The potential effect of Garlium GEM HC™ as a tick control agent in cattle

by

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Abstract

As resistance to chemical acaricides increases amongst tick populations worldwide, the investigation of natural remedies that have an acaricidal or tick repellent effect has become essential in the search for alternatives for sustainable tick control. Garlium GEM HC^{TM} (Garlium) is a natural concentrated garlic product that was fed to eight of 16 Bonsmara steers (average live weight of 215 kg) supplemented at a rate of 2 g Garlium per animal per day in their daily feed ration over a period of 43 days. The other eight steers served as controls and were not supplemented with Garlium.

All animals were infested with the same number of *Rhipicephalus appendiculatus* adults and *Rhipicephalus microplus* larvae by placing the ticks in cotton bags attached to the ears and the body of the steers, respectively. Engorged female ticks were collected after detachment and kept in a temperature and humidity-controlled mini-acaridarium to lay eggs. The number of attached ticks (both male and female) was also determined, as well as the hatchability of the eggs that were laid. Blood samples were collected from the animals intermittently on predetermined days to determine the effect of Garlium on whole blood composition. The results were analysed by a two-sample t-test method.

In general, the results did not show a significant advantage of dietary supplementation of Garlium to Bonsmara steers. Most of the tick parameters were numerically improved, but due to the large standard deviations observed in the results of the different parameters, the results were not statistically significant ($P > 0.05$). When calculating the number of larvae produced by female *R. appendiculatus* ticks per host, a 19.1% reduction ($P > 0.05$) in the number of larvae was observed. Garlium treatment tended to reduce the number of *R. microplus* larvae produced per host by 42.3 % ($P = 0.10$). Supplementing Garlium to the cattle had no adverse effect on their haematocrits or haemoglobin concentrations. Thus, there is no evidence that supplementation of Garlium at the rate used in this study would cause anaemia. Garlium supplementation, however, significantly ($P = 0.02$) reduced the segmented neutrophil count in

the treatment group, suggesting that the immune system was challenged to a lesser degree in the treatment group compared to the control group. There was also a tendency ($P = 0.10$) for haematocrits and lymphocyte counts to increase with Garlium supplementation.

Although the potential repellent effect of Garlium supplementation on tick infestation under field conditions was not investigated in this study, and although there were no statistically significant differences in tick parameters between the treatment and control groups, the effect of reducing the number of larvae produced per host by approximately 20% and 40%, respectively, for the two tick species, may be of economic benefit to commercial cattle farmers.

Key words: Cattle; tick control; natural remedies; garlic; *Rhipicephalus appendiculatus*; *Rhipicephalus microplus*

Chapter 1. Introduction

For research institutes and commercial enterprises, controlling ticks and tick-borne diseases has been a point of focus for many years. Tick infestation on cattle may have a negative effect on the productivity of the animals. Little (1963) reported that blood loss caused by ticks explains about 25% of total loss in live weight gain of cattle. Several scientists have suggested that loss of production was due to suppression of appetite (Riek, 1957; O'Kelly and Seifert, 1970). A series of studies confirmed the importance of a reduction in feed intake by tick-infested cattle (O'Kelly et al., 1971; Springell et al., 1971; Seebeck et al., 1971).

Continuous research and product development are needed to remain abreast of the growing development of acaricide resistance in certain tick species. Resistance of ticks to certain acaricides was already reported in the 1980s when resistance to organophosphates was reported by Aguirre et al. (1986). Miller et al. (1999) also reported pyrethroid resistance in *R. microplus* strains. One of the first cases of amitraz resistance was reported in 1981 (Nolan,1981).

It has been suggested that the alternation of acaricides or their use in combination may delay the development of resistance. Thullner et al. (2007) conducted a study where acaricides (organophosphates and pyrethroid) were rotated. Resistance against deltamethrin was detected after five tick generations when only deltamethrin was used (resistance factor [RF] = 9.2). When the use of deltamethrin was rotated with coumaphos, resistance to deltamethrin varied from one generation to the next, but no significant resistance developed. After ten generation rotations, the RF value of deltamethrin was 1.6. No consistent change in resistance to coumaphos was observed.

Jonsson et al. (2010) evaluated the efficacy of an acaricide rotation program using both amitraz and spinosad alone or in a rotation programme. Spinosad is from the spinosyn family, and its mode of action differs from that of amitraz. Amitraz-resistant tick larvae were used in the study. Treatment with spinosad alone or with a rotation between amitraz and spinosad resulted in reduced levels of resistance to amitraz. When the cattle were treated only with amitraz, ticks increased their level of resistance to amitraz (Rodriguez-Vivas et al., 2006).

Using acaricides in a rotational programme seems to work well; however, there are still too many producers that unfortunately do not use acaricides responsibly, thereby increasing the risk of ticks developing resistance to certain acaricides.

Pressures from both the consumer and the increase in resistance of ticks against certain acaricides increase the need for more research to be conducted to find suitable alternative products or products that can be used in a rotation programme with the proven acaricides to reduce the risk of acaricide resistance. Natural alternatives to current commercial acaricides have been the focus of some of the research conducted (Pereira Junior et al., 2019; Quadros et al., 2020). For this reason, Garlium GEM HC (Garlium), a natural product consisting of garlic powder, garlic oil and salt, was evaluated in the study to determine the effect of Garlium on reproduction and possible repellent effect on *R. appendiculatus* and *R. microplus* when supplemented in a feedlot ration to beef cattle.

Chapter 2. Literature review – Tick species

2.1. Introduction

Ixodid ticks (Acari: Ixodidae) species can be divided into three categories: one-host, two-host, and three-host ticks (Nava and Guglielmone, 2013). As the description indicates, the ticks will use either one, two or three hosts to complete their life cycle (eggs, larva, nymphs and adults). One-host species include *Rhipicephalus microplus*, *Rhipicephalus decoloratus* and *Margaropus winthemi.* Two-host species include *Hyalomma rufipes, Hyalomma truncatum* and *Rhipicephalus evertsi evertsi.* Three-host species include *Amblyomma hebraeum, Ixodes rubicundus* and *Rhipicephalus appendiculatus* (Walker et al., 2003). The greater the number of different hosts a tick uses to complete its life cycle, the more difficult it may be to control.

Two tick species, *Rhipicephalus appendiculatus* (a three-host tick) and *Rhipicephalus microplus* (a one-host tick) were used in this study to represent the different extremes of tick life cycles, both having a significant economic impact on profitable beef production. The rationale for using a one-host tick species was that one-host ticks spend the most prolonged period persistently feeding on cattle, with all three life cycle stages occurring on the same animal in quick succession over three to four weeks. Whereas each life cycle stage in three-host ticks can complete its engorgement in less than a week, each feeding stage is interrupted by long periods of living freely in the environment.

2.2. *Rhipicephalus appendiculatus*

Rhipicephalus appendiculatus is one of the most important vector species of *Theileria parva,* a protozoa, that can cause East Coast fever in cattle (Radley et al., 1974). The disease is fatal in cattle and has been reported in more than ten African countries (Norval et al.,1992). More than one million cattle die yearly from theileriosis (Mukhebi and Perry, 1992). *Rhipicephalus appendiculatus* can be found at different altitudes, from sea level and upwards until 2000 m above sea level. (Walker et al., 2000).

Rhipicephalus appendiculatus is a three-host tick with nymphs and adults which feed primarily on cattle and antelope. Several studies on *R. appendiculatus* in South Africa indicated that it completes a single life cycle per annum (Horak et al., 2018). The life cycle of *R. appendiculatus* under laboratory conditions is presented in Table 1 (Tukahirwa, 1976).

Table 1. *Rhipicephalus appendiculatus's* life cycle under laboratory conditions (Tukahirwa, 1976).

Activity	Development period (days)	Host species
Females feed	6 -11	Host 1
Pre-oviposition	5 – 8	
Larva hatch	28 - 33	
Larva feed	3 - 6	Host 2
Nymphs pre-moult	10 – 15	
Nymphs feed	4 - 7	Host 3
Adults pre-moult	15 - 19	

Each stage will start with ticks climbing onto vegetation and waiting for a host to pass. Hatching will start 23 – 38 days after eggs are laid, and the larva will take between 30 and 70 days to develop into the nymph stage (Tukahirwa, 1976). The moulting period from larva to nymph depends on the day length, with longer days resulting in a shorter period (Randolph, 1997). The larvae will detach from the host and moult on the ground until the nymph finds a new host. Most nymphs will attach to the head, bottom part of the neck and on the legs of the host (Baker and Ducasse, 1967).

On cattle and other antelope, during the immature stages, ticks feed rapidly in all development stages and require 4 to 7 days to engorge (Walker et al., 2003). Adults are found on the ears, eyelids, neck and around the anus. Once detached, the engorged female lays 3 000 to 5 000 eggs (Randolph, 1997). These eggs hatch in 20 to 90 days. The life cycle can be completed in 3 months, but it probably takes a year to complete under southern African conditions.

Rhipicephalus appendiculatus can also transmit benign bovine theileriosis caused by *Theileria taurotragi*. When lots of ticks attach to an animal, the saliva of *R. appendiculatus* that contains a toxin may influence the host's immune processes, increasing the host's susceptibility to diseases like anaplasmosis and heartwater (Walker et al., 2003). As a parasite, the tick may cause considerable direct damage to its hosts (Mwambi et al., 2000). Severe infestations of ticks can damage the ear, and fly larvae infestations may then increase the damage to the ear. In a study conducted by Norval et al. (1997), adult *R. appendiculatus* caused damage to the ears of tick-infested cattle.

Controlling *R. appendiculatus* is usually labour intensive, difficult and expensive. Farmers who do not have dip tanks or cattle spray facilities must apply acaricide in and around each ear of the animals. Pour-on acaricides are not always adequate to control *R. appendiculatus* as it might not get into contact with all the ticks. Resistance of *R. appendiculatus* against certain acaricides has been assessed for many years. Baker (1978) reported resistance to toxaphene, which has been banished since the ’80s, in *R. appendiculatus* in eight African countries. Although not yet familiar in southern Africa, emerging resistance to cypermethrin was recorded in *R. appendiculatus* by Ntondini et al. (2008) when evaluating the resistance of ticks from communal areas in the Eastern Cape province of South Africa. This contrasts with a survey by Mekonnen et al. (2002), where they evaluated the acaricide resistance of one, two and three-host ticks against three different acaricides. Ticks collected from both commercial and communal land were used in the study. There appeared to be no acaricide resistance in *R. appendiculatus* against any of the three acaricides (amitraz, cypermethrin and chlorfenvinphos). Several other studies reported acaricide resistance in *R. appendiculatus* (Luguru et al.,1987; Kagaruki, 1991; Muyobela et al., 2015; Vudriko et al., 2016) from which it is evident that, although not widespread, acaricide resistance in *R. appendiculatus* is of concern and alternatives to control *R. appendiculatus* should be evaluated to reduce the risk of acaricide resistance.

2.3. *Rhipicephalus microplus*

Rhipicephalus microplus can be found in many parts of southern Africa and prefers savannah-type vegetation and temperate climates, typically in grassland with trees often used as cattle pasture. The number of *R. microplus* in southern Africa starts to increase in spring (October), then their numbers decrease towards December (Baker et al., 1989).

Rhipicephalus microplus can cause the transmission of *Anaplasma spp.* and *Babesia spp*., negatively influencing livestock health (Van Wyk et al., 2016). Economic losses due to *R. microplus* include a reduction in milk production and live weight gain, blood loss, mortality, hide damage and morbidity. It also includes the cost of controlling the effects of haemoparasites like *Babesia bigemina*, *Babesia bovis* and *Anaplasma marginale* (Rodriguez-Vivas et al., 2018). *Babesia spp.* are transmitted by larvae or nymphs, whereas *Anaplasma spp* are transmitted intrastadially by male ticks (Vannier et al., 2015).

Rhipicephalus microplus is a one-host tick with the parasitic part of the life cycle lasting approximately 21 days. The engorged females lay between 1000 to 2500 eggs. In three to six weeks, the eggs hatch, larvae attach to a host, engorge, and moult to the nymphal stage. The nymphs then moult to adults on the host after a week. The adults attach and start to consume blood, whereafter the male and female mate. Females, once fully engorged, will drop from the host after seven days. The ticks, therefore, spend about 21 days on the host, and the life cycle can be completed in two to three months. More than one life cycle can be completed per year (Walker et al., 2003). The life cycle of *R. microplus* is presented in Table 2 (Tukahirwa, 1976).

Table 2. The life cycle of *Rhipicephalus microplus* under laboratory conditions (Tukahirwa, 1976).

Activity	Days after larval attachment
Larvae feed	1 – 7
Nymphs pre-moult	5 – 9
Nymphs feed	6 – 20
Adults pre-moult	11 – 16
Females feed	14 – 20
Females detach	21 – 30

Rhipicephalus microplus will attach itself to different parts of the body of the host, like the shoulder, neck and side of the body. During the immature stages, ticks may be found on the ears and the legs. Adults prefer the area under the tail and ventral part of the body, including the scrotum. (Horak et al., 2018).

Chapter 3. Tick control

3.1. Introduction

Most farmers worldwide use acaricides, like amitraz, deltamethrin, cypermethrin and chlorfenvinphos. These chemical products are applied to the skin of the host to achieve direct contact with the ticks.

Amitraz has an alpha-adrenergic agonist activity, interacts with the tick's central nervous system's octopamine receptors, and inhibits prostaglandin synthesis. It results in overstimulation, paralysis and mortality in ticks (Bonsall & Turnbull, 1983). Deltamethrin is a neurotoxin that can temporarily affect a tick's nervous system when exposed. Ticks may develop resistance to acaricides. This includes thickening of the cuticle to limit infiltration of the insecticide, metabolic resistance via overexpression of metabolizing glutathione-S-transferases, and sodium channel mutations (Müller et al., 2008).

Resistance of R. microplus against chemical acaricides was already reported in the 1980s. Aguirre et al. (1986) reported resistance against organophosphates. Soon other studies indicated an increase in resistance. Pyrethroid resistance was detected in the 1990s, as reported by He et al. (1999) and Miller et al. (1999). In the Eastern Cape province (South Africa), the resistance of R. microplus to amitraz, cypermethrin and chlorfenvinphos was reported by Ntondini et al. (2008). They collected ticks from 59 dip tanks over two years. Although the resistance of ticks against the acaricides was below 10%, it is still a concern that R. microplus exhibits resistance to all three acaricides tested. Rodriguez-Vivas et al. (2006) conducted a study to determine the association between management and *R. microplus* ticks' resistance to amitraz. They reported that 19.4% of farms evaluated had *R. microplus* that were resistant to amitraz. The intensive use of amitraz, high interval of acaricide application and method of application might influence the appearance of amitraz resistance.

3.2. Natural tick control

From the studies referenced in the previous sections, it is clear that relying only on chemical acaricides will increase the risk of acaricide resistance. To extend the use of the currently available chemical acaricides, one should consider other methods to control tick numbers. One way in which this might be achieved is by the alternation of chemical acaricides with natural tick control products and the application of different management systems. Natural acaricides/repellents may be more beneficial than synthetic acaricides because they might reduce the risk of acaricide resistance. With fewer ticks remaining on the host, it might increase the natural resistance of cattle to ticks as the immune system of the host is not severely challenged, allowing it to increase immune cells, like T cells, to respond to ticks more efficiently (Tabor et al., 2017). If cattle are often dipped with an acaricide, the improvement in immunity may be compromised (Pegram et al., 1993).

A review on natural acaricides was published by Quadros et al. (2020). Over 200 plant species with acaricidal and tick-repellent properties have been documented worldwide. Essential oils and plant extracts are the primary forms of plant-based products that can have an effect on ticks. Most of these plant-based products have been evaluated using a direct application approach. For example, Carvacrol is an essential oil with acaricidal properties and it can reduce egg hatching in *R. microplus* ticks (Pereira Junior et al., 2019), probably negatively affecting oogenesis (Konig et al., 2019). Oocyte development was also negatively affected after low doses of acetylcarvacrol were applied to *R. sanguineus*.

Two approaches can be followed to control ticks with natural acaricides/tick repellents. The first is to apply the natural acaricide by dipping or spraying the product onto the animal, and the second is to supplement the tick repellent via animal feed.

3.2.1. Dipping or spraying of natural acaricides/tick repellents

Madzimure et al. (2011) evaluated the efficacy of *Lippia javanica* (Fever tea/Lemoenbossie) leaf extracts in controlling ticks on cattle. *Lippia javanica* plants were soaked in water and solutions of different concentrations were prepared (5%, 10% and 20% leaf extract). Negative control, and positive control (12.5% amitraz-based compound), were used as two of the five treatments. The cattle were sprayed once a week with the different treatment products and attached ticks were counted on predetermined days on the hosts. The number of attached ticks was compared to the negative control group and percentage efficacy regarding tick repelling of the 10% and 20% *L. javanica* concentrations were significantly higher than negative control from week three until the end of the trial (week 7). From the results, it can be concluded that spraying of aqueous extracts from *L. javanica* leaves onto cattle has a repellent effect against ticks. The interaction between time and different *L. javanica* treatment concentrations demonstrate that both dose level and duration of treatment may influence the effect of the repellent product (Belmain et al., 2001).

The effect of essential oils from cumin seeds (*Cuminum cyminum*), allspice berries (*Pimenta dioica*) and basil leaves (*Ocimum basilicum)* on ticks were investigated using *R. microplus* tick larvae (Martinez-Velazquez et al., 2011). Larvae were placed onto filter paper containing different concentrations of the essential oils. Larvae were kept on the filter paper for 24 hours, after which the number of live and dead larvae was determined. The cumin essential oil killed the tick larvae at all five different concentrations. The allspice essential oil had a 100% mortality at higher concentrations, but not at a concentration of 1.25%. No acaricidal effect was observed when the basil essential oil was evaluated.

Brown et al. (1998) demonstrated that the application of a hexane extract from different parts of the *P. dioica* plant (leaf, bark, twigs and berries) resulted in acaricidal effects on engorged adult *R. microplus* ticks. Leaf hexane extract resulted in 10% mortality and 24% inhibition of egg production. The berry essential oil was more effective, resulting in 100% mortality.

Fernandes et al. (2007) investigated the acaricidal effect of extract from copaiba tree (*Copaifera reticulata). Rhipicephalus microplus* larvae were exposed to copaiba tree oleoresin extract mixed with water and dimethylsulfoxide. Larva mortality increased with an increase in oleoresin concentration and reached 100% mortality with a 4000ppm oleoresin concentration.

Tests were conducted by Pereira & Famadas (2006) to evaluate the effect of *Dahlstedtia pentaphylla* plant roots against *Boophilus microplus*. Thirty cattle were divided into three groups and artificially infested with B. microplus larvae once a week. Two different solutions of *D. pentaphylla* extract were compared to control. The best results were obtained by spraying *D. pentaphylla* extract solution (an efficiency rate of 76%) and observed at three days after applying the extract. No effect was observed of the extract solution on laying or hatching of eggs of female ticks.

Lavandula angustifolia essential oil was evaluated to determine the potential acaricidal effect (Pirali-Kheirabadi and da Silva, 2010). Engorged female *R. annulatus* ticks were immersed in different concentrations of essential oil and then incubated. *L. angustifolia* essential oil had acaricidal effects against *R. annulatus*. The mortality rate differed at different dose levels. At higher concentrations, *L. angustifolia* essential oils increased mortality and reduced egg production compared to the control group.

From these studies, it can be concluded that a number of plants show potential for the development of acaricides or tick repelling products. Applying the plant extracts/oils directly onto the ticks is an effective way to reduce the number of ticks. It appears that none of the plant extracts/oils were evaluated over an extended period of time to determine if any acaricidal resistance may develop, however, these results suggest that these products may be of value in future as the acaricidal resistance of some ticks against certain commercial acaricides becomes more common. None of the studies reported any economic benefits of using the plant extract/oil as an acaricidal product.

3.2.2. Supplementation of natural tick repellent via animal feed

Apart from the use of garlic as an in-feed tick repellent agent, data on the supplementation of natural tick repellents via the feed are limited. Fourie et al. (2005) tested the efficacy of powdered aloe juice (Aloe ferox) against ticks by supplementing it to cattle and dogs via the feed. Calves were infested over 25 days with *R. decoloratus* larvae and were supplemented daily with two different levels of powdered aloe juice (5 mg/kg body weight and 25 mg/kg body weight). The powdered aloe juice was supplemented in-feed to the animals. The number, weight and fertility of female ticks were determined. The in-feed supplementation of powdered aloe had no effect on the number of attached ticks on the calves or the fertility of the ticks.

3.3. Garlic as tick control agent

Different garlic components have been evaluated to determine the effect on internal and external parasites. These studies include *in vitro* studies, applying garlic components to attached ticks and supplementing garlic-based products in-feed.

To evaluate the efficacy of a potential acaricide, *in vitro* testing is usually the first step. Aboelhadid et al. (2013) investigated the effect of *Allium sativum* and *Allium cepa* oils on *Rhipicephalus (Boophilus) annulatus* ticks. *B. annulatus l*arvae were collected from naturally infected cattle. Garlic and onion oils were dissolved at different concentrations in ethanol, methanol alcohols and water. Adult, larva, and egg immersion techniques were used to determine the acaricidal effect. Different percentages of garlic oil dilutions in ethanol and methanol killed all adult ticks and larvae. Similar results were observed with garlic oil dissolved in water.

A study was conducted by Nchu et al. (2015) to determine the repellent effect of garlic. A dichloromethane (DCM) extract of garlic bulbs was evaluated to assess its effect against *Hyalomma rufipes.* Different concentrations of DCM were evaluated. A tick repelling bioassay, based on the climbing behaviour of ticks, was applied in the study. Ticks were placed on a base with a glass rod in the middle. Filtered paper with either a garlic oil solution or just the paper (control) was placed at the top of the glass rod. The number of ticks that crawled onto the filtered paper was used to assess the repellent effect of the garlic solution. Tick repellency increased significantly with increased concentrations (40% – 86%). DCM concentration of 1.4% resulted in a repellence index of 87% for male and 87.5% for female ticks. The result of this study indicated garlic extract has a repellent activity.

Shyma et al. (2014) evaluated the effect of different concentrations of methanolic extract from *Allium sativum* cloves on *R. microplus.* At 100 mg/ml concentration, tick mortality was 73%. Inhibition of egg production was the highest at 85% *A. sativum* concentration. Larvae treated with different *A. sativum* concentrations showed significantly higher mortalities ($P < 0.001$) than that of control tick larvae, and at 100 mg/ml, it was 69.%.

From these publications it is evident that garlic has acaricidal/repellent properties. Further studies were conducted to evaluate the acaricidal effect of garlic in a commercial farming system. Mgocheki (2017) conducted a field experiment where 25 cattle were infested with *Rhipicephalus spp (R. decoloratus* and *R. microplus*). Cattle were sprayed with an aqueous garlic extract. Cattle were observed once a day for one week for any remaining ticks on the cattle. Fifty percent of the ticks were killed within 16 hours with different concentrations of garlic solution. Ninety percent of the ticks were killed within 24 hours. Tick numbers were reduced by 99% and 94% two days after the garlic spray, using the 25% and 50% concentrations, respectively.

In a further study conducted by Mgocheki (2017), un-infested cattle treated with different garlic concentrations, grazed on infested pasture and grazed together with non-treated infested cattle. Ticks were counted on each of the treated animals once a day for seven days. At 10% or higher garlic solutions, the garlic extract repelled ticks within seven days, while tick numbers increased in the 5% or lower concentrations. These *in vivo* studies on commercial conditions confirmed the *in vitro* results.

However, spraying cattle with a natural acaricide is still labour intensive. Therefore, it would be useful to supplement the cattle with a plant-based product, like garlic, in-feed and potentially reduce the number of ticks attached. However, as garlic is digested in the rumen, the garlic metabolites will be absorbed into the blood and distributed to different organs of the body. A few studies have been conducted to determine the efficacy of supplementing garlic-based products in-feed to ruminants.

Firstly, it is important to determine the effect of garlic supplementation on the digestibility of animal feed. Busquet et al. (2005) incubated different concentrations of garlic oil and other garlic compounds in diluted ruminal fluids for 24 hours. Garlic oil and some of the garlic metabolites changed the volatile fatty acid (VFA) composition in the rumen, increasing propionic and butyric acid production. Increasing these two VFA's may improve the growth performance of ruminants. Garlic oil also reduced the methane production in the rumen fluid, leading to a possible improvement in animal production. To determine the effect of garlic extract on the performance of pre-weaned calves, Ghosh et al. (2011) supplemented pre-weaned calves with garlic extract at 250 mg/kg body weight/day. There were significant increases in body weight gain and feed intake. The severity of scours in the treatment group was reduced compared to the control group.

Garlic was also evaluated to determine the effect of supplemented garlic on internal parasites. Hasan et al. (2015) mixed 25 or 50 ml of a 10% garlic oil-water solution with the feed fed to goats. Over 60 days, the number of gastrointestinal parasite eggs per gram of faeces was significantly higher in the control groups compared to the treatment groups. Weight gain was improved in the treatment groups. Blood samples were also collected over time, and no blood parameters were negatively affected.

Moriel et al. (2017) supplemented a combination of garlic oil and cinnamaldehyde extract to beef cattle, grazed on different pastures, and supplemented with or without feed concentrate. Cinnamaldehyde and garlic oil reduced horn fly counts of heifers grazing limpo grass, but not heifers grazing bahia grass. These results are likely attributed to the poorly drained soils where limpo grass pastures were established, which created conditions (i.e., standing water) that promoted the growth of the horn fly population.

Costa-Junior & Furlong (2011) conducted a study to determine the effect of a garlic extract product (mineral sulphur and *Allium sativum* extract mix) on *R. microplus* that were placed on 24 crossbred calves. The garlic-based product was fed to the cattle at 20 g/h/d over a period of five months and *R. microplus* larvae were placed on the calves at intervals of two weeks. Engorged females were collected every two weeks and were allowed to lay eggs. They observed a 64% reduction in the number of engorged females, a 22% reduction in weight of females and a 12% reduction in egg production by the females that were engorged and detached from the hosts supplemented with the garlic extract.

3.3.1. Garlic metabolism

For many years, people have consumed garlic for its taste and nutritional and health benefits. Avesta, a collection of Zoroastrian holy writings compiled during the sixth century BC, already referred to garlic as a medicinal plant (Dannesteter, 2003). Garlic has been used to address a variety of health issues in humans. These include cardiovascular diseases (Chan et al., 2013), tumours (Capasso, 2013), diabetes (Sheela et al., 1995), anti-microbial effect (Adler and Beuchat, 2002), anti-protozoal properties (Lemar et al., 2002), antifungal properties (Yousuf et al., 2011) and antiviral properties (Weber et al., 1992).

There are different types of garlic, the main two being hard- and soft-neck garlic. Garlic consists of 65% water, 28% carbohydrate, 2.3% organosulfur compounds, 2% protein and 1.5% fibre (Santhosa et al., 2013).

For medicinal use, garlic oil is mainly prepared by a distillation process. The composition of steam-distilled garlic oil is diallyl, allyl methyl, and dimethyl mono to hexa sulfides (Lawson and Bauer, 1998).

Allicin is the primary bioactive compound present in garlic. When garlic is crushed, the allinase enzyme is activated and converts alliin to allicin (Bayan et al., 2014). The major volatile molecules from garlic and garlic oil are diallyl disulfide (DADS), diallyl sulfide (DAS), diallyl trisulfide (DTS), methyl allyl disulfide, methyl allyl trisulfide, vinyl dithiins and ajoenes (Amagase, 2006).

The breakdown path of organosulfur compounds is reported in Figure 1. Allyl mercaptan (AM) is formed by the reduction of disulfides and is further broken down by S-adenosylmethionine synthetase into allyl methyl sulfide (AMS), Gao et al. (2013).

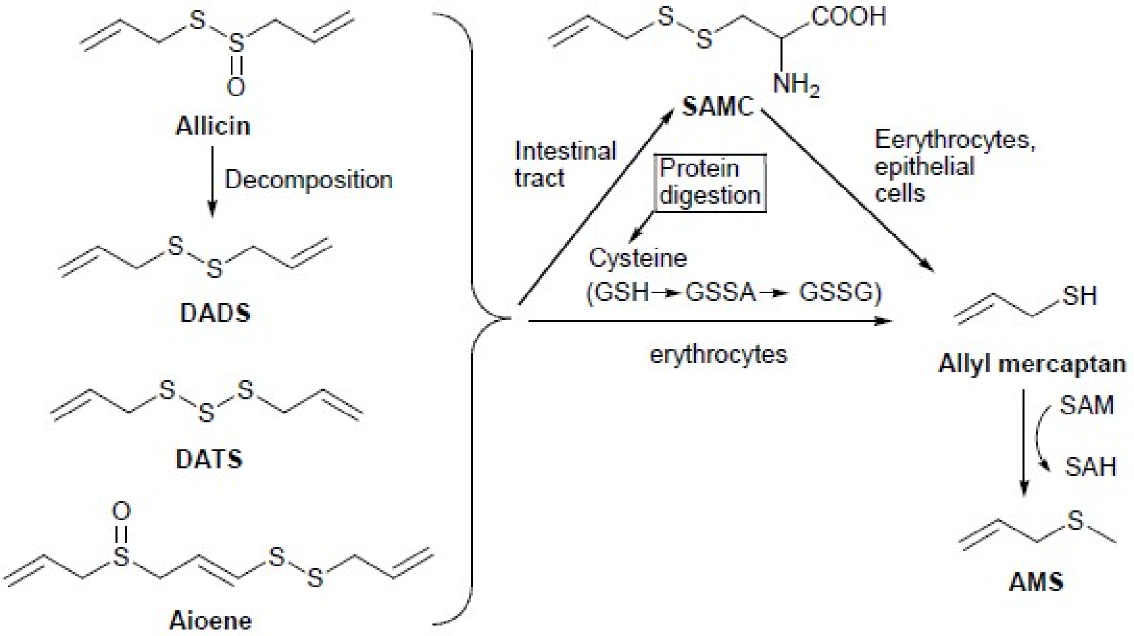

Fig 1. Metabolic pathways of organosulfur compounds derived from garlic. Gao et al. (2013).

Vinyl dithiins could be detected after 24 hours in the serum, kidney and fat tissues only, once it is orally consumed. Diallyl sulfoxide and diallyl sulfone were detected in rat liver, blood, and urine after daily supplementation of diallyl sulfide (Egen-Schwind et al., 1992).

In a study conducted with rats using alliin, allicin and vinyl dithiines, within 10 minutes, the alliin concentration peaked, and no traces were observed in the blood after 6 hours, while the allicin and vinyl dithiines concentrations were the highest at between 30 and 120 minutes (Gao et al., 2013).

An *in vivo* study was conducted to determine the uptake and metabolic fate of DADS in mice. It was injected at a sublethal dose of 100 mg/kg. DADS was rapidly absorbed (Gao et al., 2013).

There are oil- and water-soluble organosulfur compounds in garlic. Water-soluble compounds from garlic consist of S-allyl cysteine and S-allyl mercaptocysteine. The pharmacokinetic behaviour differs between the groups of compounds. The pharmacokinetic behaviour of S-allyl cysteine was investigated after supplementing it in rats, mice, and dogs. S-allyl cysteine was absorbed quickly in the gastrointestinal tract, and it was detected in the plasma, liver, and kidney. The bioavailability of S-allyl cysteine was above 85% for mice, rats, and dogs (Nagae et al.,1994). The plasma concentration-time curves of 4 primary metabolites of diallyl disulfides after single oral administration to male rats are presented in Figure 2.

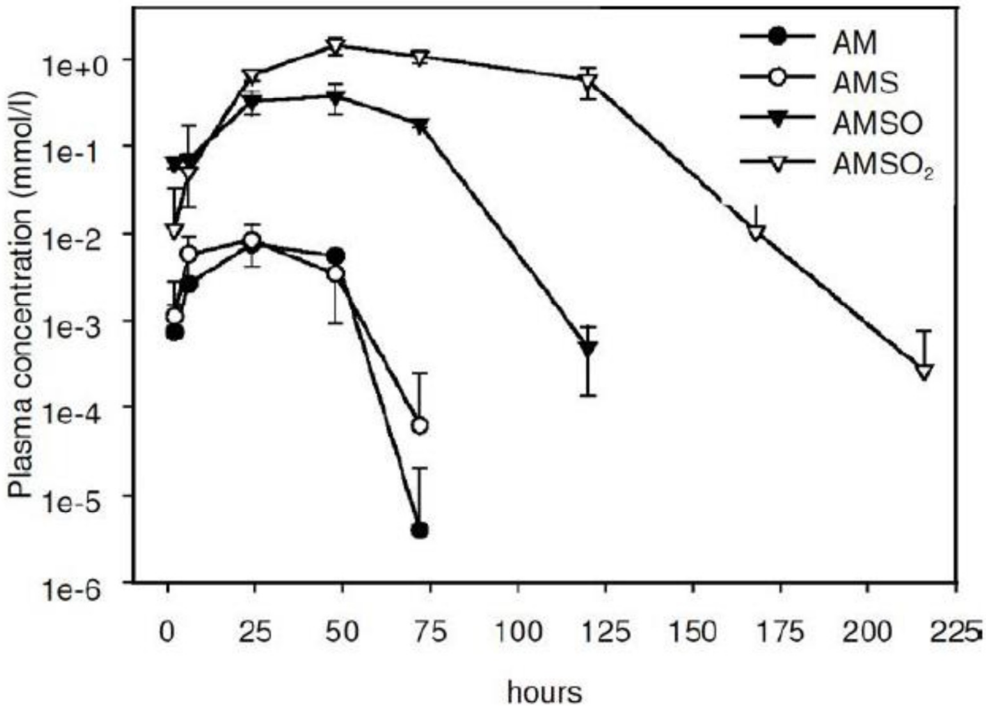

Fig 2. Plasma concentration-time curve of metabolites of DADS after one oral administration of DADS (200 mg kg-1) to male rats (Gao et al., 2013).

3.4. Garlium GEM HC

In the present study, Garlium GEM HC™ (Act 36 of 1947 reg nr: V27580) was used to determine if the product has any tick repelling characteristics. Garlium GEM HC (Garlium) is manufactured by Pancosma SA (Switzerland) at the manufacturing site in Spain. Garlium consists of garlic oil, allyl disulfide, sodium chloride and garlic powder. It is compounded in a powder form and can be supplemented to animal feed. Garlium contains about 36% garlic compounds. Regular garlic contains about 0.7% garlic oil; thus, Garlium is a very concentrated *allium* source. The salt in Garlium acts as both a carrier and preservative agent. Garlium has a shelf life of 12 months, after which the concentration of different sulphur compounds starts to decrease due to the volatility characteristics of sulfur-based compounds.

Garlium was developed as a fly repelling agent that can be supplemented to horses at 4 g/horse/day. Supplementing dried/milled garlic to horses has been used for many years (Horton et al., 1991). The effectiveness of garlic in repelling flies from horses is still not clear. As it is difficult to accurately determine how effective a repelling agent functions against flies, it was decided for this study to evaluate Garlium for its potential tick repelling effect that is more easily measured.

Chapter 4. Material and Methods

4.1. Hypothesis

H0: Supplementation of Garlium to cattle will not have a controlling effect on the attached ticks.

H1: Supplementation of Garlium to cattle will have a controlling effect on the attached ticks.

4.2. Objectives

The objectives of this trial were: 1) To determine the repellent effect of in-feed supplementation of Garlium on the number of ticks attached to beef cattle, and 2) To determine the effect of in-feed supplementation of Garlium on the reproductive success of female ticks collected from beef cattle.

4.3. Approvals

The following regulatory bodies approved the study:

1. DALRRD. Section 20 of the Animal Disease Act 1984 (Act 35 of 1984). Project no: REC 079 – 18. Reference no: 12/11/1/1
2. Animal Ethics Committee, University of Pretoria Veterinary Faculty. Project no: V068–18
3. Research Ethics Committee, University of Pretoria Veterinary Faculty. REC 079 – 18

Animals were cared for according to the guidelines for the Care and Use of Animals in Agriculture, Research and Teaching (1999).

4.4. Experimental design, animals, and diets

The study was conducted from February to July 2020. The experimental study was done in two stages. Stage 1 was conducted at the Experimental Farm of the University of Pretoria using 16 beef feedlot cattle. Stage 2 of the study was conducted at the Ectoparasitology laboratory of the Department of Veterinary Tropical Diseases, Veterinary Faculty, University of Pretoria.

Stage 1

Sixteen male Bonsmara cattle were grouped according to their live weight into two groups of eight animals each. Both groups had a similar average live weight. Each group was then allocated to a treatment regimen. The two treatments were as follows:

1. The control group received a feedlot starter diet with no supplementation of Garlium (CON)
2. The Garlium group received the control diet, supplemented with 2.0 g Garlium/animal/day (GAR)

There were two periods for Stage 1 of the study. Period A was the adaptation period (Day 1 – 21), and Period B (Day 21 – 43) was the assessment period. During Period A, the cattle had time to adjust to the trial facility (single pen for each animal) and the trial feed.

Animals

All Bonsmara bulls were sourced from a Bonsmara stud breeder for the study. All cattle had individual ear tags for identification. The cattle were about seven months old at the start of the trial. The average starting weight of the cattle was 215 kg at the onset of the trial and all cattle were properly examined to ensure that only healthy animals entered the study. Cattle were dipped with Eraditick 125 (Amitraz 12.5% m/v; registration nr G3583 of Act 36, 1947), a short-acting acaricide, seven days before arriving at the trial facility. This was done to remove ticks attached to the animal from field exposure and to use a compound with minimal residual actions that might interfere with the experimental infestations. All animals had a very low tick infestation at the start of the study. During the adaptation period, all ticks that were observed were individually sprayed with Eraditick 125. This acaricide treatment was stopped seven days before the start of Period B. No other external parasite products were used during the study.

Cattle were individually weighed at the start and end of the study. This was to determine if the cattle gained or lost weight during the study, as an indicator of their general health.
Animals were monitored daily. Animals that exhibited any health problems were treated according to the SOP of the Experimental Farm. A veterinarian from the Faculty of Veterinary Science, UP, was on stand-by and assisted when required.

Housing

Sixteen individual brick-paved pens measuring 14 m^2 were used during the trial. Each pen had its own feeding trough, and two pens shared a water trough. Pens were cleaned regularly to prevent the build-up of manure. Water and feeding troughs were cleaned regularly, and all animals had access to shade. A custom-made crush with head and body clamp was situated next to the pens.

To prevent the escape of ticks from the trial facility, a tick perimeter was constructed. A small continuous trench (rain gutters) was placed around the 16 pens and the area housing the crush with body and head clamp. The tick perimeter was out of reach for the cattle. The tick perimeter was filled with a mixture of an acaricide, Deltaforce 100 (Deltamethrin 10% m/v; registration no G4367 of Act 36, 1947) and water (1 litre Deltaforce 100:2222 litres water). The deltamethrin/water mix was replenished every seven days to ensure that the tick perimeter remained effective. Any ticks that might escape would be contained by the tick perimeter.

A pilot study was conducted to determine the long-term effectiveness of the Deltaforce 100/water mixture that was exposed to the weather elements. Deltaforce 100/water mixture was poured into the tick perimeter. Samples were collected on days 1, 4 and 7 of the pilot study. Duplicate samples were stored in amber glass sample bottles, and one sample per day was sent on the sample collection day to AirSep Laboratories (an independent analytical laboratory situated in Johannesburg). Samples were analysed for the deltamethrin concentration. A sample from the deltamethrin and water solution with the lowest deltamethrin level was taken to a commercial beef farm in the Limpopo area. *R. appendiculatus* and *R. microplus* adult ticks were collected from cattle on the farm. Ticks not injured and alive were put into the Deltaforce 100/water mixture. The time period from putting the ticks into the acaricide mixture until they were confirmed dead was determined.

Feeding

Animals were adapted over 21 days (Period A) from a roughage-based diet to the experimental feed (feedlot starter diet). Compositions of the diet are presented in Table 3 and Table 4.

Table 3. Ingredient composition of the feedlot starter diet fed to the cattle during the study.

Ingredient	% DM
Maize	44,77
Molasses syrup	4,26
Soya oilcake	4,74
Rice bran	22,13
Wheat straw	14,68
Lucerne hay	5,01
Cattle Feedlot Premix V1[1]	0,11
Limestone	2,23
Salt	0,62
Urea	0,90
Acid Buf	0,32
Ammonium sulphate	0,23

[1] Cattle Feedlot Premix V1 contain per kg mix: 3 000 000 IU of vitamin A, 25 g Mn, 65 g Zn, 10 g Cu, 0.2 g Co, 0.5 g I, 0.15 g Se, 15 g Mg and 30 g Na-monensin.

Table 4. Nutrient composition of the feedlot starter diet fed to the cattle during the study.

Nutrient	Unit	DM
DM	%	88,08
ME	MJ /kg	11.17
CP	%	14,32
peNDF	%	16,06
ADF	%	14,49
Starch	%	36,39
Ether extract	%	6,44
Ca	%	0,93
P	%	0,61

DM: Dry Matter; ME: Metabolizable energy; CP: Crude Protein; peNDF: effective neutral detergent fibre; ADF: acid detergent fibre; Ca: Calcium; P: Phosphor.

Feed was mixed by a commercial feed mill that specializes in mixing experimental diets for research purposes. Both groups of cattle received the experimental diets during both Period A and Period B to ensure that the animals were adapted to the diets. The Control group received the feedlot starter diet, whereas the Garlium group received the same feedlot starter diet, but with 0.028% Garlium mixed into the diet. This resulted in an intake of 2 grams of Garlium/animal/day if the animal consumed 7 kg (as is) of concentrate/day. The decision to supplement 2 g Garlium/animal/day was based on previous commercial experience with Garlium as well as keeping in mind the financial cost of supplementing Garlium to beef cattle. Animals were fed 8 kg feedlot starter/day to ensure that the feed intake was at least 7 kg with ± 1 kg excess to compensate for spillage of feed.

Green and red plastic pellets (size ± 2 – 3 mm) were also mixed into the respective diets as tracers to distinguish between the two diets. The control diet received 500 g green plastic pellets/ton final feed, whereas the Garlium diet received 500 g red plastic pellets/ton final feed.

Additional *Eragrostis curvula* hay was fed in the afternoon to all cattle that had already consumed the allocated 8 kg concentrate before 17:00. This was to ensure that all animals were well fed and played a role in preventing potential cases of sub-acute rumen acidosis.

Orts were collected and weighed back each morning and recorded to ensure that all animals consumed at least 7 kg of concentrate/day during Period B. Clean water was available *ad libitum.*

4.5 Tick placement and collection

Laboratory-reared *Rhipicephalus appendiculatus* adults and *Rhipicephalus microplus* larvae were sourced from Clinvet International, Bloemfontein, South Africa (www.clinvet.com).

Rhipicephalus appendiculatus

Two custom-made ear bags (fabric tube), one for each ear, were glued to the base of both ears of each animal, using a contact adhesive (Fig 3). The other end was open. Fifty unengorged male and 50 unengorged female *R. appendiculatus* ticks were placed inside each ear bag. The open end of the bag was closed with a strong elastic band (sheep castration band) to prevent the ticks from escaping from the ear bag. The *R. appendiculatus* ticks remained in the ear bag and were allowed to attach to the ear freely.

Fig 3. Placement of ear bags.

Engorged female *R. appendiculatus* ticks were collected and counted on Day 6 to Day 8 after the unengorged adult ticks were placed inside the ear bags. Access was gained by removing the elastic band to collect engorged females that had detached from the animal. Attached male and unengorged female *R. appendiculatus* ticks were counted on Day 8 after tick placement. Engorged females for each animal were stored in a separate ventilated sample bottle.

Rhipicephalus microplus

A rectangular cloth was glued onto the back of each of the cattle (Fig 4). A small space was left open to place an average of 3200 *R. microplus* tick larvae per animal between the cloth and the skin of the cattle. The opening was then closed with glue to prevent the larvae from escaping. This ensured that the larvae attached to the cattle. The cloths were removed from the cattle four days after placement to allow the larvae to move around on the body to find more suitable attachment sites, like the ventral part of the body.

The *R. microplus* larvae remained on the animals and moulted to nymphs and then to adults after about 21 days. Engorged females began to detach from Day 20 after placement of the larvae. Engorged females (dead and alive) were collected from the floor of the enclosure from Day 20 to Day 23 after infestation. Detached females were collected twice a day (09:00 and 16:00). This was to ensure that most of the ticks were collected and to reduce the number of ticks that became damaged by the cattle trampling them. Fully engorged females were also collected from the animal from Day 21 to Day 23 after placement of larvae. Unengorged adult females and attached adult males were counted on 23 days post-infestation. Engorged females for each animal were stored in a separate marked ventilated sample bottle. No larvae and nymphs were collected as they were too small to detect.

Fig 4. Placement of rectangular cloth on back of cattle.

After collection each day, all engorged females from both *R. appendiculatus* and *R. microplus* were taken to the laboratory on the experimental farm. The ticks were then placed in a sieve and gently rinsed with water to clean the ticks from debris. Wet ticks were then placed on dry paper towels to dry and to count them. Records were kept of the number of dead and live engorged females. Live, clean, and dry engorged female ticks were then transported to the Ectoparasitology laboratory at the Faculty of Veterinary Science of the

University of Pretoria, where they were placed into a mini-acaridarium in a temperature-controlled room.

Stage 2

Before the arrival of the ticks at the Ectoparasitology lab, a designated area was identified in the laboratory. Double-sided tape was placed on the perimeter of the designated area to prevent any ticks from escaping. Two custom-built mini-acaridariums were partially filled with a 40% saline solution to ensure relative humidity of approximately 85%. The laboratory temperature was kept constant at 25°C (± 1°C).

On arrival at the laboratory, all engorged female ticks, per species, per animal, were weighed using a laboratory scale (Sartorius) recording in grams (accurate to 5 decimals). The average weight per tick species per animal was calculated. All *R. appendiculatus* ticks were stored in one acaridarium, and all *R. microplus* ticks were held in the other acaridarium. Between one and five 50 ml ventilated plastic sample bottles were used per animal/tick species, evenly distributing the female ticks among them. For the *R. appendiculatus* tick, the average number of ticks per plastic sample bottle was not more than ten ticks per sample bottle.

The number of live engorged *R. microplus* tick females collected was, on average, about 215 per animal. Due to limited space in the acaridarium, the average weight of the total number of *R. microplus* ticks per animal was determined. Forty female *R. microplus* ticks were then collected per animal with an average weight representing the average weight of all the *R. microplus* ticks for a specific animal. These 40 *R. microplus* ticks were then divided into groups of 10 and put in four ventilated 50 ml sample bottles. The sample bottles containing the ticks were then stored in the acaridarium (Figure 5)

Fig 5. Acaridarium containing engorged females in sample bottles

Engorged females remained in the sample bottles until they began to lay eggs. On average, female ticks started to lay eggs nine days after detachment. Once the female ticks began to lay eggs, they were allowed to lay eggs for a minimum of 12 days. Twelve days after the last ticks began laying eggs, the female ticks were removed from the sample bottles. The female ticks were then stored in larger plastic containers and were then put into a freezer (-20°C) to ensure that all female ticks were killed.

The number of eggs in each of the 50 ml sample bottles was then determined as follows: 0.02 g of eggs were weighed on the laboratory scale and then counted using a light microscope. This procedure was repeated three times to determine the average weight of a tick egg. Once this was done, all the eggs per sample bottle were weighed to determine the total number of eggs produced by female ticks per animal.

The eggs then remained in the ventilated 50 ml sample bottle until hatching. Sample bottles containing the eggs were stored inside the mini-acaridariums. From the start of hatching, 21 days were allowed for the eggs to hatch. This was to ensure that enough time was allowed for all the eggs to hatch.

Twenty-one days after the eggs began to hatch, all sample bottles containing the larvae and eggs were stored in the freezer (-20°C) for three days to ensure that all larvae were killed. The number of hatched larvae for each animal was determined as follows: Both eggs and larvae were collected from the sample bottles for an animal and put into one container. All eggs and larvae were then placed into a 50 ml plastic syringe, and a mixture of water and liquid dishwashing soap was added to the eggs and larvae. A 1 ml plastic syringe was connected to the outlet of the 50 ml syringe. The outlet of the 1 ml syringe was closed by hand. Both 50 ml and 1 ml syringes were shaken to allow for an even distribution of eggs and larvae in the 50 ml syringe. A representative sample was then collected from the 50 ml syringe into the 1 ml syringe. The 1 ml sample was then spread onto filter paper. This allowed the water to filter through the filter paper, leaving the eggs and larvae on the filter paper. Both larvae and eggs were counted, and the numbers were used to calculate the hatching percentage. The total number of larvae produced from ticks per animal was then calculated.

4.6. Blood sampling and analyses

Blood samples were collected from the cattle on three different days during the study. The sampling took place at the start of the adaptation period, one day after tick infestation and again 21 days after tick infestation. Blood samples were collected to determine if Garlium supplementation had any significant effect on the primary blood parameters. Blood samples were also collected to determine the presence of any garlic metabolites in the blood.

Cattle were restrained in a crush, and blood was drawn from the jugular vein using 20-gauge BD Vacutainer® needles. 10 ml and 4 ml capacity EDTA BD Vacutainer® K2E tubes were used for blood collection. Blood samples were stored in a cooler box filled with ice and transported immediately after collection to the Veterinary Diagnostic Laboratory at the Faculty of Veterinary Science, University of Pretoria.

Whole blood analyses were conducted on the 4ml blood samples by the Veterinary Diagnostic Laboratory. The 10ml blood samples were centrifuged to separate the blood plasma from the rest of the blood sample.

Four millilitres of plasma was then placed into two cryotubes (2ml / cryotube) and stored at -80°C. These samples are stored for future garlic metabolite analyses.

4.7. Statistical analyses.

Data were analysed using the statistical software Genstat® (VSN International, 2017). Each animal represented an experimental unit. All parameters, except feed intake, were measured on a predetermined day of the trial. Animals were allocated according to live body weight to the treatments to ensure that the average live weight of the two groups was similar at the beginning of the trial. A two-sample t-test method was used to analyse the data. Predicted means were separated using Fisher's protected least significance difference test (LSD) at the 5% level. In all tests, significance was declared at $P \leq 0.05$ and tendencies accepted if $P \leq 0.10$.

Chapter 5. Results and discussion

5.1. Animal production performance

When evaluating a feed supplement, it is customary to determine the effect of the feed additive on the production performances of the animals. Reducing the number of attached ticks but decreasing animal performance is counterproductive and may result in economic losses to the producer. From an animal ethics point of view, it is important to monitor the production performance of the experimental animal to ensure that animals are not negatively affected by the treatment. Although the aim of the trial was not to determine the effect of Garlium on production performance, the production parameters were recorded and are presented in Table 5. Although animals were grouped according to starting live weight, there was nevertheless a numeric difference in starting weights between the two groups. This was due to some variation in starting live weights amongst the 16 cattle. There was very little difference in net live weight gain between the two groups over the trial period, indicating that Garlium supplementation does not affect the production performance of the cattle. The average daily live weight gain of the cattle on 1.42 kg/day for both groups and is well within the norm for beef cattle on a starter diet (Meissner et al., 1995)

Table 5. The effect of daily supplementation of 2 g Garlium to beef cattle on their production parameters.

Parameter	Control	Garlium	P-value
Starting live weight (kg)	219,4	211,5	0,57
End live weight (kg)	279,6	273	0,68
Net live weight gain (kg)	60,2	61,5	0,86
Aver feed intake (As is; kg/d)	8,0	7,9	0,34

As was expected, there was no significant difference with regard to the daily feed intake between the two groups as feed intake was restricted to a maximum of 8 kg/day, which is about 90% of unrestricted feed intake. Therefore, one would not expect a significant difference in growth performance, as was the case in this trial.

It is important to note that, although the Garlium group consumed on average 100 g less feed/day, they were still consuming sufficient Garlium per day (7 kg feed contain ±2 g of Garlium)

5.2 Acaricide efficacy evaluation (Pilot study)

To obtain approval from Section 20 (DALRRD) to proceed with the trial, it was requested that the long-term efficacy of Deltaforce 100 (10% deltamethrin) mixed with water at a ratio of 1: 2222 be tested. This was to ensure that when detached ticks attempted to escape the tick perimeter, they would not survive the exposure within the tick perimeter. Therefore, samples of the Deltaforce 100/water mix were collected on different days and were analysed for the deltamethrin concentration. The results of the analyses are presented in Table 6.

Table 6. The effect of the environment over time on the stability of Deltaforce 100 + water mixture

Sample	Deltamethrin (mg/kg)
Sample A (Day 1)	17.0
Sample B (Day 4)	10.7
Sample C (Day 7)	20.4

As expected, the level of deltamethrin decreased from Day 1 to Day 4. The increase in deltamethrin levels between Day 4 and Day 7 can be explained by the period between Day 4 and 7 characterized by very hot weather. A significant amount of water evaporated from the tick perimeter, resulting in a higher concentration of deltamethrin.

The duplicate samples from Day 4 were split into three sample bottles and taken to a commercial farm to determine if the deltamethrin concentration was sufficient to kill both *R. appendiculatus* and *R. microplus.* Twelve adult *R. appendiculatus* and 12 adult *R. microplus* were collected from beef cattle on a commercial farm. All ticks collected were alive and with no visible damage. Twelve ticks per species were divided into three groups, and each group was placed into one of the three Deltaforce 100/water mixtures. The length of time that elapsed for the ticks to die was recorded. The acaricidal property results are presented in Table 7.

Table 7. The killing effect of the Deltaforce 100/water mixture (Sample B)

Type of tick	Nr of ticks put in mixture	Nr of ticks dead after 2 minutes	% killing rate
R. appendiculatus	12	12	100%
R. microplus	12	12	100%

From the results in Table 7, it is clear that the deltamethrin/water mixture (10.7 mg deltamethrin/kg solution) was still effective in killing all ticks. It can therefore be concluded that the tick perimeter was effective in preventing any ticks from escaping the trial area.

5.3. Tick production performance

The study aimed to determine if supplementing 2 g Garlium/animal/day will reduce the number of ticks attaching and the success rate of their engorgement. Repellency may be assessed under field conditions, where cattle are supplemented with Garlium while grazing on pasture/veld. Natural tick infestation will occur, and the unsupplemented group can be compared to the Garlium supplemented group over time. The benefit of such a trial is that the ticks and cattle are in their natural habitat, and ticks have the choice to attach, remain attached or detach and attempt to find another host. The challenge with a field study is that it is difficult to accurately determine the tick challenges and the number detaching from the host. Conducting a field study would indicate if the Garlium supplementation is beneficial in a commercial environment but won't necessarily provide accurate data. It would also be almost impossible to collect the engorged female ticks that have detached from the host to determine the egg production and hatchability of the eggs.

The alternative is to conduct a controlled study in a controlled environment, as was done in this study. The benefit of a controlled study is that data collection is more accurate, especially on the reproduction parameters of the female ticks. This will allow for a more comprehensive study as more parameters can be measured. The challenge with a controlled study is that the environment where both the cattle and the ticks are kept is unnatural, which will likely affect how both the ticks and the host will react. This is especially relevant for the *R. appendiculatus* ticks used in this study. They were confined to the ear (ear bag used) and did not have the choice of finding another host. They were allowed to detach from the host but remained confined to the ear, increasing the opportunity to re-attach to the same host. This increased the possibility of the ticks re-attaching and completing engorgement. Another potential limitation to the trial design was that only adult *R. appendiculatus* attached to the host and that both the larvae and nymphs were not exposed to the Garlium supplemented host. There were fewer limitations regarding *R. microplus* as the larvae were placed on the host, and after a few days, the larvae were allowed to move around on the host. Therefore, all three life cycle stages were exposed to the Garlium supplementation, and they had the choice to detach from the host.

Supplementing Garlium at 2 g/animal/day had a limited effect on *R. appendiculatus* (Table 8). Based on the total collection of ticks over the collection period, the number of engorged females was reduced by 11.9%, and males were decreased by 12.6%. As with the other *R. appendiculatus* parameters measured, the differences were not statistically significant (P > 0.05), most probably due to the high standard deviation per parameter within a treatment.

Table 8. The effect of daily supplementation of 2 g Garlium to beef cattle on average *Rhipicephalus appendiculatus* parameters per host.

Parameter	Control	Garlium	P-value
No engorged females	20,0 (± 10.95)	17,6 (± 9.80)	0,66
Total weight of engorged females (g)	5,13 (± 3.973)	5,05 (± 4.606)	0,97
Average weight of engorge female (g)	0,243 (± 0.1008)	0,273 (± 0.1681)	0,67
No of non-engorged attached females	7,0 (± 3.81)	8,5 (± 3.34)	0,42
No of live males attached	53,6 (± 13.60)	46,9 (± 13.17)	0,33
Average no of eggs per female	2135 (± 1230)	1756 (± 1597)	0,60
Total eggs per host	44420 (± 37158)	39115 (± 46440)	0,81
Average hatchability of eggs (%)	79,5 (± 14.11)	71,6 (± 21.16)	0,40
No of larvae per host	38182 (± 35780)	30871 (± 40865)	0,71

Limited research has been conducted on the effect of Garlium on tick infestation; therefore, some unpublished studies are referenced and discussed for comparative purposes. Our results are in contrast with results obtained from a commercial trial conducted in South Africa (Hagg, 2017, unpublished). In this commercial trial, 80 Bonsmara cattle were allocated to either a control or three Garlium treatments (0.5 g, 1.5 g and 4 g Garlium/animal/day) over a trial period of 64 days. Cattle grazed on natural veld and were naturally infested with ticks. Tick counts and identification were done on Days 1, 7, 14, 21, 42 and 64. All visible ticks on the left side of the animal body were counted. Supplementing Garlium at 1.5 g/animal/day reduced the number of attached *R. appendiculatus* by 72% ($P < 0.001$) over the 64 days compared to the unsupplemented control treatment. Some of the results of this commercial study of the effect of Garlium on *R. appendiculatus* are presented in Table 9.

Table 9. The effect different inclusion levels of Garlium had over time on the average *Rhipicephalus appendiculatus* load on beef cattle (Hagg, 2017, unpublished).

	Control	0.5 gram	1.5 gram	4.0 gram	P-value
Day 1	2,15	3,9	4,55	2,3	0,532
Day 7	21.70 [a]	18.45 [a]	7.95 [b]	8.80 [b]	< 0.001
Day 14	42.80 [a]	16.53 [b]	8.90 [c]	12.16 [d]	< 0.001
Day 21	39.5 [a]	13.11 [b]	6.9 [c]	9.5 [bc]	< 0.001
Day 42	28.53 [a]	5.11 [b]	6.11 [b]	6.47 [b]	< 0.001
Day 64	16.29 [a]	1.65 [b]	12.22 [a]	4.93 [c]	< 0.001
Day 7 - 64	30.73 [a]	11.59 [b]	8.37 [c]	8.70 [c]	< 0.001

When comparing the results of the commercial beef trial to the current study, it is clear that the environment may play a role in how effective Garlium can repel ticks. Even at 1.5 g Garlium/animal/day, it resulted in a much better tick repelling effect than the results of supplementing 2 g of Garlium/animal/day in the current study. With the commercial trial, the ticks had the option of not attaching or detaching from the host and thus giving an indication of the repellent effect. With the current experimental trial, the ticks were confined to the ears, and therefore it might have limited the tick repelling effect of the Garlium.

It was also observed in the commercial beef study (Hagg, 2017, unpublished) that supplementing 1.5 g Garlium/animal/day reduced the number of *Amblyomma hebraeum* (South African bont tick) by 39% ($P < 0.01$) over the 64-day period. Some of the results of this commercial study of the effect of Garlium on *A. hebraeum* are presented in Table 10.

Table 10. The effect different inclusion levels of Garlium had over time on the *Amblyomma hebraeum* load on beef cattle (Hagg, 2017, unpublished).

	Control	0.5 gram	1.5 gram	4.0 gram	P-value
Day 7	3	2.7	2	1.95	0.467
Day 14	4.5	3.53	3.65	2.37	0.233
Day 21	8.10 a	5.47 ab	5.7 ab	3.5 b	0.056
Day 42	12.94 a	5.94 b	5.94 b	3.94 b	< 0.001
Day 64	15.18 a	6.82 b	8.28 b	3.2 c	< 0.001
Day 7 - 64	8.43 a	4.84 b	5.16 b	3.04 c	< 0.001

Amblyomma hebraeum is a three-host tick, similar to the life cycle of *R. appendiculatus.* Supplementing 4 g of Garlium/animal/day reduced the number of attached *A. hebraeum* by 64% (P< 0.01) over 64 days, indicating that supplementation level of Garlium may play a role in the efficacy of Garlium. Again, in a natural environment, it seems that the repelling effect of Garlium is more noticeable compared to the current experimental study.

Another study with Garlium was conducted by Fitte & Steyl (2020). These researchers evaluated the repelling effect of supplementing 1 g Garlium/springbok/day on *Rhipicephalus evertsi evertsi,* which is a two-host tick where the larvae and nymph stages are completed on the same host, and the adult stage is completed on a different host (Walker et al., 2003). Twenty-four young springbok (*Antidorcas marsupialis*) males were randomly allocated to either a control or Garlium treatment group. Animals were kept in two small enclosures with no natural pasture. Springbok in both groups received the same feed *ad lib,* except for the Garlium group that was supplemented with 1 g Garlium/animal/day via the feed.

As was done in the present study, ear bags were placed on the ears of the springbok, and *R. evertsi evertsi* larvae were placed inside the ear bags. *Rhipicephalus evertsi evertsi* is a 2-host tick of which the larvae and nymphs attach in the ear canal. Ear bags were removed

11 days later, and all ticks were collected. The number of engorged nymphs was reduced by 67% (P = 0.02) when the Garlium treatment group was compared to the control group. The number of attached *R. evertsi evertsi* was decreased by 50% (P = 0.01)

Although this latter study (Fitte & Steyl, 2020) was designed to place larvae deliberately into the ears and confined by ear bags, there was still a significant difference between the control group and Garlium treatment group. Although the *R. evertsi evertsi* were confined to the ear (similar to the present study), the amount of Garlium/kg live weight of the host was much higher. Two gram Garlium supplemented to a 240 kg beef animal = 0.008g Garlium/kg live weight and one gram Garlium for a 25 kg springbok = 0.04 g Garlium/kg live weight. Thus the dosage of Garlium was more than five times that administered to the cattle. This again suggested that increased levels of Garlium supplementation might have a more pronounced tick repelling effect. It can therefore be argued that if Garlium supplementation was increased in the current study, one might also have seen a significant difference between the control and Garlium treatment groups. Increasing the supplementation level of Garlium will increase the cost per animal per day, and that may become uneconomical for the farming industry.

An interesting observation was the increase of 12.7% (Table 8) in the average weight of the engorged females collected from the Garlium supplemented cattle. The number of attached males was also reduced by 12.6% in the Garlium group, similar to the effect of Garlium on the number of engorged females. The average number of eggs produced per female also decreased by 17.8% when Garlium was supplemented. This was unexpected as an increase in body weight of female ticks usually increases the number of eggs produced by the female tick (Chilton, 1992).

The hatchability percentage of the *R. appendiculatus* eggs was only reduced (9.8%) in the Garlium group. However, when calculating the number of larvae produced by the female ticks per host, a 19.1% reduction in larvae was observed.

A study by ValcArcel et al. (2015) in which they supplemented rabbits in-feed with spinosad and then naturally and artificially infested the rabbits with *H. lusitanicum* ticks produced some interesting results. Under field conditions, Spinosad was supplemented in-feed to rabbits during peak infestation and reinfestation periods. Spinosad is an insecticide and acaricide, and is produced during the fermentation process of *Saccharopolyspora spinosa* bacteria. With naturally infested rabbits, the number of ticks collected from treated rabbits was significantly lower than those collected from untreated rabbits. Although this study was not conducted with a garlic-based product, it supports the idea of in-feed supplementation of a natural product to animals, resulting in a significant reduction in the number of attached ticks.

Supplementing Garlium at 2 g/animal/day had a more profound effect on *R. microplus* than on *R. appendiculatus* (Table 11). Engorged females were collected both from the host and from the pen floor. Both intact and dead (trampled) ticks were counted, assuming that the dead ticks on the pen floor were engorged females that had detached from the host and then had been stepped on by the host. The number of engorged female ticks (dead and alive) was numerically reduced by 13.9% in the Garlium treatment. As with the parameters on *R. appendiculatus*, the differences were not statistically different, most probably due to the high standard deviation per parameter within each experimental group. Costa-Junior & Furlong (2011) supplemented a sulfur-garlic-based product (20 g/animal/day) via the feed to beef cattle over five months. *Rhipicephalus microplus* larvae were placed on the cattle twice a week. They observed a significant reduction (67%, $P < 0.05$) in the number of engorged females over the trial period. This response is much higher compared to results obtained in the current study. Possible reasons for the difference in response might be the number of larvae placed on the host (± 8000 twice a week), whereas only ± 3200 larvae were placed once on the host in this study. A cloth bag was not used to confine larvae in the study reported by Costa-Junior & Furlong (2011) on the body; thus, ticks had more opportunity to avoid attaching to the host compared to the larvae that were confined for a few days.

Table 11. The effect of a daily supplementation of 2 g Garlium to beef cattle on average *Rhipicephalus microplus* parameters per host.

Parameter	Control	Garlium	P-value
No of engorged females (dead /alive)	234 (± 78.3)	202 (± 79.4)	0,42
No of engorged females (alive)	173 (± 64.5)	157 (± 71.7)	0,63
No of unengorged females	177 (± 66.5)	121 (± 47.4)	0,07
Total weight of engorged females (alive) (g)	46,1 (± 16.30)	42,3 (± 22.27)	0,71
Average weight of engorged female (alive) (g)	0,111 (± 0.0237)	0,129 (± 0.0193)	0,13
Average nr of eggs per female	2 781 (± 269.4)	2 739 (± 137.6)	0,71
No of eggs per host	652 336 (± 219 519)	550 551 (± 215 274)	0,37
Average hatchability of eggs (%)	30,4 (± 13.00)	21,0 (± 10.81)	0,14
No of larvae per host	194528 (± 114 704)	112201 (± 62 149)	0,10

There was an observed reduction of 31.9% (P = 0.07) in the number of attached unengorged females when the control group was compared to the Garlium supplemented group. This is especially important as the number of engorged females was also reduced. This appears not to be due to decreased number of unengorged females and an increased number of engorged females but a decrease in both numbers. This may be the result of the repelling effect of Garlium since the unengorged females did not fully engorge, despite having sufficient time to do so.

Although Mgocheki (2017) sprayed cattle with an aqueous garlic solution and did not supplement the garlic via the feed, it also supports the potential tick repelling effect of garlic observed in this study. In their study, the 25 cattle were infested with *Rhipicephalus spp. (decoloratus* and *microplus*), both one-host ticks as used in the present study. The number of attached ticks was reduced by more than 90% compared to the negative control group. A higher tick repellent effect can be expected when garlic is applied directly to the ticks, compared to ingestion and digestion of garlic and subsequent the absorption of the garlic metabolites into the blood and to the skin of the host. This also suggests that even with the breakdown of the garlic to different sulfur compounds, it is still effective to either repel ticks or to influence the feeding behaviour of the attached tick.

Interestingly, the average weight of the engorged females (only live female ticks), although numbering fewer in the Treatment group, was higher in the Treatment group than the Control group. This contrasts with the study conducted by Costa-Junior & Furlong (2011), where they observed a reduction (21.6%; $P < 0.05$) in the average weight of the engorged female *R. microplus*. There was no difference in the number of eggs produced per female. This is in line with what Costa-Junior & Furlong (2011) observed. They observed a numerical reduction of 11.7% on the number of eggs produced per engorged female. The average number of eggs produced was around 2700 per female, which is the upper limit of the number of eggs expected to be produced per female (Walker et al., 2003). When combining the number of engorged females and multiplying it by the average number of eggs produced per engorged female, the number of eggs produced per host was reduced by 15.6% in the treatment group.

The hatching of eggs commenced around the beginning of July 2020 in southern Africa, which is the winter period. Although the temperature and humidity in the acaridariums were maintained at 25°C and 85% humidity, the shortening of daylight length could have had a negative effect on the hatchability of the *R. microplus* eggs. Hatchability was between 20 and 30% which is lower than expected. Bharkad et al. (2019) observed a hatchability percentage of 93% and Perinotto et al. (2012) observed a hatchability percentage of 95% for *R. microplus* eggs under laboratory conditions. Average hatchability was decreased by 30.9% when the Garlium supplemented group was compared to the control group. When considering all the parameters to calculate the number of larvae produced per host, Garlium treatment reduced the number of larvae by 42.3 % ($P = 0.10$). Although this is only a tendency,

a 42% reduction in larvae produced will, over time, significantly affect the tick population in a commercial farm setting. Comparing these results with other studies is difficult as little data are available for natural in-feed tick repelling agents. Most studies have been conducted with acaricide products that are applied to the skin of animals.
Spraying and dipping of acaricides onto the host is effective in reducing not only the number of ticks attached but also in reducing the number of eggs produced per female (Kagaruki, 1996; Mehlhorn et al., 2011).

However, as mentioned in the introduction and literature review, there is an increase in acaricide resistance in tick species, like *R. microplus* to amitraz (Davey et al., 2008). Although the results in this study did not indicate a significant response by reducing the number of attached ticks, it is believed that when taking the other two studies (Hagg, 2017, unpublished and Fitte et al., 2020) into consideration, a garlic-based product like Garlium can play a role in controlling the tick infestations on cattle and game. An approach that could be considered is to supplement Garlium to cattle during the tick season and to only dip/spray cattle with an acaricide when the tick numbers on the cattle become too high. This will most probably reduce the number of times one needs to treat the animals with an acaricide, thereby reducing the risk of ticks developing resistance to the acaricide.

It is also important to create conditions where cattle can build resistance to ticks when naturally exposed. This can be achieved by limiting the tick load below the economic threshold and allowing enough ticks to attach and stimulate the host immune system. Supplementing Garlium to cattle may be beneficial in this regard.

Another benefit of supplementing Garlium may be that it could reduce the number of times animals need to be handled to apply acaricides by dipping or spraying. This will reduce the stress and the risk of injury, thus improving production performance.

Supplementing garlic to animals may also have health benefits for ruminants. Githiori et al. (2006) reported nematocidal activity when evaluating garlic supplementation in small ruminants. Rochfort et al. (2008) reported that supplementing garlic may improve the immune system of the ruminant. Cardozo et al. (2005) conducted an *in vitro* study to examine the effect of different plant extracts on the rumen biome content. Supplementing garlic increased total volatile fatty acids, decreased acetic acid and increased both propionic and butyric acid. This resulted in an increase in available energy for the ruminant to be used for both growth and immune support.

Another possible benefit of using Garlium to reduce the number of attached ticks is supplementing animals that are difficult to access or handle. During the summer months, the wildlife industry in southern Africa struggles to control tick numbers. Different applicators are used in the industry to reduce tick infestation, but with limited success (Sonenshine et al., 2006). Supplementing Garlium via the game lick to game may assist in reducing the number of attached ticks, as suggested by Fitte & Steyl, (2020).

5.4. Cattle blood analyses

Toxicity from grazing *Allium* plant species, including onion and garlic, have been associated with haemolytic anaemia in ruminants (Katsogiannou et al., 2018). A case study by Van der Kolk, (2000) where dairy cows consumed a large number of onions (*Allium cepa*) resulted in some cows dying. The remaining sick cows were evaluated and revealed signs of haemolysis, severe haemoglobinuria and the appearance of Heinz bodies.

Heidarpour et al. (2011) supplemented 12 adult female goats with different levels of onions: control, 30% and 60% onions for 60 days. Packed cell volume and haemoglobin concentrations were reduced, suggesting oxidative damage in the destruction of red cells in goats feeding on onions. However, it seems that goats can consume up to 60% onions in their diet without noticeable clinical anaemia.

To ensure that the supplemented Garlium had no adverse effect on the blood, whole blood samples were collected from the cattle on different days during the study.
Whole blood analyses results are presented in Table 12.

Immune parameters in the first blood sample collected, e.g. white blood cells, segmented neutrophils, lymphocytes and platelets in both control and Garlium groups, were above the normal range, suggesting that the animals were stressed on arrival at the beginning of the trial. As the trial progressed, the immune blood parameters reduced to within the normal range for both control and Garlium groups.

Supplementing Garlium to cattle at 2 g/animal/day had no negative effect on the red blood cell count, haemoglobin and haematocrit concentrations in the blood. Therefore, there was no indication that supplementation of Garlium at this level may cause anaemia. Garlium supplementation significantly (P = 0.02) reduced the segmented neutrophil count in beef cattle at the end of the trial. Segmented neutrophils are part of the immune system and act as phagocytes, consuming specific pathogens. Increased levels of segmented neutrophils are an indication of infection or inflammation in the body (Baydar and Dabak, 2014). Segmented neutrophil values were above the normal range in the control group on the last day of the study, indicating that the animals in the control group were possibly immunologically challenged (Bedenicki et al., 2014). Supplementing Garlium significantly reduced the segmented neutrophil counts in the treatment group, indicating that the Garlium supplemented animals were either less challenged immunologically than the control group, or the Garlium group was able to handle the immune stress better. There were tendencies (P = 0.10) of increased haematocrit and lymphocyte counts on the last day of the trial when Garlium was supplemented to beef cattle.

Table 12. The effect of Garlium supplementation on the whole blood analyses of beef cattle

Parameter	Control	Garlium	s.e.d.	P-value	Ref range
Day 1 of trial					
Haemoglobin (g/L)	92,6	100,6	5,81	0,19	80 - 140
Red blood cell count (10ˆ12/L)	6,90	7,76	0,801	0,30	5,5 -9
Haematocrit (L/L)	0,263	0,283	0,0146	0,20	0,24 -0,4
White blood cell count (10ˆ9/L)	16,84	16,89	2,757	0,99	4 - 10
Segmented Neutrophil (10ˆ9/L)	5,61	5,85	1,054	0,82	0,6 - 4
Lymphocyte (10ˆ9/L)	9,83	9,31	1,687	0,76	2,5 -7,5
Platelet count (10ˆ9/L)	754	651	78,1	0,21	200 - 600
Tick placement day					
Haemoglobin (g/L)	105,2	115,0	6,53	0,16	
Red blood cell count (10ˆ12/L)	7,52	8,30	0,802	0,35	
Haematocrit (L/L)	0,291	0,315	0,0174	0,20	
White blood cell count (10ˆ9/L)	14,43	13,33	1,648	0,52	
Segmented Neutrophil (10ˆ9/L)	5,12	4,31	0,869	0,37	
Lymphocyte (10ˆ9/L)	8,17	8,22	1,314	0,97	
Platelet count (10ˆ9/L)	473	489	72,8	0,83	
Last day of trial					
Haemoglobin (g/L)	114,1	124,0	5,98	0,12	
Red blood cell count (10ˆ12/L)	8,13	8,86	0,720	0,33	
Haematocrit (L/L)	0,311	0,340	0,0161	0,10	
White blood cell count (10ˆ9/L)	14,38	13,74	1,500	0,68	
Segmented Neutrophil (10ˆ9/L)	5,77	3,53	0,838	0,02	
Lymphocyte (10ˆ9/L)	7,07	9,21	1,173	0,10	
Platelet count (10ˆ9/L)	384	404	64,3	0,77	

Chapter 6. Conclusion and Critical evaluation

6.1 Conclusion

Supplementing 2 g Garlium/animal/day to beef cattle had a limited effect on the number of ticks attaching. Although not statistically significant, Garlium treatment tended to reduce the number of *R. microplus* larvae produced per animal by 42%, as well as resulting in a 19% reduction in the number of *R. appendiculatus* larvae produced per animal. Garlic has a direct tick repellent effect, as reported by Nchu et al. (2015). Although the Garlium was supplemented via the feed, the garlic metabolites, in very low concentrations in the blood, have some repellent effect that could also impact the egg production of the female ticks. This is supported by the research conducted by Costa-Junior and Furlong (2011).

From a commercial point of view, the decrease in the number of larvae produced per host may positively impact the production performance parameters of the host. If one considers the other two Garlium trials (Hagg, 2017, unpublished and Fitte & Steyl, 2020) and the findings of Costa-Junior and Furlong (2011), there appears to be value in continuing research on Garlium supplementation and its effect as a tick repelling agent. Reducing the number of attached ticks on cattle with in-feed supplementation of Garlium may reduce the frequency of acaricide application. This in turn may result in a lower risk of acaricide resistance of ticks, resulting in a more sustainable tick control program.

It is also encouraging to see that supplementing Garlium to beef cattle at 2 g/animal/day had no significant negative effects on the blood parameters.

In dairy cows, it would be of value to determine if different supplementation levels of Garlium will affect the taste and/or smell of the milk. Ticks, but more importantly, flies, can also have a negative effect on the production of dairy cows. Stable fly infestation can result in average annual animal production losses of 139 kg of milk per lactation for dairy cows, and bodyweight loss for pre-weaned calves, grazing beef cattle, and feeder cattle. (Taylor et al., 2012). Another area of interest is to investigate the possible effect that garlic metabolites in the blood of a ruminant may have on the physiology of ticks feeding on them. *In vitro* tick studies would likely be able to shed some light on this.

6.2 Critical evaluation of the study.

It is believed that if the study had been repeated with a larger group of beef cattle, there might have been a better opportunity to observe significant differences. This is because the standard deviation of the different parameters per experimental unit in this study was high. It is believed that a similar trial, but performed under field conditions, may result in more pronounced differences where repellency may play a larger role in reducing the number of ticks infesting an animal, especially considering the commercial trial data (Hagg, 2017, unpublished).

Another factor that might have influenced the results is the time of the year that the trial was conducted. The trial was initially planned to commence in Oct/Nov 2019, but due to delays from regulatory bodies, the ticks were only placed onto the cattle in March 2020. *Rhipicephalus microplus* peak seasons are during spring and summer (Walker et al., 2003). If the study began in October/November, it would have most likely affected the hatchability of the *R. microplus* in particular, as the days during the hatching period would have been longer, resulting in a higher hatchability percentage.

Adult *R. appendiculatus* are most abundant during the rainy season, larvae in the cooler late summer to winter period after the rains and nymphs in the winter and early spring (Walker et al. 2003). The adult *R. appendiculatus* ticks in this study were placed on the animals during the second part of March, thus within the normal period of peak abundance of these ticks.

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Addendum

Garlium was sourced from the supplier, Pancosma SA, which is based in Switzerland. Garlium is registered at the South African Department of Agriculture, Land Reform and Rural Development (DALRRD) in accordance with Act 36 of 1947: V 27580.

Approval documents attached:

1. DALRRD. Section 20 of the Animal Disease Act 1984 (Act 35 of 1984). Project no: REC 079 – 18. Reference no: 12/11/1/1
2. Animal Ethics Committee, Faculty of Veterinary Science, University of Pretoria: Project no: V068 – 18
3. Research Ethics Committee, Faculty of Veterinary Science, University of Pretoria. REC 079 - 18