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**Effects of dietary *Acacia mearnsii* tannin
additives as methane mitigation agents on the
carcass characteristics of lambs**

By

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Preface

1.1 Project theme

Investigation into the effect of dietary supplemented tannins as methane mitigation agents on the meat quality of Dohne merino lambs

1.2 Project title

Effects of dietary *Acacia mearnsii* tannin additives as methane mitigation agents on the carcass characteristics of lambs

This study forms part of a larger departmental research project on the effects of dietary tannins as methane mitigating supplements on nutrient digestibility, growth, and changes in methane production. In order for this treatment to reduce methane emissions to be accepted it should not have any negative effects on the products of the animals supplemented.

This project was divided into two phases:

The first phase was completed by another student who studied the nutritional aspects such as feed intake, nutrient digestibility, growth and changes in methane emission. This study spans from the time of weaning of the lambs until a slaughter weight of approximately 50kg was reached. The aim of this study was to determine if the supplementation of diets with tannins would significantly decrease the methane emissions of the lambs and if the encapsulation of the tannin would promote higher levels of intake due to the encapsulation process masking the bitter taste associated with tannins. These results were reported in a separate PhD thesis.

The current report involves the second phase of this which focused on the effects of these dietary tannin supplements on the carcass and meat quality of the animals. This study was specifically based on the effects of *Acacia mearnsii* on carcass characteristics of lambs when used as a methane mitigation agent. This explains the novelty of the study as *Acacia mearnsii* tannins have not been as thoroughly studied as other crude tannin supplements. This study is crucial because although this tannin supplement may have potentially beneficial effects on reducing methane emissions there is a need to know how it affects the meat quality. On the day of slaughter, the perirenal fat was dissected and packaged to be frozen at -20 °C. The next day a three-rib cut was obtained from the left side of each carcass, for dissection and carcass composition analysis, meat colour analysis, fat content and fatty acid profile analysis. These samples were all stored at -20 °C until the ethical application was approved for research.

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Declaration

I, Megan Venter, declare that this dissertation submitted in partial fulfilment of the requirements for the degree MSc (Agric) Animal Science: Animal nutrition in the Department of Animal Science at the University of Pretoria is my own work and has not been previously submitted by me for a degree at this or any other tertiary institution.

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Abstract

This study formed part of a larger research project funded by the NRF about methane emissions, digestibility of feed, intake and growth performance of Dohne Merino rams supplemented with encapsulated *Acacia mearnsii* tannins as potential methane mitigating additives. Livestock, particularly ruminants, are responsible for emitting methane which is a concern worldwide as it is one of the greenhouse gases responsible for affecting global warming, and hence the need for the development of methane mitigating dietary additives. Over time, many forms of methane mitigating additives have been developed and tested although, such substances need to adhere to certain rules that stipulate that they cannot be harmful to animals or cause any other negative effects on animal products. Before supplemental methane mitigation strategies can be implemented, their risks and benefits on carcass quality need to be considered. Past research has suggested that the addition of tannins to the diet as a methane mitigation option could potentially have co-benefits in terms of meat quality and carcass characteristics. The present study, however, focused specifically on *Acacia mearnsii* tannin, which has not been researched as other dietary tannin additives have been. Hence, this study investigated the effects of the dietary tannin additive on the meat quality and carcass characteristics of the Dohne Merino sheep, which included initial meat colour and shelf-life preservation over refrigeration time, carcass composition, intramuscular fat percentage as well as long chain fatty acid (LCFA) profile of the carcass (intramuscular and subcutaneous). Forty Dohne Merino ram lambs were selected according to their weaning weight and randomly assigned to one of the four treatment diets, this meant there were 10 animals per treatment group. The diets consisted of high fibre diets made up of total mixed ration (TMR), which were supplemented with different dietary additives. Treatment diet one consisted entirely of TMR (negative control group) (C); diet two consisted of TMR with 75 mg/kg of Rumensin additive (positive control group) is a widely used additive with methane mitigation potential (M); diet three consisted of TMR and crude *Acacia mearnsii* tannin (20 g/kg feed) (CT) and finally diet four consisted of TMR and encapsulated *Acacia mearnsii* tannin (20 g/kg feed) (ECT). Ethics approval for this study was obtained from the University of Pretoria with animal ethics number EC075-17. The sheep were slaughtered at approximately 50kg at a commercial abattoir (Klipleiland Butchery Abattoir in Bronkhorstspruit), where all the samples necessary for this study were collected. After 24 hours in the cold room the three-rib cut samples were dissected from the carcasses. These were used to determine the carcass composition of the animals, initial meat colour, intramuscular fat percentage and fatty acid profiles of the intramuscular and subcutaneous fat tissue. Meat colour coordinate measurements were taken on Day 1 and Day 7 of refrigeration. The tannin treatment groups were not successful at maintaining the red colour coordinate (a^*) of meat, although all

treatments showed a significant reduction in red colour over the seven-day refrigeration period, and the C treatment group had the lowest reduction. There were no significant differences between treatments on Day 1 or Day 7 of a^* colour measurement, thus it seems regardless of treatment the a^* coordinate deteriorated at a similar rate for all treatment groups. Hue angle (H^*) was also calculated on days one and seven. All treatments except for treatment C had a significant change in H^* over the seven-day refrigeration. Treatment M was most significantly different from treatment ECT on Day 1 of measurement and from treatment C on Day 7 of measurement for H^* . The fatty acids (FA) profiles obtained in this study only differed significantly for a few FA across all four treatments groups. The highest concentration of linoleic acid was found in CT treatment animals, which was 16.7% higher than the C treatment group, and lowest in M treatment animals, where there was a 24% difference. A larger range of significant differences were seen in the intramuscular FA of treatment groups, this followed the trend of previous research. C18:2n6t (linoleic acid), C18:3n3 (linolenic acid), C22:6n3 (DHA) and C21:0 (heneicosanoic acid) all showed significant differences ($p < 0.05$) between treatments, with both tannin treatments having the highest concentration and treatment M the lowest concentration of FA. From these findings it seems that tannin treatments had a significant effect at increasing the concentrations of UFA and one SFA found in intramuscular fat. Most significantly C18:3n3, which is considered beneficial for human health, was only 14.6% higher in CT as compared to the C group and 28.9% higher than treatment M which had the lowest concentration. If methane mitigation strategies through dietary tannin additives are successful in lowering the carbon footprint significantly, it can confidently be concluded that *Acacia mearnsii* tannin treatment groups showed significant effects on intramuscular FA composition, specifically for beneficial UFA, although numerically these values were minor and probably negligible in some cases. No significant negative effects on carcass characteristics or FA mean there should be no resistance from consumers for the use of tannins as a dietary methane mitigation additive.

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Abbreviations

ADG - Average daily gain

BH – Biohydrogenation

C – Control

CH₄ - Methane

CLA - Conjugated linoleic acid

CT – Crude tannin

DHA- Docosahexaenoic acid

DM - Dry matter

ECT – Encapsulated crude tannin

EPA - Eicosapentaenoic acid

EU – European Union

FA - Fatty acid

FAO - Food and Agriculture Organization

FCR – Feed conversion ratio

H₂ - Hydrogen

LCFA - Long chain fatty acid

MUFA - Monounsaturated fatty acids

NDF – Neutral detergent fibre

NRF - National Research Foundation

n-3 - Omega-3 fatty acid

n-6 - Omega-6 fatty acid

PEG - Polyethylene glycol

SFA - Saturated fatty acids

UFA – Unsaturated fatty acid

Chapter 1

1.1 General introduction and background

Greenhouse gas emissions from livestock have in the past been described as one of the most significant sources of greenhouse gases estimated at 18% of the world's emissions (Steinfeld *et al.*, 2006). In the last decade further research has determined that this value was overestimated, and the real value is closer to 5-10% (Meissner *et al.*, 2012). Global warming is still escalating and thus there is a need to find solutions to reduce greenhouse gas emissions. Reducing livestock emissions therefore appears necessary if we are to reach the global mitigation target of below 2°C temperature increment in the next century (Okuah, O. *et al.*, 2019). Guyader *et al.* (2014) states that between 2.3–10.8% of feed energy is lost to emissions by ruminant animals depending on the type of diet. Along with the effect on the environment, methane emissions from ruminants specifically is the result of inefficient utilization of feed energy (Eckard *et al.*, 2010). Thus, as described by Eckard *et al.* (2010) decreasing these emissions will have a positive effect on increasing energy utilisation efficiency for production purposes while lowering the effect on global warming.

Nutritional methods of methane mitigation seem to be the best short-term option, thus in the last decade there has been a movement towards using additives to animal feeds to decrease methane emissions. Cottle *et al.* (2011) described the methane mitigation options to include changing the microbial population in the rumen or the gastrointestinal tract environment by inhibiting methanogens through the use of antibiotics, probiotics, acids or oils. The microbial population can be changed by inoculating the rumen with additives that increase the level of acetogenic species or feeding highly digestible feeds to encourage an increase in propionate population, which causes a decrease in methanogenesis (Cottle *et al.*, 2011). It has been found that most current methods of methane mitigation such as the use of ionophores, acids, oils or probiotics are expensive and may have effects that waiver after long-term use (Woodward *et al.*, 2006). O'Kelly *et al.* (1992) stated that there are varying results when looking at the effectiveness of the most common ionophore, Rumensin, when feeding high forage-based diets as compared to concentrate-based diets. There may also be room for concern in antibiotics accumulating in animal products over long-term use as the dose is increased to overcome the animal's adaption to the antibiotic (O'Kelly *et al.*, 1992).

Tannins are phenolic compounds found in fibrous shrubs and unpalatable plants, and act as a defence mechanism for the plant by deterring herbivores (Cooper, 2001). There have been studies that reported antioxidant effects of tannin-containing plants such as carob pulp,

acacia leaves or sulla fresh herbage when ingested by animals (Priolo *et al.*, 2007). Due to the large abundance of tanniferous plants it has been hypothesised that they could become a more cost-effective approach to methane mitigation.

Before any interventions for methane mitigation can be implemented into commercial practices there needs to be certainty that there will be no negative effects on animal products. Thus, this study was taken up to determine what, if any, effects will be relayed to meat products of animals given Acacia tannin additives. It is hypothesized that the tannin dietary additive in this study will have a beneficial effect at improving the FA profile of the Dohne Merino lambs and possibly improve meat colour stability.

1.2 Motivation

Agriculture is responsible for between 5-10% of the global anthropogenic greenhouse gas emissions (Grobler *et al.*, 2014). Grobler *et al.* (2014) also continues to emphasise that livestock only contributes about 65% of agricultural greenhouse gas emissions, and enteric fermentation accounts for about 90% of this. Due to livestock contributing the largest percentage to greenhouse gas emissions in the agricultural sector there has been movement towards methane mitigation strategies for livestock. The most developed and easily implemented forms of mitigation are the nutritional strategies, which have been considered promising movements towards lower methane emissions from livestock. Although according to Grobler *et al.* (2014), most of the methods currently being used can be effective through increased feed conversion ratio (monensin) or through the manipulation of the rumen environment with supplementation of plant or animal oils, probiotics or organic acids, the effects of these methods are still somewhat unclear as the lowering methane levels could be due to lower fibre digestion or due to the inhibition of methanogenic bacteria. For this reason, any methane mitigation strategies not only need to be effective but also should not cause any negative effects on animal performance and animal products.

In this study the effects of tannins as methane mitigation strategies on the intrinsic carcass characteristics will be investigated. The colour of red meat is one of the biggest influences on how consumers perceive and choose products (Kerry *et al.*, 2000) therefore this is an important meat quality attribute that needs to be kept consistent or improved. In the past there has been evidence that tannins may help meat stay redder for longer due to antioxidant interactions in the meat inferred by tannins therefore extending its shelf life. Tannins have also been investigated for their ability to alter the fatty acid profile of ruminants by including increased unsaturated fats which will contribute to producing a potentially healthier meat product (Luciano *et al.*, 2011).

1.3 Aim

The aim of this study was to determine if the dietary supplementation of tannins as a methane mitigation agent to lambs has any significant effects on carcass characteristics and meat quality.

1.4 Specific objectives

Objectives:

1. To determine the effect of supplementation of *Acacia mearnsii* tannin on carcass composition of the rib cut samples of Dhone Merino lambs.
2. To determine whether the addition of *Acacia mearnsii* tannins will have an effect on meat colour of rib cut samples of Dhone Merino lambs.
3. To determine whether the addition of *Acacia mearnsii* tannins will have an effect on the levels of fatty acids found in subcutaneous as well as intramuscular fat of Dhone Merino lambs.

1.5 Hypotheses

- H01: The addition of *Acacia mearnsii* tannins to the diets of Dhone Merino sheep has no significant effect on meat colour.
- HA1: The addition of *Acacia mearnsii* tannins to the diets of Dhone Merino sheep has a significant effect on meat colour.
- H02: The addition of *Acacia mearnsii* tannins to the diets of Dhone Merino sheep has no significant effect on carcass composition.
- HA2: The addition of *Acacia mearnsii* tannins to the diets of Dhone Merino sheep has a significant effect on carcass composition.
- H03: The addition of *Acacia mearnsii* tannins to the diets of Dhone Merino sheep has no significant effect on medium and long chain fatty acid profile of subcutaneous or intramuscular fat.
- HA3: The addition of *Acacia mearnsii* tannins to the diets of Dhone Merino sheep has a significant effect on medium and long chain fatty acid profile of subcutaneous or intramuscular fat.

Chapter 2

Literature review

2.1 Introduction

As of February 2020, the global population is already sitting at over 7.8 billion people (<https://www.worldometers.info/world-population/>. Accessed on the 26th of February 2020). The livestock industry is under pressure to almost double its production by 2050 (Ilea, 2009) to keep up with population growth. The need for increased livestock production is critical to be able to meet higher food demands and deal with food insecurity thus the movement toward more intensive farming structures has been necessary. The Cambridge dictionary describes intensive livestock farming as “increasing production through increased feed utilisation and efficient land management “although this leads to increased methane production as there is an increase in the number of animals farmed and animal welfare is more difficult to uphold.

Global warming and climate change are real threats that the world is only now starting to take seriously and slowly implementation of strategies to reduce global warming are being considered (Weber, 2006), and movement towards more environmentally friendly approaches to farming practices may become necessary. The contribution of anthropogenic methane emission from animals, comprising of 26% worldwide, is the result of enteric methane production by ruminants such as cattle, sheep and goats (U.S. EPA ,2012). Nutritional methods of methane emission mitigation from ruminants are without doubt the most developed and ready to be practically applied in the field as compared to other approaches (Martin *et al.*, 2010). Most livestock in developing countries are farmed in extensive farming practices and are fed mainly low-quality high roughage diets with little or no concentrate supplementation, which ultimately promotes more methanogenesis. In extensive farming practices it will be difficult to implement methane mitigation strategies that involve frequent handling of animals thus nutritional intervention could be the most viable option, and with appropriate nutritional and management practices it may be possible to achieve significant decreases in methane emissions (Boadi *et al.*, 2004).

However, most interventions that control methane production in ruminants are expensive and in some cases can lead to loss of farm profits (Thamo *et al.*, 2013). Ionophores are considered as established and readily used medications to inhibit enteric methane production, but the feeding of antibiotics has been banned by the European Union since 2006, and along with increased consumer concern over the use of antibiotics there has been growing interest in alternative supplementation as feasible alternatives for manipulating rumen fermentation (Calsamiglia *et al.*, 2007; Benchaar *et al.*, 2008; Hart *et al.*, 2008). Further examples of methane mitigation options are the use of probiotics, vaccines, grains, fats, oils, acids or salts

to change either the rumen microbial environment or the gastrointestinal tract environment but this can lead to decreased utilization of low quality feeds (Cottle *et al.*, 2011). Farmers may not be willing to adopt new products that are effective in reducing methane emissions if it will negatively affect the production of their animals or if it is not cost-effective and so more research is needed to find a middle ground.

In this review some of the promising methane mitigation strategies are discussed and the justification for the use of natural products is provided. Along with the positive results from the use of current methods of methane mitigation that have been well documented, there are few articles outlining the effects of these mitigating agents on meat characteristics. Of these, fewer are specific to Acacia tannins which are specific to countries in the southern hemisphere where Acacia species are widely distributed (Impson *et al.*, 2008). There is a need to bridge the gap in information on the impact of Acacia tannins as dietary supplements on meat characteristics.

In a few studies done on tannin supplemented diets and the effect on meat characteristics it was found that they may have a positive effect on meat redness, which ultimately leads to an extension in shelf life of red meat products (Luciano *et al.*, 2011; Gesteira *et al.* 2018). This is due to the resistance of oxidation in the meat due to tannins having antioxidant capabilities (Luciano *et al.*, 2011). There is also research done by the same authors that suggests that due to changes in biohydrogenation in the rumen brought on by tannins, the fatty acid profile of meat could be altered (Vasta *et al.*, 2009; Gesteira *et al.*, 2018)

Recent research has also brought about information on the other physical benefits tannins could have on ruminants. Internal and external stressors have negative effects on animal production and thus eliminating or controlling the stressors will ultimately lead to improved production. Heat stress is one such stressor that is common in the southern hemisphere and causes negative effects on meat quality over extended exposure (Marai *et al.*, 2007). The use of tannins as supplements could have the potential to decrease the effects of heat stress on the meat quality as well as the growth of the animals (Liu *et al.*, 2016). Another common stressor found in the livestock industry is internal parasites and in recent research it has been hypothesized that tannins could be used as natural anthelmintics, especially for the treatment of nematode infection (Zhong *et al.*, 2015). These are examples of how useful tannins are in livestock production when correctly used as feed additives and why further research is necessary.

2.2 Methane mitigation strategies in livestock

2.2.1 Methane formation from ruminal degradation

Hydrogen gas (H₂) is formed through microbial fermentation of feed and as a result is used by methanogenic archaea as an energy source. Even though H₂-utilising methanogens only make up a minor population in terms of rumen microbial biomass they form a leading role in rumen fermentation (Hungate, 1967). This means that they are essential for optimal rumen function and animal nutrition (Janssen *et al.*, 2008). Efficient H₂ removal is important in order to improve the rate of rumen fermentation and to eliminate the repressive effect of H₂ on the microbial degradation of feed material (Wolin, 1979; McAllister *et al.*, 2008). The rate of methane formation is quantified by the rate at which H₂ passes through the rumen pool (Janssen, 2010). The methane produced by methanogenic archaea in the rumen is released through eructation by the ruminant to the atmosphere and it is known that individual cattle produce between 150–420 L of methane per day and sheep between 25–55 L per day (Czerkawski, 1969; Holter *et al.*, 1992; McAllister *et al.*, 1996). This methane from ruminants collectively accounts for about a quarter of all anthropogenic methane emissions worldwide and has been implicated as one of the drivers of global warming and climate change (Wuebbles *et al.*, 2002). Methane formation also represents feed energy that is wasted during digestion and degradation in the ruminant animal. Blaxter (1967), Johnson *et al.* (1995) and Guyader *et al.* (2014) state that between 2.3–10.8% of feed energy is lost to emissions by ruminant animals depending on diet type. Development of methane mitigation strategies to reduce anthropogenic emissions from ruminants has been at the height of scientific and consumer discussion (Iqbal *et al.*, 2008; Leslie *et al.*, 2008; Martin *et al.*, 2010).

2.2.2 The key mitigation agents

The solution to the problem of wasted feed energy as well as methane emissions can be solved by implementing mitigation strategies. These include changes in diet, direct rumen manipulation and systematic changes (Hopkins *et al.*, 2007). Hopkins *et al.* (2007) defined systematic changes to take into consideration breed, livestock numbers and level of intensiveness of production. There is an inverse relationship between production intensity and methane emissions, but intensive farming may be less desirable in terms of other environmental implications. Although changes in terms of farm operations are more desirable in the long term, dietary mitigation strategies are more likely to make the biggest short term improvement (Lascano *et al.*, 2010).

Manipulation of nutrients in the diet by including more concentrates reduces the protozoa population in the rumen through faster passage rate and by causing the rumen pH to decline. This alters the acetate: propionate ratio to favour propionate production and will ultimately decrease the amount of methane produced per unit of feed intake (Hopkins *et al.*, 2007). Organic acids have been considered safe to use as methane mitigation agents by acting as a hydrogen sink and competing with the methanogenic archaea. These acids produce no noticeable abnormal residues in meat (Castillo *et al.*, 2004) but their biggest downfall is that they are expensive, specifically malate (Martin, 1998; Carro *et al.*, 2002). Another concern with the use of organic acids is that they can cause pH problems in the rumen that will lead to increased levels of butyrate and propionate. This problem can be overcome through encapsulation of the organic acid (Wallace *et al.*, 2005).

Manipulation of rumen environment through supplementation of vegetable and animal oils is one of the most researched mitigation methods. A positive attribute to the use of essential oils is that they have the ability to portray antimicrobial properties (Benchaar *et al.*, 2008). In some studies, it was found that 27% of methane production was decreased with only 500 mg/d of fish or sunflower oil supplementation, although after an extended period the effectiveness wavered (Woodward *et al.*, 2006). It is still unclear whether the effects of oil supplementation on methane production is due to the inhibition of microflora, reduced intake or the reduction of fibre digestion (Lascano *et al.*, 2010). Furthermore, most studies on essential oils have been conducted *in vitro* and further investigation into the *in vivo* effects is needed to determine the mode of action of the various essential oils, their compounds and the concentrations that favourably alter ruminal fermentation (Benchaar *et al.*, 2008). According to a study done by Bessa *et al.* (2005) when lipids were supplemented there were no differences in meat quality parameters such as tenderness and consumer preference as compared to control treatments. In previous trials the same authors concluded that dietary

lipid supplementation had minor effects on consumer preference of lamb meat (Santos-Silva *et al.*, 2003; Santos-Silva *et al.*, 2004). Bessa *et al.* (2007) also determined that when lambs were supplemented with linseed oil and sunflower oil they showed increased levels of biohydrogenation intermediates that lead to increased levels of conjugated linoleic acid (CLA) found in muscle, and these results were supported by Ivan *et al.* (2001). Another study reported that using oils high in linoleic acid can cause repartition of fats on the carcass leading to a more evenly distributed subcutaneous and intramuscular fat profile, and according to Marinova *et al.* (2001) this increase in carcass quality of goats specifically was due to their normally lower level of intramuscular fat.

Ionophores such as monensin which are included in products such as Rumensin are antibiotics that have been used for a long time in animal production that carry a co-benefit of reducing methane in addition to them also being growth promotants (Castillo *et al.*, 2004). Their modes of action include increasing conversion efficiency of feed thus decreasing CH₄ production as well as reducing CH₄ through their direct effect on rumen fermentation (Lascano *et al.*, 2010). The effect of ionophores is more noticeable when feeding concentrate-based diets. Although dry matter intake may be decreased with supplementation, the feed efficiency is increased (Raun *et al.*, 1976; Goodrich *et al.*, 1984; van Nevel *et al.*, 1996.). However, firstly, the CH₄ mitigating effect of monensin has not been maintained for repeated applications (Omar, 2004) as some studies have shown that ionophore-induced enteric CH₄ suppression is temporary (Johnson *et al.* 1994; McCaughey *et al.* 1997). Secondly, the authors hypothesised that the short-term effectiveness of ionophores is due to the bacteria becoming adapted to the dosage within 45 days. Although, increasing the dosage can sustain the effect it also causes concern in the long term as these antibiotics could accumulate in the animal products (O'Kelly *et al.*, 1992). Thirdly, less data is available for the use of ionophores in grazing ruminants consuming high forage diets (O'Kelly *et al.*, 1992). In the few studies documented, it was found that there is a large range at which ionophores can affect methane emissions (0-76%), and their effectiveness is subject to dosage and type of ionophore as well as the diet fed to the animal (van Nevel *et al.*, 1996).

Probiotics have also frequently been used as alternative methods of methane mitigation. Lascano *et al.* (2010) state that probiotics were developed to directly influence rumen fermentation, but many studies have found inconsistent results in their efficiency. They have to be supplemented daily, which is inconvenient and their effects on CH₄ reduction has been limited (Lascano *et al.*, 2010). Live yeast cultures are the most readily used probiotic in ruminant production, but they have not been substantially tested for their effect on CH₄ production (Chaucheyras-Durand *et al.*, 2008). The few reports that are available on yeast strains selected for effects other than CH₄ reduction, and the results were contradictory with

either increases, decreases or no effects reported (Doreau *et al.*, 1998; Chaucheyras-Durand *et al.*, 2008). The use of probiotics, comprising of acetogens or live yeasts, remains a potentially interesting option for methane mitigation. Although, results have been either unsatisfactory, inconclusive, or have yet to be confirmed through *in vivo* experimentation, further research is needed to confirm their anticipated beneficial properties (Martin *et al.*, 2010).

Secondary metabolites from certain plants have also been used in an attempt to mitigate methane production and compounds such as condensed tannins (CT) could be toxic to methanogenic microbes (Wallace *et al.*, 2004). Results have been variable as the methanogenic effects produced are not the same for all condensed tannins. The extent of the methanogenic effect depends on the concentration, molecular weight and structure of the condensed tannin being fed (Martin *et al.*, 2010).

2.3 Tannins are potentially important for ruminant nutrition

2.3.1 Changing views on tannins

In the past tannins were classified as anti-nutritional products due to the negative effects of tannins on nutrient utilization and feed intake (Morales *et al.*, 2013). In plants the primary role they play is self-defence to deter herbivores, bacteria or fungi from ingesting the plant (Šliviňsk *et al.*, 2002). In the last decades, the perception around them has changed to include them as beneficial compounds as they can be used to alter rumen environments and microbial activity favourably in addition to their potential antioxidant effect. Tannins are phenolic compounds found in many tropical plants and they have a potential effect on enteric methane emissions in ruminants due to their antioxidant capabilities (Goel *et al.*, 2012). Due to the abundance of forages containing secondary metabolites such as condensed or hydrolysed tannins, an increased interest in the use of these plants as a natural feed supplement to mitigate methane has been considered. In their abundance the tannin-containing trees or shrubs do not compete with human food production (Makkar, 2003) making them even more attractive as methane mitigating options.

The effect of tannins on the rumen environment depends on the chemical composition, concentration of tannins and the type of tannin being used (Patra *et al.*, 2011). Although hydrolysable tannins could be more effective in terms of methane mitigation in addition to not compromising feed digestibility, they could have a possible toxic effect in the animals while CT have a lower risk of causing toxicity (Waghorn, 2008). Hydrolysable tannins tend to decrease methane emissions more than CT through the direct effect of inhibiting the growth and activity of methanogens (Goel *et al.*, 2012). The inhibition of microbial activity has been attributed to ability of CT to bind to nutrients and the direct interaction with the microbial cell wall which renders nutrients unavailable for microorganisms to use (Smith *et al.*, 2005; Patra *et al.*, 2011). Tannins also bind to proteins in the rumen and reduce their breakdown, essentially converting them to rumen protected proteins (Woodward *et al.*, 2001). Several studies have found that CT also demonstrate their mode of action on animals through possible suppression of certain enzymes in the digestive tract and by inhibiting bacterial growth and metabolism caused by the binding of proteins to tannins (Vasta *et al.*, 2008). Overall improvements in animal performance have been seen after increased dietary protein in the small intestine binds to CT in the rumen and later dissociates in the abomasum, which ultimately leads to increased protein digestibility in the small intestine (Naumann *et al.*, 2013).

Carulla *et al.* (2005) saw a reduction of approximately 12% in methane emissions of cattle when 2.5% condensed tannin from *Acacia mearnsii* was supplemented, although, it was thought to be due to a 5% reduction in the total-tract neutral detergent fibre (NDF) digestion.

Negative effects brought on by supplementing tannin-containing forage can be neutralized by adding a binding agent called polyethylene glycol (PEG) (Priolo *et al.*, 2007). Therefore, Priolo *et al.* (2007) suggested adding a smaller amount of the tannin additive in order to avoid the negative effects of CT on fibre digestibility while still reaping the benefits of methane mitigation. PEG has been used in addition to tannin additives for increased efficiency in utilisation of diets high in tannin additives by binding to the tannins and diminishing their negative effects on digestion (Priolo *et al.*, 2007). However, the addition of PEG increases the bioavailability of nutrients in the feed while also decreasing the efficiency of their utilisation for microbial protein synthesis (Makkar, 2003). Makkar (2003) thought this was due to spillage or uncoupling of released energy. Further research is necessary to determine the exact concentration range of tannins to be included in the diets of ruminants due to the variability of response to tannin depending on the molecular weight and degree of complexity of different types of tannins.

Carulla *et al.* (2005) supplemented the sheep diet with 2.5% of condensed *Acacia mearnsii* tannins that resulted in decreased digestibility of organic matter (OM), crude protein (CP), NDF, and acid detergent fibre (ADF). Beauchemin *et al.* (2007) reported that feeding 1 to 2% of dietary DM as CT from Quebracho trees depressed apparent total-tract digestibility of CP and acid detergent insoluble nitrogen (ADIN) in beef cattle, indicating that post ruminal availability of amino acids may have been reduced. A more recent study by Adejoro *et al.* (2019) determined that the use of dietary encapsulated *Acacia mearnsii* tannins were found to decrease methane emissions while CP and NDF digestibility were higher than when the CT extract was fed. The reason for encapsulation was initially to hide the bitter taste of tannins but it was later discovered that there are other benefits such as slower release rate (later degradation) in the mouth and rumen, and this is beneficial as it lowers the binding of tannins to proteins before the rumen (Adejoro *et al.*, 2019). Gesteria *et al.* (2018) also suggested that tannins ability to protect excessive protein degradation in the rumen results in lower methane emissions (Makkar, 2003 ; Vasta *et al.*, 2011) while disturbing some ruminal organism activity (Caçõo *et al.*, 2012) and could cause improve protein utilization by the animal.

Eckard *et al.* (2010) reported that higher faecal nitrogen (FN) and lower urinary Nitrogen (UN) is associated with feeding tannin containing diets. Similar results were recorded by Perez-Maldonado *et al.* (1996) when they demonstrated that FN increased by 14% when goats and sheep were given diets containing two different types of tannin-containing legumes (*D. intortum* and *C. calothyrsus*) at a concentration of 300 g/kg DM. Eckard *et al.* (2010) goes on to explain that this shift will have a positive effect on the long-term environmental welfare due to FN being in its organic form while UN is most commonly in the form of urea. Urea has the potential to be hydrolysed to form ammonia and nitrified to form nitrate, which is a more potent and undesirable greenhouse gas. Nitrate also leaches into groundwater as a pollutant

or could be converted to nitrous oxide, which is another harmful greenhouse gas (Eckard *et al.*, 2010). Thus, it is expected that the condensed tannin protein complexes found in the faeces may help improve manure quality. This statement is supported by the studies done by Śliwiński *et al.* 2004 who determined that the effects of tannins in this way could have the potential to reduce the amount of easily volatile UN as well as maintaining their protein-binding activity during manure storage.

2.3.2 Tannins as natural antioxidants

Thermal changes as a result of global warming have an impact on pasture growth and quality. The increase in temperature is implicated in changes in animal behaviour, immunological function and physiological function (Chauhan *et al.*, 2014). Chauhan *et al.* (2014) explains that the oxidative state of an animal is very important in terms of health thus the balance of the cellular oxidants and antioxidants ratio is crucial to ensure the animal does not experience oxidative stress that will damage cells, proteins and DNA. There is a growing consciousness of consumers to favour antioxidant-rich foods with an association to lower risk of autoimmune thyroid diseases with a higher selenium status, improved male and female reproductive health and antiviral effects in humans (Rayman, 2012). The use of tannins as an additive may provide opportunities for the livestock industry to deliver production of antioxidant rich milk and meat products.

There have been several studies based around tannin consumption in animals and their potentially beneficial effects on humans (Okuda, 2005) in terms of their antioxidant (Amarowicz *et al.*, 2000; Smirnova *et al.*, 2009), bactericidal (Akiyama *et al.*, 2001; Bansa *et al.*, 2007), insecticidal (Ayres *et al.*, 1997; Barbehenn *et al.*, 2009) as well as their nematocidal (Hoste *et al.*, 2006; Alonso-Díaz *et al.*, 2008) properties. All of these properties are desirable and thus further investigation of co-benefits of dietary tannins is important.

Although many articles describe the useful effects of dietary tannins on methane mitigation potential and possible production benefits, there has been minimal research done on the effects that tannins have on the meat characteristics especially with regards to Acacia tannins. The available literature has described that phenolic compounds such as tannins have had positive effects by improving the oxidative sustainability of meat from different livestock.

Dietary tannins are considered as antioxidants, and this could result in a lower oxidation level of meat during storage. The antioxidant capability of polyphenols such as tannins has not fully been studied but, in some cases, the number of hydroxyl groups of polyphenols has been positively related to their degree of polymerisation, thus tannins could be potentially

important antioxidants (Luciano *et al.*, 2011). Besides the use of tannins in *in vivo* studies it was also found that the direct supplementation of polyphenol-containing plant extracts to meat has delayed metmyoglobin formation. Metmyoglobin is formed through the oxidative degeneration of polyunsaturated fats in the muscle, which causes the meat colour to turn from bright red to brown (Faustman *et al.* 2010). It has been thought that increased levels of endogenous α -tocopherol concentrations are responsible for keeping the colour of red meat stable (McDowell *et al.* 1996; Coronado *et al.* 2002) and antioxidants play a major role in how readily the substrate is available in the meat. This would explain why polyphenols may have an inhibitory effect on metmyoglobin formation. Meat colour has been described as the single most important sensory trait affecting consumer purchasing decisions of red meat products due to a bright red colour being associated with “freshness” by consumers (Liu *et al.*, 1995). The haeme-containing proteins, haemoglobin and myoglobin, which are the main pigments found in muscle tissue associated with colour, rapidly start to oxidise from slaughter and cause the colour of the meat to change from red to brown (Chauhan *et al.*, 2014). This change in colour is not only visually unappealing to consumers but also represents how long the meat has been on the shelf thus meat that is no longer red but brownish is perceived to be older and no longer fresh.

The effects of tannin-containing feeds on small ruminant meat colour is thought to be due to a reduction in microbial biosynthesis of vitamin B12 which is a precursor for the synthesis of haem pigments (Liu *et al.*, 2016). Other hypotheses are that the resistance of meat to oxidation could also be due to the ability of tannins to modify muscle FA composition (Luciano *et al.*, 2011). Due to this, it might be difficult to determine whether the effects on meat preservation are due to direct or indirect effects, and additional research is needed to determine the mode of action.

2.4 Tannins effect on meat oxidation and fatty acid composition

2.4.1 Meat colour importance to consumers

Meat colour is the most important factor affecting customer preference in meat products because redness symbolises freshness (Kerry *et al.*, 2000). Many dietary strategies have been tested to determine what additives can improve shelf life, one being that fresh herbage could increase shelf-life due to the presence of natural antioxidants (Wood *et al.*, 1997). Meat colour is measured using the following coordinates: L* (lightness), a* (redness) and b* (yellowness). In a study conducted by Luciano *et al.* (2011) meat colour coordinates were compared between meat samples of different treatment diets. The authors documented that a* in the tannin and herbage fed meat samples were higher than that of concentrate-fed animals over 14 days post-mortem storage. The b* coordinate was higher in the concentrate-fed sheep while the results of other test diets stayed similar to measurements at slaughter. These results advocate that tannins have a significant effect on extending the shelf-life in red meat animals. Gesteira *et al.* (2018) concluded that the presence of CT in the diet resulted in an antioxidant effect on the meat products and reduced lipid oxidation between 25- and 50-days post-mortem. Luciano *et al.* (2011) also observed results similar to this in a study where the colour stability and oxidation of lamb meat products were tested after tannin-containing diets were given. The authors concluded that the lamb meat had increased antioxidant activity due to an increase in the phenol concentration in muscle. This contributed to an increased ability to eradicate radicals while increasing the resistance of myoglobin to oxidize to metmyoglobin, which results in meat colour changing from red to a brown colour. Although there has been some research to support these hypotheses as discussed above there is still limited research on this topic and thus further studies are needed in order to have more comparison in results.

2.4.2 Role of biohydrogenation on meat colour

Due to the action of biohydrogenation (BH) of UFA in the rumen, fats of ruminant animals tend to be more saturated than those of non-ruminant animals (Jenkins *et al.*, 2008). BH is a series of enzymatic reactions performed by ruminal microbes that adds hydrogen to the double bonds of unsaturated fatty acids resulting in saturated fats (Gesteira *et al.* 2018). During ruminal BH, C18:2 n6 (linoleic acid) and C18:3 n3 (linolenic acid) are steadily hydrogenated to form saturated C18:0 (stearic acid) (Vasta *et al.*, 2009 b). Some BH intermediates are CLA and trans11 C18:1, which are formed by cellulolytic bacteria such as *Butyrivibrio fibrisolvens* (Kepler *et al.*, 1967). This saturation of fats in meat makes it less healthy for human consumption due to saturated fats being associated with health problems such as coronary

heart disease (Jenkins *et al.*, 2008). The manipulation of BH in the rumen has become a popular topic of research in the recent years in order to try improving the nutritive value of ruminant products and making them more attractive to consumers (Morales *et al.*, 2013). It has been hypothesized that the use of tannins as supplements could potentially cause variations in the FA composition in meat due to the interference with BH pathways in the rumen by the tannins and this, in turn, could affect the oxidation process of fats in meat (Luciano *et al.* 2011).

The presence of cis-9 trans-11 CLA in meat has been considered to be beneficial to human health (Priolo *et al.*, 2007). The fatty acid CLA, commonly known as rumenic acid, has been reported to lower cancer risks, reduce arteriosclerosis, improve immune response and improve lean tissue deposition (Jenkins *et al.*, 2008; Dilzer *et al.*, 2012). Priolo *et al.* (2007) also state that it is well known that the synthesis of CLA is generated by BH by certain ruminal microbial populations although unpublished *in vitro* studies by Vasta *et al.* (2009) found that the biosynthesis of CLA in the rumen was negatively affected by tannins in the diet. Bearing in mind that tannins inhibit the normal activity of ruminal microorganisms (Molan *et al.*, 2001) it is probable that tannins cause reduced ruminal BH, resulting in a higher accumulation of C18:2 n6, total polyunsaturated acids (PUFA) and lower levels of C18:0 in the meat lambs supplemented with tannins compared to the animals given control diets. Although the synthesis of CLA in the rumen is affected by tannins, the largest quantity of CLA present in meat and milk is generated endogenously by the action of the enzyme Δ^9 -desaturase in the muscle tissues from the precursor vaccenic acid (Vasta *et al.* 2009). Due to tannins having the ability to interfere with BH, they have also been known to increase the flow of UFA and vaccenic acid to the duodenum for absorption (Gesteira *et al.* 2018). Tannins found in *Acacia angustissima* and *Acacia salicina* were able to reduce microbial protein synthesis in both buffer rumen fluid and in pure ruminal bacteria strains (Getachew *et al.*, 2000). It was hypothesized by Priolo *et al.* (2005) that because the addition of tannins interferes with BH through inhibiting ruminal microbial activity which forms CLA, it is thought to have effects on the FA compositions found in muscle. The results completed by Vasta *et al.* (2009) contradict past results (Gesteira *et al.*, 2018; Priolo *et al.*, 2005; Vasta *et al.*, 2007) as no change in total CLA concentration was found with the addition of tannin containing diets. Nevertheless, in a recent study Gesteria *et al.* (2018) found that the deposition of CLA in the meat was not affected by the use of tannins in the diet, although the nutritional quality of meat was improved with increased linolenic acid deposition, which ultimately minimises the risk associated with cardiovascular disease (Parodi, 2016). In the same study done by Vasta *et al.* (2009) VFA production was not affected by the concentration of CT when compared to diets supplemented with PEG and condensed tannins. However iso-butyrate and iso-valerate, which are formed from the breakdown of feed

amino acids by ruminal bacteria, were significantly reduced by the presence of tannins in the buffer rumen fluid for the different dose levels (Vasta *et al.*, 2009).

Priolo *et al.* (2005) were able to determine that the level of CLA and vaccenic acid found in meat was less in animals fed tannins than those with no tannin supplements or fed with PEG along with tannin supplements. Vasta *et al.* (2009) suggested that it was due to tannins inhibiting the major ruminal micro-organism activities that are involved in BH. Tannins act directly by being toxic to microbes as well as indirectly by forming complexes with other molecules. Specifically Acacia was responsible for a decline by 74% of protozoal microbes (Getachew *et al.*, 2000). In this same study it was found that only *Acacia mearnsii* was effective in modifying ruminal BH. Although there are variable reports on the effects of Acacia on the microbial population, Carulla *et al.*, (2005) found that there was an increase in total protozoal counts, allowing bacterial counts to increase slightly. There have been conflicting results from studies showing different results from *in vitro* to *in vivo* experiments on CLA accumulation thus further studies need to be conducted to confirm the results (Priolo *et al.*, 2007).

Vasta *et al.* (2009) determined that certain steps in the BH sequence are influenced by tannins at different rates. For example, the hydrogenation of vaccenic acid to stearic acid is more readily affected than the conversion of linoleic acid to CLA. Some authors have hypothesized that tannin additives could reduce or completely inhibit BH of vaccenic acid to stearic acid, which results in the accumulation of vaccenic acid bypass in the duodenum (Priolo *et al.*, 2007; Patra *et al.*, 2011). This accumulation could lead to milk and meat products becoming healthier as the endogenous biosynthesis of CLA in the muscle and mammary gland by the action of the enzyme Δ^9 -desaturase is increased (Vasta *et al.*, 2009). In this respect, Whitney *et al.* (2011) and Rana *et al.* (2012) observed that Δ^9 -desaturase activity increased from 47% to 58% with tannin supplementation, which resulted in an increased total CLA content in muscle. Further *in vivo* studies are needed to better understand the changes in BH and the accumulation of vaccenic acid caused by tannins.

2.4.3 Other important meat quality attributes affected by tannins

The effect of increased UFA in meat is thought to have a desired effect on meat tenderness. In the study of Gesteira *et al.* (2018), it was thought that tenderness might have been improved with tannin supplementation but that this was due to the perception that juicier meat appears more tender to a tasting panel. The tasting panel also confirmed that the meat from the animals given tannin additives had a better flavour as compared to the other test diets, and this was thought to be due to the reduction in ruminal proteolysis and lipid oxidation brought on by the tannin supplements (Watkins *et al.*, 2013).

Cooking weight loss is measured by weighing a meat sample before cooking and after meat has been cooked in an oven at 170°C and the core temperature of meat has reached 72°C. The cooking losses recorded during this experiment were lower for meat of animals fed with tannins, which may contribute to increased juiciness of meat and appear as tenderness to panel tasters (Gesteira *et al.* 2018). Cooking weight loss is associated with meat fat content and the level of UFA in the meat, greater UFA and lower melting points of fat increase the fat loss and consequently the cooking losses will increase (Carvalho *et al.*, 2014). Although in this experiment the cooking weight loss decreased with increasing levels of tannin inclusion in the diet.

2.4.4 Suckling lamb meat quality can be influenced by addition of tannin in the ewe diet

In a recent study there was evidence to suggest that the addition of tannins to diets of ewes could be used to improve meat shelf life and colour of their suckling lambs' meat (Lobón *et al.*, 2017). In the last few decades, the movement away from high fat diets has become apparent due to the negative association of high fat diets with higher risks of coronary heart disease (Keys, 1970). Meat and milk have varying levels and composition of fats that can be influenced by the diets fed, and FA profiles can also be changed by altering diets fed to animals (Wood *et al.*, 1997; Wood *et al.*, 1999). Crude tannins can be used to alter BH pathways and, thus, change the FA profiles of the ewe's milk, which consequently affects lamb meat FA profile (Carreño *et al.* 2015). However, the majority of knowledge on this topic is based on the effects that CT have on the meat FA profiles of feedlot lambs and little research is based on suckling lambs. Similarly, one can expect that modified milk FA profiles of ewes fed tannins will result in changes in FA profile of the lambs that are suckling these ewes (Velasco *et al.*, 2001; Valvo *et al.*, 2005). Suckling lambs do not have a fully functional rumen and therefore the BH of FA may not be very effective (Lane *et al.* 2000) and the FA profile of the lamb meat may not be directly related to the ingested milk (Valvo *et al.* 2005; Osorio *et al.* 2007).

Lobon *et al.* (2010) were able to determine that the meat of lambs suckled from tannin supplemented ewes was more resistant to lipid oxidation over a period of two to seven days of storage. Grape pomace (GP) is a by-product resulting from the wine and juice industries and is a rich source of polyphenols and fibre. This by-product is costly to dispose of (Yi *et al.*, 2009; Crupi *et al.*, 2018) but it could replace part of the forage portion of ruminant diets. Previous studies have shown that polyphenols from the diet can be transferred to the milk of cows, ewes and goats (O'Connell *et al.*, 2001; Jordán *et al.*, 2010; Chiofalo *et al.*, 2012), and this has led to increased antioxidant potential in the milk. These phenolic compounds resulting in increased antioxidant capacity of milk can also relay the beneficial effects to the lamb's meat (Gómez-Cortés *et al.*, 2018). In the study by Gómez-Cortés *et al.* (2018) the a^* coordinate values were higher in R. abdominis muscle from lambs fed with 5% GP diet compared with the control treatments. In the Longissimus muscle, the b^* coordinate as well as hue angle (H^*) parameters were lower in the groups that received GP additives than in the control animals. When meat oxidises, its water holding capacity (WHC) is diminished between muscle myofibrils, which increases liquid loss as well as L^* (Huff- Lonergan *et al.*, 2005). Estévez (2011) determined that antioxidants could improve WHC by slowing down oxidation, and this slows the loss of protein cross-links and membrane integrity. WHC is measured by cooking losses and the WHC was recorded to be higher in lambs fed with vitamin E and GP at different levels compared with the control group. These results are similar to what Morán *et al.* (2012) found. The meat in their experiment had higher WHC when antioxidants such as vitamin E or polyphenols were given as additives in the lamb diets. In terms of the FA composition of the lambs in the study, it was determined that the proportions of vaccenic acid was high in the lambs fed 5% GP, and highest in lambs fed 10% GP in their diets. This in turn could result in increased capacity for endogenous CLA formation thus increasing the healthiness of meat.

In a recent study conducted by Lobon *et al.*, (2019), supplementation of quebracho tannin additives to ewes decreased the total percentage of saturated fatty acids (SFAs) significantly but did not alter the individual proportions of the other FA groups. The lower SFAs proportions found in the meat of the suckling lambs due to the dietary quebracho tannin additives was not anticipated because the SFAs concentration found in the milk of ewes fed quebracho only differed by negligibly. Therefore, the existing results have emphasised a potential role for CT in altering lamb FA profiles, beyond the expected reduction of PUFA BH in the rumen. Inserra *et al.* (2015) investigated carob pulp, a legume by-product that is also rich in CT, and discovered that it decreased the SFAs content of pork (monogastric similar to suckling lambs) meat despite there not being a difference in the concentration of the FA group between the diets. CT additives could cause decreased synthesis of SFAs (Inserra *et al.*,

2015). Vasta *et al.* (2009) found a reduced percentage of SFAs due to the addition of CT in lamb diets. Although the study showed positive results, further research will need to be done to confirm the beneficial effects of ewe milk on the meat quality of their suckling lambs as this is still a novel study topic.

2.5 Animal stress and tannin supplementation

2.5.1 External stressors

Stress, in this context is a term used to describe a generalized negative physiological effect on the animal as a result of extreme internal or external factors (Mitchell *et al.*, 2010). Heat stress (HS) is a well-known problem that influences the livestock industry and leads to economic losses globally especially during the summer months (St-Pierre *et al.*, 2003; Bernabucci *et al.*, 2010) and is of growing concern due to the ever-changing climate (Silanikove *et al.*, 2015). HS can result in oxidative stress either through increased production of oxidative radicals or a decreased production of the animal's natural antioxidant defences (Trout *et al.* 1998; Bernabucci *et al.*, 2002; Saker *et al.*, 2004; Di Trana *et al.*, 2006; Chauhan *et al.*, 2014).

High temperatures have been a concern in the sheep farming industry in areas where ambient temperatures exceed the animal's thermal neutral zone, in this case 5-25°C, and the animals are unable to balance the heat production and dissipation (Al-Dawood, 2017). It causes negative biological effects on normal bodily processes, which include decreased feed intake, weight gain, meat quality, and general health (Marai *et al.*, 2007). The cellular response of HS starts with the activation of heat shock factors which produce heat shock proteins. This increases glucose and amino acid oxidation, reduces fatty acid metabolism, and activates endocrine and immune responses via extracellular secretion of heat shock proteins (Collier *et al.*, 2008). The reason for this is that animal cells undergo lipid oxidation when exposed to high ambient temperatures and this, in part, is thought to be the result of oxidative stress (Bollengier-Lee *et al.*, 1998; Sahin *et al.*, 2003). This oxidation in the cells has been reported to cause unpleasant flavours and odours in meat (Fernandez *et al.*, 1997).

There are some common methods that are used to try alleviating HS in small ruminants, a lot of them including changes to housing conditions and a few including nutritional strategies (Morrison, 1983; Caulfield *et al.*, 2014; Al-Dawood, 2015). One of the easiest ways to reduce the effects of HS is shade. It is cost effective, easy to implement and in most cases very efficient, but it is only applicable in extensive farming conditions (Silanikove, 2000 a; Al-Tamimi, 2007). A sufficiently shaded area can reduce heat load by 30–50% (Muller *et al.*, 1994). Shelters also do not need to be intricate or expensive. Trees and bushes can be effective at sheltering animals from solar radiation (Onyewotu *et al.*, 2003). One of the best strategies to reduce HS is to provide *ad libitum* fresh and cool drinking water (Silanikove, 2000 a; Atrian *et al.*, 2012). When animals experience HS their water requirements increase; and it is thus critical that animals always have access to fresh water. Ration modifications can also

greatly reduce the negative effects of HS, and these adjustments may include changes in feeding schedules to feed animals during cooler hours or changing grazing time, feeding intervals and ration composition. High-quality fibre forage and increasing the energy density of feed or the use of feed additives such as buffers, niacin or antioxidants can greatly help animals reduce HS (Al-Dawood, 2017).

In this regard, diets rich in natural antioxidants can help with the over production of free radicals due to lipid oxidation and therefore mitigating the undesired effects of HS on the animal (Liu *et al.*, 2011). Liu *et al.* (2016) found that the negative effects of lipid oxidation caused by HS in lambs were ameliorated by the dietary supplementation of condensed tannins. Moreover, when animals are subjected to HS there is a large production of glucocorticoids from the adrenal cortex and this can result in catabolic effects in skeletal muscle and will negatively affect the welfare and performance of animals, and their products (Tomas *et al.*, 1980; Odedra *et al.*, 1983). The results of Liu *et al.* (2016) confirmed the findings by Odedra *et al.* 1983 and Tomas *et al.* 1980, that CT decreased the cortisol levels in lambs. The results of the study by Liu *et al.* (2016) indicated that the desired effects of CT on growth performance are most likely due to the reduced stress and minimized catabolic effects of cortisol in the skeletal muscle of lambs. Liu *et al.* (2016) found that with condensed tannin supplements the meat colour of heat-stressed lambs was improved. Zhong *et al.* (2015) observed that CT increased L*, a*, and b* values in the muscle of lambs, which indicates improved colour stability in fresh meat. Similarly, Luciano *et al.* (2009) demonstrated that quebracho tannin additives improved meat colour stability in sheep in general without heat stress being a factor. The physiological functions of condensed tannins on meat colour can be attributed to the high levels of antioxidants, which delay myoglobin oxidation in meat (Luciano *et al.*, 2011).

2.5.2 Internal stressors

Internal parasite infections can cause great stress to animals and can lead to decreased productivity along with declining health. One of the most common internal parasite infections that impact the livestock industry is the nematode *Haemonchus contortus* (Zhong *et al.*, 2015). It has been described as one of the biggest factors affecting sheep production in many climates due to recent strains becoming drug resistant due to overuse of chemical treatments (Jackson *et al.*, 2000; Tsiboukis *et al.*, 2013). Thus, the negative perceptions associated with synthetic additives has piqued the interest in the use of plant-derived phenolic compounds such as CT, and in recent years the use of feed containing CT has been considered as a viable alternative to commercially and commonly used manufactured anthelmintics (Kahiya *et al.*, 2003; Max *et al.*, 2007). The use of natural bioactive compounds might offer additional benefits which include low toxicity levels to mammals and are significantly more environmentally friendly (Batish *et al.*, 2008; Rosado-Aguilar *et al.*, 2010). Animals infected with *H. contortus* showed a decrease in meat colour stability and antioxidant capacity, and supplementation of condensed tannins to the diet was able to reverse these negative effects (Zhong *et al.*, 2015).

The use of many current chemical treatments for internal parasites has resulted in the parasites developing a resistance and thus there has been interest in seeking alternative, more sustainable management strategies such as the use of nutraceuticals (Hoste *et al.*, 2015). Andlauer *et al.* (2002) describe nutraceuticals as a substance that is considered a food as well as having health benefits that prevent or treat diseases. The effect of tannins on internal parasitic infections has provided a viable alternative to synthetic therapeutic prevention methods. Waghorn *et al.* (1994), Niezen *et al.* (1995) and Robertson *et al.* (1995) observed that lambs being supplemented with tannins exhibited higher growth rates and lower faecal egg loads compared to lambs not treated with any anthelmintics although the potency of effect on parasites seems to depend on the plant source of tannins fed. Kahiya *et al.* (2003) observed that goats given a diet containing *Acacia karoo* experienced reduced faecal egg counts (FEC); while goats given a diet containing *Acacia nilotica* experienced no drop in FEC. The results of this study suggest that the protein-precipitating activity of certain CT in the diet may be the factor that influences whether or not a CT from specific browse possesses anthelmintic properties. Many factors could determine whether a CT has anthelmintic properties but the most likely cause seems to be molecular weight as well as concentration of CT in the diet (Naumann *et al.*, 2013).

Low concentrations of tannins can protect plant proteins against ruminal degradation and thus increase protein availability to the small intestine for improved absorption of amino acids

(Waghorn *et al.* 1987). Hence dietary tannins may benefit parasite infected sheep by improving their protein nutrition which may result in improved animal immune response to the parasitic infection (Molan *et al.*, 1999). According to Molan *et al.* (1999), increasing levels of tannins in the diets lowered levels of hatched eggs in the faecal collections of infected lambs. Tannins are able to interfere with the life cycle of the parasites by limiting the development of eggs and decrease the development of first-stage larvae (Molan *et al.*, 1999). In the study by Fernández-Salas *et al.* (2011) acaricidal effects of tannin containing forage extracts were confirmed by using PEG as an effect blocker of tannins, it binds to and renders tannins inactive thus showing what effects tannins have when PEG isn't used. The authors revealed the mortality percentages of parasites dropped to values similar to the controls after PEG addition indicating that tannin additives were involved in the acaricidal effect against a parasite infestation of *R. microplus*. The biological activity of tannins has therefore been compared to the medicinal properties of some plant extracts against gastrointestinal nematode infections in small ruminants (Hoste *et al.*, 2006; Alonso-Díaz *et al.*, 2008).

Anthelmintic drugs are absorbed in the digestive tract (Sanyal *et al.* 1995) while tannins are not and will leave the body with faecal excretions. This means that even the eggs shed by the worms in the digestive tract will continue to be exposed to the effect of tannins throughout development making tannins more effective with prolonged treatment (Molan *et al.*, 1999).

2.6 Conclusion

Significant research has brought about change of the perspective of tannins to become beneficial nutritional additives for ruminant nutrition. With the movement away from antibiotic use in animal production, natural methods have become more attractive. In many studies tannins have shown methane mitigation potential and some of those studies have shown that tannins also have the potential to exhibit co-benefits to meat quality characteristics but is often limited to certain condensed tannin varieties. Meat colour and FA proportions can be altered positively by the presence of tannins in the diet. Shelf life can potentially be extended and more UFA than SFA could be deposited in intramuscular fat. In some cases, tannins have also shown to have acaricidal effects on internal parasites, minimise the effects of heat stress on meat quality of animals, and referred antioxidant potential from ewes' milk to suckling lamb meat. Acacia tannins are of interest as they are found in abundance and if their co-beneficial properties can be proven in the following research, a movement towards their commercial use can be made.

Chapter 3

Research methodology

In this section the methodology and techniques followed for this study will be stated. The methodology on the animals from this study will be outlined before the specifics will be elaborated on. For this study, the start was signified by the slaughter of the lambs and the dissection of rib cuts for investigation, this was followed with lab work to determine the results of the specific objectives below.

3.1 Methods and materials

This study was conducted at the small stock section of the Hatfield experimental farm at the University of Pretoria (UP) Hillcrest campus. The animals were born and raised there and were selected from weaning to be used in the experiment. In 2019 the animals entered the first phase of the project, which was part of a PhD project that aimed to determine whether the addition of *Acacia mearnsii* tannins to the diets of the Dohne Merino ram lambs decreased methane emissions without affecting digestibility of low-quality forage.

The weaned lambs selected for this trial had an average weight of about 31.93 ± 3.92 kg. The lambs were stratified according to their weight from minimum weight 23.90 kg to maximum weight 37.40 kg and then randomly assigned to one of the four treatment diets.

The second phase of the project that makes up this MSc project started in 2020 and focused on determining how the tannin included in the diet affected or influenced the product quality of the animals.

3.2 Ethical approval

Ethical approval was obtained for the study from the university Animal Ethics Committee (Ref no EC075-17) to conduct a growth trial and measurement of digestibility and methane emissions from sheep receiving different tannin additives.

The second phase of the study involved laboratory analysis of meat samples collected at an abattoir to investigate the effect of tannin based dietary additives on carcass quality under ethical approval number (NAS201/2020). Both ethical approvals are attached in Appendix A.

3.3 Experimental design

A total of 40 Dohne Merino ram lambs with an average weight of approximately 32 kg were used for the growth trial that made up part of Mr Shehu L. Ibrahim's PhD study. The animals were bred and raised at the small stock section on the experimental farm of UP. The animals underwent an adaptation period of one month where they were all housed in one pen, to ensure adaptation of all experimental animals to the total mixed ration (TMR) diet before experimental diets were given. After the one-month adaptation period, all ram lambs were moved to the metabolic house where they were separated into pens and underwent another 8-day adaptation period in order to acclimatize to the experimental pens. A completely randomized block design was used to assign animals to the 20 pens and dietary treatments. There were four treatments and thus each treatment diet had 10 animals per replicates. Each pen housed two animals that were receiving the same experimental treatment. The pens were considered experimental units and the individual sheep within a pen were considered as observational unit for that treatment.

The experimental treatments were as follows:

1. TMR for maintenance and growth (composition in Table 1 below) (Negative control) **(C)**
2. TMR with an addition of Rumensin (Monensin being the active ingredient) at 75mg/kg of feed (Positive control) **(M)**
3. TMR with the addition of raw condensed Acacia tannin at 20g/kg of feed. **(CT)**
4. TMR with an addition of encapsulated condensed Acacia tannin at 20g/kg of feed. **(ECT)**

A high forage based TMR was formulated in order to meet the maintenance and growth requirements of the growing lambs and formed the negative control diet in the growth experiment. The composition of TMR diet is summarized in Table 1. The animals were fed their respective treatment diet twice daily, the pens were cleaned, and water troughs checked once daily until a slaughter weight of about 50 kg was reached. Lambs were taken to an abattoir for slaughtering as specified by the UP experimental farm. Feed refusals from the two mealtimes were weighed and recorded each morning so that this feed wastage will not be taken into account when determining FCR.

Table 1 Total mixed ration composition (provided from parallel PhD. projects laboratory data)

Ingredient	Composition (%)
Soybean meal	17.0
Yellow maize	28.0
Alfalfa hay	20.0
Eragrostis curvula hay	22.2
Molasses	6.0
Wheat	5.0
Urea	0.8
Vitamin premix	0.5
Chemical composition	
Total volume	100.0
Dry matter (%)	89.7
CP (%)	17.2
Starch (g/kg)	64.9
NDF (g/kg)	340
ADF (g/kg)	241.6
Lignin (g/kg)	24.6
Ash (%)	6.5
ME (MJ/kg)	9.

3.4 Pen layout

The four treatments were divided among the animals and thus each treatment diet had 10 animals assigned to it as replicates. Each pen housed two animals that were receiving the same experimental treatment. The pens were considered experimental units and the individual sheep within a pen were considered as observational unit for that treatment. This representation is shown in appendix A.

3.5 Sampling:

There were four different slaughter days as the animals reached the specified slaughter weight at different times. The days on feed ranged from 65 – 129 days with an average of 101 days on feed. The slaughter dates ranged from the first slaughter date on 4 December 2019 to the last slaughter date on 30 January 2020.

During the course of the trial, one lamb was removed due to a mouth defect that resulted in poor eating ability and another lamb died. All 38 remaining lambs were slaughtered after approximately 101 days at a mean slaughter weight of 52.20 ± 4.05 kg.

The meat sample that was taken in the abattoir after 24 hr for this study was bought from a commercial abattoir (Klipleiland butchery abattoir, Bronkhorstspuit). These animals, that were part of the PhD study, were slaughtered according to standard procedures, after negotiation and agreement. The slaughtering was arranged by the University farm as part of the normal farm activity.

After 24hrs of the carcass being kept in a cold room at 4°C, the rib cut sample (rib 8-10) was taken from the left side of each of the lambs. The three-rib sample was cut using the ventral extremity of the sample, which is a line drawn from the pubic symphysis to the middle of the first rib, to obtain an estimate whole carcass composition (Casey *et al.*, 1988). All samples were sealed in polypropylene bags labeled with animal ear tag numbers and placed in a cooler box with ice to be transported to the university, and they were then frozen at -20 °C until further analysis of meat samples took place.

3.6 Sample preparation and analysis:

3.6.1 Meat colour analysis

The three-rib cut samples dissected from sample animals, were left to bloom at room temperature and then divided into individual rib cuts for colour analysis. The meat colour analysis was done in the Food Science laboratory of the University of Pretoria using the Konica Minolta CM-600d colour measuring spectrophotometer. The colour readings were taken from the same side of each longissimus muscle from each sample in triplicate, thereafter they were individually vacuum packed and labeled to be stored at 4°C for seven days. The colour coordinate readings that were taken were as follows: coordinate a* which represents “Redness”, coordinate b* which represents “Yellowness” and coordinate L* which represents “Lightness or Brightness”. After the 7 days storage the samples were re-evaluated for colour coordinates to determine whether the treatment diets could have influenced the preservation of colour.

3.6.2 Carcass composition

Each rib cut sample, that consisted of three-rib cuts, were weighed as a whole, then dissected into bone, fat and muscle. Each component was weighed separately and recorded in order to calculate the percentage composition for each component. The meat, fat and bone were vacuum packed and stored in the freezer at -20°C until further analysis.

3.6.3 Ether extract analysis

Ether extracts were done in order to determine the percentage of intramuscular fat found in longissimus muscle samples. The method used was the SOXTEC method which involved boiling the freeze-dried meat samples (± 1 g) in petroleum ether for two hours and then oven drying until all the petroleum ether has evaporated. The samples were weighed afterwards and shown as a percentage of the whole sample.

3.6.4 Fatty acid profile analysis

The lipid extraction method by (Ways and Hanahan, 1964) was used with some modifications of the chloroform: methanol (2 : 1, v/v) proportion in the method (Folch *et al.*, 1957). Butylated hydroxytoluene (2.6 DI-tertBUTYL-P-CRESOL) was included as an antioxidant. Methyl esters of the FA component of the neutral triglycerides were prepared according to the NaOH/ methanol method (AOAC, 1975). These esters were separated on a polar phase SP2330 column (2 m X 3 mm, packed with Silar 10C coated on Gas Chrom Q) fitted to a Varian 3700 gas chromatograph with a flame ionization detector as previously described by Webb *et al.* (1994a). Profiles of the cis-trans FA's from subcutaneous adipose tissue were obtained from fat samples that were treated with n-Hexane at 35°C for 24 h, after which the fatty acids were esterified according to the method of Van Wijngaarden (1967). The cis-trans fatty acids isomers were then separated on a SP2560 fused silica capillary column (10 mm X 0.2 mm) fitted to a Varian 3700 gas chromatograph (Webb *et al.*, 1994a). Standards for the FA's were obtained from Nu-Chek-Prep. Inc. (Elysim, MI, USA). Fatty acids were expressed in both normalised (molar proportion) and gravimetric (milligrams per gram of fresh tissue) formats (Slover and Lanza, 1979; Huerta-Leidenz *et al.*, 1993).

3.7 Statistical analysis of data

All sample analysis in the laboratory was replicated in duplicate in order to avoid high variances between results. The data was recorded in Excel and checked for mistakes before statistical analysis continued. Due to the animals not being slaughtered on the same day or at the same weight these differences were taken into account as random factors when determining the statistical data. The data was sent to a statistician in order to determine the best way to correct for these differences. With the statistical analysis a normality test was performed first to ensure the data from the study came from a normal population and for this a goodness of fit or Shapiro-Wilk test was done. Later on the original or transformed data were subjected to analysis of variance (ANOVA) using in Genstat (VSN International, 2017) to determine whether the experimental treatments had a significant effect ($p < 0.05$) on colour coordinates, carcass composition (bone, meat or fat percentage) as well as the FA profile of subcutaneous and intramuscular fat. For the FA analysis, the FA that showed significant difference, a Tukey 95% confidence intervals test was done to determine between which treatment groups the differences lie. A treatment with the same letter is not significantly different while a treatment group where the letters differ are significantly different from each other. In some cases, there was a significant difference found between treatments but where Tukey's connecting letters report was done all treatments were not significantly different. This is because Tukey's test controls the experiment wise error rate, part of the cost is that individual pairwise comparisons are significant at a $p < 0.05$. Thus a pair wise comparison that is significant with a t-test at a p-value of < 0.05 will be non-significant with Tukey's test.

Subsequently average values were summarized in tables and figures and significant co-benefits and where necessary trends were discussed further.

Chapter 4

Results and discussion

This initial aim of the study was to determine the methane mitigating ability of tannins in lambs, in order for this treatment to be considered a successful mitigation treatment. For this purpose, evidence-based results are also required about the effects of such treatments on carcass and meat quality of the animals.

4.1 Days on feed and average daily gain (ADG)

From Table 2 it is apparent that the treatments represented as **C**- control (TMR), **M** – Rumensin additive (Monensin as the active ingredient) treatment, **CT** – raw crude tannin treatment and **ECT** - encapsulated tannin treatment had no significant effect on the ADG of animals or the composition of each carcass component. The mean ADG for all treatment groups was $0.222\text{kg} \pm 0.083$.

Table 2 The effects of Acacia tannin treatments as a methane mitigating agent on growth and carcass composition.

Parameter	Mean (Kg) \pm SD					Significance (p<F)
	Pooled	C	M	CT	ECT	
ADG	0.222 ± 0.083	0.207 ± 0.080	0.232 ± 0.088	0.241 ± 0.095	0.209 ± 0.079	0.369
Fat %	40.41 ± 4.543	41.45 ± 4.575	40.36 ± 4.151	39.73 ± 4.964	40.03 ± 4.986	0.792
Bone %	15.48 ± 2.038	14.76 ± 2.345	15.83 ± 2.054	14.78 ± 2.148	16.53 ± 1.148	0.093
Muscle %	44.11 ± 4.155	43.79 ± 3.631	43.81 ± 4.074	45.49 ± 4.125	43.45 ± 5.031	0.731

Past research has shown that *Acacia mearnsii* tannin additive used for methane mitigation can often result in lower feed intake of animals due partly to the bitter taste of tannin (Ma *et al.* 2014), however, this does not seem to be the case in this study as the ADG of the CT treatment was the highest over all of the treatments.

However, the ECT treatment did not have the same desired result as the CT treatment group on ADG. This differs from past research by Adejoro *et al.* (2019), which found that encapsulation of tannin additives did not have negative effects on digestibility of feed and may amplify the effects of tannin additives to be localised to the rumen without affecting the small intestine. Encapsulation may slow the tannin release in the rumen before the establishment of new linkages with protein and other feed nutrients (Adejoro *et al.*, 2019). While CT protects proteins from excessive ruminal degradation and reduces enteric methane, it can also inhibit

the absorption of the nitrogen in the intestine or reduce fibre digestion (Waghorn *et al.*, 2008). This may have been the case in this current experiment when looking at the ADG results.

The slaughter weight of sheep for treatment groups are presented in Figure 1. There were no significant differences in the slaughter weights of lambs between treatment groups.

4.2 Slaughter weights and total weight gained

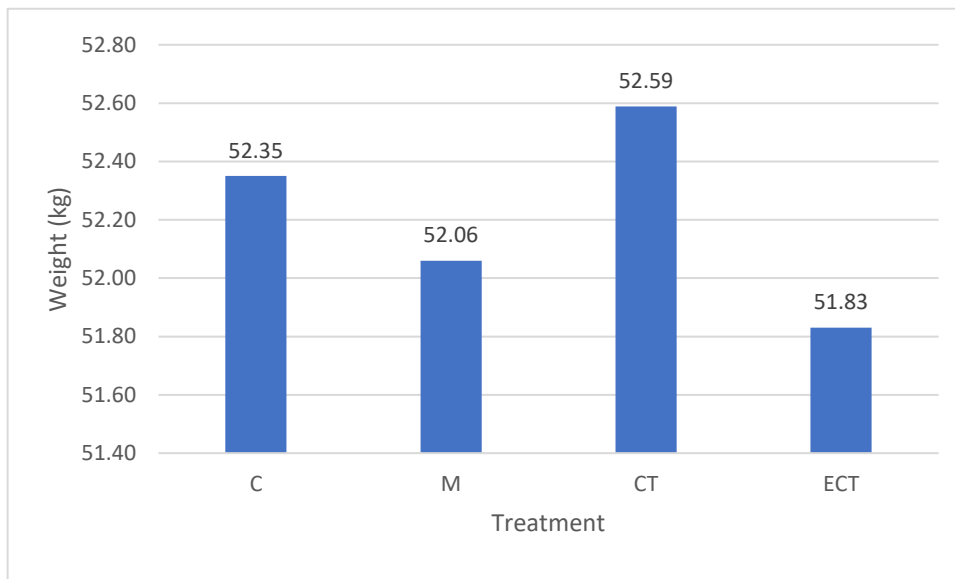


Figure 1: Slaughter weight means for treatment groups

Figure 2 shows the mean total weight gained over the mean days on feed for each treatment group.

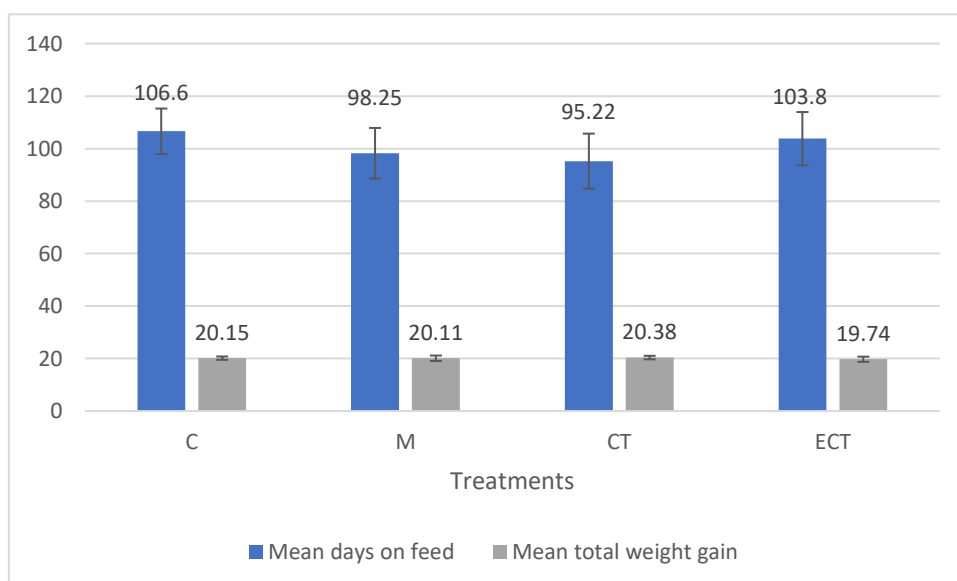


Figure 2. Total days on feed vs total weight gained.

During slaughter, the animal carcasses were classed according to the standards of the South African red meat classing guidelines and these classes were taken into account. Most of the lambs had an A3 grade with only two having grade A2 (one from C and one from ECT treatment group) and three having A4 (two from ECT and one from the M treatment groups). The animals that had A4 were all part of the last slaughtering group and thus they had time to gain an extra fat layer during the additional feeding period.

Dohne Merinos are intermediate maturing breeds which means that during the time leading up to maturity the growth of muscle is at its highest, leading to these animals having higher muscle mass as maturity compared to early maturing types, after which the animals enter a fattening phase (Butterfield *et al.*, 1983). As the animal gains meat and fat between early development and slaughter weight the proportions of FA change (Wood *et al.*, 2008).

This could explain why the last animals left for slaughter were generally given a higher fat classification number as compared to the animals slaughtered in the first cycle.

4.3 Ether extracts

Ether extract analysis was done on the muscle samples in order to determine whether the addition of tannins to the diets had an effect on the level of intramuscular fat.

Figure 3 represents the total intramuscular fat % of lambs on different treatments. There was no significant difference between treatments in terms of % intramuscular fat found in meat samples.

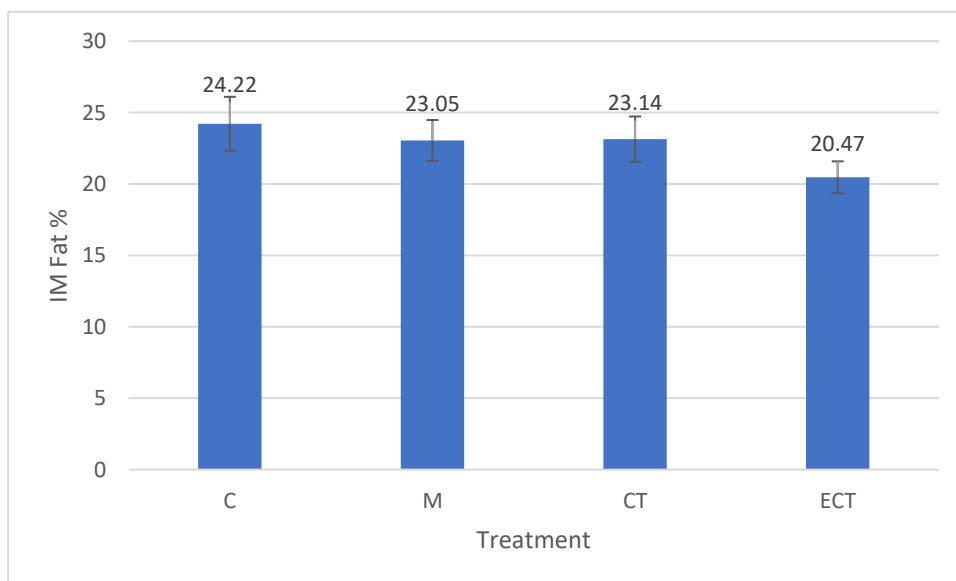


Figure 3. Mean total intramuscular fat % for treatment groups

4.4 Meat colour

Meat colour analysis was done on the longissimus muscle from the rib-cut samples. On the day of thawing the first measurement was taken in triplicate. Afterwards the samples were vacuum packed and left in the refrigerator at 4°C for seven days where after the second measurement was taken.

Tannins have antioxidant potential that can lead to improvement of meat shelf life by preserving meat colour for longer through oxidative stability (Luciano *et al.*, 2011). Based on previous research (Karabagias *et al.*, 2010; Luciano *et al.*, 2011), diets high in polyphenols such as tannins have the ability to maintain meat colour for a longer period of time. Karabagias *et al.* (2010) found that although the L* and b* values tended to change drastically over a period of refrigeration, the a* coordinate stayed relatively unaffected for tannin additive containing diets. The first and second measurement of colour coordinates (a*, b* and L*) was compared between treatments as well as the H* values using ANOVA (Table 3)

Table 3 Colour coordinates as affected by treatment groups and refrigeration for 1 or 7 days

Colour coordinate reading	Treatment	Day 1	Day 7	% Difference	Significance (p < F)
a*	C	15.031 ±2.006	12.576 ±1.006	16.33	0.0028
	M	14.964 ±1.861	12.316 ±0.644	18.87	0.0005
	CT	15.627 ±1.583	12.542 ±1.882	19.74	0.0017
	ECT	15.039 ±2.06	12.153 ±1.373	17.97	0.0033
	P value	0.5185	0.5605		
b*	C	7.101 ±1.627 ^{ab}	5.911 ±1.23 ^b	16.76	0.0817
	M	7.64 ±1.49 ^a	7.367 ±1.31 ^a	4.94	0.5696
	CT	7.31 ±1.12 ^{ab}	6.751 ±1.5 ^{ab}	7.65	0.3842
	ECT	6.465 ±2.19 ^b	6.355 ±1.155 ^{ab}	1.7	0.8899
	P value	0.0469	0.0134		
L*	C	38.26 ±2.129	38.086 ±0.673	0.45	0.8296
	M	39.362 ±1.936	39.039 ±1.764	0.97	0.6659
	CT	37.839 ±3.932	38.074 ±2.71	+0.62	0.8849
	ECT	37.974 ±3.239	38.419 ±1.33	+0.94	0.7489
	P value	0.1649	0.0391		
H*	C	25.24 ±3.585 ^{ab}	26.01 ±4.611 ^b	+3.05	0.4151
	M	27.0 ± 3.274 ^a	30.074 ±4.863 ^a	+11.39	0.0060
	CT	25.0185 ±4.351 ^{ab}	28.744 ±7.465 ^{ab}	+14.89	0.0382
	ECT	22.777 ±6.496 ^b	28.154 ±5.531 ^{ab}	+23.61	0.0019
	P value	0.0083	0.0522		

a*= "Redness"; **b***= "Yellowness"; **L***= "Lightness/Brightness"; **H***= Hue angle

Means in the same row with different subscript letters differ (p < 0.05)

C = Control; M = Monensin treatment group; CT= Crude tannin treatment group; ECT= Encapsulated tannin treatment

The ability of tannins to preserve colour through antioxidant action was tested after seven days of refrigeration when the coordinates were measured again and compared to the previous value. After which the H* was determined, it was found that the H* value is more representative of the meat colour as opposed to looking at the singular colour coordinates.

Table 3 shows that there was a significant difference in coordinate a* values between the treatments after the seven-day refrigeration period. All treatments dropped significantly ($p < 0.05$) in coordinate a* during the seven-day refrigeration but CT and M treatments dropped most significantly. As compared to the control the M, CT and ECT treatments had a greater significant difference in a* colour coordinate values. Among the treatments, CT seemed to have the greatest decrease in a* value when compared to M and ECT after seven days (Table 3) although these values were not significantly different from each other. Past research revealed that tannin additives had the ability to slow colour deterioration (specifically with coordinate a*), but that was not the case in this study. There were no significant differences between treatments on Day 1 and Day 7 of measurement. The change in fresh meat colour is the result of the net balance between oxidative, antioxidative and reducing systems in meat, and discolouration of fresh meat indicates dominance of oxidative processes (Bekhit *et al.*, 2019). This significant decrease in a* is understandable as the red pigmentation is the fastest colour coordinate to deteriorate. This is due to the red pigmentation in meat being largely attributed to the presence of haemoglobin, myoglobin and cytochromes, which are readily leached from meat samples over time. Myoglobin is the biggest contributor to red colour in red meat (Bekhit *et al.*, 2019) thus the colour holding ability is dependent on the oxidative stability of myoglobin (bright red in colour) to forming metmyoglobin (brown in colour) (Faustman *et al.* 2010). In past research, dietary tannin supplements have been considered to reduce oxidative deterioration in red meat samples through their antioxidant properties (Luciano *et al.*, 2010). This was, however, not the case in this experiment.

Yellow colour coordinate values (b*) did not differ significantly after 7 days refrigeration time across all the treatments. The lack of significant change was also observed by Luciano *et al.* (2010). It is interesting to note that in previous research (Luciano *et al.*, 2010; Bekhit *et al.*, 2019; Priolo *et al.*, 2000) b* values increased in value while in this study, the b* values decreased. Ideally a lower level of yellow pigment is desired as a decrease in red pigment and increase in yellow pigment will result in brown colour. When comparing treatments at Day 1 and Day 7, there was a significant difference between the treatments. On Day 1, treatment M and ECT were most significantly different and on Day 7, treatment C

and M were most significantly different (Table 3). On both days, treatment M had the highest value and was most significantly different from treatment C on Day 1 and treatment ECT on Day 7. These results suggest that monensin may have had an influence at maintaining a higher b^* coordinate value than the other treatments, which would cause meat to turn from red to brown faster.

Changes in L^* were not significant over the seven-day refrigeration thus the increase in some values and decrease in other values for the treatments may have been incidental. Luciano *et al.* (2008) reported that all treatments showed an increase in L^* coordinate over time while in this study none the treatments showed this trend in L^* coordinates. Priolo *et al.* (2000) also recorded higher L^* readings in lamb supplemented with a diet containing carob pulp (a tannin-containing feed) when compared to the control diets. Priolo *et al.*, (2000) suggested that lower levels of pigments (haemoglobin and myoglobin) in lambs that were tannin supplemented could have been responsible for the differences in colour readings. The differences in the findings could be attributed to different cuts of meat used to measure colour coordinates, Luciano *et al.* (DATE) used longissimus muscle tissue while Priolo *et al.* (2000) used semimembranosus muscle tissue. From these results it can be assumed the tannin treatments will not have an effect on the L^* colour coordinate.

Changes in a^* and b^* over a period of time are the main coordinates used to describe meat browning (Mancini and Hunt, 2005; Insausti *et al.*, 2008). However, increases in H^* values over time, which are the result of decreases in a^* relative to b^* , are able to better describe meat discolouration instead of single colour coordinates (Renerre, 2000). In the present study, with the exception of C treatment the H^* colour coordinate increased with time for M, CT and ECT dietary treatments.

H^* was calculated as follows: $H^* = \tan^{-1} (b^*/a^*) \times (180/\pi)$

When comparing all Day 1 H^* to Day 7 H^* there was a significant difference between the means across M, CT and ECT treatment groups. Table 3 shows that the ECT treatment had the biggest increase in H^* while treatment C was most stable after seven days. The greater the H^* is the more colour deterioration there was over time. There was also a significant difference found between treatments on both Days 1 and 7. Again, treatment M was most significantly different from treatment ECT on Day 1 and from treatment C on Day 7. This was similar to the results found by Luciano *et al.* (2009) where they saw H^* increase with time regardless of dietary treatment but diet did significantly affect this colour coordinate ($p < 0.003$). After 7 days of storage, meat from the animals given a conventional concentrate diet had higher H^* values than that from animals given tannin additives and herbage (Luciano *et al.*, 2009). Treatment M had the highest value on both days which indicates that Monensin

was not able to keep H* as stable as the other treatments. These results indicate that the dietary tannin additives did not have a significant co-beneficial effect at improving meat colour stability as seen in previous studies

4.5 Fatty acid composition

Tables 4 and 5 represent the results of subcutaneous and intramuscular FA profiles, respectively.

Dietary additives of *Acacia mearnsii* tannins as a methane mitigation agent in the diet of Dohne Merino sheep were expected to result in co-benefits regarding the composition of certain FA. However, there was no effect of dietary M, CT and ECT on subcutaneous FA profile. The exception being C18:2n6C that showed a tendency ($p < 0.09$) to differ between the dietary treatments. In line with the findings of this study, Gesteira *et al.* (2018) found that feeding tannins can lead to a change in BH in the rumen and subsequently a shift in FA profile of the intramuscular fat but not the subcutaneous FA profile. CLA is one of many unsaturated FA that have been reported as beneficial dietary FA as it can reduce cancer risks and arteriosclerosis, improve immune response and improve lean tissue deposition in humans (Jenkins *et al.*, 2008; Dilzer *et al.*, 2012). The reason for this shift is believed to not be directly related to an increase in CLA production but due to an increase vaccenic acid production in the rumen, which leads to the increased action of Δ^9 -desaturase and thus increases the level of linoleic and CLA in the muscles (Vasta *et al.*, 2009).

The FA profile of subcutaneous and intramuscular (IM) fat samples was broadly similar, but IM showed an increased diversity in FA types, which was expected. Significant differences were seen between treatments containing tannins compared to non-tannin containing treatments.

Table 4 The effects of *Acacia mearnsii* tannin treatment diets on fatty acid profile of subcutaneous samples

Subcutaneous FA	Mean Molar % [X ± SD]				Significance (p = F)
	C	M	CT	ECT	
Saturated FA					
C10:0	0.103 ± 0.022	0.094 ± 0.021	0.110 ± 0.031	0.107 ± 0.023	0.453
C12:0	0.094 ± 0.050	0.077 ± 0.033	0.125 ± 0.079	0.093 ± 0.034	0.217
C14:0	2.813 ± 0.613	2.711 ± 0.308	3.058 ± 0.741	2.882 ± 0.580	0.732
C16:0	25.23 ± 1.535	26.11 ± 1.165	25.52 ± 2.117	24.91 ± 2.019	0.233
C17:0	2.277 ± 1.141	2.371 ± 0.571	2.266 ± 0.640	1.946 ± 0.498	0.775
C18:0	23.34 ± 4.091	23.56 ± 3.750	22.46 ± 5.113	22.82 ± 4.683	0.991
C20:0	0.103 ± 0.017	0.101 ± 0.021	0.103 ± 0.022	0.101 ± 0.018	0.985
C21:0	0.383 ± 0.127	0.284 ± 0.089	0.328 ± 0.132	0.394 ± 0.151	0.212
C22:0	0.045 ± 0.015	0.045 ± 0.015	0.038 ± 0.012	0.049 ± 0.011	0.366
Unsaturated FA					
C14:1	0.074 ± 0.037	0.057 ± 0.025	0.076 ± 0.039	0.073 ± 0.047	0.813
C16:1	1.362 ± 0.213	1.306 ± 0.619	1.266 ± 0.358	1.261 ± 0.373	0.868
C18:1n9t	2.936 ± 0.811	3.074 ± 0.363	2.888 ± 0.563	3.327 ± 0.865	0.346
C18:1n9c	38.56 ± 4.353	37.71 ± 3.624	38.63 ± 6.203	39.15 ± 4.883	0.947
C18:2n6t	0.165 ± 0.035	0.152 ± 0.060	0.163 ± 0.052	0.194 ± 0.049	0.144
C18:2n6c	2.111 ± 0.487	1.962 ± 0.446	2.498 ± 0.305	2.270 ± 0.508	0.090
C20:1	0.041 ± 0.017	0.050 ± 0.010	0.055 ± 0.014	0.057 ± 0.035	0.414
C18:3n3	0.264 ± 0.060	0.240 ± 0.060	0.308 ± 0.051	0.269 ± 0.063	0.111
C20:2	0.031 ± 0.003	0.037 ± 0.013	0.035 ± 0.005	0.035 ± 0.005	0.249
C20:4n6	0.064 ± 0.027	0.063 ± 0.021	0.066 ± 0.019	0.062 ± 0.017	0.959

C = Control; M = Monensin treatment group; CT= Crude tannin treatment group; ECT= Encapsulated tannin treatment

4.6 Subcutaneous fatty acid profile results

One of the biggest themes in this study is based on whether the addition of tannins to the diet of lambs could result in a co-benefit in the form of increased beneficial FA found in the animal products. The main beneficial FA found in red meat are stearic acid (C18:0), oleic acid (C18:1), linoleic acid (C18:2), α -linolenic acid (C18:3), EPA (C20:5) and DHA (C22:6). Oleic acid (18:1cis 9) is formed from stearic acid (18:0) by action of $D\Delta^9$ -desaturase and is a major component in ruminant FA formation (Wood *et al.*, 2008). This is the same enzyme that forms CLA, which is considered an important nutrient in human nutrition and health.

In concordance with previous research on sheep by Chikunya *et al.* (2004) and Castro *et al.* (2005), the most abundant FA found in the subcutaneous fat of Dohne Merino sheep IM meat samples was oleic acid (C18:1n9c) at 38.53 molar % average, which was followed by palmitic acid (C16:0) and stearic acid (C18:0) respectively.

Table 4 shows that the diet given to the animals had no significant effect on the FA profile found in the subcutaneous fat samples. It can therefore be assumed that when *Acacia mearnsii* tannins are given as dietary methane mitigators there will be no significant adverse effects on the FA profile of subcutaneous fat.

Figure 4 shows the representation of SFA, monounsaturated fats (MUFA) and PUFA in the subcutaneous fat of experimental lambs.

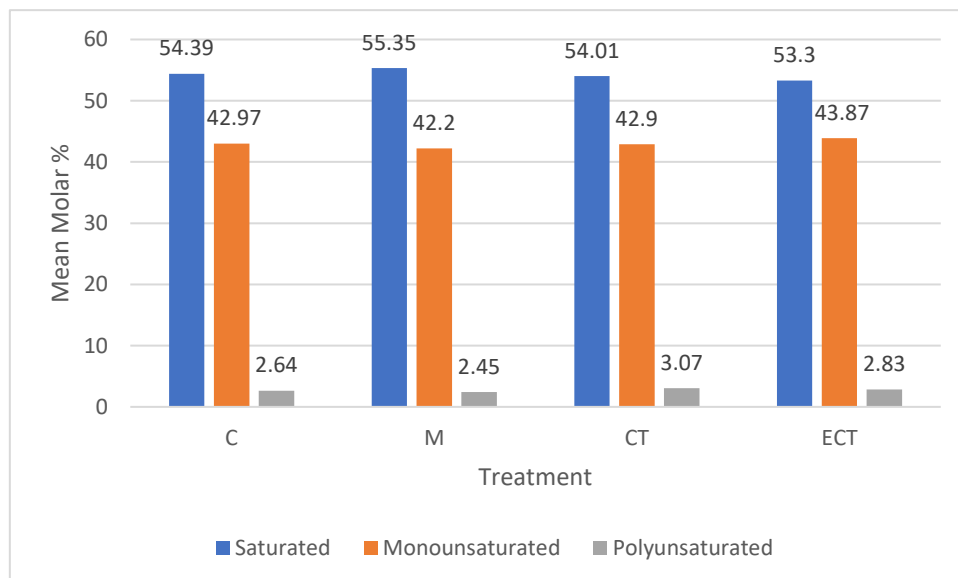


Figure 4. Pooled representation of fatty acids found in subcutaneous fat

These values were within the normal ranges for subcutaneous FA values as represented by Castro *et al.* (2005). Although the differences were not significant across all FA saturation levels, the Acacia tannin treatment groups had slightly higher concentrations of PUFA and MUFA while the control treatments had highest SFA values. Due to the tannin interactions with BH this could have increased tissue MUFA content and could have caused the slight activation of their biosynthesis in the $D\Delta^9$ -desaturase catalysed reaction (Vasta *et al.*, 2009). This could indicate that in the rumen the pathways of SFA formation were partially interrupted and thus caused higher UFA to be present in the subcutaneous fat of the sheep that received tannin-containing treatments.

Table 5 The effects of *Acacia mearnsii* tannin treatment diets on fatty acid profile of intramuscular samples

Intramuscular FA	Mean Molar % [X±SD]				Significance (p = F)
	C	M	CT	ECT	
Saturated FA					
C10:0	0.108 ± 0.013	0.116 ± 0.023	0.120 ± 0.024	0.119 ± 0.019	0.592
C12:0	0.095 ± 0.044	0.096 ± 0.018	0.148 ± 0.097	0.099 ± 0.025	0.063
C14:0	2.198 ± 0.269	2.398 ± 0.327	2.580 ± 0.549	2.362 ± 0.298	0.179
C16:0	27.16 ± 1.250	28.52 ± 1.860	28.04 ± 1.238	27.88 ± 1.506	0.132
C17:0	1.016 ± 0.165	0.987 ± 0.062	0.992 ± 0.123	0.921 ± 0.070	0.210
C18:0	17.65 ± 1.649	17.67 ± 1.950	16.35 ± 2.131	17.62 ± 1.392	0.618
C20:0	0.069 ± 0.006	0.069 ± 0.008	0.074 ± 0.010	0.073 ± 0.007	0.391
C21:0	0.302 ± 0.062	0.278 ± 0.044	0.352 ± 0.109	0.347 ± 0.071	0.032
C22:0	0.181 ± 0.050	0.170 ± 0.048	0.158 ± 0.056	0.203 ± 0.052	0.118
C24:0	0.027 ± 0.016	0.026 ± 0.005	0.026 ± 0.011	0.032 ± 0.006	0.517
Unsaturated FA					
C14:1	0.055 ± 0.011	0.058 ± 0.016	0.071 ± 0.027	0.057 ± 0.012	0.487
C16:1	1.374 ± 0.225	1.517 ± 0.246	1.362 ± 0.268	1.494 ± 0.303	0.553
C18:1n9c	45.05 ± 2.106	43.91 ± 2.349	44.96 ± 1.993	43.62 ± 1.504	0.285
C18:2n6t	0.170 ± 0.029 ^{ab}	0.154 ± 0.030 ^b	0.178 ± 0.044 ^{ab}	0.191 ± 0.031 ^a	0.040
C18:2n6c	3.067 ± 1.075	2.774 ± 0.490	3.209 ± 0.554	3.405 ± 0.651	0.210
C20:1	0.046 ± 0.005	0.044 ± 0.005	0.049 ± 0.003	0.046 ± 0.007	0.384
C18:3n3	0.277 ± 0.057 ^{bc}	0.240 ± 0.042 ^c	0.321 ± 0.049 ^a	0.287 ± 0.039 ^{ab}	<.001
C18:3n6	0.028 ± 0.008	0.024 ± 0.009	0.027 ± 0.009	0.031 ± 0.006	0.212
C20:2	0.049 ± 0.009	0.049 ± 0.008	0.048 ± 0.008	0.048 ± 0.010	0.983
C20:3n6	0.067 ± 0.025	0.058 ± 0.011	0.064 ± 0.022	0.076 ± 0.021	0.254
C20:4n6	0.899 ± 0.499	0.764 ± 0.173	0.770 ± 0.231	0.972 ± 0.274	0.382
C20:5n3	0.034 ± 0.015	0.041 ± 0.018	0.049 ± 0.023	0.043 ± 0.019	0.087
C24:1	0.021 ± 0.011	0.020 ± 0.007	0.020 ± 0.009	0.023 ± 0.007	0.773
C22:6n3	0.039 ± 0.019 ^{ab}	0.029 ± 0.009 ^b	0.034 ± 0.013 ^{ab}	0.048 ± 0.015 ^a	0.037

Means in the same row with different superscripts differ (p < 0.05)

C = Control; M = Monensin treatment group; CT= Crude tannin treatment group; ECT= Encapsulated tannin treatment

4.7 Intramuscular fatty acid profile

Table 5 shows the IM FA profile of the Dohne Merino sheep that received different dietary additives. There were only a few FA that showed significant differences between dietary treatments.

In recent years there has been increased interest in the effects of various tannins in the diets of small ruminants due to its effect on meat quality traits. Previous research indicated that tannins affect meat colour and meat FA composition (Priolo *et al.*, 2000; Priolo *et al.*, 2005; Vasta *et al.*, 2007). According to Vasta *et al.* (2009), BH, the process whereby FA are altered to form more SFA, can be interrupted by the presence of tannins in the rumen. This causes more incorporation of UFA in the adipose tissue of ruminants, ultimately making these meat products healthier for human consumption. Similarly in this study dietary tannin additives resulted in higher concentrations of UFA in IM samples.

Figure 5 shows the distribution of SFA, MUFA and PUFA found in the intramuscular FA profile.

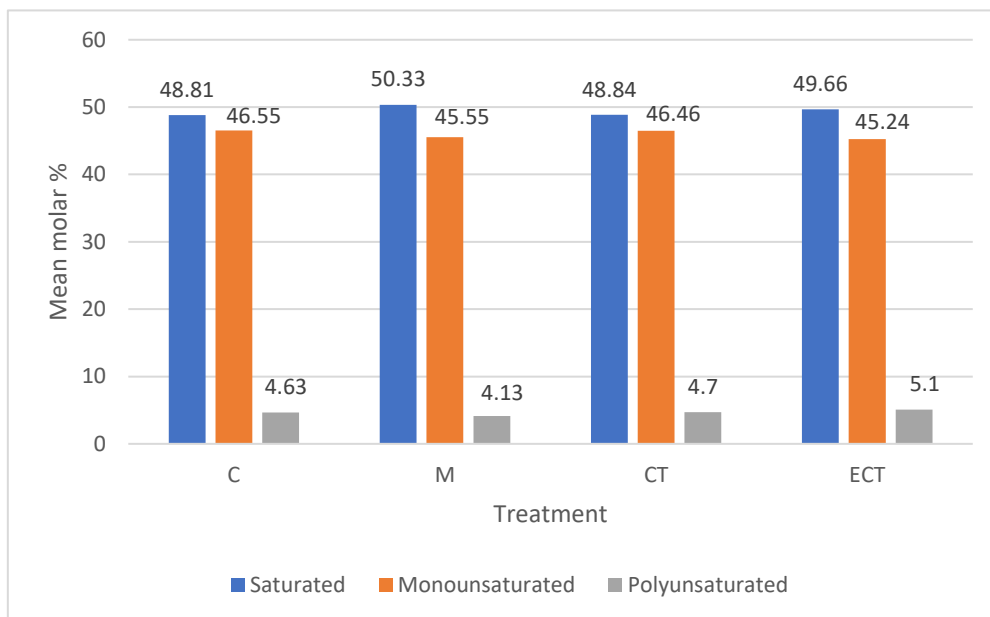


Figure 5. Pooled representation of fatty acids found in the intramuscular fat

The graphed results show that the tannin containing treatments did not have the same effect as seen in the subcutaneous FA representation. The CT treatment had the lowest level of SFA while dietary tannin additives did not have a desirable effect in terms of increasing

MUFA concentrations. It appears that the ECT treatment had the highest concentration of PUFA, with the CT treatment following closely in concentration. The differences between results were NS thus the effects from tannin treatments can be considered negligible.

It appears that dietary tannin additives (ECT and CT) have resulted in higher levels of individual FA. Figure 6 shows C21:0 or heneicosanoic fatty acid compared between dietary inclusion of Monensin and the control group.

In Figure 6, superscripts that differ represent significant differences between treatments.

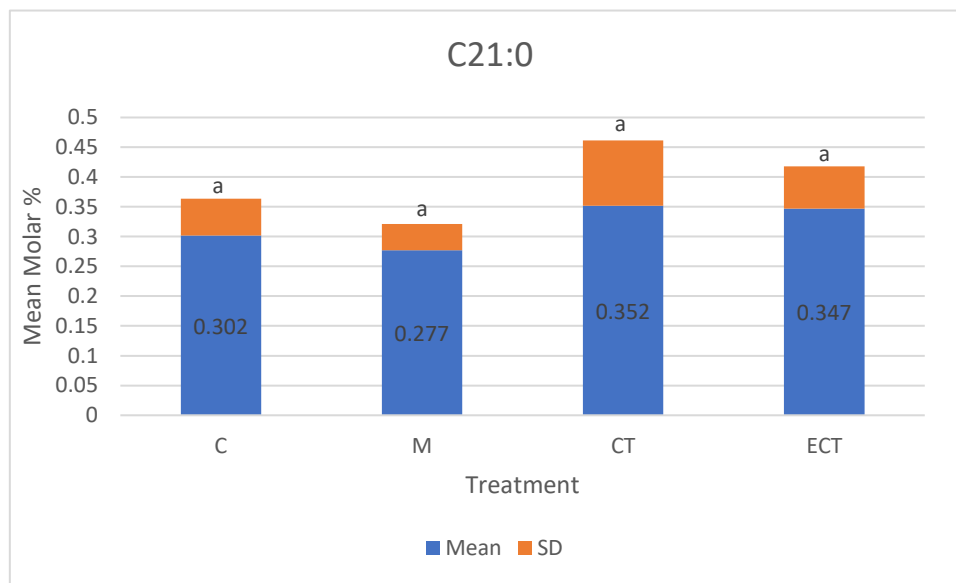


Figure 6. Concentration of C21:0 found in intramuscular fat of each treatment group

Heneicosanoic acid is not considered to be one of the beneficial FA in terms of human health so the increase in C21:0 concentrations for the tannin treatment groups were not necessarily regarded as a co-benefit of dietary tannin additives

In Figure 7, superscripts that represent significant differences between treatments. The C18:3n3 fatty acid concentration in the intramuscular fat of SA Merino sheep is shown in Figure 7. This FA is known as α -linolenic acid, an omega – 3 FA. Its concentration in the intramuscular fat was elevated due to the inclusion of dietary tannin as a methane mitigation additive in the diet of sheep. This can be considered a co-benefit to the methane mitigating effects of dietary tannin additives. C18:3n3 is listed as a beneficial FA for human health and its increase in meat samples could contribute to healthier red meat products. In contrast the lowest concentration for C18:3n3 was seen in the M treatment (Figure 7).

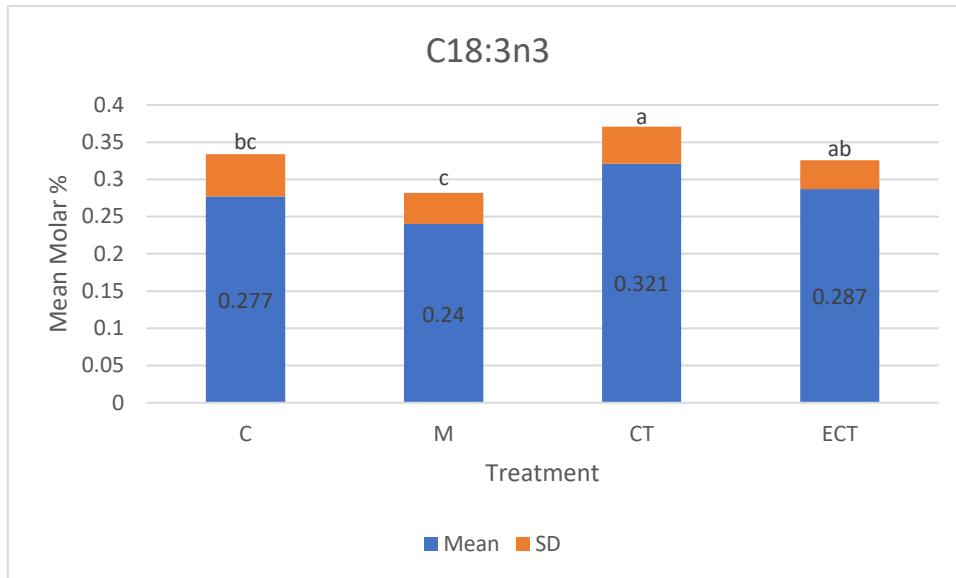


Figure 7. Concentration of C18:3n3 found in intramuscular adipose tissue of each treatment group

In Figure 8, superscripts that differ represent significant differences between treatments.

The C18:2n6t or linoleic acid concentration in intramuscular fat of Dohne Merino sheep is represented in Figure 8. This FA is among the beneficial FA as it falls within the omega - 6 FA group. Inclusion of dietary encapsulated tannin additives increased the concentration of this FA as compared to the M treatment. Again, the M treatment seems to have the lowest C18:2n6t concentration, which means it is less likely to have health benefits for consumers when consuming the meat from sheep that received dietary Monensin additive as compared to a diet containing tannin additives.

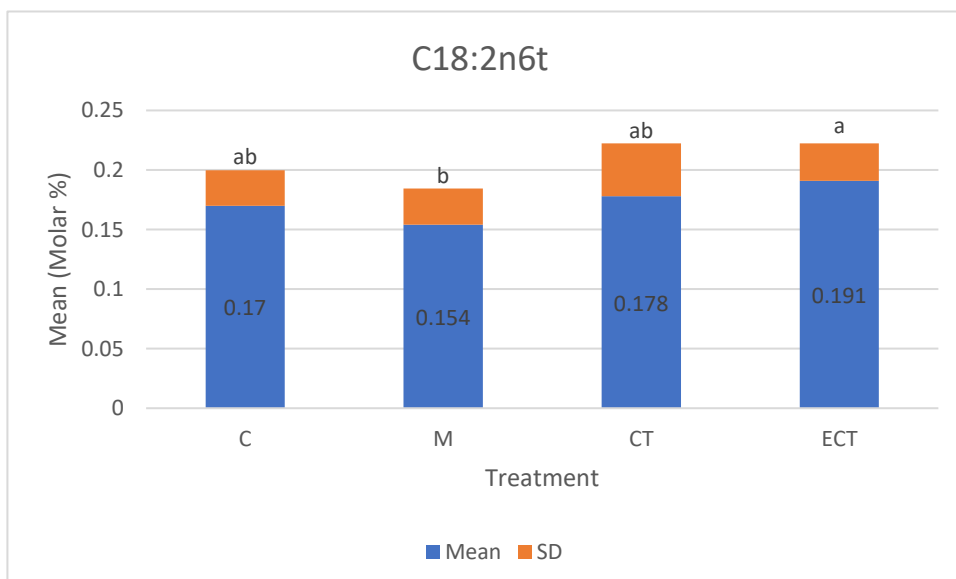


Figure 8. Concentration of C18:2n6t found in intramuscular fat of each treatment group

In Figure 9, superscripts that differ represent significant differences between treatments. C22:6n3 also known as docosahexaenoic acid (DHA) is another FA that is considered beneficial as it falls within the omeg-3 FA group. Dietary inclusion of encapsulated tannin additives resulted in a higher C22:6n3 FA concentration than those sheep on Monensin additive but not as high as the C treatment. The M treatment concentration was most significantly lower than the ECT treatment, suggesting that Monensin might have a negative association with UFA concentration of IM fat.

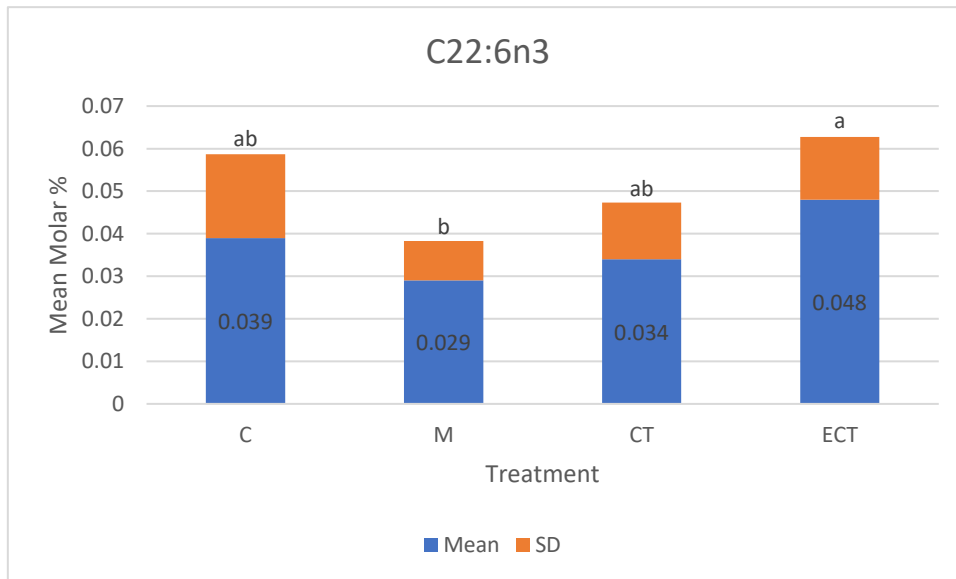


Figure 9. Concentration of C22:6n3 found in intramuscular fat of each treatment group

Chapter 5

Conclusions and critical review

From the results presented in this study the null hypotheses are rejected for some of the parameters monitored.

Acacia mearnsii tannin supplementation had significant effects on FA profile of IM adipose tissue. Whereas dietary inclusion of *Acacia mearnsii* tannin did not have a significant effect on ADG, carcass composition and % total IM fat.

In terms of meat colour, there were significant differences in colour coordinate retention over refrigeration time, but this was seen for all dietary treatment groups. There were also significant differences seen between treatments on both Days 1 and 7 of refrigeration. On both days, treatment M had the highest values for b^* and H^* , denoting greater colour deterioration, and was most significantly different from the other treatments suggesting that Monensin may negatively affect colour stability over time. This could indicate that tannin treatments are more beneficial in terms of meat colour stability than the conventional methods such as Monensin being used at present.

When considering the use of *Acacia mearnsii* tannins as methane mitigation agents, other effects on the animal and animal products should be considered. In this study positive effects associated with the use of tannins as dietary methane mitigation agents were noted. Some of the observed co-benefits include increased UFA concentrations such as linoleic acid, linolenic acid and DHA.

The FA profile for all treatment groups were similar for the subcutaneous FA profile as there were no significant differences found although significant differences were detected in the IM FA profile. Tannin containing treatment groups tended to have significant effects on the beneficial UFA, and in most cases the CT treatment group resulted in most significantly raised concentration.

The *Acacia mearnsii* tannin treatments significantly affected the intramuscular fatty acid profiles of lambs, which included linoleic acid (C18:2n6t), α -linolenic acid (C18:3n3) and DHA (C22:6n3). In all of the UFA where tannin supplementation had a significant effect, an increase in concentration was observed. This could be considered a co-benefit to methane mitigation as the higher concentration of UFA contributes to healthier red meat products. The CT treatment group had the most significant effect at increasing desirable UFA concentration apart from C22:6n3, whereas the ECT treatment showed the most significant increase. This indicated that the tannin treatments had significantly improved meat quality of the treatment

lambs.

Trends of increased concentration in IM FA profile were seen for lauric acid (C12:0) and heneicosanoic acid (C21:0), although not considered significant. Overall, where a significant increase in UFA was seen it was from the tannin containing treatment groups (CT and ECT).

If the use of *Acacia mearnsii* tannins as dietary additive for methane mitigation are successful in lowering the carbon footprint significantly it can be concluded that they have a co-beneficial effect on improving the IM FA profile of meat. Lambs in the CT treatment groups took fewer days to reach target weight, and while the ECT group took a few days longer they did not take as long as the C treatment group animals. Differences between treatment groups for ADG, % fat, % bone, % muscle, % total IM fat were considered negligible. From the above results and with no negative effects on other carcass characteristics observed, consumer resistance would not be expected when implementing *Acacia mearnsii* tannins as methane mitigation agents and could become a co-beneficial treatment for improvement of meat quality.

Critical review and recommendations

Methane mitigation and meat quality research can have limitations as seen in this study. The limitation of 10 lambs per treatment group was accommodated by choosing lambs with the greatest similarity and using a completely randomized design to assign them to treatments. Ideally more lambs per treatment group with more replicates would have been more statistically relevant and accurate. The use of cattle, although more labour intensive than sheep, may be beneficial in terms of recording methane mitigation capacities of treatments as cattle produce higher levels of methane and are more relevant for the use of methane mitigation strategies. In future studies should include a mixture of male and female animals as experimental units and higher concentrations of tannin treatments should be tested.

Some recommendations for future studies related to this topic:

During the growth trial the feed was often not homogenously mixed and in some cases the encapsulation process caused clumps in the feed that had to be mixed by hand, which was ineffective. For future studies it may be better to pellet the feed to ensure consistent ingestion of additives and lower spilling of feed between animals. The feeding troughs were also unstable and easily knocked over causing feed to be wasted and not recorded.

The animals that were ready for slaughter could not be slaughtered at the same time due to the abattoir having capacity for only a small group at a time. This could have affected

the consistency of results of the study. As a result, there were many anomalies seen in the meat colour results and one of these could be attributed to the fact that the meat samples were frozen prior to analysis. This process could have affected meat colour as during the thawing process pigmented fluid is lost from the meat. The reason for freezing prior to analysis was due to the wait for the ethical approval certificate before any analysis could begin.

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Appendix A

Table 6 Representation of animal to block and treatment assignment

<i>Animal ID</i>	<i>Treatment</i>	<i>Block</i>	<i>Pen</i>
19-348	1	1	1
19-381	1	1	1
19-321	1	2	5
19-315	1	2	5
19-341	1	3	9
19-374	1	3	9
19-365	1	4	13
19-334	1	4	13
19-366	1	5	17
19-318	1	5	17
19-380	2	1	2
19-377	2	1	2
19-382	2	2	6
19-373	2	2	6
19-343	2	3	10
19-316	2	3	10
19-349	2	4	14
19-337	2	4	14
19-336	2	5	18
19-327	2	5	18
19-323	3	1	3
19-378	3	1	3
19-310	3	2	7
19-331	3	2	7
19-347	3	3	11
19-373A	3	3	11
19-335	3	4	15
19-344	3	4	15
19-353	3	5	19
19-367	3	5	19
19-379	4	1	4
19-346	4	1	4
19-361	4	2	8
19-358	4	2	8
19-342	4	3	12
19-325	4	3	12
19-364	4	4	16
19-362	4	4	16
19-357	4	5	20
19-322	4	5	20

Appendix B



UNIVERSITEIT VAN PRETORIA
UNIVERSITY OF PRETORIA
YUNIBESITHI YA PRETORIA

Animal Ethics Committee

PROJECT TITLE	Effects of <i>Acacia mearnsii</i> Condensed Tannin Encapsulated in different oils on rumen fermentation, methane emission, nitrogen balance and performance of merino sheep
PROJECT NUMBER	EC075-17
RESEARCHER/PRINCIPAL INVESTIGATOR	SL Ibrahim

STUDENT NUMBER (where applicable)	U_16270542
DISSERTATION/THESIS SUBMITTED FOR	PhD

ANIMAL SPECIES	Merino	
NUMBER OF ANIMALS	54	
Approval period to use animals for research/testing purposes		November 2017- November 2018
SUPERVISOR	Dr. A Hassen	

KINDLY NOTE:

Should there be a change in the species or number of animal/s required, or the experimental procedure/s - please submit an amendment form to the UoP Animal Ethics Committee for approval before commencing with the experiment

APPROVED	Date	9 January 2018
CHAIRMAN: UoP Animal Ethics Committee	Signature	



Faculty of Veterinary Science
Animal Ethics Committee

6 October 2020

**Approval Certificate
New Application**

AEC Reference No.: NAS201/2020
Title: Effects of dietary Acacia mearnsii tannin additives on the carcass characteristics of lambs
Researcher: Miss M Venter
Student's Supervisor: Prof EC Webb

Dear Miss M Venter,

The **New Application** as supported by documents received between 2020-09-15 and 2020-10-02 for your research, was approved by the Animal Ethics Committee on its quorate meeting of 2020-10-02.

Please note the following about your ethics approval:

1. The use of species is approved:

Species and Samples	Number
Sheep	
Meat cut (stored /retrospective samples)	38 (EC075-17)

2. Ethics Approval is valid for 1 year and needs to be renewed annually by 2021-10-06.
3. Please remember to use your protocol number (NAS201/2020) on any documents or correspondence with the AEC regarding your research.
4. Please note that the AEC may ask further questions, seek additional information, require further modification, monitor the conduct of your research, or suspend or withdraw ethics approval.
5. **All incidents** must be reported by the PI by email to Ms Marleze Rheeder (AEC Coordinator) within 3 days, and must be subsequently submitted electronically on the application system within 14 days.
6. As part of your approval, the committee requires that you record a **short video footage** of major animal procedures approved in your study. **The committee may request them for monitoring purposes at any later point.**

Ethics approval is subject to the following:

- The ethics approval is conditional on the research being conducted as stipulated by the details of all documents submitted to the Committee. In the event that a further need arises to change who the investigators are, the methods or any other aspect, such changes must be submitted as an Amendment for approval by the Committee.

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