers and veterinarians is the trichostrongyles, common nematode parasites that cause major production loss and disease. They possess a direct life cycle, where the adults in the host produce eggs that are shed in the feces. The larvae develop from egg to third stage larva, or L3, in the feces. The L3 is the infective stage and is ingested by the host to finish the life cycle (Sutherland and Scott, 2010). One species stands out as particularly pathogenic due to the fact it feeds on blood. This parasite, *Haemonchus contortus*, is an abomasal species that can cause weight loss, lethargy, submandibular edema, anemia, and death in heavily infected individuals. Lambs and kids are at the greatest risk of developing disease due to their immature immune response (Getachew et al., 2007).

Until recently, this parasite was controlled almost exclusively with anthelmintics. Unfortunately, the wide and frequent use of these drugs has created selection pressure that favors individual worms resistant to these anthelmintics (Kaplan, 2004). The offspring of the individuals that survive treatment make up greater and greater portions of the worm population as more treatments are administered, leading to a resistant population (Jackson and Coop, 2000). Resistance in small ruminant GI nematodes has been reported to all widely available anthelmintics. For each class of anthelmintic, resistance was reported within a decade of the drug becoming commercially available (Kaplan, 2004). Due to the rise of resistance, the current reliance on anthelmintics as the primary means of control is a situation that must be altered.

A potential alternative treatment could be found in the bacterium, *Bacillus thuringiensis* (*Bt*). Most strains of *Bt* produce a parasporal crystal next to the spore during the sporulation phase of the bacteria. This crystal is composed of proteins, referred to as Cry proteins (Bravo et

al., 2007). Some of these proteins are protoxins that can be proteolytically cleaved, releasing pore-forming toxins. There are many Cry proteins with insecticidal properties, lethal to members of the orders Coleoptera, Diptera, Lepidoptera, and Hymenoptera (Roh et al., 2007). The Cry protein must be ingested by the insect, where it is cleaved in the midgut, releasing the pore-forming toxin that binds to glycolipid receptors in the insect intestine. Death occurs due to cell lysis and possible sepsis (Bravo et al., 2007). Some Cry proteins are lethal to only a few species, while others can span insect orders in their effectiveness. Certain proteins have also been shown to be toxic to nematodes, which possess the same glycolipid receptors in their intestine (De Lara et al., 2016; Kotze et al., 2005; Wei et al., 2003)

Previous work has shown that *H. contortus* is vulnerable to Cry proteins (De Lara et al., 2016; Kotze et al., 2005). One protein, Cry5B, has been demonstrated to affect a range of parasitic species (Hu and Aroian, 2012). Hu et al. (2010), observed a 70% reduction in *Heligmosomoides bakeri* numbers in mice treated with Cry5B. Cappello et al. (2006) found a 90% reduction in the number of *Ancylostoma ceylanicum* in hamsters and Urban et al. (2013) reported a 97% reduction in *Ascaris suum* numbers in pigs after Cry5B treatment. Our previous study (Chapter 2) using live *Bt* with cytosolic Cry5B protein (BaCC) demonstrated the impact of Cry5B on *H. contortus*. In the BaCC study, we administered 4 doses over 4 days of ~40 mg/kg of Cry5B orally to sheep and saw a 98% reduction in female worm count and a 94% reduction in FEC after 72 hours, with no signs of toxicity in any of the treated animals.

Based on these studies, as well as the experiment conducted previously using BaCC, we hypothesized that inactivated *Bt* with cytosolic Cry5B (IBaCC) would also be effective against *H. contortus* in sheep. Another experimental goal was to determine whether an increased dose of Cry5B would have a more significant effect on the number of *H. contortus* in the treated animals.

2. Materials and methods

2.1. Animals

The lambs used for the study were Dorset, Suffolk, and Dorset/Suffolk cross lambs aged 7-8 months. The six female and six castrated male lambs had been previously been pastured, but were maintained in housing for the duration of the study to prevent additional natural *H. contortus* exposure. All animals were orally administered albendazole (7.5 mg/kg), ivermectin (0.2 mg/kg), and levamisole (8.0 mg/kg) sequentially to remove existing strongylid infection. The sheep were euthanized at the end of the study with an intravenous injection of 100 mg/kg pentobarbital. Exsanguination was used to confirm death following the cessation of vital signs. All protocols in the study were approved by the VT Institutional Biosafety Committee (Protocol #17-006) and the Institutional Animal Care and Use Committee (Protocol #18-135, #18-141).

2.2. Haemonchus contortus

The third stage infective *H. contortus* larvae (L3) used for infection were obtained from a sheep experimentally infected with *H. contortus*. Feces were collected and incubated at room temperature for 14 days to allow L3 to develop. Larvae were harvested using a modification of the Baermann test (Zajac and Conboy, 2012), and stored in water at 4°C.

2.3. Cry5B and control

The *B. thuringiensis* and Cry5B protein used in this experiment was provided by the laboratory of Dr. Raffi Aroian at the University of Massachusetts Medical School (Hu et al., 2010). The preparation provided was inactivated *B. thuringiensis* with cytosolic crystal protein (IBaCC) in water. Food grade terpene was used to inactivate the bacteria, but leave them intact. Food grade, or Food Contact Substance, defined as any substance or material that may come into contact with food, and is safe for consumption (USDA, 2018). The *B. thuringiensis* were

genetically modified to be sporulation deficient and to produce Cry5B in the cytosol during vegetative growth that encapsulated the protein. The IBaCC was stored at -80 °C and thawed the night before administration at 4 °C.

2.4. Haemonchus contortus infection

Haemonchus contortus L3 were diluted to a concentration of 1,000 L3/mL of water. Ten mL of this mixture was administered orally using a syringe for each animal. Ten mL of tap water was subsequently administered using the same syringe. After the infection, FAMACHA© scores were obtained for each animal weekly to monitor for development of anemia. Fecal samples were collected once weekly to determine FEC for each animal, and these FEC were used to determine mean FEC when sorting animals into experimental groups. Method for determining FEC described in the next section.

2.5. Fecal egg count determination and larval cultures

Fecal samples were collected rectally from the sheep and fecal egg count (FEC) was determined using the Mini-FLOTAC® test (University of Naples Federico II, Naples, Italy) with a detection limit of 5 eggs per gram of feces (epg). For the larval cultures, composite fecal samples were prepared by mixing 10 grams of feces from each animal in each experimental group. Larval cultures were created from the composites with 10 grams of feces in each culture, set up for 14 days to allow L3 to develop (Zajac and Conboy, 2012).

2.6. Worm count

The sheep were euthanized, their abomasa removed, placed in trays, and cut along the greater curvature. The abomasa were washed with warm tap water, and the contents collected. A 20% aliquot was collected for each animal. An equal volume of 10% formalin was used to fix the worms. The adult worm count was determined using a dissecting microscope and a square petri

dish with gridlines. Lugol's iodine was added to the washings to stain the worms, assisting in their identification. The counts obtained were multiplied by five to determine the total worm burden for each animal. Unfortunately, during collection, one control animal's abomasal contents were disposed of before the aliquot was obtained. Therefore, there was one more aliquot in the treatment group than the control.

2.7. Larval cultures

Fecal samples were collected from each animal, and composite samples were created for both experimental groups to be used in larval cultures. The average of the individual FEC of each group member was used to estimate the number of eggs in each culture. The culture FEC was determined in this way due to limited fecal sample volume. Three replicate cultures were created for each experimental group for the first four treatment days. After 14 days, the cultures were harvested by a modification of the Baermann test (Zajac and Conboy, 2012) and the collected liquid containing L3 allowed to sediment for an hour at 4°C. The supernatant was drawn off, leaving 50 mL containing L3 that was transferred to a 50 mL conical centrifuge tube and allowed to sediment for an hour. The sediment was transferred to a 15 mL tube, and the process was repeated to result in a final volume of 2 mL. After mixing, the number of larvae in five 20-microliter aliquots was counted and the average used to calculate the total number of larvae in each culture.

2.8. Experimental protocol

The experimental protocol is shown in Fig. 3.1. Sheep were infected with *H. contortus* six weeks prior to beginning treatment. The day before treatment began, sheep were weighed to determine the treatment dose required for each animal and divided into experimental groups balanced for mean FEC and sex. Mean FEC was determined using the previous three weeks FEC

data for each sheep. Each animal in the treatment group received 60 mg/kg of Cry5B, administered orally by syringe. The control group received 200 mL of water by oral administration. Treatments were administered at approximately the same time each day. FEC were determined daily. Fecal samples on Days 1-3 were collected before treatment was given. Larval cultures were created on treatment Days 1-4. On Day 8, the sheep were euthanized, and their abomasa removed to collect the contents containing adult worms. Fecal samples for the Day 8 FEC were collected from the rectum when the abomasa were processed.

2.9. Statistics

The FEC and adult worm burden data were log transformed [10] to normalize the data, and the geometric means used for analysis. The FEC data was analyzed with a mixed model ANOVA, and the slice option used to compare the two groups on each day. The worm counts for the two experimental groups were compared using a pooled T-test, and the sex differences between groups analyzed using a mixed model ANOVA. Statistical significance was established at $P \leq 0.05$ for all tests.

3. Results

3.1. Fecal egg counts

There were no signs of toxicity in the sheep treated with Cry5B. The arithmetic means for the two experimental groups are shown in Fig. 3.2. FEC were determined from feces collected in the morning (before treatment was administered on Days 1-3). On Day 1, the control group mean FEC (±S.E.) was 240 (±89.8), while the treatment FEC was 555 (±193.5). On Day 8, the control group mean FEC was 777 (±370), while the treatment FEC was 97 (±44.6). The treatment group

mean FEC was significantly lower than the control group mean starting on Day 3 (P=0.009), and the treatment group mean FEC remained significantly (P=0.005 to P=0.02) lower than the control group for the duration of the study.

3.2. Worm counts

The arithmetic mean worm count (±S.E.) for the two experimental groups are displayed in Fig. 3.3. The mean total worm count of the treatment group was significantly lower than the mean control group worm count (P=0.05). The sex specific counts are displayed in Fig. 3.4, and the number of male worms 490 (±140.1) was significantly higher than the number of females 194 (±111) in the control group (P=0.04). There were significantly fewer female worms 9 (±6.3) than males 229 (±50) in the treatment group (P=0.0001). There were also significantly fewer treatment group female worms 9 (±6.3) than control group females 194 (±111) (P=0.001). There was no significant difference between the control group males and treatment group males.

3.3. Larval cultures

The L3 counts and percent of eggs that developed in the larval cultures are shown in Table 3.1. In samples collected before treatment (Day 1), only 10% of the control group *H. contortus* eggs developed to the L3 stage while over 95% of the treatment group eggs developed to the L3. In Day 2 samples, >95% of the control group eggs developed compared to only 38% in the treatment cultures. Day 3 had 41% of the control eggs develop and 68% of the treatment eggs, and on Day 4 over 95% of eggs in both groups developed to the L3.

4. Discussion

The results of this study provide further support for the hypothesis that Cry5B is effective against *H. contortus* infection in sheep. As with our previous study, Cry5B in IBaCC significantly reduced fecal egg counts and the number of female worms. There was a significant reduction in the total worm burden of the treated animals compared to the control animals, which was not seen in the BaCC study. This could be explained by the increased dose of Cry5B in this study. A greater impact on males was seen than in the previous study, which may have not been significant by itself, but contributed to the overall significant reduction in worm burden. However, the live bacteria in the BaCC study may have been able to produce additional Cry5B in the animal, making it impossible to know accurately how much Cry5B was delivered. Based on the FEC study, the Cry5B may have begun to have an impact on the adult worms after just one or two doses. More Cry5B being introduced to the abomasum would allow for both more worms to be exposed, and those that were exposed to potentially consume more material, ensuring their death and removal from the abomasum. This suggests that the most important factor in designing treatment dosage and duration is the concentration of the initial doses.

Surprisingly, there was a significant difference between the mean number of male and female worms in the control animals with 68% of the control population being male. Roberts and Swan (1981) looked at the sex ratio of *H. contortus* infections in 61 sheep from nine different producers and found that 45.25% of all the worms were male, but the individual sex ratio in each animal could vary wildly. They found that the smaller the population, the greater the chance of variation in the ratio due to individual variation. This would explain the uncharacteristically higher number of males in the control group of five sheep.

The impact of the IBaCC on the FEC is similar to that seen in the BaCC study. There was a significant difference in the mean FEC of the two experimental groups after just two doses, and

a 91% reduction in the treatment group FEC after three doses. The impact seen on the FEC reflects the lethal effect of the Cry5B on the adult worms. While the percent reductions in this study are lower than those seen in the BaCC study, the differences are not dramatic. There were only six animals in each group in this study, and five in the BaCC study. If there were more animals in each group, smaller differences in the effectiveness of the two preparations may have been more evident. The difference in the total worm burden of this study may not have been significant if only five sheep were used like the previous study. However, even with the low number of animals, the effectiveness of the Cry5B remained highly significant, regardless of preparation.

In this study, there was not a clear impact on larval development in the feces. Unlike the BaCC study, where there was a clear reduction in the percentage of eggs that developed to the L3 after treatment, the percentage of eggs that developed in this study fluctuated by day in both treatment and control groups. While the changes in the treatment group from Day 1 to Day 2 and 3 could potentially be related to the Cry5B, that conclusion cannot be drawn since the control cultures also fluctuate. There are a few possible explanations for the unexpected variation in larval culture results. One is that the Cry5B in the inactivated bacteria did not survive the passage through the animal to the feces to be consumed by the L1 and L2. The Cry proteins are delicate and will rapidly degrade in acidic environments. Just 15 minutes of exposure to solutions with a pH of 1.0 and 3.0 caused irreversible damage to the proteins, and significantly reduced their efficacy (Kotze et al., 2005). Hu et al. (2010) showed that Cry5B in simulated gastric fluid almost completely broke down in just four minutes. Based on how rapidly Cry5B degrades in these low pH environments, it is possible that encapsulating the protein in the vegetative *Bt* cell provided enough protection for the protein to be consumed by the worm in the

abomasum, but not make it through the rest of the digestive tract and into the feces, contributing to the fluctuation seen in larval development.

Another explanation is based on the sheep immune response. The same number of L3 were used to infect the sheep in this study as in the BaCC study, but the number of worms retrieved from the control animals was only half of those retrieved in the previous study, resulting in lower FEC as well. Factors that could contribute to this difference include a stronger immune response by infected sheep. If the host immune response was starting to clear out the infection naturally, the survivability of eggs produced by remaining worms could have been reduced (Nisbet et al., 2016). The use of Mini-FLOTAC® for FEC determination could have played a role as well. The lower detection limit of this method compared to the 50 epg limit of the modified McMaster's Test (Zajac and Conboy, 2012) used in the previous study may have revealed more fluctuations in egg counts than in the previous study. At very low FEC, a lower detection limit allows greater accuracy. The previous study may have overestimated the effect of the Cry5B on the larval development. Whether the eggs produced had lower survivability, or the FEC determination method was more accurate than in the last study, the fluctuating egg counts and development percentages in the control group make it impossible to draw any conclusions from the cultures.

The results of this study compare favorably to other studies using Cry5B against parasitic nematodes in a variety of hosts. A dose of 60 mg/kg Cry5B in the sheep over 3 days resulted in the results described above. In the study by Hu et al. (2010), one dose of 90-100 mg/kg resulted in ~70% reduction in worm burden in mice infected with *H. bakeri*. In Cappello et al. (2006), a dose of 1 mg repeated for 3 days caused a ~90% reduction in worm burden in hamsters infected with *A. ceylanicum*. Finally, Urban et al. (2013) reported that 2 doses of 20 mg/kg caused a 97%

reduction in the *A. suum* L4 burden of pigs. While the total worm burden was not reduced as significantly as in these published studies, none of those hosts were ruminants. The digestive system of the ruminant is much more complex, with four stomachs compared to the single stomachs of the hamster, mouse, or pig. The Cry5B in those studies had to reach the small intestine to reach the worms but moved through the stomach in a shorter amount of time than the Cry5B given to the sheep. Based on these studies, as well as the previous BaCC study, the Cry5B appears to act quickly, with only a few doses being required to cause significant reduction in worm counts. The dose required needs to take into consideration the digestive system of the host animal, as well as the location of the parasite within the host. The method used to protect the protein would also likely differ based on the gastrointestinal system of the host, with a ruminant host requiring the protein be protected from the effects of the rumen.

While the larval cultures did not provide clear evidence of an effect of Cry5B on larval development in the feces, there was a clear impact on the adults in the abomasum, and a reflected effect on FEC. The experiment showed that Cry5B in the inactivated bacterial preparation was as effective at treating *H. contortus* in sheep as in the live bacteria. Due to the rapid degradation of the protein in the stomach, it is likely that only a portion of the Cry5B administered reached the worms, was consumed, and could act on the gut of the nematode. These previous studies with Cry5B in other hosts support the theory that the Cry5B is impacting the worms with only one or two doses, and the FEC results show a delay based on the eggs being shed and taking time to leave the host in the manure. Therefore, future studies should continue to determine if there is a dose that can be administered once that will still have significant anthelmintic affects. The WAAVP recommends that for new anthelmintics to be considered effective, they need to achieve 90-98% efficacy in worm reduction (Sutherland and Scott, 2010). While the effectiveness of

Cry5B in sheep against *H. contortus* has not yet reached that level, the results of this study, as well as the BaCC study indicate that *B. thuringiensis* Cry5B protein could one day be a safe and effective novel anthelmintic for use in small ruminants.

References

Bravo, A., Gill, S.S., Soberón, M., 2007. Mode of action of *Bacillus thuringiensis* Cry and Cyt toxins and their potential for insect control. Toxicon. 49, 423-435.

Cappello, M., Bungiro, R.D., Harrison, L.M., Bischoff, L.J., Griffitts, J.S., Barrows, B.D., Aroian, R.V., 2006. A purified *Bacillus thuringiensis* crystal protein with therapeutic activity against the hookworm parasite *Ancylostoma ceylanicum*. Proc. Natl. Acad. Sci. U.S.A. 103, 15154-15159.

De Lara, A.P., Lorenzon, L.B., Vianna, A.M., Santos, F.D.S., Pinto, L.S., Berne, M.E.A., Leite, F.P.L., 2016. Larvicidal activity of *Bacillus thuringiensis* var. israelensis Cry 11Aa toxin against *Haemonchus contortus*. Parasitology. 1, 1-7.

Getachew, T., Dorchies, P., Jacquiet, P., 2007. Trends and challenges in the effective and sustainable control of *Haemonchus contortus* infection in sheep. Review. Parasite. 14, 3-14.

Hu, Y., Georghiou, S.B., Kelleher, A.J., Aroian, R.V., 2010. *Bacillus thuringiensis* Cry5B protein is highly efficacious as a single-dose therapy against an intestinal roundworm infection in mice. PLoS Negl. Trop. Dis. 4, e614.

Hu, Y., Aroian, R.V., 2012. Promise of *Bacillus thuringiensis* crystal proteins as anthelmintics. In: Selzer, P.M., Caffrey, C.R. (Eds.), Parasitic Helminths. Wiley-Blackwell, Hoboken, NJ, pp. 267-281.

Jackson, F., Coop, R.L., 2000. The development of anthelmintic resistance in sheep nematodes. Parasitology. 120, 95-107.

Kaplan, R.M., 2004. Drug resistance in nematodes of veterinary importance: a status report. Trends Parasitol. 20, 477-481.

Kotze, A.C., O'Grady, J., Gough, J.M., Pearson, R., Bagnall, N.H., Kemp, D.H., Akhurst, R.J., 2005. Toxicity of *Bacillus thuringiensis* to parasitic and free-living life-stages of nematode parasites of livestock. Int. J. Parasitol. 35, 1013-1022.

Nisbet, A.J., Meeusen, E.N., Gonzalez, J.F., Piedrafita, D.M., 2016. Immunity to *Haemonchus contortus* and vaccine development. Adv. Parasitol. 93, 353-396.

Roberts, J.L., Swan, R.A., 1981. Quantitative studies of ovine haemonchosis. I. relationship between faecal egg counts and total worm counts. Vet. Parasitol. 8, 165-171.

Roh, J.Y., Choi, J.Y., Li, M.S., Jin, B.R., Je, Y.H., 2007. *Bacillus thuringiensis* as a specific, safe, and effective tool for insect pest control. J. Microbiol. Biotechnol. 17, 547-559.

Sutherland, I., Scott, I., 2010. Gastrointestinal nematodes of sheep and cattle. West Sussex (UK): Wiley-Blackwell, 242 pp.

Urban, J.F. Jr., Hu, Y., Miller, M.M., Scheib, U., Yiu, Y.Y., Aroian, R.V., 2013. *Bacillus thuringiensis*-derived Cry 5B has potent anthelmintic activity against *Ascaris suum*. PLoS Negl. Trop. Dis. 7, e2263.

USDA, 2018. Food ingredient & packaging terms. Available at: https://www.fda.gov/food/food-ingredients-packaging/food-ingredient-packaging-terms

Wei, J.Z., Hale, K., Carta, L., Platzer, E., Wong, C., Fang, S.C., Aroian, R.V., 2003. *Bacillus thuringiensis* crystal toxins that target nematodes. Proc. Natl. Acad. Sci. U.S.A. 100, 2760-2765.

Zajac, A., Conboy, G.A., 2012. Veterinary clinical parasitology. Chichester, West Sussex: UK:Wiley-Blackwell, 354 pp.

Tables and Figures

Table 3.1: Effect of inactivated *Bacillus thuringiensis* with Cry5B protein on development of *H. contortus* eggs to third stage larvae based on experimental group. Treatment on Days 1-3, culture created on Day 1 used feces obtained before treatment administered.

	Day 1 Cultures		Day 2 Cultures		Day 3 Cultures		Day 4 Cultures	
	Control	**Treatment**	**Control**	**Treatment**	**Control**	**Treatment**	**Control**	**Treatment**
Estimated Total Egg Number	925	1694	860	1868	2343	634	2233	89
Estimated L3 Harvested	96	1768	861	712	965	404	2200	88
% of Eggs Developed	**10**	**>95**	**>95**	**38**	**41**	**68**	**>95**	**>95**

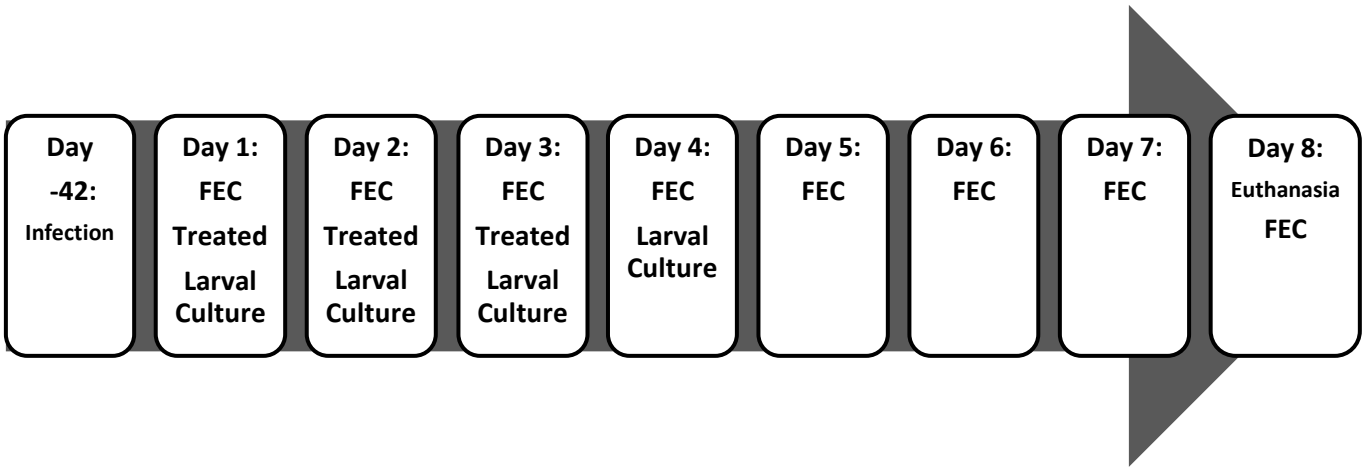

Fig. 3.1. Experimental timeline. Infection of all sheep with 10,000 *H. contortus* L3 occurred 6 weeks prior to treatment. FEC and larval cultures used feces collected prior to treatment administration. Treated animals received 60 mg/kg Cry5B, and controls received an equal volume of water. Euthanasia and abomasa removal one week after treatment to determine worm burden.

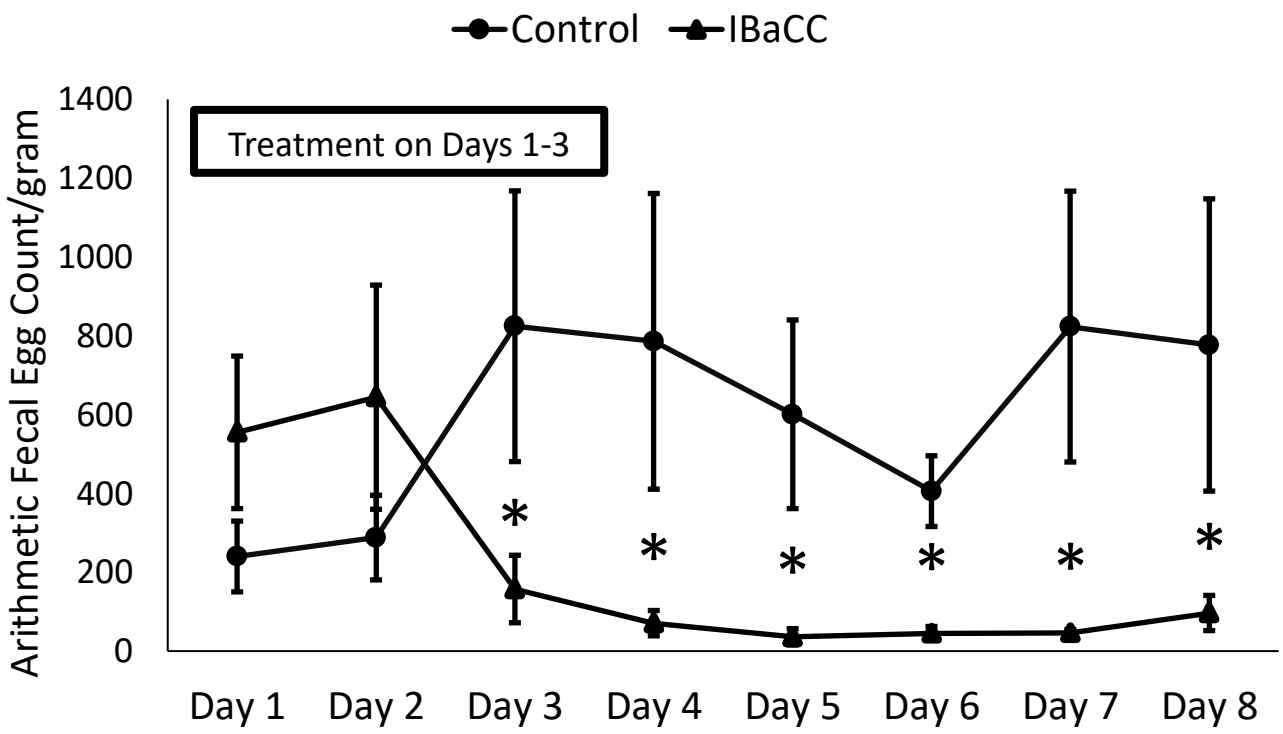

Fig. 3.2. Arithmetic mean FEC (±S.E.) of control sheep and sheep treated with 60 mg/kg of Cry5B (IBaCC) by day. Significant difference denoted by asterisks (P=0.009 on Day 3, P=0.02 on Day 8).

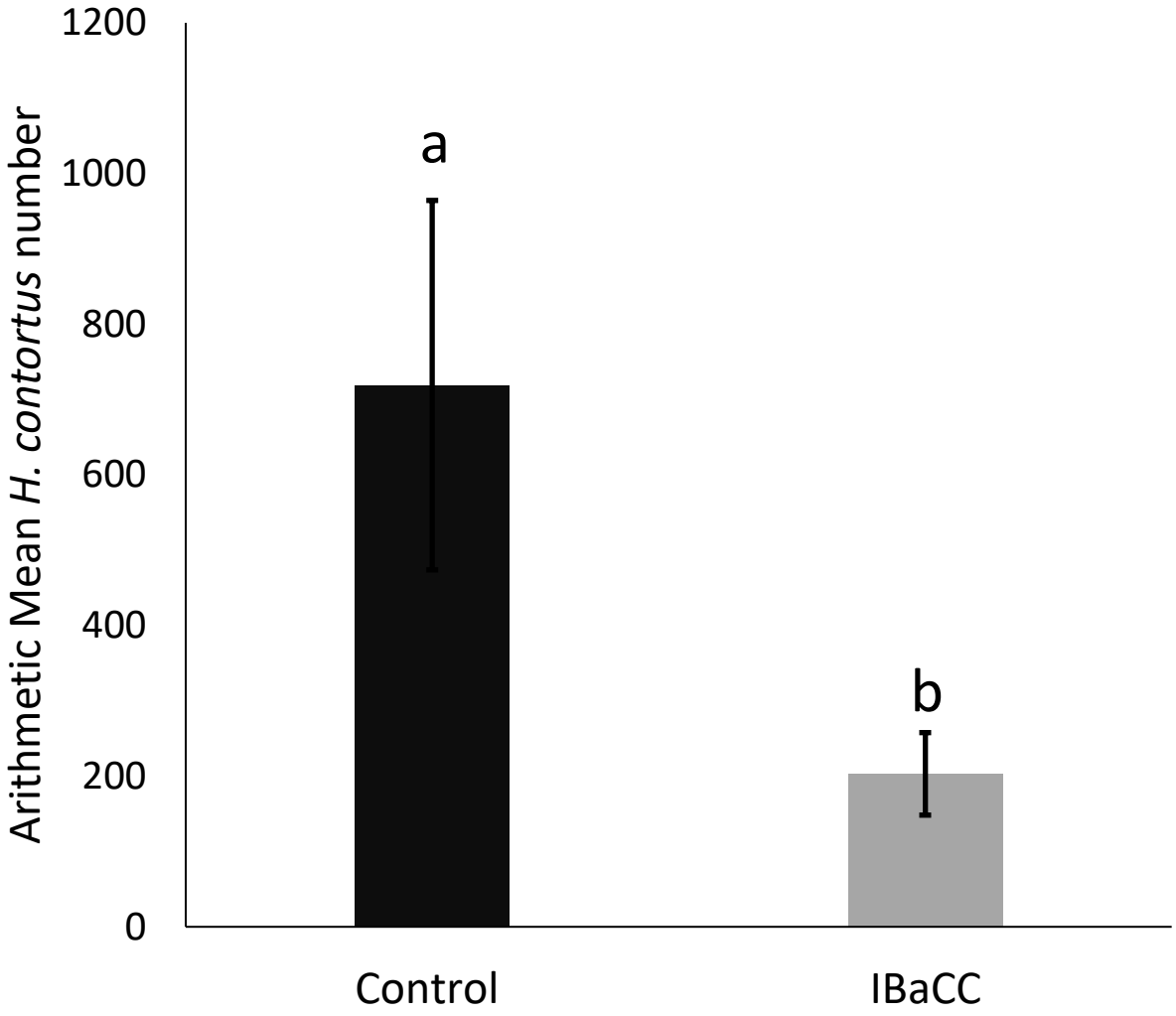

Fig. 3.3. Arithmetic mean adult worm burden (±S.E.) for control sheep and sheep treated with 60 mg/kg of Cry5B (IBaCC). Columns with different letters are significantly different (P=0.05).

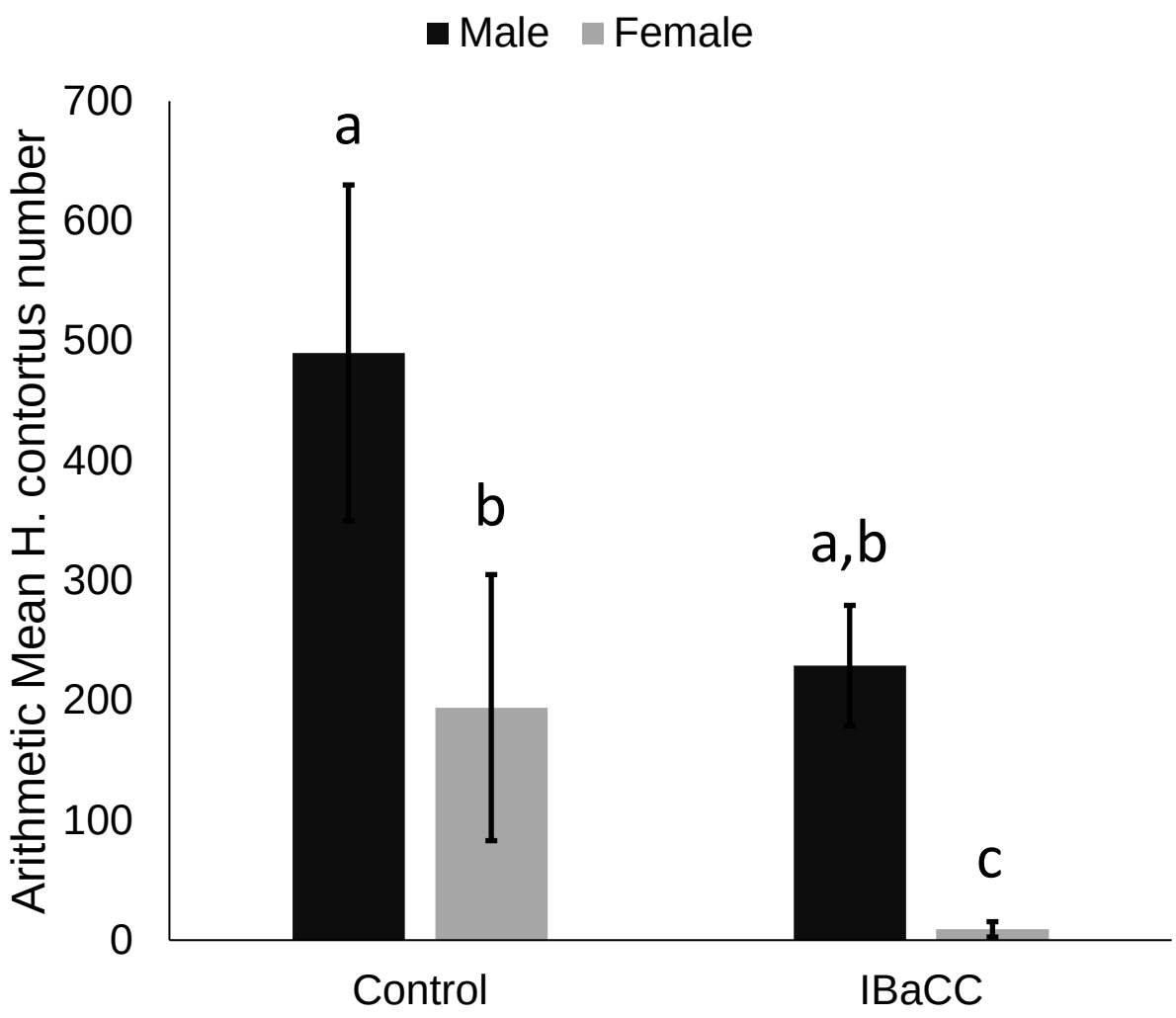

Fig. 3.4. Arithmetic mean male and female adult worm burden (±S.E.) for control sheep and sheep treated with 60 mg/kg of Cry5B (IBaCC). Columns with different letters are significantly different(a and b, P=0.04; b and c, P=0.001).

CHAPTER 4: Effect of *Bacillus thuringiensis* Cry5B protein on the development of strongylid and *Strongyloides papillosus* eggs in sheep feces

ABSTRACT

Small ruminants are parasitized by many species of gastrointestinal nematodes. These parasites have a direct life cycle, and the eggs are shed in the feces of the host. While the main treatment used for gastrointestinal parasites is anthelmintics targeted at killing the adult worms, the eggs that are shed in the feces can quickly contaminate the pasture and lead to reinfection. Therefore, management programs should also address how to prevent these eggs from developing to the infective larval stage. Previous studies have demonstrated the anthelmintic properties of *Bacillus thuringiensis* Cry 5B crystal protein on multiple parasitic nematode species. This study aims to investigate the level of larval development inhibition that this protein can provide against a natural sheep infection, using two formulations. One is BaCC, which is live *B. thuringiensis* with Cry5B inside the cytosol of the bacteria. The other is IBaCC, dead *B. thuringiensis* with cytosolic Cry5B.

Fecal cultures were set up to measure the effect of two preparations of Cry5B on strongylid and *Strongyloides papillosus* larval development. Ninety-six 10 g cultures were set up with four experimental conditions, BaCC treatment, BaCC control, IBaCC treatment, and IBaCC control. Half were kept inside a laboratory with a controlled climate, and the other half kept outside subject to natural environmental fluctuations. Fifteen mg of either BaCC or IBaCC were added to treatment cultures. Yogurt was used to replicate the bacterial mixture in control cultures. On Days 3, 5, 9, and 12 of the study, larvae were harvested from cultures, counted, and

identified to evaluate larval development. After 12 days, BaCC and IBaCC treated cultures maintained both in the laboratory and outside reduced the number of strongylid larvae by 99%. BaCC was significantly effective against *S. papillosus* on all days both in the laboratory and outside. The IBaCC was also effective, but the larval reduction varied. These results suggest that *B. thuringiensis* Cry5B protein could be effective at slowing the buildup of strongylid and *S. papillosus* larvae on the pasture if applied to feces.

1. Introduction

Gastrointestinal nematodes are common parasites found widely in small ruminants, and a single host can be infected with multiple species simultaneously. For example, *Haemonchus* and *Teladorsagia* are abomasal genera, while *Cooperia* and *Nematodirus* are found in the small intestine (Sutherland and Scott, 2010). These genera are members of the superfamily Trichostrongyloidea in the order Strongylida. They share a common direct life cycle, with adult females in the host shedding eggs that are passed in the host feces. The larvae develop to the infective third stage (L3), and these L3 are ingested by the host and make their way to the stomach or intestines. The pathogenic potential of the different genera varies, depending on factors such as feeding habits, length of life cycle, and geographic location of the host and parasite. Anthelmintic resistance contributes to the problems caused by the pathogenic potential, making it much more difficult for producers and veterinarians to control these parasites (Kaplan, 2004). *Haemonchus contortus* is a common parasite of sheep and goats, and can be highly pathogenic because it is a blood feeder. It also displays high levels of resistance to multiple anthelmintic drug classes. These factors make *H. contortus* the most important of the strongylid

nematodes of small ruminants.

Another common, but unrelated, nematode species of small ruminants is *Strongyloides papillosus* (Order Rhabditida). The life cycle of *S. papillosus* is more complex than the direct life cycle of strongylids. The third stage larvae in the environment can infect their host by either being ingested, or by penetrating the skin. The larvae then can move either to the intestines or the udder, where the larvae can be transmitted to lambs and kids in colostrum. The adult female in the small intestine reproduces through parthenogenesis and produces embryonated eggs that are shed in the feces of the host. The eggs develop to the L3, which can either develop into free-living adults in the environment or continue the parasitic life cycle and find a new host (Thamsborg et al., 2017). While *S. papillosus* has a more complex life cycle than the strongylids, it is generally a species of low veterinary importance for ruminants. Infections are most common in lambs and kids, but clinical signs of diarrhea, dehydration, and anorexia occur infrequently. They can be controlled easily with anthelmintics, as anthelmintic resistance has not yet been reported in *S. papillosus* (Thamsborg et al., 2017).

Because of the worldwide increase in anthelmintic resistance, greater reliance is being placed on other methods of control. One of these strategies is the reduction of larvae on the pasture. Strongylid eggs develop from the first to third larval stages in the feces. Once they reach the infective third larval stage they move out of the feces and onto the pasture. The development of infective *S. papillosus* L3 is similar. While treating infected animals with anthelmintics will kill adults in the host, this will not have an impact on eggs that have left the host and are in the manure, or the larvae already on the pasture (Barger, 1999). Other products, like the fungus, *Duddingtonia flagrans*, offer a method to target the eggs and larvae. When fed to the host, the fungal chlamydospores travel in the feces alongside the eggs into the environment. The fungus

develops in the manure and the hyphae create net-like structures that trap the larvae, allowing the fungus to consume the nematodes (Fontenot et al., 2003). Another microbe, with a different mode of action that could be used to target the larvae in a similar fashion in the feces is the bacterium *Bacillus thuringiensis*.

Bacillus thuringiensis (*Bt*) can produce a parasporal crystal during sporulation that is made of crystal (Cry) proteins. Not all strains of *Bt* will produce the parasporal crystal, and not all the Cry proteins that can compose the crystal are insecticidal. One crystal can be made of multiple Cry proteins, with each protein possessing lethal affects against one or more species in the insect orders Coleoptera, Diptera, Hymenoptera, and Lepidoptera (Roh et al., 2007). The proteins (protoxins) when cleaved produce pore-forming toxins that target the epithelial cells of the intestine (Wei et al., 2003). In insects, the toxins bind to glycolipid receptors in the midgut causing death by cell lysis or sepsis as bacteria from the midgut invade the body cavity through the new openings. Cry protein toxins can have a high affinity for glycolipid receptors in the nematode gut as well (Griffitts et al., 2005).

One Cry protein that has received attention for its nematocidal properties is the protein Cry5B. This protein has been shown to be effective against several parasitic species. When *Ascaris suum* fourth stage larvae were exposed to a range of Cry5B concentrations (0.1 µg/mL-1000 µg/mL) *in vitro*, all were dead by day 3 (Urban et al., 2013). Cry5B has also been tested against *Ancylostoma ceylanicum* larvae and adults *in vitro*, with adult worms displaying less than 50% motility after 40 hours exposure to varying concentrations (5µg/mL-200 µg/mL) and larval motility declining by 20% when exposed to 5 µg/mL (Cappello et al., 2006). The protein was shown to be effective against these parasitic species *in vivo* as well. There was a 90% reduction of *A. ceylanicum* worm burden in hamsters (Cappello et al., 2006), and a 97% reduction in the

worm burden of *A. suum* in pigs in the study by Urban et al. (2013). While Cry5B has been shown to be effective at treating adult parasites in the host, if the Cry protein could be delivered to feces containing eggs and larvae, it may also be effective since the first and second stage strongylid larvae must feed while in the feces, before reaching their infective stage (Craig, 1986). Since the Cry protein needs to be consumed by the worm to create pores in the intestine, larvae would be exposed when they consume the bacteria or protein in the feces.

A study by De Lara et al. (2016) tested the larvicidal effectiveness of another *Bt* protein, Cry11Aa, on *H. contortus* L2/L3 in sheep feces. Both natural *Bt* expressing Cry11Aa as well as *Escherichia coli* modified to express the protein were tested, with two mL of the bacterial suspensions containing 10^8 CFU mL^{-1} added to four grams of feces. They found 62% fewer larvae in the feces treated with natural *Bt*, and 81% fewer larvae in the feces treated with engineered *E. coli* compared to the control groups. Therefore, based on this study, as well as the previous Cry5B studies, it is likely that Cry5B could also be lethal to the larvae developing in the feces.

The Cry proteins are delicate and can be destroyed by high temperatures or highly acidic environments in just a few minutes (Kotze et al., 2005). While there have been no studies addressing the durability of Cry5B specifically in soil, other Cry proteins like Cry1F have been shown to break down rapidly in the soil with one study showing that significant degradation can occur in as little as one day (Herman et al., 2001), which may lower effectiveness if Cry protein is directly applied to feces. Moisture, temperature, and oxygen level are all important factors that affect the development of strongylid and *S. papillosus* larvae (Eysker et al., 2005). These factors should be considered in relation to the protein as well. While higher temperatures may speed up the development of the larvae, those conditions could also breakdown the protein. Therefore,

while Cry5B could be effective against the larvae in stable laboratory conditions, the same may not apply once under environmental conditions.

The goals of this study were to determine the effect of Cry5B, both in live and inactivated *Bt*, on the development of strongylid L3 in ovine manure, and whether certain species of strongylid nematodes would be more affected than others. Because *S. papillosus* was also present in the sheep feces, the effect of Cry5B on that species was also examined. Lastly, the effect of Cry5B was examined under both laboratory and environmental conditions.

2. Materials and methods

2.1. Animals and feces

Fresh feces collected directly from an eight-month-old Suffolk male lamb with a natural infection of *H. contortus*, *Trichostrongylus* spp., *Oesophagostomum* spp., and *S. papillosus* species. All protocols followed in the study were approved by the Virginia Tech's Institutional Animal Care and Use Committee, and the Institutional Biosafety Committee.

2.2. Cry5B and control

The *B. thuringiensis* Cry5B protein used in the experiment was obtained from the laboratory of Dr. Raffi Aroian at the University of Massachusetts Medical School (Hu et al., 2010). The Cry5B was provided as a cytosolic protein in two forms: live *B. thuringiensis* with cytosolic crystal protein (BaCC) and inactivated *B. thuringiensis* with cytosolic crystal protein (IBaCC). The *Bt* had been genetically modified to produce the protein in the cytosol of the vegetative cell, rather than during sporulation. The IBaCC was produced by inactivating bacteria

with food grade terpene, which left the vegetative bacterial cells containing encapsulated Cry5B intact. The control used for the BaCC was Chobani® non-fat plain Greek yogurt, and the control used with the IBaCC was Dannon® non-fat plain yogurt. Yogurt was used as a control to replicate the effect of adding a thick, bacteria filled material to the feces. Since the BaCC was a much thicker consistency than the IBaCC, Greek yogurt was chosen to replicate it.

2.3. Larval culture, quantification, and identification

The study was conducted by monitoring larval development in 10 g fecal samples placed in containers made using 2.54 cm diameter PVC piping, with a 5.08 cm mesh glued to one end, allowing water to drain out of the container. Individual containers were placed together in wire baskets. For other culture conditions, see the experimental protocol.

Larvae from individual cultures were harvested using a modification of the modified Baermann test (Zajac and Conboy, 2012). Samples were wrapped in cheesecloth and suspended over a plastic, hollow stemmed wineglass that was filled with tap water. The liquid and larvae were collected after 12 hours, and the feces and fluid from the bowl of the glass were discarded. Fluid and larvae were collected from the hollow stem, moved to a 50 mL conical centrifuge tube, and allowed to sediment for an hour in a 4° C refrigerator. This step was repeated and the remaining liquid with larvae was moved to a 15 mL conical centrifuge tube. Finally, the supernatant was drawn off until only two milliliters of liquid were left, and all larvae present. The two milliliters were mixed using a vortex mixer, and all larvae present in four 20 µL aliquots were counted and identified. The approximate total number of larvae harvested from the 10 g sample was calculated based on the number counted in the aliquots.

All larvae present in the aliquots were identified (Van Wyk and Mayhew, 2013), and sorted into the following categories: strongylid L1/L2, *Haemonchus* L3,

Trichostrongylus/Teladorsagia L3, *Oesophagostomum* L3, *S. papillosus* L1/L2, and *S. papillosus* L3. For the Day 12 cultures, another 100 strongylid L3 from each culture were identified to genus and species level after the total number of larvae was calculated from the aliquots. This was done to determine what the species composition was of the strongylid L3 population on the final day. If there were fewer than 100 strongylid L3 in the entire culture, then all strongylid L3 were counted, and species composition was determined using that number. Because Lugol's iodine was added to kill larvae and allow identification, numbers of live versus dead larvae could not be determined.

2.4. Experimental protocol

Feces were collected from a naturally infected donor sheep, homogenized, and the number of parasite eggs in three random samples were evaluated with a modified McMaster test (Zajac and Conboy, 2012) with a detection limit of 50 eggs per gram (epg). The mean fecal egg count (FEC) results were 6,750 strongylid epg, and 300 *S. papillosus* epg. Homogenized feces were used to prepare cultures on Day 0 of the study. The culture containers were each filled with 10 g of sheep feces and calculated to contain 67,500 strongylid and 3,000 *S. papillosus* eggs based on the FEC.

There were eight experimental groups to test the effects of BaCC and IBaCC under laboratory and outside environmental conditions:

-BaCC laboratory control

-BaCC laboratory treatment

-BaCC environmental conditions control

-BaCC environmental conditions treatment

-IBaCC laboratory control

-IBaCC laboratory treatment

-IBaCC environmental conditions control

-IBaCC environmental conditions treatment

Three cultures were harvested from each group on Days 3, 5, 9, and 12 for a total of 12 cultures per experimental group. Every day at 9:00 am and 6:00 pm the cultures were moistened by spraying distilled water onto the top of the feces with a spray bottle until the feces had a light sheen.

Cultures in the BaCC and IBaCC treatment groups had 15 mg of Cry5B mixed into the feces. However, the concentration of Cry5B in the BaCC and IBaCC provided by the Aroian lab differed due to the preparation process and the amount of water used to dilute the mixture. The Cry5B concentration in the BaCC preparation was 3 mg Cry5B/mL, so 5 mL of BaCC was added to each treatment culture. For the BaCC control cultures, 5 mL of Greek yogurt was added to the feces. The IBaCC Cry5B concentration was 15mg Cry5B/mL, requiring that only 1 mL of IBaCC be added to each treatment culture. To equal the volume added to the BaCC cultures, four mL of plain yogurt was also added to each treatment culture. For the IBaCC control cultures, 5 mL of plain yogurt was added.

The laboratory temperature remained at 18 °C for the duration of the study. The outside environmental cultures were kept in a wire basket covered with wire mesh to prevent fly and animal access. To prevent destruction of the small 10 g samples by heavy rain, the basket was placed under a roof awning, which allowed feces to be exposed to ambient temperature and

humidity while being protected from direct rainfall. Environmental temperature, humidity, and rainfall conditions are described in section 3.2. The experiment was performed from October 4th-15th, 2019. Samples from each of the treatments were harvested in the evening on Days 3, 5, 9, and 12 for both the laboratory and environmental cultures.

2.5. Statistics

All larval count results were log transformed [10] and analyzed using a two-way ANOVA. Slices were taken to compare the total counts for each day and by experimental condition. The contrast function was used to compare BaCC to BaCC control, IBaCC to IBaCC control, and BaCC to IBaCC when the controls were not significantly different for each of the days. All statistical analysis was performed on the calculated total number of larvae harvested from each entire 10 g sample. Significance was established at $P \leq 0.05$.

3. Results

3.1. Laboratory conditions- strongylid data

Under laboratory conditions, both the BaCC and IBaCC treated cultures showed significant total larval reduction when compared to the control cultures on all days tested (Fig. 4.1). On all days, the BaCC treated cultures had over a 96% reduction ($P<0.0001$) when compared to the BaCC control cultures. The IBaCC treated larval counts were also significantly lower than the IBaCC control cultures on all days, with a reduction of 95% ($P<0.002$) on Day 3, a reduction of 96% ($P<0.0004$) on Day 5 and 99% reductions on Days 9 and 12 ($P<0.0001$). There were significantly fewer larvae present in the BaCC treated cultures than the IBaCC

treated cultures on Days 3 and 9 ($P<0.0001$; $P<0.02$). There were significantly fewer larvae in the BaCC control cultures than the IBaCC control on Day 5 ($P=0.008$).

3.2. Environmental conditions- strongylid data

The environmental conditions for the study are displayed in Fig. 4.2. Over the 12 days, the temperature ranged from 34.4 °C to 2.2 °C. The average humidity ranged from 54% to 100%. (NOAA Local Climatological Data: https://www.ncdc.noaa.gov/cdo-web/datatools/lcd). Under environmental conditions, there were significantly fewer total larvae that developed in both the BaCC and IBaCC treated cultures when compared to control cultures on all days, except for the Day 5 IBaCC culture (Fig. 4.2). For the BaCC treated cultures, there was a total larval count reduction of over 95% on all days ($P<0.0001$) when compared to the BaCC control cultures. For the IBaCC treated cultures, there was a 86% reduction ($P<0.002$) on Day 3, a 79% reduction ($P<0.053$) on Day 5, a 98% reduction ($P<0.0001$) on Day 9, and a 99% reduction ($P<0.0001$) on Day 12 compared to the IBaCC control cultures. There were significantly fewer total larvae present in the BaCC treated cultures than the IBaCC treated cultures on Days 3, 5, and 9 ($P<0.005$, $P<0.004$, $P<0.009$), but not on Day 12 ($P<0.73$). No significant difference between BaCC control or IBaCC control cultures on any day.

3.3. Strongylid population composition

Under both the laboratory and environmental conditions, the predominant strongylid species present in the feces was *H. contortus*. In the laboratory, there were very few *Oesophagostomum* spp. larvae present, with this species making up only 1% of the population in the IBaCC control cultures. As seen in Fig. 4.3, the laboratory BaCC and IBaCC control larval populations were made of 85% and 87% *H. contortus*, respectively. The BaCC treated cultures

were composed of 92% *H. contortus*, while in the IBaCC treated cultures *H. contortus* was only 69% of the population. The other 31% of the IBaCC treated larval population was composed of *Trichostrongylus* spp. In the treated cultures exposed to environmental conditions, the larval populations were more diverse compared to the BaCC and IBaCC control cultures which consisted of 92% and 88% *H. contortus*, respectively. The BaCC treated cultures had the most *Oesophagostomum* spp. larvae (mean of 17% of the population), and 21% of the population was composed of *Trichostrongylus* spp. Thirty-one percent of the environmental IBaCC treated population consisted of *Trichostrongylus* spp.

3.4. Laboratory conditions- Strongyloides papillosus data

There were significantly fewer total larvae of *S. papillosus* harvested from both the BaCC and IBaCC treated cultures when compared to the control cultures on all days except for the Day 12 IBaCC culture (Fig. 4.4). For the BaCC treated cultures, there was a 100% decrease ($P<0.0001$) compared to the BaCC controls on Days 3, 9, and 12. On Day 5, there was a 98.7% decrease ($P<0.0001$). The IBaCC treated cultures displayed a significant reduction between the treated cultures and the IBaCC controls on Days 3, 5, and 9 ($P<0.01$; $P<0.02$; $P<0.002$). There was a 90.6% reduction on Day 3, an 84.6% reduction on Day 5, a 95% decrease on Day 9, and 68.8% reduction on Day 12. There were significantly fewer larvae in the BaCC treated cultures than the IBaCC treated cultures on Days 3 and 9 ($P<0.0001$). Significantly fewer larvae in BaCC control cultures than IBaCC controls on Day 12 ($P=0.005$).

3.5. Environmental Conditions- Strongyloides papillosus data

On all days, larvae in the BaCC treated cultures were significantly reduced compared to the BaCC controls (Fig. 4.4). The number of larvae was reduced by 90% on Day 3 ($P<0.005$),

and by 99% on Days 5, 9, and 12 ($P<0.0001$). For the IBaCC cultures, the number of larvae in the treated cultures were significantly reduced on Day 3 (83%, $P<0.01$), Day 9 (96%, $P<0.001$), Day 12 (96%, $P<0.01$), but not on Day 5 (79%, $P<0.13$). There were significantly fewer larvae harvested from the BaCC than the IBaCC treated cultures on Day 5 ($P<0.004$) and Day 12 ($P<0.01$). No significant difference between BaCC and IBaCC controls on any day.

4. Discussion

Cultures treated with *B. thuringiensis* Cry5B protein exhibited significant larval development inhibition under both environmental and laboratory conditions when compared to controls. For the strongylids, all cultures treated with BaCC displayed a ≥95% reduction in larval count from Day 3 onward. The IBaCC treated environmental cultures did not reach >95% reduction until Day 9. For both BaCC and IBaCC treated cultures in laboratory and environmental conditions, the number of strongylid larvae present was reduced by 99% compared to the controls on the final harvest date.

Because all the culture data was analyzed using a Two-way ANOVA, it was possible to compare the BaCC and IBaCC controls directly for each day in each environmental condition. When the difference between the controls was not statistically significant, the BaCC and IBaCC treated culture results could then be compared. The treated cultures were not compared on days when the controls were significantly different. There were significantly fewer strongylid larvae in the BaCC treated cultures on Days 3 and 9 compared to the IBaCC treated cultures both in the laboratory and outdoors, potentially due to additional Cry5B produced by the live bacteria in BaCC. Even though the initial concentration of Cry5B was the same for the two treatments, the

BaCC treated cultures could have a higher quantity after a day or two, which caused the rapid reduction in larval count. However, by Day 12, both BaCC and IBaCC were equally effective at inhibiting strongylid larval development, with 99% reductions when comparing treated cultures to controls in both laboratory and outside environmental conditions.

As seen in Fig. 4.3, on Day 12 in the treated cultures, there were slightly more *Trichostrongylus* and *Teladorsagia* L3 present than in the control cultures, except for BaCC treated cultures in the laboratory. This may suggest that these two genera possess higher resistance to effects of Cry5B than *H. contortus*, or that *H. contortus* L1 were able to hatch much faster from the eggs and consumed more of the Cry5B present before the other species. It could also be because in many of the cultures there were fewer than 100 L3 harvested, and small variations would be magnified when determining the percentage of L3 based on very few larvae.

Against *S. papillosus*, the BaCC treated cultures showed highly significant reductions (≥98%) on all days in the laboratory conditions, and 99% reductions on Days 5, 9, and 12 in the environmental conditions. The IBaCC treated cultures did not display such reductions, instead ranging from 61% to 95% in the laboratory conditions and 79% to 96% in the environment. In both the laboratory and environment, the BaCC was more effective at reducing the number of *S. papillosus* harvested than the IBaCC and remained highly significant as the experiment continued.

Against the strongylid larvae, in both laboratory and environmental conditions, BaCC treated cultures exhibited significant reductions in larval numbers with over 96% and 95% reductions, respectively, for all days. The IBaCC was less effective in the environment as the Day 3 and Day 5 environmental cultures dropped to 86% and 79% reductions, respectively, compared to the ≥95% reduction for all days in the laboratory. The BaCC treatment was highly

significant against the *S. papillosus* larvae in the laboratory, with ≥99% reduction for every day, while in the environment, the Day 3 BaCC treated cultures had only a 90% reduction, which increased to 99% for the rest of the days. The IBaCC treatment was more varied in the effectiveness, with the percent reductions of *S. papillosus* ranging from 69% to 95% in the laboratory, and 79% to 96% in the environmental conditions. The environmental cultures experienced more variation in the percent reduction of larval numbers. This variation could be due to the smaller number of larvae seen in the environmental cultures and the use of aliquots. The aliquots were used to determine the total number of larvae harvested, resulting in an estimation. For example, in the laboratory BaCC treated cultures, Days 3 and 9 exhibited 100% reductions in *S. papillosus* larvae while the Day 5 cultures were only 99% reduced. Rather than the treatment losing and then regaining its effectiveness, it is more likely the sampling methods allowed for some variation in the numbers of larvae retrieved by day and group. The smaller number of larvae harvested from the environmental cultures would have magnified that variation, resulting in larger differences in the percent reductions seen.

There were more total larvae harvested in the laboratory conditions compared to the environmental conditions. The fluctuations in temperature and humidity in the environmental conditions could have slowed or impaired the development of the larvae resulting in fewer total larvae. The strongylid genera have different optimum temperature and humidity ranges for development, with *H. contortus* preferring a temperature range of 25-37 °C, *T. colubriformis* preferring 22-33 °C, and *T. circumcincta* preferring 16-30 °C (O'Connor et al., 2006). *Haemonchus contortus* is especially susceptible to low temperatures (<10 °C) at all egg and larval stages. *Trichostrongylus colubriformis* is hardier to the cold than *H. contortus*, with mortality requiring even lower temperatures (<5 °C). From Day 5 onward, the minimum

temperature dropped, reaching 2.2 °C on Days 11 and 12. These low overnight temperatures would have slowed the metabolism and hindered the development of the larvae in the environmental cultures, whereas the laboratory cultures stayed steady at 18 °C.

Another issue could have been the thickness of the BaCC preparation and Greek yogurt. Based on the lower number of larvae harvested in the BaCC control cultures compared to the IBaCC controls on most days under both environmental conditions, the Greek yogurt itself may have had a reductive effect. This effect can be seen clearly in the strongylid larvae numbers in Fig. 4.1. The larvae in the feces need oxygen to survive. Normally, pelleted feces provide enough surface area to allow adequate oxygen exposure for the developing larvae. However, in the BaCC treatment and control cultures, the thick BaCC and Greek yogurt prevented oxygen from reaching the larvae. Another explanation would be that the amount of material added influenced the larval development. Five mL of material was added to each of the 10 g cultures, regardless of treatment or control. Such a relatively large amount of material added to the cultures could have altered the fecal environment, making it less favorable for larval development. This altered fecal environment may have been harder to survive in with the reduced oxygen caused by the thicker yogurt and BaCC mixture.

Some aspects of the experimental methodology may have had an impact on the results. In order to identify the stage and genus/species of larvae harvested from the cultures, Lugol's iodine was added to each aliquot, which killed any living larvae. Therefore, the total number of larvae in each sample includes both live and dead larvae recovered by the harvesting technique. However, it is unlikely that many dead larvae were present before the iodine was added. The modified Baermann test depends on the movement of larvae to free them from the fecal mass into the surrounding water where they can be collected. Also, dead larvae in the cultures would

be expected to deteriorate relatively rapidly. As a result, it seems likely that the larvae counted and identified in each culture were predominantly live larvae at the time of harvest. Therefore, the methodology used makes the differences in the total larvae harvested a good indication of the effect of Cry5B treatment.

Also, because of the large number of larvae in many of the samples it was necessary to use aliquots of the harvested material to calculate total larval numbers instead of counting the entire sample. This means that the values used in this study are estimates, which could allow for some variation by day and condition. That would help explain why some treatment cultures were not significantly different from the controls on one day, when they were significantly different the days before and after. Overall, however, the statistical significance seen between the treatment and controls groups makes it unlikely that false conclusions were drawn about the effectiveness of either the BaCC or IBaCC preparation.

The effectiveness of Cry5B against strongylids is similar to another larva targeted treatment, *Duddingtonia flagrans*. In a study by Waghorn et al. (2003), fecal cultures from sheep and goats were created with a mixed strongylid infection. The addition of the higher of two doses of chlamydospores was able to reduce the number of *H. contortus* and *T. colubriformis* L3 in the sheep feces by at least 90%. In the BaCC treated cultures in our study, there was over 95% reduction in the L3 in all conditions and on all days.

The results of this culture study provide support for the use of Cry5B against both strongylid nematodes and *S. papillosus* of small ruminants. BaCC was more effective than IBaCC against *S. papillosus* larvae and was faster acting against strongylid larvae. This may be due to production of Cry5B by the live bacteria in the BaCC formulation. These results suggest that *B. thuringiensis* Cry5B protein could aid in the control of both strongylid and *S. papillosus*

larvae on the pasture. The control of pasture contamination is just as important for proper parasite control programs as treating the adults already inside the host. By decreasing the number of larvae in the field, the rate of reinfection or new infections would also decrease.

References

Barger, I.A., 1999. The role of epidemiological knowledge and grazing management for helminth control in small ruminants. Int. J. Parasitol. 29, 41-47.

Cappello, M., Bungiro, R.D., Harrison, L.M., Bischoff, L.J., Griffitts, J.S., Barrows, B.D., Aroian, R.V., 2006. A purified *Bacillus thuringiensis* crystal protein with therapeutic activity against the hookworm parasite *Ancylostoma ceylanicum*. Proc. Natl. Acad. Sci. U.S.A. 103, 15154-15159.

Craig, T.M., 1986. Epidemiology and control of gastrointestinal nematodes and cestodes in small ruminants. Vet. Clin. North Am. Food Anim. Pract. 2, 367-372.

De Lara, A.P., Lorenzon, L.B., Vianna, A.M., Santos, F.D.S., Pinto, L.S., Berne, M.E.A., Leite, F.P.L., 2016. Larvicidal activity of *Bacillus thuringiensis* var. *israelensis* Cry 11Aa toxin against *Haemonchus contortus*. Parasitology. 1, 1-7.

Eysker, M., Bakker, N., Kooyman, F.N.J., van der Linden, D., Schrama, C., Ploeger, H.W., 2005. Consequences of the unusually warm and dry summer of 2003 in the Netherlands: poor development of free living stages, normal survival of infective larvae and long survival of adult gastrointestinal nematodes of sheep. Vet. Parasitol. 133, 313-321.

Fontenot, M.E., Miller, J.E., Peña, M.T., Larsen, M., Gillespie, A., 2003. Efficiency of feeding *Duddingtonia flagrans* chlamydospores to grazing ewes on reducing availability of parasitic nematode larvae on pasture. Vet. Parasitol. 118, 203-213.

Griffitts, J.S., Haslam, S.M., Yang, T., Garczynski, S.F., Mulloy, B., Morris, H., Cremer, P.S., Dell, A., Adang, M.J., Aroian, R.V. 2005. Glycolipids as receptors for *Bacillus thuringiensis* crystal toxin. Science. 307, 922-925.

Herman, R.A., Evans, S.L., Shanahan, D.M., Mihaliak, C.A., Bormett, G.A., Young, D.L., Buehrer, J., 2001. Rapid degradation of Cry1F delta-endotoxin in soil. Environ. Entomol. 30, 642-644.

Kaplan, R.M., 2004. Drug resistance in nematodes of veterinary importance: a status report. Trends Parasitol. 20, 477-481.

Kotze, A.C., O'Grady, J., Gough, J.M., Pearson, R., Bagnall, N., H., Kemp, D.H., Akhurst, R.J., 2005. Toxicity of *Bacillus thuringiensis* to parasitic and free-living life-stages of nematode parasites of livestock. Int. J. Parasitol. 35, 1013-1022.

O'Connor, L.J., Walkden-Brown, S.W., Kahn, L.P., 2006. Ecology of the free-living stages of major trichostrongylid parasites of sheep. Vet. Parasitol. 142, 1-15.

Roh, J.Y., Choi, J.Y., Li, M.S., Jin, B.R., Je, Y.H., 2007. *Bacillus thuringiensis* as a specific, safe, and effective tool for insect pest control. J. Microbiol Biotechnol. 17, 547-559.

Sutherland, I., Scott, I., 2010. Gastrointestinal nematodes of sheep and cattle. West Sussex (UK): Wiley-Blackwell, 242 pp.

Thamsborg, S.M., Ketzis, J., Horii, Y., Matthews, J.B., 2017. *Strongyloides* spp. infections of veterinary importance. Parasitology. 144, 274-284.

Urban, J.F., Hu, Y., Miller, M.M., Scheib, U., You, Y.Y., Aroian, R.V., 2013. *Bacillus thuringiensis*-derived Cry 5B has potent anthelmintic activity against *Ascaris suum*. PLoS Negl. Trop. Dis. 7, e2263.

Van Wyk, J.A., Mayhew, E., 2013. Morphological identification of parasitic nematode infection larvae of small ruminants and cattle: a practical lab guide. Onderstepoort J. Vet. Res. 80, 1-14.

Waghorn, T.S., Leathwick, D.M., Chen, L.Y., Skipp, R.A., 2003. Efficacy of the nematode-trapping fungus *Duddingtonia flagrans* against three species of gastro-intestinal nematodes in laboratory faecal cultures from sheep and goats. Vet. Parasitol. 118, 227-234.

Wei, J.Z., Hale, K., Carta, L., Platzer, E., Wong, C., Fang, S.C., Aroian, R.V., 2003. *Bacillus thuringiensis* crystal toxins that target nematodes. Proc. Natl. Acad. Sci. U.S.A. 100, 2760-2765.

Zajac, A., Conboy, G.A., 2012. Veterinary clinical parasitology. Chichester, West Sussex, UK: Wiley-Blackwell, 354 pp.

Figures

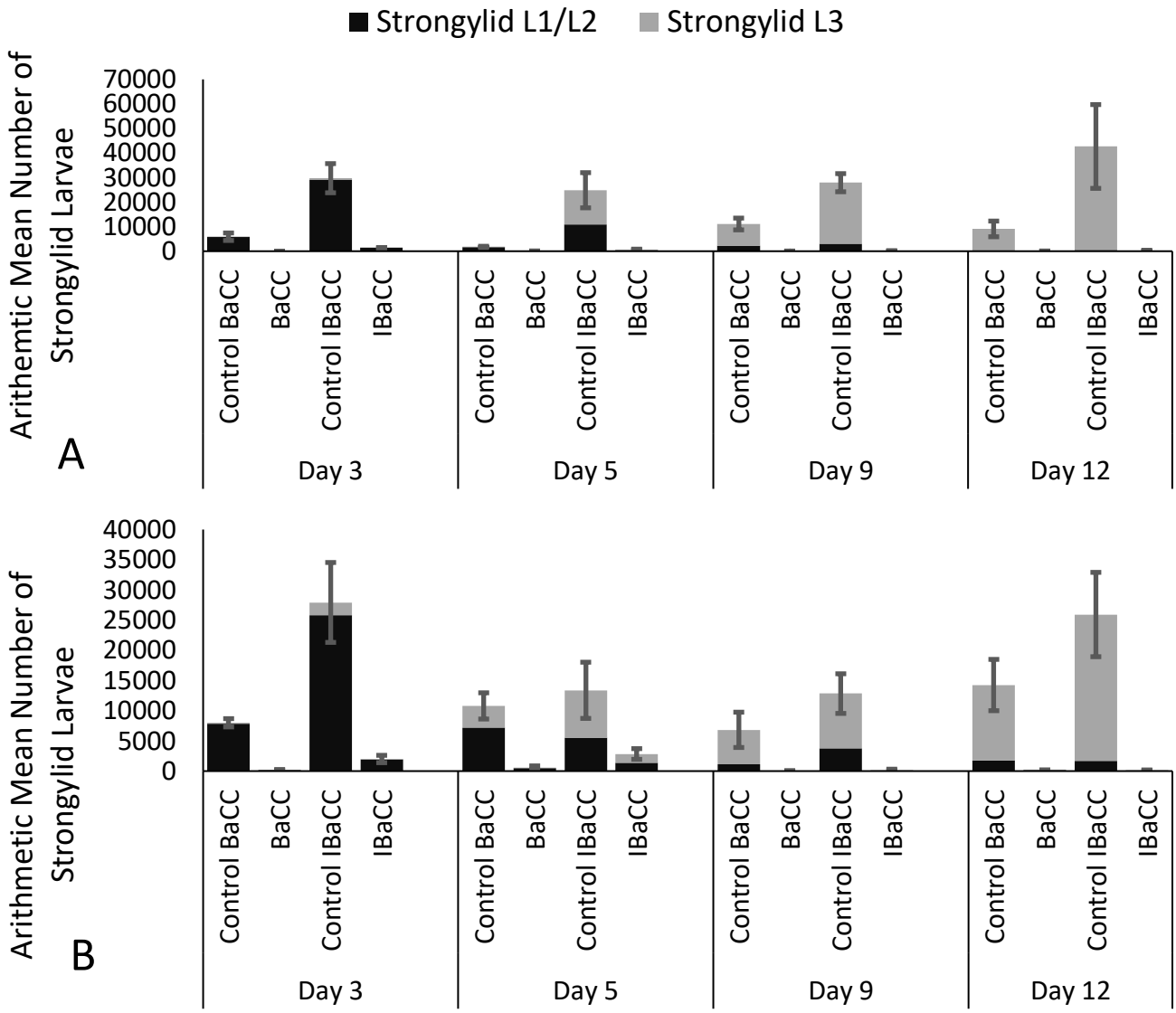

Fig. 4.1. A. Arithmetic mean number (±S.E.) of strongylid larvae in fecal cultures maintained in the laboratory by day and treatment (15 mg Cry5B). For all days, larval numbers in treated groups were significantly lower than in their respective controls. There were significantly fewer larvae in BaCC treated than IBaCC treated on Day 3 and 9. Significantly fewer larvae in c ontrol BaCC compared to control IBaCC on Day 5. **B.** Arithmetic mean number (±S.E.) of strongylid larvae in fecal cultures maintained in the environment by day and treatment. Significantly fewer larvae were present in BaCC and IBaCC treated cultures than respective control except for the Day 5 IBaCC culture. Significantly fewer larvae present in BaCC than IBaCC treated cultures on days 3-9.

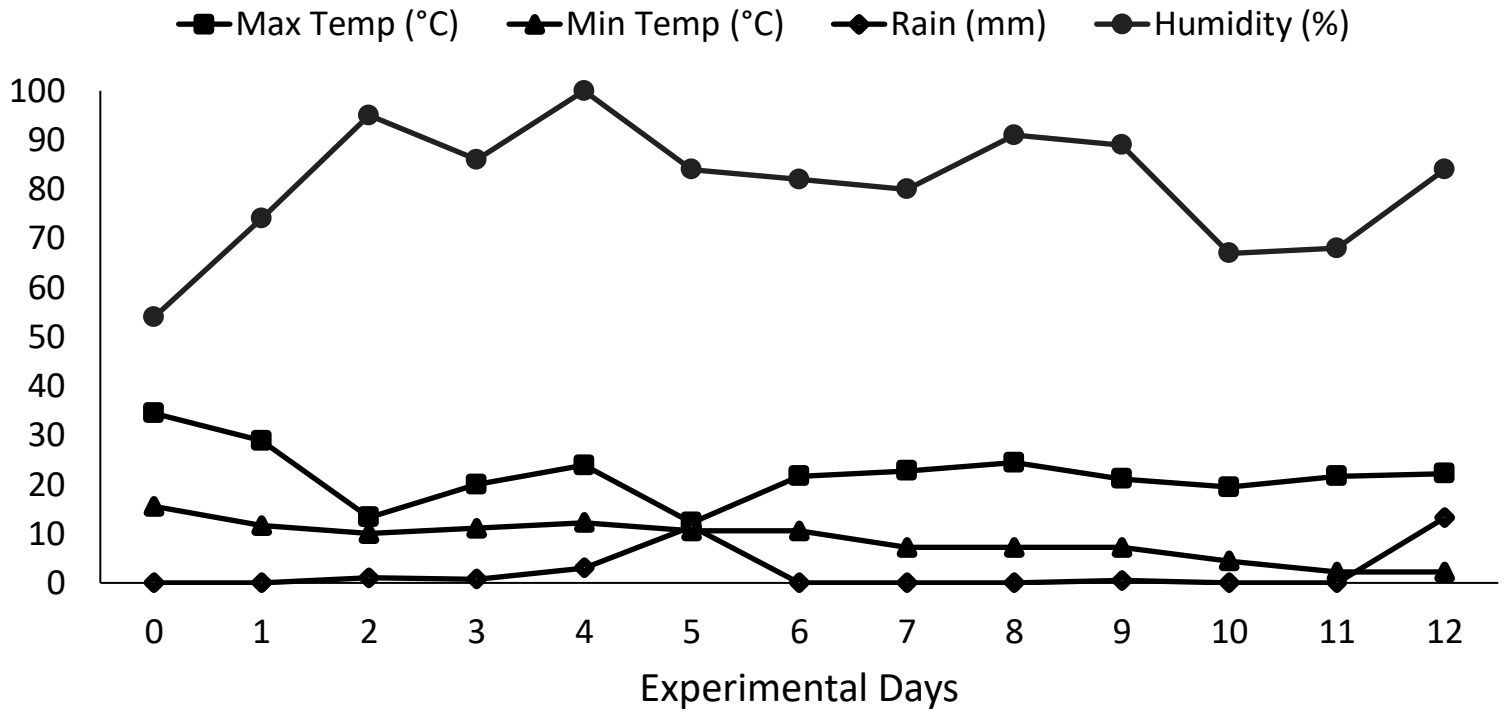

Fig. 4.2. Average daily weather data for each experimental day. All values obtained from NOAA Local Climatological Data: https://www.ncdc.noaa.gov/cdo-web/datatools/lcd.

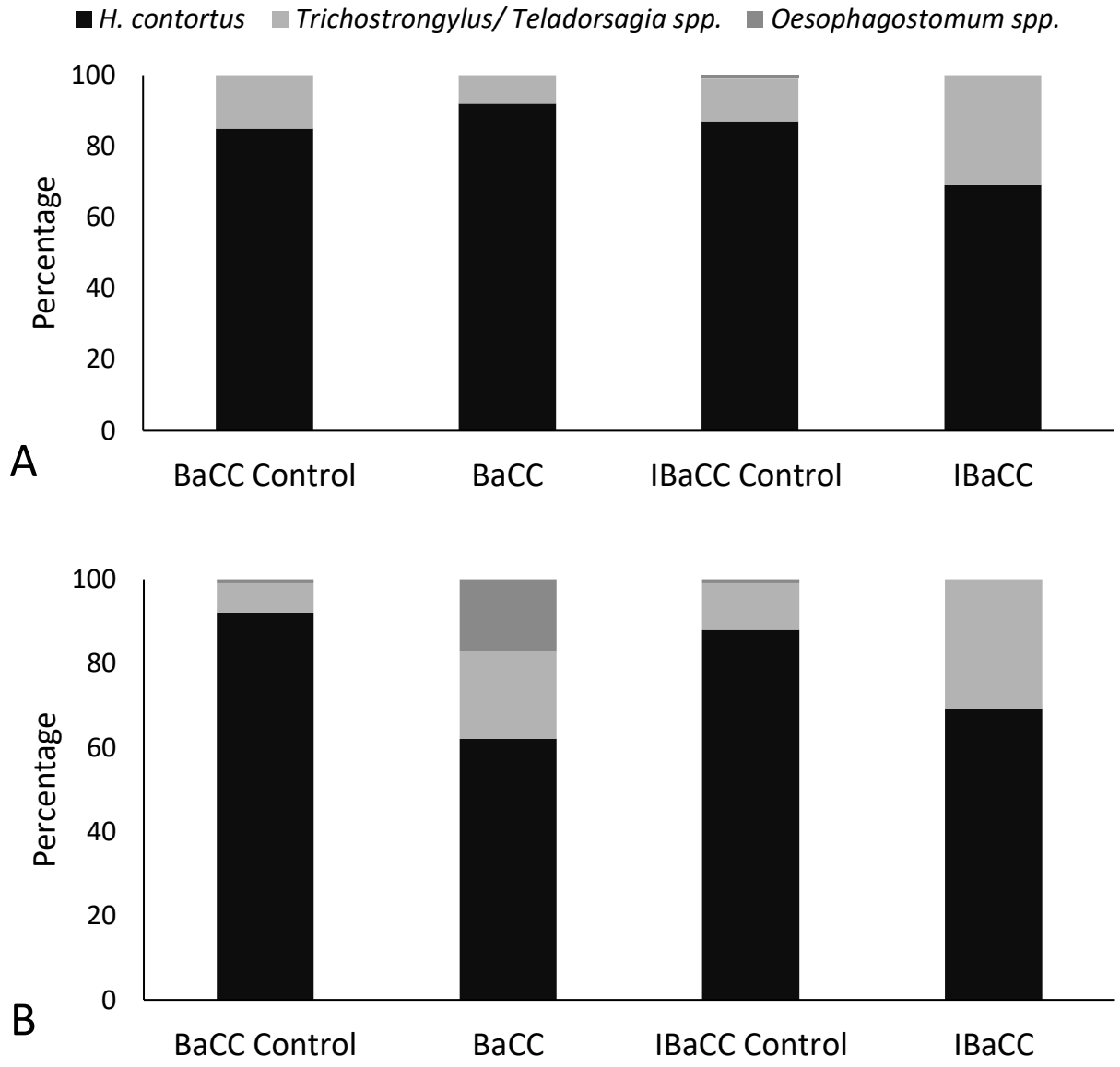

Fig. 4.3. A. Percentage of strongylid genera L3 harvested from laboratory cultures on Day 12 by experimental group (treatment cultures received 15 mg Cry5B). **B.** Percentage of strongylid genera L3 harvested from cultures maintained outdoors on Day 12.

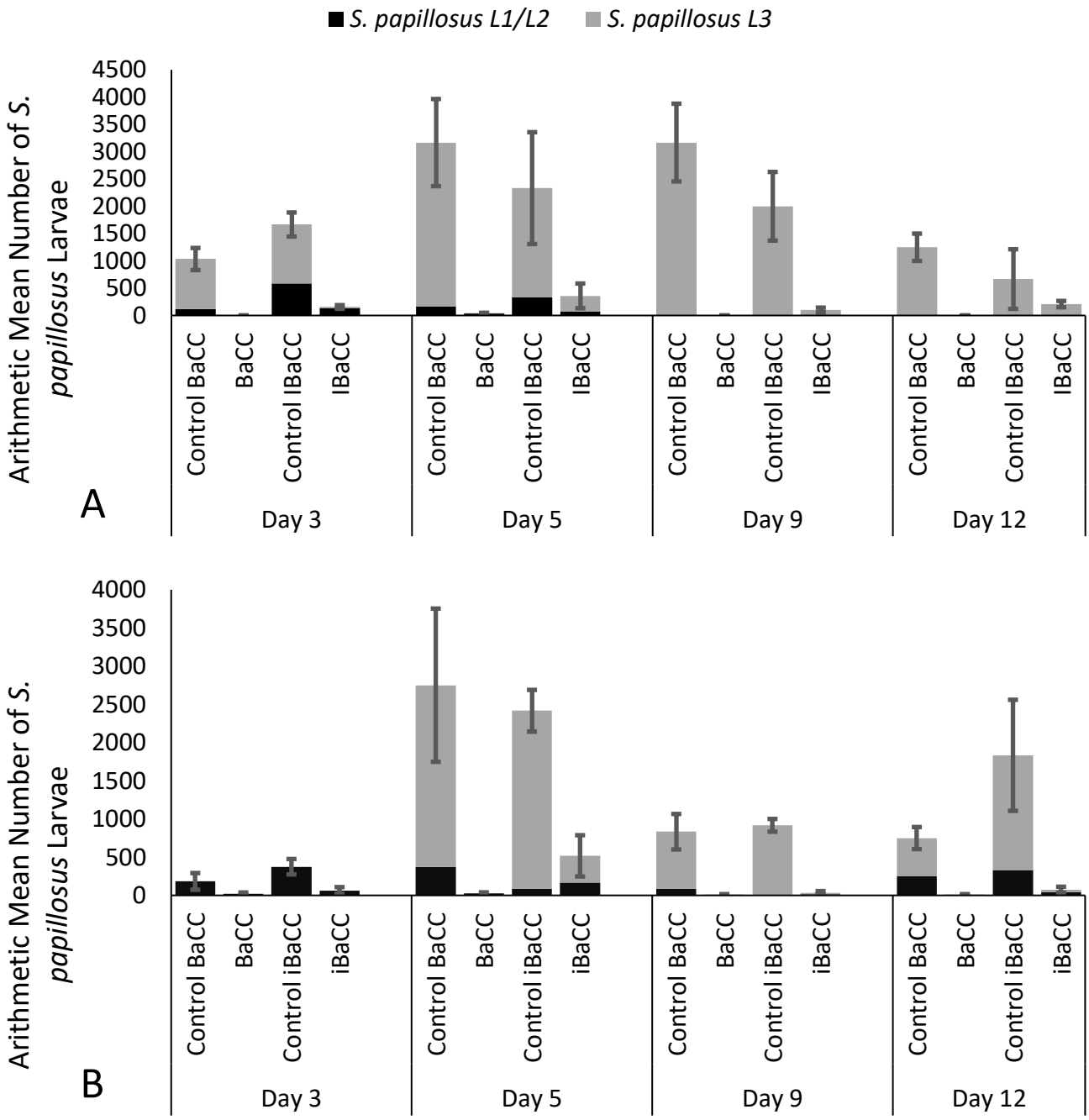

Fig. 4.4. A. Arithmetic mean number (±S.E.) of *Strongyloides papillosus* larvae in 10 grams of feces by day and experimental condition (treated cultures received 15mg Cry5B) for laboratory cultures. Significantly less larvae in BaCC and IBaCC treated cultures compared to controls on all days except Day 12 IBaCC culture. Significantly less larvae in BaCC cultures compared to IBaCC on Days 3 and 9. Controls significantly different on Day 12. **B.** Arithmetic mean number (±S.E.) of *S. papillosus* larvae for cultures maintained outdoors. Significantly fewer larvae in BaCC and IBaCC treated cultures compared to controls on all days except Day 5 IBaCC culture. Significantly fewer larvae in BaCC treated cultures than IBaCC cultures on Days 5 and 12.

CHAPTER 5: Conclusions

Haemonchus contortus is a parasite of major importance in sheep and goat production. Resistance to multiple anthelmintic drug classes seen in *H. contortus* has created a demand for alternative control methods. In both the BaCC and IBaCC studies, *Bacillus thuringiensis* (*Bt*) Cry5B protein demonstrated a significant anthelmintic effect against *H. contortus*. Cry5B was highly significant in its effect on females, but not on males. The number of females was reduced by over 95% in both studies, with these results reflected in the FEC of the treated animals. These results were achieved with no signs of toxicity for any treated animals, which is crucial for a potential treatment.

Future studies should increase the number of sheep in each experimental group if possible. One extra sheep was added to each experimental group in the IBaCC study, and there was a statistically significant difference in the mean total worm burden between the groups. That difference may not have been detectable if the sample size was smaller. Further studies could also be designed to determine when the lethal effect on the adults occurs. It is important to know whether one dose of Cry5B would be enough to kill and remove the adults, or if repeated exposure would be required. Although FEC were significantly lower in treated animals after two doses in both studies, there may have been a suppressive effect on egg production before adults were killed. The impact on the adults was not immediately seen due to the time the eggs take to travel through the intestine. A new study could administer one dose to one group, two doses to a second, and so on to determine the number of doses needed for lethality to occur. The dosage could also be further increased in future studies. The effect on the males in the treatment animals was not significant in the IBaCC study, but it was greater than seen in the BaCC study, possibly due to the increased dose of Cry5B. A significant reduction in male worm burden needs to be

seen for Cry5B to be more useful as an alternative treatment. Further studies could also test the protein against other strongylid species *in vivo*, like *Trichostrongylus colubriformis*. The protein was able to have an impact in the abomasum, but it may not be as effective against small intestinal species.

While both the BaCC and IBaCC were effective against larval and adult *H. contortus*, the formulations tested were difficult to administer. The mixture of bacteria, protein, and water was an extremely thick paste, which had to be given slowly. The concentration of Cry5B meant that lambs were receiving over 200 mL of the mixture, repeated over multiple days. The current formulation is not practical for large scale administration. If Cry5B were to offer an alternative to current anthemics, an easily administered one dose treatment should be formulated. However, multiple days of treatment may be required for enough protein to be consumed and cause the lethal effects seen. The protein, when unprotected, degrades in just minutes in simulated gastric fluid. The protein would likely need to be encapsulated to maximize the time it would be present and effective when in the digestive system. The BaCC and IBaCC mixtures both encapsulated the protein in the vegetative bacterial cell and may have contributed to the survival of the protein until it reached the abomasum, where use of pure protein might not have been as effective.

Both the BaCC and IBaCC were extremely effective against the strongylid larvae present in the fecal cultures when applied directly to the feces. These results support the potential use of Cry5B to manage pasture contamination. Further work would be needed to make the Cry5B deliverable to the feces as a feed additive like *Duddingtonia flagrans*. The Cry5B would also only work on the first and second stage, as the third stage larvae would be unable to consume the protein due to their sheath. The protein could be applied at a time of year when egg counts begin to rise to target the newly hatching larvae.

These are the first studies specifically testing Cry5B against *H. contortus in vivo* in sheep. The results of these studies further support the evidence that *B. thuringiensis* Cry5B may be a useful animal anthelmintic that could also work in ruminants. Cry5B has already been studied against parasite species in mice, hamsters, and pigs. Sheep possess a more complex gastrointestinal system, and it is encouraging to see that the protein was able to get past the rumen and into the abomasum without fully degrading. The effectiveness should also be investigated in goats, which are generally more susceptible to parasitic disease. No signs of toxicity were present in the treated animals as expected. Decades of consumption of *Bt* crops have illustrated the safety of these proteins, as well as studies specifically looking at the effect of feeding Cry proteins to ruminants. The results described in these experiments, along with other studies using Cry5B, suggest that this protein could someday become an effective alternative treatment to current anthelmintics.