Effect of plant extracts used as a methane mitigation additive on carcass fat content and fatty acid composition of South African Merino sheep

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ABSTRACT

There is growing apprehension about the use of antibiotics such as monensin in the reduction of enteric methane emissions. This has initiated the utilisation of multipurpose medicinal plants as an alternative, although there are limited efforts to understand the effect of medicinal plant extracts on animal product quality. This research used *Moringa oleifera* and *Azadirachta indica* (A. Juss) leaf extracts to replace monensin with aim of assessing the effects of the plant extract on tissue fat content and fatty acid composition of South African (SA) Mutton Merino sheep. The experimental treatments involved 40 ram lambs in a randomised complete block design into four feed additive treatment levels (i.e., negative-control, *Azadirachta indica*, *Moringa oleifera,* and monensin). Twenty-four sheep were randomly selected at 60 - 65kg bodyweight, slaughtered, and the *post mortem* samples were collected over a 23 weeks trial period.

The univariate analysis revealed a significant increase ($p < 0.05$) in the carcass fat content of the *Azadirachta indica* treatment group as compared to the monensin and *Moringa oleifera* groups, even as the groups were adjusted for any difference in the initial body weight. On the other hand, the multivariate analyses on the subcutaneous fatty acids showed a significant rise ($p < 0.05$) in polyunsaturated fatty acids of the monensin treatment group. The *Moringa oleifera* group resulted in a significant increase ($p < 0.05$) in monounsaturated fatty acids molar composition. The interaction effect of days-on-trial with lipid pigment absorbance was significantly higher in the negative control group compared to monensin and *Azadirachta indica* treatment groups. These results indicate that the addition of either *Moringa oleifera* or monensin reduces carcass fat and improves the unsaturated fatty acid proportion in SA Mutton Merino sheep. *Azadirachta indica* treatment increased the carcass fat content and the non-essential fatty acid proportion. However, both monensin and *Azadirachta indica* groups caused limited deposition of carotenoid (or lipid pigment) in the adipose tissue.

TABLE OF CONTENTS

ACKNOWLEDGMENTS .... **i**
ABSTRACT .... **ii**
TABLE OF CONTENTS .... **iii**
LIST OF TABLES .... **vi**
LIST OF FIGURES .... **vii**
CHAPTER 1: INTRODUCTION **1**
1.0 Background 1
1.1 Problem statement 2
1.2 Objectives 3
1.2.1 General objective 3
1.2.2 Specific objectives 3
1.3 Hypotheses 3
CHAPTER 2: LITERATURE REVIEW **4**
2.0 Constraints of the use of dietary additives such as monensin sodium in feedlot animals 4
2.1 Effect of monensin sodium on ruminant animal 5
2.2 Medicinal properties of *Moringa oleifera* and *Azadirachta indica* (A. Juss) 5
2.2.1 *Moringa oleifera* (Moringa or horseradish tree) 5
2.2.2 Azadirachta indica (A. Juss) (Neem or Siamese Neem tree) 6
2.3 Potential bioactivity of the plant extracts on ruminal microflora 7
2.4 Carotenoid as natural antioxidants 8
2.4.1 Nature and bioavailability of carotenoid 8
2.4.2 Antioxidative properties of carotenoid 9
2.4.3 The concept of fat colour determination 10
2.4.4 Effect of additive plant extracts on the pigment of SA Mutton Merino sheep subcutaneous fat 11
2.4.5 Biosynthesis of isoprenoid: a cholesterol overview 11
2.4.6 Brief history of the mevalonate pathway 11
2.4. Overview of cholesterol biosynthesis 12
2.5 Lipid metabolism in ruminant animal 12
2.5.1 Fatty acid synthesis in the liver and adipose tissue of ruminant 13
2.5.2 Long-chain fatty acids desaturation and chain elongation in ruminant 14
2.5.3 Esterification and deposition of lipid in ruminant adipose tissue 15
2.5.4 Non-essential fatty acids in ruminant animal 16
2.5.5 Essential fatty acids in ruminant animal 16

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2.6 The concept of aggregate lipid extractable 17

CHAPTER 3: MATERIALS AND METHODS 18

3.0 Ethics approval statement 18

3.1 Experimental site 18

3.2 Diet 18

3.3 Preparation of test ingredients 18

3.4 Experimental design and management 19

3.5 The Sampling of South African Mutton Merino sheep rib-cut, subcutaneous fat, ... 19

and intramuscular fat samples 19

3.6 Soxhlet total lipid determination 19

3.6.1 Sample preparation 19

3.6.2 Procedure 20

3.6.3 Dry material determination 21

3.6.3.1 Intramuscular fat percentage on a DM basis converted to As-is basis 21

3.7 Fat pigment determination. 21

3.7.1 Preparation of reagent 21

3.7.2 Procedure 21

3.7.3 Fat colour chart 22

3.8 Long-chain fatty acid analysis 22

3.8.1 Sample handling and preparation 22

3.8.1.1 Digestion of subcutaneous fat sample 22

3.8.1.2 Sample extraction 23

3.8.1.3 Methylation of lipid extract 23

3.8.2 Identification and quantification of fatty acids 23

3.9 Statistical methods 24

3.9.1 Bivariate correlation analysis 24

3.9.2 Factor analysis 24

3.9.3 Transformed standard score 25

3.9.4 One sample chi-square (χ^2) test 25

3.9.5 One-way analysis of covariance (ANCOVA) 26

3.9.6 One-way multivariate analysis of variance (MANOVA) 26

CHAPTER 4: RESULTS 28

4.1 Regularity or covaried relationship among pairs of variables 28

4.2 Principal component index solution 29

4.2.1 Exploratory data analysis of physiological parameters 29

4.3 SA Mutton Merino sheep (subcutaneous) fat colour characteristics goodness of fit tests 30

4.3.1 Before lipid extraction 30

4.3.2 After lipid extraction 31

4.4 Relationship between soluble lipid pigment absorbance and treatment levels following covariate interaction 31

4.5 Between-the-group difference in one-way ANCOVA for fat content component scores 34

4.6 Linear combinations and between-the-group difference in SA Mutton Merino sheep subcutaneous fatty acid composition 36

4.6.1 Fatty acids molar percentage (%) 36

4.6.2 Gravimetric fatty acids content (mg/g) 39

CHAPTER 5: DISCUSSION 43

5.1 Discussion approach 43

5.2.1 Differences in fat-soluble pigment absorbance relative to the additive treatments used along with days-on-trial 43

5.2.2 The implication on the carotenoid antioxidative capability 46

5.2.3 Body weight and fat component (*T*) score 46

5.2.4 Potential effect of *Moringa oleifera* and *Azadirachta indica* (A. Juss) on the South African Mutton Merino fatty acid synthesis. 48

5.2.5 The implication on meat quality 50

5.3 Effect of feed additives treatment on SA Mutton Merino sheep subcutaneous fatty acid composition 50

5.3.1 Interaction between carcass fat and polyunsaturated fatty acid ratio (PUFA n-6/n-3) 52

5.3.2 The implications for consuming public 53

5.3.3 Overview of feed additive treatment main effect on physiological parameters 54

CHAPTER 6: CONCLUSION AND RECOMMENDATIONS 57

REFERENCES 58

APPENDICES 63

LIST OF TABLES

Table 4.1 Correlations between dependent variables (N = 24) 29

Table 4.2.1 Means and standard deviation of subcutaneous fat-soluble pigment absorbance 33

Table 4.2.2 Estimated means and absorbance pairwise comparisons of the days-on-trial levels 33

Table 4.3 Descriptive statistics and pairwise assessment of fat content component T-scores 35

Table 4.4.1 Descriptive statistics and multiple comparisons of fatty acids molar percentage 39

Table 4.4.2 Descriptive statistics and multiple comparisons of fatty acids gravimetric content 42

LIST OF FIGURES

Figure 4.6: Adjusted mean estimates across the treatment levels 36

Figure 5.1: The graphical illustration of the feed additives treatment distribution 47

Figure 5.2: The interaction effect of PUFA ratio on carcass fat due to feed additive treatment 54

Figure 5.3: The feed additive treatment main effect on two-dimension PCA-plot 56

CHAPTER 1: INTRODUCTION

1.0 Background

In recent years there have been concerns about the use of antibiotics in the livestock industry, and this has initiated the search for natural feed additives with antimicrobial properties. The medicinal plants with antimicrobial properties have the potential to modify ruminant animal gut fermentation with positive or adverse aftereffects. In this way, a Phytochemical or plant bioactive agent capability lies in its potential pharmacological or toxicological effects in humans and animals (Durmic & Blache, 2012). However, the current knowledge on many bioactive plants of ethnoveterinary importance is inadequate, and reports of customary practices in a lot of developing nations are either incomplete or lost (Durmic & Blache, 2012).

In contrast to the natural feed additives, conventional feed-based antibiotics' effect on ruminant animal performance and product quality are well documented (Benchaar *et al.*, 2006; Duffield *et al.*, 2008a; Fatahnia *et al.*, 2010). Thus, understanding the effect of natural medicinal plant extracts as a potential substitute for conventional feed-based antibiotics is also crucial. There are several phytochemical plants of tropical species, of which only two were considered in this study namely: Horseradish/drumstick tree (*Moringa oleifera*) and Neem tree (*Azadirachta indica* A. Juss).

Horseradish and Neem tree are multipurpose trees of industrial, medicinal, and economic significance, which are both native to Asian countries like India, Pakistan and also found in some African countries such as Nigeria. Several studies (Makkar & Becker, 1996; Landau *et al.*, 2009; Lim, 2014; Gopalakrishnan *et al.*, 2016) have reported some phytochemicals present in both plants such as tannins, sterols, terpenoids, flavonoid, saponin, which affect the rumen microorganisms and may alter rumen fermentation products.

Moringa and Neem plant parts contain lipid and its derivatives, with a vast range of functional groups that could be utilised as substrates for microbial fatty acid biosynthesis. Microbial biohydrogenation of dietary polyunsaturated fatty acids (PUFA) is constrained by the amount, type of lipid supplement, basal diet, and the activity of the bacteria colony, leading to differences in tissue fatty acid deposition (Bessa *et al.*, 2007). Thus, a complex interrelationship exists between dietary factors and rumen metabolic processes, which impact the fatty acid (FA) composition of animal tissue.

Another unique effect of phytochemical plants on tissue fatty acids (FA) is their antioxidant properties in tissue. Horseradish and Neem extracts are rich in carotenoid, flavonoid, vitamins, and mineral elements (i.e. Fe^{+}, Cu^{2+}), which form part of apoproteins and antioxidant enzymes in the blood (Kumar *et al.*, 2012; Fahey, 2017). Ordinarily, antioxidants are situated in the lipid

membrane in trace amounts, but, the susceptibility of FA to free-radical is more or less proportional to the degree of unsaturation (Cadenas *et al.*, 1977). In as much as all components of free radical oxidation are present in a living cell, the mechanism of antioxidizing agents is to protect the structural integrity of the cell.

On the contrary, since the disruption of structural integrity according to Halliwell (2011) initiates the activity of free radicals thus, the protection of structural integrity does not seem to be the overall mechanism of antioxidants.

1.1 Problem statement

The use of phytochemical feed additives in the livestock industry has received a lot of attention lately. This is as a result of the positive response observed on animal health, animal performance, and reduced impact of animal waste on the environment. However, comparative efforts have not been made to assess the effect of phytochemical feed additives on animal meat quality, especially concerning fatty acid content and composition. Even so, there is an increasing demand for quality animal products with a substantial reduction in the use of livestock synthetic chemicals (Durmic & Blache, 2012).

1.2 Objectives

1.2.1 General objective

- To assess the effect of *Azadirachta indica* (A. Juss) and *Moringa oleifera* extracts on the tissue fat content and fatty acids composition of South African Merino sheep.

1.2.2 Specific objectives

a. To evaluate the effects of selected plant extracts on tissue fatty acid synthesis.

b. To determine the effect of selected plant extracts on fatty acids composition of subcutaneous fat.

c. To evaluate the effect of selected plant extracts residue on Merino sheep tissue fat.

1.3 Hypotheses

a. H_0 = The plant extracts additives will not influence the fat content of South African Mutton Merino sheep

   H_1 = The plant extracts additives will influence the fat content of South African Mutton Merino sheep.

b. H_0 = The plant extracts additives will not influence the fatty acid composition of South African Mutton Merino sheep.

   H_1 = The plant extracts additives will influence the fatty acid composition of South African Mutton Merino sheep

CHAPTER 2: LITERATURE REVIEW

2.0 Constraints of the use of dietary additives such as monensin sodium in feedlot animals

According to Adesogan (2007) feed additives are generally non-nutritive components of animal feed, added to optimise nutrient utilisation, improve animal performance, reduce the incidence of metabolic disease, and minimise the impact of animal waste on the environment. In this way, a typical diet contains feed preparations together with feed additives intended for desired objectives, essentially those used as a growth promotant, disease prevention, and control (Fajt 2007). One of the most conventionally used feed additives in ruminant animal husbandry is monensin sodium (i.e., Rumensin™). Fajt (2007) further stated that feed additives utilised as therapeutic drugs to achieve a reduction in the population of microorganisms or pathogen load are commonly referred to as growth promotants.

Similarly, Monensin was described by Ipharraguerre & Clark (2003) as an organic fermentation product of actinomycetes, which was first developed in the 1950s to prevent and control coccidiosis in poultry. The use of Monensin sodium (i.e. monensin) as an additive for modifying ruminal microbiota, became common practice in the late 1970s among dairy cattle farmers (Adesogan, 2007). This implies that the therapeutic use of monensin was originally intended for poultry, whereas the use of monensin in ruminant nutrition (from the mid-1970s) was described by Ipharraguerre & Clark (2003) as subtherapeutic use. This was why Fajt (2007) argued that subtherapeutic use of antibiotics in livestock production is inappropriate. As the treatment could potentially lead to the emergence of antibiotics resistance to microbes, and this may be passed on to the consuming public.

Perhaps, these drug-resistant-strain microbes or pathogens from a host animal would consequently make the human disease more difficult to treat with antibiotic therapy. For instance, resistant strains of *Enterococcus faecium* and *Enterococcus faecalis* had been discovered in pigs and broiler chickens respectively (Fajt, 2007). Despite the ban on monensin sodium and several other growth promotants by the European Union on the 1st of January, 2006, debates on the danger posed by the additive antibiotics continue to generate discussions around the world. Fajt (2007) also noted concerns regarding animal care and welfare, and this propelled further the yearnings for improved livestock practice and quality products free of synthetic chemical inputs.

2.1 Effect of monensin sodium on ruminant animal

Monensin is generally known as a carboxylic polyether ionophore produced by fermentation of *Streptomyces cinnamonesis* or actinomycetes, and it is supplied in the ruminant diet as a sodium salt (Ipharraguerre & Clark, 2003; Adesogan, 2007; Duffield *et al.*, 2008b; Fatahnia *et al.*, 2010; Vendramini, 2019). Vendramini (2019) described monensin as a cation-proton antiporter that causes a constant change in the cell membrane ion by employing sodium (Na^+) and potassium (K^+) substitution.

Similarly, monensin's profound antimicrobial action holds sway over Gram-positive bacteria, as the bacteria lack a protective barrier that can prevent the outflow of intracellular K^+ and inflow of extracellular proton (H^+) and Na^+ (Adesogan, 2007; Vendramini, 2019). More specifically, Vendramini (2019) discussed the modeling of *Streptococcus bovis* (i.e., a Gram +ve ruminal bacteria), which revealed the influx of H^+ in exchange for intracellular Na^+ ion. Consequently, the intracellular pH of the Gram +ve bacteria becomes acidic due to the high affinity of monensin for Na^+ leading to the eventual death of the bacteria cell, and a decrease in the population of Gram +ve bacteria (Adesogan, 2007; Duffield *et al.*, 2008b; Vendramini, 2019).

It is known that monensin additive increases the efficiency of energy utilisation in ruminant animals, which ultimately gives rise to reduced production of greenhouse gas such as methane (Adesogan, 2007; Fatahnia *et al.*, 2010; Vendramini, 2019). Similarly, Adesogan (2007) explained that monensin improves energy utilisation via inhibition of ruminal microflora (such as Gram +ve bacteria & methanogens) responsible for the less desirable metabolites produced in the rumen (i.e., H^+, acetate, lactate, formate). Thereby creating a sink for hydrogen by reducing the acetate to propionate ratio in the rumen. Duffield *et al.* (2008) & Fatahnia *et al.* (2010) also reported studies on monensin additive in the total mixed ration (TMR) fed to the lactating dairy cows which effect a significant increase in milk yield, conjugated linoleic acid, and unsaturated fatty acid contents. The improvement in dairy animal performance was because ionophore such as monensin increases the post-ruminal supply of nutrients including the product of incomplete biohydrogenation such as conjugated linoleic acid.

2.2 Medicinal properties of *Moringa oleifera* and *Azadirachta indica* (A. Juss)

2.2.1 *Moringa oleifera* (Moringa or horseradish tree)

Moringa oleifera tree is generally found in all tropical regions of the world, as it is popularly known as the horseradish tree, miracle tree, or Moringa tree (Fahey, 2017). The leaves of *Moringa oleifera* are rich in minerals (such as potassium, iron, zinc, magnesium & copper), water-soluble vitamin (i.e., vitamin C), fat-soluble vitamin (i.e., vitamin A, D & E), vitamin B

complex (i.e., niacin, pyridoxine), and phytochemicals (such as tannins & flavonoids) (Kumar *et al*., 2012; Gopalakrishnan *et al*., 2016; Fahey, 2017). Moringa is also rich in carotenoid and soluble protein yet, the most important medicinal bioactive compound of *Moringa oleifera* is pterygospermin and glucosinolates. As reported by Fahey (2017) a group of scientists from the University of Bombay, Travancore University, and the Indian Institute of Science in Bangalore isolated a compound named pterygospermin in *Moringa oleifera*, which can be broken down into two molecules of benzyl isothiocyanate.

Similarly, *Moringa oleifera* glucosinolates are readily transformed into isothiocyanate by myrosinase enzyme, meanwhile, benzyl isothiocyanate is known for its antimicrobial properties against a wide range of bacteria (such as *Staphylococcus aureus*), and fungi (Kumar *et al*., 2012; Gopalakrishnan *et al*., 2016). Kumar *et al.* (2012) also reported rhamnosylated glucosinolates phytochemicals contained in the aqueous and ethanolic Moringa leaf extract to effect hypotensive, antibacterial, and antioxidant activity in guinea pig ilea and frog heart. Apart from rhamnosylated glucosinolates, other phytochemicals that constitute the total antioxidant capacity of *Moringa oleifera* include β-carotene, phenolic compounds, vitamins, and minerals.

2.2.2 Azadirachta indica (A. Juss) (Neem or Siamese Neem tree)

Azadirachta indica (A. Juss) or Neem tree is native to Southeast Asia (which include Pakistan, India, Bangladesh, Myanmar, Sri Lanka), even although the Neem tree had been established in the tropics and the sub-tropical regions of the world (especially in Africa and Middle-east) (Tarbell, 1957; Sithisarn *et al*., 2006; Lim, 2014). The phytochemicals in *Azadirachta indica* (A. Juss) are separated into two groups namely; isoprenoids and non-isoprenoids, over 150 isoprenoid compounds were isolated from several parts of the Neem tree (Tarbell, 1957; Lim, 2014). The phytochemical compounds unique to *Azadirachta indica* (A. Juss) leaves (including isoprenoid and non-isoprenoid) are nimbolide (such as meliacin), tetranortriterpenoid, salannin, β-carotene, Nimbin, quercetin glycosides, pentanortriterpenoids, nimbaniol, isopenylated flavanone, azadirachtin, stigmasterol, scopoletin (coumarin), isoazadirolide, nimocinol, limonoids, tri-and tetra sulphide, and kaempferol (Lim, 2014).

Furthermore, the phytochemicals obtained in the form of extracts were reported by Tarbell (1957) & Lim (2014) which featured a wide range of pharmacological properties, including antimalarial, antimicrobial, antifungal, anti-inflammatory, antioxidant, nematocidal, antitubercular, antiparasitic, antiulcerogenic, and immune-modulatory. Lim (2014) also reported the reduction in the degree of lipid peroxidation as a result of Neem treatment, which indicates the antioxidative and anticarcinogenic effect of *Azadirachta indica* (A. Juss) extracts.

2.3 Potential bioactivity of the plant extracts on ruminal microflora

Moringa oleifera and *Azadirachta indica* (A. Juss) leaf extracts, as well as monensin used as dietary feed additives exert their major effect in the rumen of ruminant animals (i.e., South African Mutton Merino sheep). This marks the essential prerequisite for any feed additive used in the ruminant animal diet as stated by Adesogan (2007). The effect of these feed additives is primarily due to the modification of the ruminal microflora-colony involved in the fermentation process.

Many bioactive compounds sequestered from the leaf and other parts of both *Moringa oleifera* and *Azadirachta indica* (A. Juss) plants were described by Kumar *et al.* (2012), and Lim (2014) as having a wide range of pharmacological mix. Most especially the antimicrobial properties of phenolic phytochemicals, which could potentially change the composition of rumen microflora and the fermentation products in an unprecedented way. Furthermore, Puupponen-Pimiä *et al.* (2001) discussed the two well-known theories of the antimicrobial mechanism of phytochemical compounds, namely genotoxicity and the degree of hydroxylation of the compound.

The mechanism of the two theories as noted by Puupponen-Pimiä *et al.* (2001) is closely related to the ionophores (such as monensin) mode of action. In this way, the variations in the susceptibility of different bacterial cell membrane structures and sensitivity to the antimicrobial agent, demonstrate the plant extracts bioactivity. Also, ionophore penetrates the outer cell membrane of Gram +ve bacteria and initiates outflow of intracellular (k^+) ion and influx of extracellular hydrogen (or Na^+) ion. This continuous transaction of ions results in bacteria cell death (Adesogan, 2007). Whereas phytochemicals such as flavonoids do not compromise the integrity of bacterial cell membranes rather the phenolic compounds cause mutation of the bacteria Deoxyribonucleic acid (DNA), as a result, the bacteria subsistence is impaired (Puupponen-Pimiä *et al.*, 2001).

Puupponen-Pimiä *et al.* (2001) discussed the antimicrobial action of phenolic compounds against (Gram -ve) *Escherichia coli* (*E. coli*) CM 871 mutant strain as well as *E. coli* CM 50 normal strain. According to Puupponen-Pimiä *et al.* (2001), the mutation caused by the phenolic compounds was more severe on the *E. coli* CM 871 mutant strain, whereas the mutagenic effect on *E. coli* CM 50 was mild in comparison. The reason suggested for such variation was that *E. coli* CM 871 lacks a DNA patching mechanism after the deleterious effect of phenolic compounds. Similarly, the rationale for selective antimicrobial action against the Gram -ve bacteria in the study carried out by Puupponen-Pimiä *et al.* (2001) was attributed to the hydrophobic (or lipophilic) nature of Gram -ve cell membrane. Notably, polyphenolic

compounds are lipophilic hence, lipophilic phytochemicals can and may infiltrate lipophilic cell surface structure.

Comparatively, the degree of hydroxylation theory was also tested with quercetin, kaempferol, and myricetin phenolic extract by Puupponen-Pimiä *et al.* (2001). It was found that the flavonoid quercetin and kaempferol were not effective against Gram -ve bacteria whereas myricetin showed effective inhibition of Gram -ve bacteria. Also, subsequent assays carried out showed contradictory results from previously tested flavonoids. Therefore, Puupponen-Pimiä *et al.* (2001) resolved that non-polar (or neutral) flavonoids express more consistent effectiveness, and the pattern of hydroxylation of the cyclic ring does not indicate efficacy.

On the contrary, the bioactive compounds found in *Azadirachta indica* (A. Juss) as reported by Tarbell (1957) and Lim (2014) are well beyond flavonoids and other phenolic compounds. Neem bioactive constituents are partitioned into isoprenoids (largely diterpenoid, triterpenoid, and limonoids) and non-isoprenoids (mainly polyphenolic compounds) (Lim, 2014). Both diterpenoid and triterpenoid are not glycerol-based lipids, but alcohol or aldehyde derivatives which are known as Essential oil (Hart *et al.*, 2008). In contrast to (Puupponen-Pimiä *et al.*, 2001) hypothesized polyphenolic mode of action, Hart *et al.* (2008) believe volatile oils damage the microbial cell membrane in a similar mode of action as monensin. The mechanism of volatile oil as stated by Hart *et al.* (2008) is either through the disruption of the microbial lipophilic cell structure or via impairment of the peptidoglycan layer to the detriment of cytosolic constituents, resulting in cellular exhaustion and eventual death.

This perhaps explains why *Azadirachta indica* (A. Juss) phytochemicals are effective against Gram +ve and Gram -ve bacteria as indicated by Lim (2014). Nevertheless, the antimicrobial actions of plant extracts are naturally synergistic or additive. This implies that many phytochemicals in consortium with yet to be identified bioactive compounds may contribute to the overall plant extract antimicrobial capacity.

2.4 Carotenoid as natural antioxidants

2.4.1 Nature and bioavailability of carotenoid

Pajkovic & van Breemen (2005) described carotenoid as a polyisoprenoid compound found in foods (i.e., fruits & vegetables) and synthesised by microorganisms and plants. Out of 600 identified carotenoids, only a few (approximately 50) are of vitamin A precursor, and this vitamin is important for cellular growth (El-Agamey *et al.*, 2004; Pajkovic & van Breemen, 2005). According to Paiva *et al.* (2013) carotenoids are categorized into two main groups namely: oxygenated carotenoid or xanthophylls (such as lutein), and hydrocarbon carotenoid (such as β-carotene). The biosynthesis of the two forms of carotenoid is similar however, the addition of oxygen molecules to the hydrocarbon carotenoid results in the realisation of

xanthophylls. Whereas, the `enzymatic cyclisation of the acylic end groups results in the formation of β-carotene (Paiva *et al.*, 2013). The prevailing enzyme determines the form and concentration of carotenoids in foods, as well as plant variety, storage condition, stage of growth, and harvesting period.

Furthermore, the metabolism of carotenoid ingested in animals or humans is impacted by the dietary source of carotenoid, interaction with other dietary resources, and the fate of carotenoid cleavage to retinol in the intestinal lumen (i.e., central or asymmetric cleavage) (Parker, 1996; Paiva *et al.*, 2013). According to Parker (1996) dissipation of carotenoid in the bulk lipid of the stomach or intestinal lumen develops into the multilamellar lipid vesicles as a result of digestive enzyme activity (such as lipase). Consequently, the structural characteristics of each carotenoid affect its inclusion into the micellar lipid composition (Parker, 1996), which implies the micellar assimilation is the rate-limiting step for carotenoid absorption in animals or humans. Parker (1996) also reported a study using an everted rat gut sac, which revealed that the passage of carotenoid from micellar lipid composition into the duodenum mucosal was via passive diffusion, and the rate was mainly affected by the carotenoid concentration gradient.

Subsequently, the chylomicron transports carotenoid from duodenum mucosal into the blood circulation through the lymphatics and delivers to the plasma lipoprotein (i.e., the exclusive carrier for carotenoid in the bloodstream) (Parker, 1996). Similarly, the dispersion of carotenoids between the lipoprotein classes as reported by Parker (1996) happens to be affected by the structural characteristics of different carotenoids, and the lipid composition of the plasma lipoprotein. However, when the concentration of hydrocarbon carotenoids was expressed per mole of lipoprotein constituents, β-carotene was not proven to be greater in low-density lipoprotein (LDL) compared to high-density lipoprotein (HDL) (Parker, 1996; Yang *et al.*, 2004). Therefore, the distribution of carotenoids in lipoprotein composition is less defined and comprehensible.

2.4.2 Antioxidative properties of carotenoid

Apart from the biotransformation to retinol, antioxidant properties of unmetabolized carotenoids are the foremost important carotenoid bioactivity in a biological system (Pajkovic & van Breemen, 2005). Its ability to act as a natural biological antioxidant is based on singlet oxygen blocking or chain-breaking properties, which consequently trap free radical oxidation (Paiva *et al.*, 2013). According to Parker (1996) and Pajkovic & van Breemen (2005), the structural properties of carotenoid concerning the conjugated polyene chain affects the ability of carotenoid to break the chain of free radical activity, and in so doing protecting the plants or lipid molecules from photooxidation and lipid peroxidation respectively. The structural

characteristics also influenced the carotenoid orientation around the lipid bilayer, and perhaps modifies the surrounding bilayer membrane permeability and fluidity (El-Agamey *et al.*, 2004).

Furthermore, the degree to which carotenoids are deposited in the adipose tissue, and the level of antioxidant protection in the biological system is not fully understood (Parker, 1996). Nevertheless, Paiva *et al.* (2013) stated that antioxidative carotenoids promptly react with lipid peroxyl radical compared to the mono or polyunsaturated acyl carbon, resulting in carotenoid adduct radical formation. The carotenoid adduct radical is very stable, relatively unreactive, and afterward as stated by Paiva *et al.* (2013) the adduct experiences degeneration to produce reduced products. On the contrary, an *in vitro* study discussed by El-Agamey *et al.* (2004) using cell culture revealed that the efficacy of carotenoid antioxidant capacity was not only reduced at high concentrations, but even the carotenoid itself also acted as a pro-oxidation mediator.

El-Agamey *et al.* (2004) also indicate that under a high concentration of β-carotene and ample oxygen, the β-carotene behaved as a pro-oxidant. This was suggested to be a result of a higher autoxidation rate of the carotenoid. Further suggestions put forward by El-Agamey *et al.* (2004) suggest that in the presence of high carotenoid concentration, the permeability of the bilipid layer membrane to toxins and reactive species is increased. As a result, there would be a potential increase in the formation of more reactive carotenoid adduct radicals, and this would further initiate lipid peroxidation. Nevertheless, the rationale behind the decrease in carotenoid antioxidant activity at high concentrations is equivocal, even as the phenomenon does occur.

2.4.3 The concept of fat colour determination

The ruminant animal fat pigmentation is a natural phenomenon that has economic significance on meat quality assessment, and the incidence is commonly referred to as yellow fat (Kirton *et al.*, 1975; Webb EC, & Casey NH, 1999; Yang *et al.*, 2004; Hill, 2018). The focus of this study is on fat coloration as a consequence of the dietary accumulation of carotenoids in animal tissue (Yang *et al.*, 2004).

Carotenoids are lipid-soluble pigments, characterized by vivid colours, and synthesized by microorganisms and plants (Parker, 1996; El-Agamey *et al.*, 2004). The natural pigment could be separated into hydrocarbon carotenoid, with enzymatic cyclization of end group (e.g. β-carotene) and oxygenated carotenoid, characterized by the extension of the acyclic group by oxygen molecule (e.g. lutein) (Pajkovic & van Breemen, 2005). Several researchers (Britton, 1995; El-Agamey *et al.*, 2004; Pajkovic & van Breemen, 2005; Paiva *et al.*, 2013) have dealt with understanding the properties of carotenoids as well as their association with biomolecules, and functions in living organisms.

The well-known physical effect of carotenoids is the ability to absorb light energy, and the absorption spectrum of carotenoids ranges from 400nm – 500nm (Pajkovic & van Breemen, 2005). Carotenoid light absorption plays an important role in photosynthetic plants, and this is noticeable with a wide range of colours in fruits, vegetables, and the colouration of birds plumage (Britton, 1995). Deposited carotenoids in animal tissues are naturally hydrophobic and prevalent in the lipophilic region such as adipose tissue (Britton, 1995; Parker, 1996). This explains the rationale for the hydrolysis of lipid as the general technique used for extracting fat pigment (Kirton *et al.*, 1975; Webb EC, & Casey NH, 1999; Christie & Han, 2016).

2.4.4 Effect of additive plant extracts on the pigment of SA Mutton Merino sheep subcutaneous fat

Since fat-soluble pigments (or carotenoids) are lipophilic in the biological milieu, the anticipated fat colour contrast between samples observed was suspected to be a result of fat-soluble pigment deposited in the adipose tissue (Krinsky, 1994; Yang *et al.*, 2004; Pajkovic & van Breemen, 2005). Similarly, the feed additives treatment was expected to influence the SA Mutton Merino sheep subcutaneous fat colour characteristics. In light of this revelation, some levels of interaction were suspected between the feed additives treatment and day-on-trial for each Merino sheep sample, due to the colour variation observed.

2.4.5 Biosynthesis of isoprenoid: a cholesterol overview

2.4.6 Brief history of the mevalonate pathway

The chemical investigation of isoprenoid started at the beginning of the 1800s as stated by Holstein (2011) during this period Isoprenoid biosynthesis pathway (IBP) or mevalonate pathway was said to be the origin of many isoprenoid compounds found in nature. Between 1884 and 1887 Otto Wallach put forward a theory known as isoprene rule alongside the discovery of many isoprene compounds at the time. Similarly, Leopold Ruzicka (in the 1950s) suggested a biogenetic isoprene rule, and both Otto Wallach & Leopold Ruzicka's isoprene rule suggested the possibility of all terpenes be originated or built from the reorganisation of isoprene benzene unit precursor (Holstein, 2011).

During the same period (in 1957) the synthesis of hydroxymethylglutaryl coenzyme A (HMG-CoA) from acetate, and the relationship between HMG-CoA and mevalonate was first established hence, the mevalonate pathway (Bhagavan & Ha, 2011; Holstein, 2011). Other intermediates of IBP were also discovered including isopentenyl pyrophosphate, farnesyl pyrophosphate, dimethylallyl pyrophosphate, and geranyl pyrophosphate (Holstein & Hohl, 2011).

2.4. Overview of cholesterol biosynthesis

The 3-hydroxy- 5, 6-cholestene or cholesterol is a steroid of cyclopentanoperhydrophenanthrene, which is made up of a 5- and 6-membered benzene ring carbon skeleton (Holstein, 2011). The biosynthesis of polyisoprenoid side-chains (such as ubiquinone & cholesterol) is initiated by acetyl-CoA via a series of reaction intermediate products in the mevalonate pathway (Holstein, 2011). The biosynthesis of cholesterol can be separated into 6 sequential phases namely:

a. Condensation of acetyl CoA to 3-hydroxy-3-methylgluteryl coenzyme A.
b. Formation of mevalonate from HMG-CoA (A rate-limiting stage).
c. Synthesis of isoprenyl pyrophosphate from mevalonate.
d. Conversion of isoprenyl pyrophosphate to form squalene.
e. Conversion of squalene to lanosterol.
f. Transformation of lanosterol into cholesterol.

It is important to note that all carbon of cholesterol originates from acetyl CoA, and the carbon source stems from the citric acid cycle as soon as mitochondria oxidation is achieved or via the activation of acetate by acetyl-CoA synthase in the cytosol (Holstein, 2011). All cholesterol production is virtually derived from hepatic synthesis, in this way, hepatic synthesis controls steroid homeostasis when dietary sterol (such as phytosterol) is supplied in humans and animals.

2.5 Lipid metabolism in ruminant animal

The formation of volatile fatty acids (such as acetate, butyrate & propionate) is one of the essential roles of the rumen, which is critical to the energy requirement of the ruminant animal as well as maintenance of normal physiological processes (Noble, 1981). The intervention of ruminal microflora in the digestive processes features another significant aspect of rumen lipid metabolism vis-à-vis hydrolysis of lipid and biohydrogenation (Hobson *et al.*, 1997). Biohydrogenation of fatty acid (FA) is an important physiological process involving the conversion of unsaturated fatty acids to the saturated fatty acid end product (i.e., stearic acid) by the rumen microbes. Dietary or supplemental unsaturated fatty acids serve as substrates for biohydrogenation and their presence inhibits methanogenesis to some extent (Bessa *et al.*, 2007).

Neem and Moringa plant extracts are rich in lipid derivatives such as oleic acid (e.g., cis-9-octadecenoic), and based on product isomerization and unsaturated FAs utilised, the bio-hydrogenating bacteria species are categorized into group A and B bacteria (Hobson *et al.*, 1997). According to Hobson *et al.* (1997) group-A bacteria largely hydrogenate α-linolenic and linoleic acids, and seemed to lack the ability to hydrogenate oleic acid. Whereas, group B

bacteria are capable of hydrogenating several octadecanoic acids in addition to linoleic acid hydrogenation to stearic. Manipulation of rumen ecology with antimicrobial agents could alter the normal pathway of biohydrogenation resulting in derived intermediate or biohydrogenation intermediate caused by incomplete hydrogenation, as well as increased PUFA escape from the rumen. Overall, group A and B bacteria must be present for ruminal biohydrogenation to be complete (Hobson *et al*., 1997).

2.5.1 Fatty acid synthesis in the liver and adipose tissue of ruminant

The ruminant adipose tissue is the most active site for fatty acid synthesis, particularly in non-lactating animals as suggested by Vernon (1980) while stating the fact that fatty acid *de novo* synthesis accounted for 90% of subcutaneous adipose tissue synthesis. Similarly, the ruminant adipose and hepatic tissues readily utilised acetic acid as carbon resources in the formation of fatty acids than glucose (except for foetal lamb adipose tissue) as reported by Vernon (1980). Meanwhile, in the event of acetate short-supply, propionate, butyrate, and lactate are mostly utilised as a close substitute for adipose *de novo* synthesis, even in the presence of glucose. Also, the lipogenesis in the liver adheres to a similar pathway as seen in the adipose tissue via hepatic extramitochondrial sources, and activation of acetate (Bell, 1979; Vernon, 1980).

Consequently, Bell (1979) & Vernon (1980) explained that the selective exclusion of glucose carbon (by the ruminant lipogenic tissues) even in a normal physiological state, underscores the adaptive coping mechanism of ruminant animals in sparing glucose where possible. This is because virtually all the glucose used in the ruminant metabolism is drawn from gluconeogenesis, whereas in the non-ruminant the glucose carbon obtained from food is employed as carbon resources for lipogenesis. Moreover, the characteristics of ruminant hepatic *de novo* synthesis are distinctive, considering the deficiency in the high concentration of hepatic glucokinase substrates (K_m), which regulates glucose uptake as seen in the monogastric (Bell, 1979). Nevertheless, the rate of ruminant hepatic lipogenesis is lower compared to the adipose tissue *de novo* synthesis. The extent of acetate carbon utilisation is also lower in hepatic lipogenesis despite the preference for acetate carbon resources (Bell, 1979; Vernon, 1980).

Furthermore, an *in vitro* study on sheep liver carried out by Bell (1979) revealed that the consumption rate of acetate carbon was subdued by the availability of propionic and butyric acids. Hence, he suggested hepatic acetyl CoA synthetase (in the mitochondria) has a greater propensity for the activation of propionate and butyrate as opposed to acetate activation. It is important to highlight that much of the simple sugar metabolised in ruminant adipose tissues is released as lactate (in the cytosol), which amounts to nothing in terms of nicotinamide

adenine dinucleotide (NADH) yield (Vernon, 1980). Similarly, lipogenesis in the adipose and hepatic tissue demands a substantial amount of reduced NADP (i.e., NADPH as hydrogen source), which equals 14 moles of NADPH (plus 8 moles of acetyl CoA) to produce one mole of palmitic acid as proposed by Vernon (1980).

Meanwhile, the glucose is poorly utilised by ruminant animals as mentioned earlier, and the bulk of the cellular NADPH is derived from malate (i.e., a reduced form of oxaloacetate) which leaves mitochondria. This explains the elevated activity of cytoplasmic NAD-malate dehydrogenase enzyme in ruminant adipocytes accompanied by a relatively constant cytoplasmic NADH: NAD ratio (Vernon, 1980). In contrast to the ruminant adipose tissue, NADH: NAD ratio is a rate-limiting step to hepatic lipogenesis by the way of oxaloacetate being wrestled between gluconeogenesis and lipogenesis pathway, given that the ruminant liver is the net exporter of glucose (Bell, 1979; Vernon, 1980; Christie, 2014). In addition to the NADP-malate dehydrogenase pathway, hepatic NADPH is also sourced from the NADP-isocitrate dehydrogenase pathway in the ruminant liver (Bell, 1979; Christie, 2014).

Apart from constraints due to NADPH availability, it is worth noting that ruminant hepatic lipogenesis is equally limited by the capacity of the liver to mitigate the demand for carbon resources between ketogenesis and lipogenesis. Therefore, the hepatic fatty acid synthesis is very low in comparison to the adipose tissue synthesis of the ruminant animals (Bell, 1979; Vernon, 1980; Christie, 2014).

2.5.2 Long-chain fatty acids desaturation and chain elongation in ruminant

According to Bell (1979) Vernon (1980) & Christie (2014), the mammalian liver cells and adipocytes are responsible for the desaturation of long-chain fatty acids (LCFAs) into their consequential 9,10-cis-monounsaturated fatty acids, in the microsomal fraction of liver and adipose tissue respectively. The microsomal desaturation takes place in the company of microsomal enzyme, malonyl CoA, and NADPH. Moreover, Bell (1979) argues that the desaturation of LCFAs in the hepatic microsomal fraction of sheep is considerably lower compared to the adipose tissue, particularly the subcutaneous depot.

Similarly, Vernon (1980) validates the assertion of Bell (1979) while discussing desaturation of subcutaneous and abdominal adipose tissue as determined by the age of the animal, which suggests a greater proportionate increase in the subcutaneous depot site than abdominal adipose tissue (in sheep and cattle) over stages of development. This explains the underlying reason for greater oleic (C18:1n9c) to stearic (C18:0) ratio in subcutaneous adipose tissue of ruminant, which results from higher desaturase activity. Moreover, Bell (1979) indicated that nearly all fatty acids synthesised in the subcutaneous adipose tissue are of endogenous origin, and LCFAs synthesised *de novo* are desaturated easily. Therefore, *de novo* synthesis of

LCFAs would increase the level of desaturase enzyme, the effect of which is expected to be greater in subcutaneous adipose tissue as aforementioned.

Meanwhile, mitochondrial elongation of fatty acids chain length takes place in the presence of acetyl CoA, NADH, and NADPH (i.e., a reduced form of NADH) (Bell, 1979). Also, Bell (1979) reported 60-70% of the LCFAs *de novo* synthesis in an *in vitro* study (using tissue slices) undergo chain elongation, which predominantly involves elongation of palmitic acid to stearic acid. In contrast to the desaturation of adipose tissue, the elongation of LCFAs is not influenced by the stage of development (i.e., age) or the nature of the lipogenic tissue or location (Bell, 1979; Vernon, 1980; Christie, 2014).

2.5.3 Esterification and deposition of lipid in ruminant adipose tissue

The synthesis of glycerol-3-phosphate is essential for the formation of triacylglyceride in ruminant lipogenic tissues, even in the presence of glucose, insulin, and long-chain fatty acid together with CoA esters were reported by Vernon (1980) to shape the rate of its synthesis. Similarly, Vernon (1980) was of the view that the most important and probably unique biochemical pathway for acyl glycerol synthesis stems from glucose carbon (or glycolytic pathway). However, Bell (1979) affirmed phosphorylation of glycerol via glycerol kinase and pentose phosphate pathway as alternatives to the glycolytic pathway for acyl glycerol synthesis. Nevertheless, this calls attention to the importance of glucose in ruminant lipid metabolism, specifically, the esterification of fatty acid aimed at the animal fat depot.

Moreover, Bell (1979) described the sheep liver homogenates as having an increased turnover of esterified fatty acids into mono-, di-, and triacylglyceride and phospholipids compared to the adipose tissue homogenates. Studies have also shown an increase in mono-acylated fatty acids in the mitochondrial as compared to the microsome of the liver, and palmitic acid was esterified promptly with acyl glycerol than linoleic acid (Bell, 1979). A similar study reported by Vernon (1980) detailed more than 95% *de novo* synthesis (such as palmitic & myristic) were esterified with triacylglycerol of 60-80% lipid proportion, while mono- and diacylglycerol constitutes 20-40%. It is important to mention that despite the disparity in the constituent fatty acids esterified by hepatocytes and adipocytes, the rate of fatty acid esterification is largely determined by LCFAs concentration and glucose supply (Bell, 1979; Vernon, 1980).

According to Vernon (1980) & Christie (2014), perirenal and subcutaneous adipose tissue in sheep possesses an active lipoprotein lipase, and studies have shown that adipose tissue lipoprotein lipase exhibit a preference for exogenous or dietary sources of LCFAs. In this way, esterification of dietary LCFAs via hepatic metabolism, and subsequent transportation by chylomicrons (as acyl glyceride) would result in the deposition of the exogenous fatty acids. Similarly, Vernon (1980) discovered that during the growth phase of Merino sheep (i.e.,

between 100-250 days of age), the ratio of fatty acids deposition increase steadily per adipocyte. Meanwhile, the extent of fatty acid esterification, *de novo* synthesis, and acyl glycerol synthesis reduce per adipocyte, along with an increase in the lipolytic rate. This indicates that the degree of lipid deposition in ruminant animal increase with the increment in the animal stage of development.

2.5.4 Non-essential fatty acids in ruminant animal

According to Webb & O'Neill (2008) non-essential fatty acids are of *de novo* synthesis origin, particularly the endogenous synthesis of ruminant lipogenic tissues, which are usually produced via activation of acetate carbon and series of enzyme activities. The major non-essential fatty acid synthesise from acetyl CoA in sheep and cattle are palmitic (C16:0), stearic (C18:0), oleic (C18:1n9c), and minor fatty acids (such as myristic & palmitoleic acid) (Vernon, 1980). Concerning the proportion of non-essential fatty acids, oleic and palmitic acids are the first & second most abundant fatty acids in the ruminant subcutaneous tissue and *longissimus* muscle respectively (Lough *et al.*, 1992).

The findings of Vernon (1980) also revealed that oleic acid was more in the subcutaneous adipose tissue of ruminant in comparison to abdominal fat depot. Whereas, the stearic acid proportion of subcutaneous adipose was less than the abdominal fat depot. Ruminant nutritionists described non-essential fatty acids as predominantly saturated fatty acids in form of glycerophospholipids and sphingolipids, which were renowned for increasing the blood serum cholesterol level (Lough *et al.*, 1992; Webb & O'Neill, 2008). Other non-essential fatty acids include odd- and branched-chain fatty acids which are of microbial and dietary origin.

2.5.5 Essential fatty acids in ruminant animal

Essential or dietary fatty acids are not synthesised by ruminant lipogenic tissues intrinsically, hence the importance of their requirement and supplementation in the diet of ruminant animals (as well as humans) (Webb & O'Neill, 2008). The most important group of essential fatty acids are 18 carbon molecules and 20 carbon molecules of polyunsaturated fatty acids (PUFA). The 18 carbon PUFA include linoleic acid (9-cis, 12-cis-octadecadienoic acid) and alpha-linolenic acid (9-cis, 12-cis,15-cis-octadecatrienoic acid) (Lough *et al.*, 1992; Webb & O'Neill, 2008). While the 20 carbon PUFA includes arachidonic (C20:4n-6) and eicosapentaenoic acid (20:5n-3), which are derived from desaturation and elongation of linoleic and α-linolenic acid respectively (Webb & O'Neill, 2008).

Supplemental unsaturated fatty acids usually reduce the accretion of tissue *de novo* synthesis leading to lower serum cholesterol levels in animals, and this desirable effect could decrease the risk of cardiovascular disease in human consumers (Lough *et al.*, 1992; Webb & O'Neill, 2008).

2.6 The concept of aggregate lipid extractable

Lipids and their derivatives usually occur in a combination of neutral or non-polar lipids (such as triacylglycerol), together with more polar lipids (such as glycolipids) (Iverson *et al.*, 2001; Christie & Han, 2016).

Adoption of lipid polarity as a means of classification is oversimplified, according to Christie & Han (2016), it is essential to note that, unesterified fatty acids are neutral lipids, meanwhile most fatty acids are considered to be polar lipids. The relative proportion of polar and non-polar functional groups in a lipid sample impacts the sample's dissolution in all organic solvents (Dekker, 2014). Lipid solubility in itself is a comparative precondition for lipid extraction, and the efficiency of the extraction technique is dependent on the solubility of the sample in a solvent. Hence, the solvent extraction technique and the nature of the lipid in terms of polarity define the concept of total lipid extractable (Manirakiza *et al.*, 2001).

In principle, the Folch method (chloroform/methanol/water phase), Soxhlet method (acetone/hexane), Bligh and Dyer method (chloroform/methanol) were the most commonly used techniques for the determination of total lipid content (Iverson *et al.*, 2001; Manirakiza *et al.*, 2001; Dekker M., 2014; Christie & Han, 2016). However, there were limitations to all methods developed initially, and several modifications were made to forestall over and underestimation of the total lipid content of a sample (Iverson *et al.*, 2001; Manirakiza *et al.*, 2001).

CHAPTER 3: MATERIALS AND METHODS

3.0 Ethics approval statement

The research project titled the effect of some medicinal plant extracts on nutrient digestibility, rumen fermentation, growth performance, and methane emission in sheep was approved on the 30th January 2018 by the University of Pretoria Animal Ethics Committee. In reference to a Master of Science degree research having the project number EC004-18 and Ms. Danah du Preez as the principal researcher.

Following the completion of her field study experiment, post-mortem samples were collected to investigate the effect of plant extracts used as a methane mitigation additive on carcass fat content and fatty acid composition of South African Merino sheep by Olaniyi, Michael Olanrewaju. As an independent Master of Science research study.

3.1 Experimental site

The experiment was carried out at the University of Pretoria experimental farm, Voortrekker Street, Koedoespoort 456-Jr, Pretoria, 0186.

3.2 Diet

A total mixed ration containing soybean meal (17%), yellow maize (28%), Alfalfa hay (20%), *Eragostis curvula* hay (22.2%), molasses (6.0%), wheat offal (5%), urea (0.8%), vitamin premix (0.5%) and salt (0.5%) was prepared as the basal diet for all the treatment groups (i.e., T_1 -T_4). Meanwhile, 75g of monensin was added to one tonne of the basal diet given to the positive control treatment (T_4), while *Moringa oleifera* and *Azadirachta indica* (A. Juss) extracts at 50mg/kg feed intake were given (employing drenching) to treatment two (T_2) and three (T_3) respectively. At the same time, negative control treatment (T_1) was not given any feed additive.

3.3 Preparation of test ingredients

The test ingredients preparation was carried out following Akanmu & Hassen (2018). Freeze-dried *Azadirachta indica* (A. Juss) and *Moringa oleifera* leaves were obtained from matured tree plants in Nigeria and milled through a 0.5 mm screen. The ground leaves sample (100g) from *Moringa oleifera* and *Azadirachta indica* (A. Juss) were extracted separately in different graduated glass bottles with 1000mL of methanol, and placed in the shaker for 96 hours (Akanmu & Hassen, 2018). Thereafter, the constituent extracts were precipitated after sieving the mixture through a 150µm screen aperture separately. The extract precipitates were freeze-dried till constant weight was achieved and stored under low temperature (4°c). Stored plant extracts were reconstituted with distilled in a ratio of 1g extract sample to 1L distilled water before drenching the sheep.

3.4 Experimental design and management

A total number of 40 sheep (newly weaned ram lambs of about 38kg average bodyweight) were used for this study in a randomized complete block design (RCBD). The animals were allotted on a weight equalization basis into four feed additive treatments, five blocks, and two animals per block in twenty pens at the University of Pretoria experimental farm.

The administration of reconstituted *Moringa oleifera* and *Azadirachta indica* (A. Juss) extracts were carried out by drenching sheep in the respective treatment groups (i.e., T_2 and T_3) both in the morning and afternoon at 50mg/kg feed intake. While 75g/tonne of monensin sodium was prepared together with the feed given to the positive control treatment (T_4). The selection of animals for slaughter commenced after 64 days-on-trial, and the sheep were selected randomly at 60–65Kg body weight across the treatment groups. Later on, animals were also selected on 78, 99, and 162 days-on-trial. The slaughtering operations were carried out at Renbro abattoir, Hammans kraal Pretoria, South Africa.

3.5 The Sampling of South African Mutton Merino sheep rib-cut, subcutaneous fat, and intramuscular fat samples

The post-mortem rib-cut samples were collected from randomly selected and slaughtered South African (SA) Merino sheep at the broadest muscle of the back (*M. latissimus dorsi*) on both left and right 8th, 9th & 10th thoracic ribs after 24 hours (of slaughtering operations) according to Webb *et al.* (1994, 1998). Following the dissection of rib-cut samples, the proportions of samples' muscle, bone, and fat were determined and expressed in a percentage ratio. Also, subcutaneous fat, as well as intramuscular fat from the centre of the rib-cut longissimus muscle were separated and stored (at -20℃) for impending laboratory analyses.

3.6 Soxhlet total lipid determination

In this study, a hot Soxhlet (Foss Tecator®) system was used for the determination of total intramuscular fat content.

3.6.1 Sample preparation

A portion of the *M. longissimus* (thoracic) muscle obtained from the sheep rib-cut samples was freeze-dried in a (Vacutec®) freeze drier for 6 days. It is worth noting that, a water-rich sample is not suited for the Soxhlet extraction technique and studies have shown lipid recoveries lower than 50% when watery samples were used (Manirakiza *et al.*, 2001). The reason suggested was that non-polar organic solvent (such as petroleum ether or diethyl ether) can extract water in addition to lipid residue. Therefore, the drying method that would prevent lipid binding to protein and carbohydrate was recommended (Manirakiza *et al.*, 2001; Dekker, 2014). Thereafter, freeze-dried samples were ground in M 20 Universal batch mill® (with cooled

grinding chamber) and this approach increases the sample surface area and ease handling (Dekker, 2014)

3.6.2 Procedure

The crude fat analysis was carried out according to Manirakiza *et al.* (2001) and Gildemeister *et al.* (2003), and some modifications in line with recommendations (Dekker, 2014).

Two Soxhlet apparatus were used (i.e., Soxtec HT6 extraction unit and Soxtec 2043 programmable unit) and each unit represents the replication of the other for different samples analysed (i.e., two replicates). The Soxhlet apparatus were put on standby mode, and the temperature set at 112℃ for the Soxtec HT6, and 105℃ for the programmable Soxtec 2043. Whatman (no 1) filter paper was used, weighed, and tared (on Sartorius® balance). The use of (Whatman) filter paper makes valid sense because ground sample particles could pass through the cellulose thimble during extraction and this was predicated on what Manirakiza *et al.* (2001) recommended.

Thereafter, 1g of the prepared sample was carefully weighed and enveloped (in filter paper) before being rolled, and inserted into different cellulose thimble (or extraction thimble). The use of a 1g sample was due to the bulky nature of the freeze-dried meat sample. Later, the thimble rack was used to transfer samples (contained in a thimble) into the Soxhlet condenser.

The hot Soxhlet system operation dwells on three important steps namely: extraction (boiling for 2 hours), rinsing (rinse for 3 hours), and drying (or concentration for 20 minutes) (Manirakiza *et al.*, 2001). After, mounting extraction thimbles into the condenser, aluminum extraction cups were taken from the oven (placed 30 min before, at 70℃) into the desiccator to cooled (for 10 min), and weighed. All thimbles and extraction cups were properly labelled before use and all precautionary measures including cooling water and extraction fans were put on. Thereafter, the thimble knobs were lowered into the boiling position (i.e., boiling solvent) to boil for 2 hours. After boiling, thimble knobs were moved to rinse position, and rinsing lasted for 3 hours.

The condenser taps were closed after rinsing, and the extraction cups were allowed to boil for an additional 20 minutes (to dry). After concentrating the lipid residue, the safety handle was lifted and the extraction cups were put into the oven (for 30min, at 70℃) to evaporate the petroleum ether that was left. The cups were later removed, cooled in a desiccator (for 10 min), and weighed (on Sartorius® balance).

The measurement taken was on a dry matter basis (DM) and the calculation of fat percentage goes thus:

$$Fat\ percentage\ (DM) = \frac{(Cup\ mass + ether\ extract)g - (Cup\ mass)g}{(Cup\ mass)g} \times 100 = \ \%\ fat$$

3.6.3 Dry material determination

The percentage of dry matter content for the longissimus (thoracic) samples was also carried out. The dry matter determination was done in line with the well-known, and adopted codex-defined method, by the Association of Official Analytical Chemists (AOAC, 2007) international.

Washed and dried crucibles were put in the oven (for 1 hour, at 105℃) in preparation for use. After 1 hour, the crucibles were removed, cooled (for 35 min) in a desiccator and the dry mass weighed. Meat samples were chopped into tiny pieces and air-dried. Thereafter, two grams of each sample (plus crucible) were weighed (on sartorius® balance) and put into the oven (for 36 hours, at 105℃). After drying for 36 hours, the crucibles (plus samples) were removed from the oven, cooled in the desiccator (for 30 min), and the final mass of respective crucibles (plus samples) was measured on the balance.

Percentage of dry material was calculated as detailed in the following:

$$Percentage\ of\ dry\ material = \frac{(mass\ of\ oven\ dried\ sample)g}{(mass\ of\ the\ air\ dried\ sample)g} \times 100 = \ \%\ muscle\ DM$$

3.6.3.1 Intramuscular fat percentage on a DM basis converted to As-is basis

Intramuscular fat as-is basis, explains the amount of fat in the longissimus muscle as-is in an intact sample (i.e., including water). It is important to point out that, the ratio of intramuscular fat (as-is basis) and the muscle dry matter content, is equal to the proportion of fat dry matter in each meat sample (Chiba, 2009).

Such that: $\frac{Fat\ percentage\ (as-is\ basis)}{Percentage\ muscle\ DM} = \frac{fat\ percentage\ (DM\ basis)}{100\%\ DM}$

$$Fat\ percentage\ (as\ is\ basis) = \frac{\%\ muscle\ DM\ \times fat\ percentage\ (DM)}{100\ \%\ DM}$$

3.7 Fat pigment determination.

3.7.1 Preparation of reagent

A potassium hydroxide (10g) was dissolved in 100mL of methanol (10% w/v)

3.7.2 Procedure

The laboratory analysis was carried out as reported in the literature with some modifications (Kirton *et al.*, 1975; Christie & Han, 2016; Hill, 2018).

The subcutaneous fat (5g) from respective samples were chopped into tiny pieces and saponified in a refluxing system (having two replications) each containing 10mL (10% w/v)

methanolic potassium hydroxide at 65℃ for 1 hour. The basis for the saponification process is to convert fat into fatty acid salt or soap. In this way, the fatty acids will exist in a non-esterified form, similar to the intestinal phase of the micellar solution which consists of bile salt and monoglyceride (Wiseman, 2014). Precautionary measures for reflux systems including cooling water and extraction fans were put on initially. After 1 hour, the thermal hydrolysis was stopped with (30mL) water. The addition of water keeps the soap solution from turning into a paste during cooling.

Thereafter, the soap solution was decanted into a separating funnel containing (20mL) diethyl ether through a glass funnel (plugged with a cotton ball), for further extraction. Subsequently, the solution separates into the ethereal and aqueous layer after agitation. The ethereal layer contains non-saponifiable materials such as long-chain alcohol, sterols, hydrocarbons, and plasmalogen (Christie & Han, 2016). At the same time, the aqueous layer contains the alkaline medium with the saponified product (i.e., fat pigment). The absorbance of respective sample alkaline solutions medium was measured at 423nm wavelength with a spectrophotometer and each measurement was replicated twice (Specord 200®) (Kirton *et al.*, 1975; Webb & Casey 1999; Hill, 2018).

3.7.3 Fat colour chart

The fat colour attributes for different samples were visually observed during the laboratory analysis (i.e., before and after the extraction process). An additive colour model selection chart was used in computing the colour properties on the Microsoft office software package.

3.8 Long-chain fatty acid analysis

3.8.1 Sample handling and preparation

Before fatty acid extraction, all samples were separated from their tissue matrices and free of non-lipid contaminants as much as possible and the tissue samples were stored in a manner that prevents considerable deterioration (at -20°c). Samples were chopped into tiny pieces with a clean table knife for every sample, and the preparation of the sample was divided into three phases namely: digestion, extraction, and methylation. The sample preparation was done according to AOAC 996.06 (AOAC 996.06, 2005)

3.8.1.1 Digestion of subcutaneous fat sample

A portion of subcutaneous fat samples (0.1g) was chopped into tiny pieces and added into clean 30mL test tubes (with Teflon lined screw cap) containing 0.1g of pyrogallol (as an antioxidant), and two glass beads as a stirrer. Thereafter, 2mL of pentadecanoic acid - C15:0 (as internal standard) was added and sequel by 2mL of ethanol (98%) together with 10mL of hydrochloric acid (HCL 32%). This was followed by gentle shaking to allow proper mixing (until the sample distributes widely) and the same procedure was carried out for all the samples

prepared. All the test tubes (containing the mixture) were placed in a water bath (at 75℃, on gentle shaking) for 40min. Subsequently, the test tubes were removed from the water bath and allow to cool to room temperature under the fume cupboard (AOAC 996.06, 2005).

3.8.1.2 Sample extraction

Following the digestion of fat samples, the content of the test tubes was removed into separate glass beakers (150mL). Thereafter, 20mL of diethyl ether was added to the mix and shake on a Rota-mix for 5 min. After 5 min of mixing, 20mL of petroleum ether was also added to the mix (and shake on Rota-mix again). All the mixtures were allowed to stay and settled for 16 hours before removing the dark-purplish-coloured layer by aspiration. Thereafter, the white flakes (i.e., lipid layer) were slowly dry in the fume cupboard (whilst in the glass beaker for up to 6-8 hours) (AOAC 996.06, 2005).

3.8.1.3 Methylation of lipid extract

After the extraction, the flakes of lipid extracts in the separate glass beakers were dissolved with 2mL of chloroform to (2mL) diethyl ether in (1:1) ratio respectively. This was followed by gentle shaking to ensure the optimal dissolution of lipid flakes (including those stuck around glass sides). Each liquid mix was later transferred into clean and separate test tubes using a glass pipette. Shortly after pipetting, all liquid-mix test tubes were evaporated to dryness under a low stream of nitrogen until visible liquid fade away. Subsequently, 2mL of sulphuric acid (2% H_2SO_4) in methanol (99% MeOH) and 1mL of toluene were added to each solid residue (in test tubes) respectively. After gentle agitation, all test tubes were properly sealed with Teflon screw caps and place into the oven for 45 mins at 100℃ (AOAC 996.06, 2005).

After the incubation, the samples were allowed to cool down to room temperature, and 5mL of distilled water was used to stop the reaction. Later on, the fatty acid methyl esters (FAMEs) were extracted by adding 1mL of n-hexane each, and vortex (with screw caps on) for approximately 1min. The layers were allowed to settled and separate, meanwhile, anhydrous sodium sulphate (1g) was used for each sample until the hexane layer water bubbles were trapped. Then, each sample's upper hexane layer was transferred into the gas chromatography (GC) vial using a glass pipette and stored in the freezer until injection.

3.8.2 Identification and quantification of fatty acids

Calibration, identification, and quantification of fatty acids were carried out on Shimadzu lab solution software. The FAME 37 component mix standard solution was diluted to 10%, 20%, 40%, 60%, 80%, and 100%. Also, the sample chromatogram peaks were identified based on the linear calibration of the standard solution and the relative retention time.

Furthermore (as mentioned) the pentadecanoic – C15:0 acid was added as an internal standard for quantification, and the differential recovery of each fatty acid molecular class was given by the area and the height of chromatogram peaks. The results of the fatty acid quantification were calculated in terms of molar percentage (%) and gravimetric content (mg/g)

The molar percentage is given as;

$$Fatty\ acid\ molar\ \% = \frac{Individual\ fatty\ acid\ area\ (\mu V)}{Total\ fatty\ acids\ area\ (\mu V)} \times 100\%$$

Similarly, gravimetric content (mg/g) is given as;

$$Fatty\ acid\ weight\ per\ sample\ (mg/\mu L) = \frac{Individual\ fatty\ acid\ area}{Area\ of\ IS}$$

$$FA\ gravimetric\ content\ = \frac{Concentration\ of\ IS\ (1000\ mg/\mu L)}{Sample\ weight\ (mg)} \times FA\ weight\ per\ sample$$

Where FA means fatty acid and IS means internal standard (i.e., pentadecanoic – C15:0 acid).

3.9 Statistical methods

Statistical techniques used for all measured variable data sets were carried out on IBM SPSS statistics, windows software package version 25 (©copyright IBM corporation 2017). Other aspects of working the data file such as defining variables, creating, editing, and importing data sets were carried out on IBM SPSS as well. The study research questions form the basis for every statistical procedure that was executed on IBM SPSS, and the following were the statistical analyses performed on the variable data sets.

3.9.1 Bivariate correlation analysis

The research question was to determine if there were covaried relationships among possible pairs of quantitative parameters measured in the study. Two different correlation coefficients Pearson's (r) and Spearman (r_S) correlation coefficient) were used to describe variables based on the variable measurement scale and assumptions, and the details thereof were reported in the result section.

3.9.2 Factor analysis

The purpose of the analysis was to assess whether a succinct factor index would define the main study hypothesis indicator from two sets of measured variable indexes.

The factor analysis technique used was the principal component factor analysis. With this statistical technique, a common factor alongside its dimension underlying the factor component statistically explained the variation and covariation between the component variables (Green & Hall, 2008).

Also, the principal component analysis (PCA) was used to describe the main effect of the feed additive treatments used on the physiological parameters measured (i.e., on fatty acid composition and tissue fat content).

3.9.3 Transformed standard score

Standard scores are unit free number statistics, expressed with a known mean (*M*) and standard deviation (*SD*) such as z-score (with $M = 0$ & $SD = 1$) and transformed standard score (or *T*-score) (Witte, Robert., 2010). The fat content component scores generated by IBM SPSS in the factor analysis procedure were identical to the conventional z-scores.

Transformed standard scores are generally considered an alternative to z-scores, especially when reporting results. This is because *T*-scores are without negative values, and in most cases with no decimal point, however, the number of decimal places depends on the original z-scores' decimal points (Witte & Witte, 2010). Therefore, the component z-scores obtained were transformed using the desired mean of 50 and a standard deviation of 10 to achieve a new standard score known as *T*-scores.

Such that; Z^{l} = desired mean + (z-score) * (desired *SD*)

Where (Z^{1}) Z prime is also referred to as *T*-score (Witte, Robert., 2010).

The transformation did not alter the original overall distribution shape or the relative position of every individual case in the distribution.

3.9.4 One sample chi-square (χ^2) test

The research questions were the following:

1. Assuming the null hypothesis is true, were the proportions of subcutaneous fat colours (before extraction) associated with floral white, antique white, papaya whip, and blanched almond colour characteristics, equally distributed?

H_0: $P_{floral\ white}$; = $P_{antique\ white}$; = $P_{papaya\ whip}$; = $P_{blanched\ almond}$ = 0.25 (or 1/4)

2. Were the proportions of yellow 3, yellow 2, and gold 1 soluble lipids colour characteristics, due to extracted pigment the same?

H_0: $P_{yellow\ 3}$; = $P_{yellow\ 2}$; = $P_{gold\ 1}$ = 0.33 (or 1/3)

The one variable chi-square test was used to check the disparities between the observed frequencies and the expected frequencies. In this way, the observed frequencies were described based on the assumption of equal proportion for each qualitative category (Wilcox, 2010; Witte, Robert, 2010). Before conducting the chi-square test, variables were coded and redefined to reflect the different categories in the data set.

3.9.5 One-way analysis of covariance (ANCOVA)

The following were the hypotheses of mean differences analysed with analysis of variance statistical tests:

1) Assuming there were no differences in the initial body weight between treatments, did fat content scores differ for South African (SA) Merino sheep given plant extracts additives (*Azadirachta indica*, & *Moringa oleifera*), compared to those given only monensin?

2) Was there any relationship between the SA Mutton Merino sheep subcutaneous fat (soluble) pigment absorbance and the feed additive treatment levels, assuming the levels of treatment were solely limited to the number of days-on-trial?

In one-way ANCOVA, each case in the sample distribution have scores on the dependent variable, independent variable, and the covariate (Wilcox, 2010). The first hypothesis consideration had a pre-test variable or covariate value (as initial body weight) and based on the pre-test scores, cases were assigned to the independent variable (or additives treatment) levels. Thereafter, outcome variables were measured as post-test scores.

The second hypothesis could be described as the paradigm of a potentially confounding research study question. Samples from the different treatments were not selected or assigned randomly into groups based on their covariate scores (i.e., number of days-on-trial) (Green & Hall, 2008). Instead, the covariate has a likelihood of masking the treatment effect. In this analysis, the days-on-trial was considered a continuous variable predictor or confounder, even though cases in the data set have finite numerical values.

The reason for the consideration was due to several levels of the finite scores in the covariate, and it is worth noting that, when dealing with predictor variables on a categorical scale, the response variable with their respective discrete values lack the order of predictor levels (Witte, Robert., 2010). As a result, the number of days-on-trial was utilized in the statistical analysis on an interval scale.

3.9.6 One-way multivariate analysis of variance (MANOVA)

The following hypotheses testing were designed to reflect the means differences of several identified long-chain fatty acids (LCFAs) molar percentage and gravimetric content in SA Mutton Merino sheep subcutaneous fat.

1. Were the means of saturated, monounsaturated, and polyunsaturated fatty acids molar percentage, and their linear combinations different for SA Mutton Merino sheep in the feed additive treatment groups?

2. Similarly, were the means of saturated, monounsaturated, and polyunsaturated fatty acids gravimetric content, and their linear combinations different for SA Mutton Merino sheep in the feed additive treatment groups?

In these hypotheses testing separate MANOVA was conducted for the individual fatty acid quantification approach, to avoid violation of multivariate normality assumption as well as non-multicollinearity of linear combination assumption. Besides, the scores on the dependent variables for Myristoleic – C14:1 (gravimetric), Palmitoleic – C16:1 (gravimetric), heptadecanoic – C21:0 (molar %), Elaidic – C18:1n9t (gravimetric), behenic -C22:0 (molar % & gravimetric), tricosanoic – C23:0 (gravimetric) and pentadecenoic – C15:1 (molar % & gravimetric) fatty acids were transformed using the appropriate arithmetic functions on IBM SPSS (i.e.,, square-root or Log base 10). This was done to rectify the skewness of the raw data and the resultant normality failure at every single level of the independent variable.

Furthermore, the Bonferroni adjustment for *p*-values was not applied in these statistical analyses due to the criticism regarding the MANOVA technique. The underlying reason that examines the multivariate hypothesis testing, followed by the multiple analysis of variance (ANOVA) tests, does not take into account the linear combinations of the dependent variables (Green & Hall, 2008). This implies that the MANOVA test and the follow-up ANOVAs statistics are two separate and independent tests. Therefore, there was no reason for Bonferroni's adjustment, as this would be impacted negatively on the statistical power. Notwithstanding, the post hoc analyses were conducted with the Bonferroni comparison procedure.

CHAPTER 4: RESULTS

4.1 Regularity or covaried relationship among pairs of variables

Before conducting correlation statistics, the bivariate normally distributed variable assumption was tested, and Shapiro-Wilk *p*-value*s* were greater than 0.05 for all variables analysed with Pearson's correlation (*r*). However, days-on-trial data sets failed the normality ($p < 0.05$) assumption, at certain levels of the respective factor variable. It is crucial to note that, days-on-trial was a discrete variable data set and the numerical transformation was not performed on the raw data. This implies that the individual case only takes a finite score, whose numerical value was achieved by counting (Witte, Robert, 2010). Thus, Spearman correlation statistics (r_s) was a reference instead of Pearson's correlation due to the robustness of Spearman-rho correlation (r_s) (i.e., non-assumption of normality). The descriptive statistics of correlated dependent variables can be seen in Table 4.1. To test for the assumption of regularity among possible pairs of dependent variables, and the Pearson product-moment correlation coefficient (*r*) was used to describe relationships.

. An r (22) of 0.41 indicates that as scores on sample rib-cut fat % increased across sample cases, so did intramuscular (IM) fat % scores increase also at a moderately constant rate. The positive relationship was statistically significant ($p \leq 0.05$) and this outcome proves to be probable if this study were to be repeated. The corresponding number of days each animal spent on trial was shown to correspond with large and small values on intramuscular fat % scores with r_s (22) = 0.54 ($p < 0.01$). The small significance value also implied that the positive correlation would reappear more likely if the study were to be repeated.

In addition, the rib-cut fat % and IM fat % samples showed positive relationship with carcass weight, r (22) = 0.32 and r (22) = 0.15 respectively. The strength of the associations was not large enough to suggest statistical significance ($p > 0.05$). Also, the relationship strength r_s (22) = 0.33 between rib-cut sample fat % and the number of days animals spent on the trial. This implied that low rib-cut fat% tends to be associated with a smaller number of days the animal spent on trial, equally animals with longer days-on-trial tend to have high rib-cut fat% score. However, the positive relationship was not statistically significant ($p > 0.05$), probably due to a lack of power. All other bivariate correlation relationships among pairs of dependent variables showed little or no relationship (positive or negative) and can be seen in Table 4.1.

It was apparent that regularity between pairs of quantitatively measured variables was identified and described. Therefore, seeing that covaried relationships exist, the null hypothesis was rejected.

Table 4.1 Correlations between dependent variables (N = 24)

Dependent variables	Rib-cut fat percentage	IM fat (%) as-is	fat color absorbance	Days-on-trial
IM fat as-is	0.41*			
Fat color absorbance	0.07	-0.02		
Days-on-trial	0.33	0.54**	0.22	
Carcass weight	0.32	0.15	0.27	0.36

*$p \leq .05$
**$p < .01$
IM means Intramuscular fat

4.2 Principal component index solution

The positive correlation matrix between rib-cut fat % and intramuscular fat % with r (22) =.41 (r means Pearson correlation, and '22' represents a *degree of freedom*) and significantly so with $p < 0.05$, validated the underlying assumption of linearly related measured variables for factor analysis (Green & Hall, 2008). The correlation statistics were also supported by Bartlett's test of sphericity, which implied that the correlation matrix was statistically different ($p \leq 0.05$) than the zero or identity matrix. Also, the assumption of normality was satisfied as aforementioned.

Extracted factor from the two correlated variable components, produced single factor variability, and total eigenvalue strength equals 1.405 (based on eigenvalue greater than one criterion). Total variance explained by one factor retained was (1.405 / 2) * 100) = 70.25 %, and due to its single-factor variability, the solution cannot be rotated. The rib-cut fat component item loads r = 0.838 on the single component factor and implies that approximately 70% of variance explained by the one component factor solution was due to the rib-cut variable contribution.

Similarly, intramuscular fat measured variables also approximate 70% of the variance (0.838 component loading) that was explained by the single component factor retained. Furthermore, the factor component scores (or component z-scores) were generated for each case in the total sample distribution data set, and the z-scores were later converted into transformed standard scores.

4.2.1 Exploratory data analysis of physiological parameters

The results of (13) physiological parameters analysed produced three principal components (PC) factors based on eigenvalue greater than one criterion. Meanwhile, the total percentage variability explained by the three PC factors retained equals 100%, in respect of 45% (PC1), 40% (PC2), and 15% (PC3) approximately. Furthermore, the two PC factors with the largest

percentage variability were employed to explore the main effect of the feed additive treatment concerning the physiological parameters measured. Hence, the results data were presented on a 2-dimension biplot (as can be seen in Figure 5.3), and the total variability explained by the two-factor (i.e., PC1 & PC2) equals 85%.

Combination of polyunsaturated fatty acids (PUFA) component loads the highest (0.374) value on PC1, followed by the combination of saturated fatty acids (SFA) (0.362), polyunsaturated-to-saturated fatty acid ratio (P:S) (0.325), day-on-trial (0.190), rib-cut bone % (0.188), rib-cut lean muscle (*LM*) % (0.171) and intramuscular (IM) fat % (0.08). With 14%, 13%, 11%, 4%, 4%, 3% and 1% explained variance respectively. Meanwhile, the fat soluble pigment absorbance (-0.364), combination of monounsaturated fatty acids (MUFA) (-0.363), unsaturated-to-saturated fatty acids ratio (U:S) (-0.348), PUFA omega-6 : omega-3 ratio (n-6/n-3) (-0.282), rib-cut fat % (-0.206), and the carcass fat content *t*-score (-0.087) showed negative component loadings or correlation on the extracted factor PC1.

Conversely, the intramuscular fat % (0.428) and the carcass fat content *t*-score (0.421) load the largest positive value on the PC2, followed by rib-cut fat % (0.344), days-on-trial (0.337), and combination of saturated fatty acids (0.146). While, the rib-cut lean muscle % (-0.293), rib-cut bone % (-0.287), polyunsaturated-to-saturated fatty acids ratio (-0.267), PUFA n-6/n-3 ratio (-0.246), U:S ratio (-0.188), PUFA (-0.166), MUFA (-0.146) and fat-soluble pigment absorbance (-0.050) all showed negative correlation with extracted factor PC2.

Therefore, considering the magnitude of variance component loadings on PC1, it could be suggested that the first principal component represents the fatty acid composition of SA Mutton Merino sheep (i.e., PUFA, SFA, and P:S ratio). Whereas, the magnitude of variance component loadings on PC2 implied that the second principal component characterised the fat content component of SA Mutton Merino sheep (i.e., carcass fat content *t*-score, intramuscular fat %, and rib-cut fat %).

4.3 SA Mutton Merino sheep (subcutaneous) fat colour characteristics goodness of fit tests

4.3.1 Before lipid extraction

Expected frequencies were greater than 5 for all observed colour categories, indicating that, the sample size was large enough for the χ^2 test to yield accurate results.

One-variable χ^2 test results revealed there was no statistically significant difference (at 0.05 significance level) between observed proportions and hypothesized values of $\frac{1}{4}$ (0.25, 0.25, 0.25, and 0.25), $\chi^2(3, N = 24) = 6.67$, $p = 0.08$. Furthermore, the effect size (6.67/24*3) of 0.10 suggests the observed frequencies (8, 4, 10, and 2) somewhat deviate from the expected

frequencies. While on the other hand, the important question was whether the disparities between observed and expected frequencies were sufficiently small enough to be considered a common occurrence, assuming that the null hypothesis is true.

Given the fact that the observed χ^2 of 6.67 was less than the critical χ^2 of 7.81 (at 5% significance level) so, the null hypothesis was retained. It was evident from the statistical results, that equal proportions of the subcutaneous fat colour categories observed, were probable in the actual population of South African Merino sheep used for the experimental trial.

4.3.2 After lipid extraction

Similarly, expected frequencies also yielded test statistics that satisfied one sample chi-square (χ^2) test assumption (i.e., expected frequencies greater than 5).

The results of one sample χ^2 test showed sample distributions were significantly different (at $p < 0.05$ significance level) from hypothesized proportions of $\frac{1}{3}$ (0.33, 0.33, and 0.33), χ^2 (2, N = 24) = 12.25, $p < 0.01$. An effect size estimate (12.25/24*2) of 0.26 suggests that observed frequencies reasonably deviated from the expected frequencies. Besides, a chi-square (χ^2) post-test was conducted to determine whether the χ^2 significant test result was not due to discrepancies between yellow-3 and gold-1 colour attributes, but rather as a result of the disproportionate number of lipid-soluble pigments that were neither yellow-3 nor gold-1 colour features. The follow-up test descriptive statistics are shown in Table 4.3.3.

Post-test expected frequencies for yellow-2 were 8 counts (i.e., one-third of the sample size), meanwhile the combination of yellow-3 and gold-1 categories equaled 16 counts, which makes up two-thirds of the sample size (24).

The outcome of the follow-up test revealed that observed proportions (8, 16) differ significantly from the hypothesized values, χ^2 (1, N = 24) = 12.00, $p < 0.01$. An effect of 0.50 indicates a high deviation from the observed proportions, and so, the null hypothesis was rejected. Overall, the χ^2 tests have shown that yellow-2 constituted the highest incidence rate of extracted lipid pigment colour categories, and explained why the hypothesized proportion of soluble lipid pigment colours were not the same.

4.4 Relationship between soluble lipid pigment absorbance and treatment levels following covariate interaction

Descriptive statistics of soluble lipid pigment absorbance or fat colour absorbance can be seen in Table 4.2.1. The distribution normality assumption was assessed and resolved to be fulfilled, as Shapiro-Wilk p-values were greater than 0.05 at all levels of factor variable for soluble lipid

pigment absorbance. However, normality was not assumed for the number-of-days-on-trial data set.

Similarly, the assumption of homogeneity of regression for ANCOVA was tested, and the result shows, the interaction between treatment levels and the number of days on trial was statistically significant, $F(3, 16) = 3.84$, $p = 0.03$ and partial $\eta^2 = 0.42$ (effect size). The result suggests that sample mean differences as regards the number of days-on-trial acted, together with the additive treatment groups, and the group varied as a function of days SA Mutton Merino sheep spent on the trial. The number of days-on-trial accounted for approximately 42% of the variance explained by the groups' sample mean differences.

Therefore, the assumption of slopes equality based on null interaction effect between treatment groups, and the covariate was rejected. This decision draws to attention a direct suggestion that one-way analysis of covariance (ANCOVA) cannot be conducted, due to heterogeneity of slopes or regression. Furthermore, knowing that ANCOVA was inappropriate, a follow-up test was conducted according to Green & Hall (2008) popularly known as "simple group main effects". The statistical procedure required at least three covariate levels to evaluate the differences in adjusted means between treatment groups of soluble lipid pigment absorbance values, concerning several stages in the study period.

From the days-on-trial data set, the overall distribution mean (M) and standard deviation (SD) in disregard for factor levels was 90.83 and 34.64 respectively. Subsequently, 56.19 ($M - SD$), 90.83 (overall M), and 125.47 ($M + SD$) typified low, medium, and high levels of the covariate mean respectively. These mean values were utilised in simple group main effects analysis. A statistical significance level of 0.017 (0.05/3) was chosen before conducting the analysis, as a correction for type-I-error. The independent variables and covariate levels (i.e., medium and high) can be seen in Table 4.2.2.

The results of the simple main effect test indicated that soluble lipid pigment absorbance values at high level ($M = 125.47$) number-of-days-on-trial were statistically different from the adjusted means of the treatment, $F(3, 16) = 4.09$, $p = 0.025$, partial η^2 of 0.43 (power = 0.55). However, the simple main effect tests for both low and medium levels of covariate were not statistically significant. For medium level, $F(3, 16) = 1.27$, $p = 0.32$, partial $\eta^2 = 0.19$ (power = 0.14), and for low level, $F(3, 16) = 0.89$, $p = 0.47$, partial $\eta^2 = 0.14$. The insignificant results as indicated for both levels may be due to weak statistical power. The results also suggested that the interaction effect size continuously increased with the increasing levels of covariate means (i.e., 14%, 19%, and 43% of the estimated variance respectively).

A pairwise comparisons test was conducted to assess the differences between means adjusted for statistical significance in the levels of factor variables, and the covariate evaluated at 125.47 days (value), based on LSD procedure (at 0.017 significance level). The adjusted means statistical differences can be seen in Table 4.2.2. An adjusted mean difference of 0.573 (1.162 – 0.589), between negative-control and Neem treatment groups, differed significantly $p = 0.009$. Similarly, the adjusted mean difference of 0.593 (1.162 – 0.569) between the negative-control and Monensin treatment group also differs significantly, $p = 0.008$. However, the adjusted mean difference of 0.300 (1.162 – 0.862) between the negative-control and Moringa group lacked statistical significance, $p = 0.145$. All other conceivable pairwise comparisons were not significantly different.

Overall, the simple-group main effect results have shown that, at a certain degree or level, a statistically significant relationship occurs between treatment groups and subcutaneous fat-soluble pigment absorbance values, if the number of days in which the SA Mutton Merino sheep spent on trial was held constant. As a result, the null hypothesis was rejected.

Table 4.2.1 Means and standard deviation of subcutaneous fat-soluble pigment absorbance

Treatment group	*M*		*SD*
Negative-control	0.857	±	0.359
Moringa	0.693	±	0.251
Neem	0.696	±	0.313
Monensin	0.639	±	0.154
p-value	0.574		

The covariate: days-on-trial M= 90.83 SD = 34.64

Table 4.2.2 Estimated means and absorbance pairwise comparisons of the days-on-trial levels

Estimated means at	56.19 days			90.83 days			125.47 days		
	M		*SE*	*M*		*SE*	*M*		*SE*
Negative-control	0.608	±	0.128	0.885	±	0.095	1.162[a]	±	0.142
Moringa	0.524	±	0.135	0.693	±	0.094	0.862[ab]	±	0.135
Neem	0.826	±	0.143	0.708	±	0.095	0.589[b]	±	0.129
Monensin	0.708	±	0.135	0.639	±	0.094	0.569[b]	±	0.135
p-value	0.469			0.317			0.025		

[a b] Means with different superscripts in the same column are significantly different ($p < 0.05$)
SE means standard error

4.5 Between-the-group difference in one-way ANCOVA for fat content component scores

The descriptive statistics associated with dependent variables across factor levels can be seen in Table 4.3. Normality assumptions were tested, and Shapiro-Wilk *p*-values were greater than 0.05 for the dependent variables used at all levels of the independent variables.

A preliminary one-way analysis of variance (ANOVA) test evaluating the mean differences of initial bodyweight among the treatment levels was conducted. The result shows that, the pre-test variable did not differ significantly between the groups, $F(3, 20) = 0.11$, $MSE = 14.97$, $p = 0.95$, $\eta^2 = 0.02$ and the equality of variance across the groups based on Levene's *F* test was similarly insignificant, $F(3, 20) = 0.46$, $p = 0.72$. This implies that before the commencement of the trial, the pre-test variable used to assign cases (i.e., sheep) into different levels of factor variable under the randomized complete block design was successfully done without bias. Therefore, the null hypothesis of no between-group difference in the levels of factor variable for the initial body weight was retained.

Before conducting ANCOVA analysis, the assumption of homogeneity of regression or slopes was tested. The test result revealed that the interaction between treatment groups and the covariate was not significant, $F(3,16) = 0.51$, $p = 0.68$, and partial η^2 of 0.09 (i.e., very low effect size). The result suggests that there may be somewhat little variation (approximately 9% var.) in South African Mutton Merino sheep fat content (*T*-scores) as a result of their pre-test values (i.e., initial BW). However, the sample distribution slopes did not vary significantly as a function of initial body weight, and consequently, the null hypothesis was retained.

Based on the outcome of the homogeneity of slopes ($p > 0.05$) assumption, the treatment main effect and covariate effect were analysed with ANCOVA. Also, the equality of variance assumption was satisfied by the fat content *T*-score based on Levene's *F* test, $F(3, 20) = 1.53$, $p = 0.24$. The one-way analysis of covariance (ANCOVA) test result yielded a significant relationship on the initial body weight and the fat content error variance, $F(1, 19) = 4.85$, $p = 0.04$, partial η^2 of 0.20. This implies that, after controlling for the levels of factor variable, the initial bodyweight account for 20% of variance explained by the South African Mutton Merino sheep fat content *T*-scores.

Similarly, the fat content (*T*-scores) ANCOVA test results were significantly different between the groups of additives used for the South African Merino sheep, $F(3, 19) = 3.13$, $MSE = 71.08$, $p \leq 0.05$, while controlling for the initial bodyweight, the partial $\eta^2 = 0.33$. The effect size presented a strong relationship between fat content, and the levels of additives treatment, accounting for 33% of the variance explained by the fat content *T*-scores.

The fat content group means adjusted for initial differences in body weight within the levels of the independent variable can be seen in Figure 4.6. The *Azadirachta indica* (Neem) group had the highest adjusted mean estimate ($M = 58.35$), followed by the negative-control group mean ($M = 50.78$). *Moringa oleifera* (Moringa) group had a smaller mean ($M = 45.44$) adjustment, and the monensin group had a similar adjusted mean estimate as to the Moringa group.

A follow-up test was conducted based on the least significant difference (LSD) pairwise comparison procedure at a 0.05 level of significance. The adjusted mean difference of 12.912 (58.348 – 45.436) between *Azadirachta indica* (Neem) and the monensin treatment group differs significantly ($p = 0.016$). Likewise, the adjusted mean difference of 12.907 (58.348 – 45.441) between the *Azadirachta indica* and *Moringa oleifera* group also showed statistical significance ($p = 0.016$) similar to the monensin group mean. Furthermore, *Azadirachta indica* and the negative-control adjusted mean difference of 7.573 (58.348 – 50.775) was not statistically significant ($p > 0.05$). All other possible combinations of the pairwise comparisons were not significantly different from each other.

After adjusting for initial body weight, the fat content scores differed statistically between the different additive treatment levels, thus the null hypothesis was rejected.

Table 4.3 Descriptive statistics and pairwise assessment of fat content component T-scores

	Control		Moringa		Neem		Monensin		
Variables	*M*	*SD*	*M*	*SD*	*M*	*SD*	*M*	*SD*	*P*
InitialBW (kg)	38.55	± 3.00	38.10	± 3.71	38.32	± 4.08	37.35	± 4.53	0.95
Carcass wgt	29.23	± 1.73	30.80	± 4.15	30.17	± 2.04	28.73	± 2.91	0.61
LM % DM	32.66	± 1.99	34.48	± 5.38	34.50	± 2.84	35.34	± 4.85	0.70
IM fat % DM	36.47	± 6.87	34.56	± 7.23	44.26	± 6.70	35.84	± 7.73	0.11
IM fat as-is	11.97	± 2.77	12.15	± 4.20	15.22	± 2.25	12.63	± 3.07	0.28
Rib-cut ratio									
Fat %	33.23	± 3.86	30.08	± 3.88	34.34	± 2.45	30.03	± 2.68	0.07
Muscle %	51.78	± 3.50	55.50	± 2.72	51.57	± 2.57	54.40	± 2.22	0.06
Bone %	14.98	± 1.25	14.41	± 1.94	14.08	± 2.28	15.56	± 1.51	0.51
CFC *T*-scores	50.27[ab]	± 7.67	45.42[b]	± 13.00	58.09[a]	± 6.80	46.22[b]	± 8.06	0.05

IM means Intramuscular fat
M = Mean
SD = Standard deviation
LM means *Longissimus* muscle
P means p-value
BW means body weight
CFC means Carcass fat content
[a b] Means with different superscripts within the same row are significantly different ($p < 0.05$) (initial BW included as a covariate/pre-test variable)

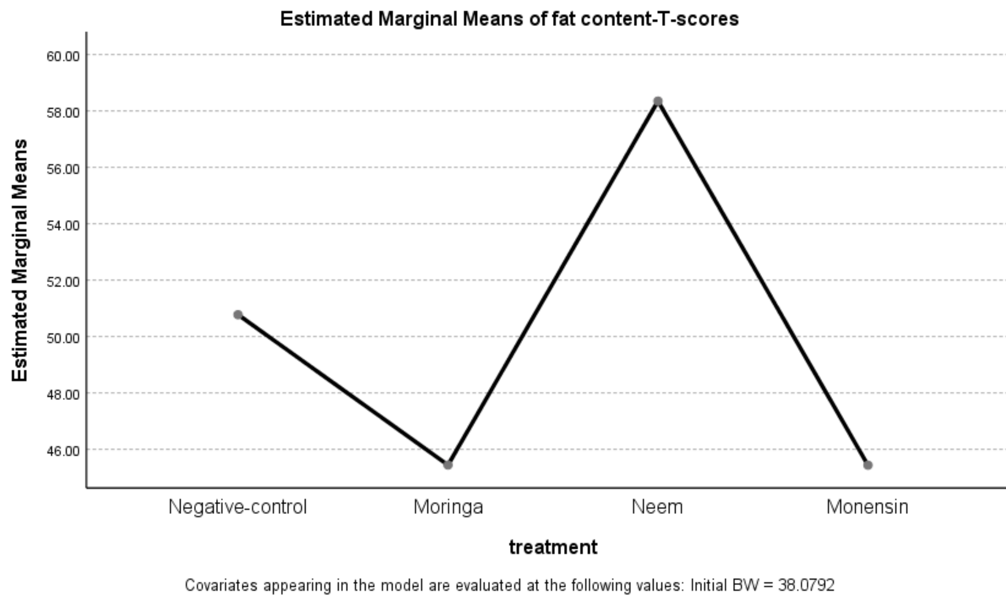

Figure 4.6: Adjusted mean estimates across the treatment levels

4.6 Linear combinations and between-the-group difference in SA Mutton Merino sheep subcutaneous fatty acid composition

4.6.1 Fatty acids molar percentage (%)

The descriptive statistics of subcutaneous fatty acids molar percentages can be seen in Table 4.4.1. The univariate normality assumption of SFA, MUFA and PUFA (molar %) were tested and Shapiro-Wilk p-values were greater than 0.05 for all dependent variables. The result of Box's F test on fatty acids molar percentage combination was significant, F (18, 1413.50) = 2.57, $p < 0.01$. This F Box's results implied that the variance and covariance between the SFA, MUFA, and PUFA molar percentages are not the same for all feed additive groups. So, the assumption of homogeneity of dispersion matrices was rejected, and this perhaps suggests the sample scores on the dependent variables can be used to predict group membership. Moreover, the MANOVA results showed there was statistical significance between feed additive treatment groups across linear combinations, Pillai's Trace = 0.74, F (9, 60) = 2.19, p = 0.04, with an effect size of approximately 0.25 (or 25% variance explained).

Multiple ANOVAs as follow-up tests were carried out on fatty acid combinations at 0.05 significance level (i.e., SFA, MUFA, and PUFA) together with every fatty acid-dependent variable on the molar percentage. The assumption of homogeneity of variance based on Levene's F test was satisfied on every dependent variable ($p < 0.05$) except for heptadecanoic – C17:0, myristoleic – C14:1, ɣ-linolenic - C18:3n6, Homo- γ -Linolenic - C20:3n6, pentadecenoic - C15:1, and behenic - C22:0 acid. The univariate ANOVA on SFA was

significant, F (3, 20) = 3.69, p = 0.03, η^2 = 0.36, MUFA also yielded a significant statistic, F (3, 20) = 4.38, p = 0.02, η^2 = 0.40, as well as PUFA, F (3, 20) = 5.32, p < 0.01, η^2 = 0.44. Thus, SFA, MUFA, and PUFA ANOVAs indicated 36%, 40%, and 44% of the variances of the dependent variable were associated with the group factor respectively.

The ANOVA on stearic – C18:0 FA molar percentage was significant, F (3, 20) = 3.13, $p \leq 0.05$, η^2 = 0.32. This also implies 32% of the stearic acid variance was explained by the feed additive treatment groups. The significant ANOVA observed on heptadecanoic acid – C17:0, F (3, 20) = 3.31, p = 0.04, was disapproved by Welch F test, F (3, 9.70) = 2.64, p = 0.11. Also, ANOVA on tricosanoic acid – C23:0 was significant, F (3, 20) = 3.24, p = 0.04, η^2 = 0.33. Furthermore, the ANOVA on eicosanoic – C20:1 and oleic – C18:1n9cis FAs were significant, F (3, 20) = 4.25, p = 0.02 partial η^2 = 38.9 (39% explained variance) and F (3, 20) = 3.9, p = 0.02, η^2 = 37 (37% explained variance) respectively. Behenic – C22:0 acid yielded marginally significant result using a robust Brown-Forsythe F test, F (3, 8.23) = 4.09, p = 0.05. The ANOVA on linoleic – C18:2n6cis and α-linolenic – C18:3n3 PUFAs also yielded statistical significance, F (3, 20) = 4.55, p = 0.01, partial η^2 = 0.41 and F (3, 20) = 5.46, p < 0.01, η^2 = 0.45 respectively. Indicating 41% and 45% of linoleic and α-linolenic variance were explained by the feed additive treatment effect respectively. All other fatty acids dependent variables tested were not statistically significant p > 0.05.

The post hoc analyses for the significant univariate ANOVAs were conducted via Bonferroni multiple comparisons to find which feed additive treatment groups altered the fatty acids molar percentage the most. As can be seen in Table 4.4.1, the Moringa feed additive group (47.258) has the largest quantitative average on MUFA, which was significantly different from the monensin group (42.629) with an estimated mean difference of 4.629 (p = 0.04). Whereas, the other feed additive groups were not significantly different from each other. Similarly, monensin feed additive group (3.363) was significantly different from negative-control group (2.526) and Neem group (2.560) on PUFA, with estimated mean differences of 0.837 (p = 0.02) and 0.803 (p = 0.02) respectively. While estimated mean differences were not significant for the Moringa group (2.661). The SFA was not significantly different among the feed additive treatment groups despite statistical significance on ANOVA. However, monensin feed additive treatment was shown to increase tricosanoic – C23:0 acid significantly, given the estimated mean difference of 0.041 (0.089 – 0.048) between monensin and negative-control group (p = 0.04). Meanwhile, Neem and Moringa groups were not significantly different from the other groups.

Oleic acid – C18:1n9c was statistically different between Moringa (45.039) and monensin group (40.450), with an estimated mean difference of 4.589 (p = 0.04). Also, there was an

apparent significant increase in monensin (2.712) group linoleic acid – C18:2n6c (as seen in Table 4.4.1). This was marked by a significant rise in monensin group mean as compared to the negative control group (p = 0.03) with an estimated mean difference of 0.657 (2.712 – 2.066), as well as the Neem group (p = 0.03) with a mean difference of 0.645 (2.712 – 2.066).

As for α-linolenic – C18:3n3, the monensin feed additive treatment was also significantly different from the negative control group (p = 0.01) with a mean difference of 0.150 (0.511 – 0.361). The significant difference further extends to the Moringa and Neem group (p = 0.03), with an estimated mean difference of 0.121 and 0.129 respectively. Furthermore, the feed additive groups were not significantly different from each other on heptadecanoic - C17:0, behenic - C22:0, stearic – C18:0, and eicosenoic - C20:1 despite significant ANOVA results. This probably suggests a lack of statistical power.

Without any doubt, the significant difference across the groups on the fatty acids means combination (i.e., SFA, MUFA, and PUFA), and the multiple ANOVAs on individual fatty acids molar percentage have shown that the SA Mutton Merino sheep (subcutaneous) fatty acid compositions were different because of the feed additives treatment used. As a result, the null hypothesis was rejected.

Table 4.4.1 Descriptive statistics and multiple comparisons of fatty acids molar percentage

	Control		Moringa		Neem		Monensin		
Fatty acids	*M*	*SD*	*M*	*SD*	*M*	*SD*	*M*	*SD*	*P*
C14:0	3.09	± 0.88	2.76	± 0.49	3.20	± 0.70	3.45	± 0.76	0.43
C16:0	27.04	± 2.10	26.02	± 2.59	27.64	± 2.01	26.63	± 1.41	0.59
C17:0	5.52	± 1.38	4.52	± 0.47	3.74	± 1.03	3.59	± 1.38	0.11
C18:0	16.73	± 4.13	15.72	± 2.01	17.59	± 2.04	19.24	± 4.40	0.05
C20:0	0.11	± 0.02	0.11	± 0.03	0.11	± 0.02	0.13	± 0.03	0.37
C21:0	0.51	± 0.10	0.47	± 0.16	0.41	± 0.11	0.53	± 0.25	0.65
C22:0	0.02	± 0.00	0.03	± 0.01	0.03	± 0.00	0.04	± 0.01	0.05
C23:0	0.05^{a}	± 0.01	0.06^{ab}	± 0.03	0.07^{ab}	± 0.02	0.09^{b}	± 0.02	0.04
C14:1	0.25	± 0.11	0.18	± 0.04	0.17	± 0.06	0.15	± 0.06	0.13
C15:1	0.03	± 0.03	0.03	± 0.03	0.02	± 0.02	0.03	± 0.02	0.70
C16:1	1.32	± 0.42	1.08	± 0.25	1.07	± 0.29	0.96	± 0.20	0.23
C20:1	0.15	± 0.04	0.14	± 0.06	0.08	± 0.01	0.09	± 0.02	0.02
C18:1n9c	44.37^{ab}	± 3.33	45.04^{a}	± 1.80	42.35^{ab}	± 2.41	40.45^{b}	± 2.53	0.02
C18:1n9t	0.79	± 0.47	0.79	± 0.55	0.59	± 0.21	0.95	± 0.64	0.65
C20:2	0.08	± 0.01	0.08	± 0.01	0.07	± 0.01	0.08	± 0.02	0.76
C18:2n6c	2.05^{b}	± 0.14	2.16^{ab}	± 0.36	2.07^{b}	± 0.49	2.71^{a}	± 0.36	0.01
C18:3n3	0.36^{b}	± 0.04	0.39^{b}	± 0.05	0.38^{b}	± 0.06	0.51^{a}	± 0.11	<0.01
C18:3n6	0.03	± 0.00	0.03	± 0.01	0.03	± 0.00	0.04	± 0.01	0.08
C20:3n6	0.03	± 0.00	0.03	± 0.01	0.03	± 0.00	0.03	± 0.00	0.87
SFA	50.09	± 3.19	49.69	± 2.19	52.79	± 1.73	54.28	± 3.66	0.03
MUFA	46.90^{ab}	± 3.23	47.26^{a}	± 2.09	44.14^{ab}	± 2.18	42.63^{b}	± 2.74	0.02
PUFA	2.53^{b}	± 0.18	2.66^{ab}	± 0.43	2.56^{b}	± 0.56	3.36^{a}	± 0.42	<0.01
UFA/SFA	0.99	± 0.13	1.01	± 0.09	0.89	± 0.06	0.85	± 0.10	0.03
PUFA n6/n3	5.86	± 0.40	5.61	± 0.46	5.47	± 0.73	5.56	± 0.98	0.78
PUFA/SFA	0.05	± 0.00	0.05	± 0.01	0.05	± 0.01	0.06	± 0.01	0.05

SFA means saturated fatty acids
MUFA means monounsaturated fatty acids
PUFA polyunsaturated fatty acids
UFA/SFA means unsaturated fatty acids-to-saturated fatty acids ratio
PUFA/SFA means polyunsaturated fatty acids-to-saturated fatty acids ratio
P means p-value
$^{a\,b}$ means with different superscripts within the same row are significantly different ($p < 0.05$

4.6.2 Gravimetric fatty acids content (mg/g)

Similar to Table 4.4.1 the descriptive statistics of subcutaneous fat gravimetric FAs content can be seen in Table 4.4.2. The univariate normality assumption of SFA, MUFA and PUFA (gravimetric content) were tested and Shapiro-Wilk *p*-values were greater than 0.05 for all dependent variables. Similarly, the Box's *F* test results on the combination of the fatty acid were nonsignificant, $F(18, 1413.50) = 1.02$, $p = 0.43$. This implies the fatty acids combination variance and covariance are the same in the feed additive treatment levels and the assumption of homogeneity of dispersion matrices was retained.

The MANOVA results revealed there was statistical significance between the feed additive treatment groups on SFA, MUFA, and PUFA combinations, Wilks' Lambda = 0.39, F(9, 43.96) = 2.33, p = 0.03, η^2 = 0.27. The results of MANOVA analysis implied that the fatty acids gravimetric mean combinations were not the same across the feed additive treatment groups and there was no equality of means on the dependent variables. Also, 27% of the variance explained by the combination of the fatty acids was due to the feed additive treatment used. However, the additional univariate ANOVA tests were not significant ($p > 0.05$) on SFA and MUFA, F (3, 20) = 2.62, p = 0.08, η^2 = 0.28, and F (3, 20) = 1.20, p = 0.34, η^2 = 0.15 respectively. Whereas, ANOVA on PUFA was statistically significant, F (3, 20) = 3.88, p = 0.02, η^2 = 0.37 (37% of variance explained).

Multiple ANOVAs tests were also conducted (at 0.05 significance level) and the assumption of the equality of variance based on Levene's F test was greater than 0.05 on each fatty acid gravimetric content-dependent variable. The ANOVA on tricosanoic acid – C23:0 was statistically significant, F (3, 20) = 4.618, p = 0.01, partial η^2 = 0.41. Similarly, the univariate ANOVA on linoleic acid – C18:2n6c showed statistical significance, F (3, 20) = 3.63, p = 0.03 η^2 = 0.35 (implies 35% variance explained). Likewise, ANOVA on α-linolenic acid – C18:3n3 was statistically significant, F (3, 20) = 4.56, p = 0.01, partial η^2 = 0.41 (i.e., 41% variance explained). All other multiple ANOVAs tests conducted were not statistically significant (p > 0.05).

The significant ANOVAs were subject to a post hoc test via Bonferroni multiple comparisons (at 0.05 level) to find where significant differences between-the-group lies as seen in Table 4.4.2. Monensin feed additive group mean (8.442) was significantly different from the negative-control group mean (4.750) on gravimetric PUFA content with an estimated mean difference of 3.692, while Neem and Moringa groups were neither different from each other nor other groups. The transformed tricosanoic acid variable (i.e.$\log_{base10}$ of C23:0) presents a significant increase in monensin group (-0.677) compared to negative-control group (-1.072), with an estimated difference of 0.395 (p = 0.01). All other estimated mean differences between the groups were not significant.

On linoleic acid – C18:2n6c the moringa and Neem groups were indifferent in comparison to other groups, while the monensin (6.808) group was significantly different (p = 0.03) from the negative-control (3.854) group, with an estimated mean difference of 2.954. Similarly, the monensin feed additive group was seen to have the highest α-linoleic acid – C18:3n3 estimated mean (1.285), which was significantly different (p = 0.01) from the negative control group (0.687) with a mean difference of 0.598. While Neem and Moringa feed additive groups

were neither different from each other nor the remaining groups. All other fatty acids dependent variables tested were not statistically significant ($p > 0.05$).

The fatty acids' gravimetric contents have also shown that SA Mutton Merino sheep subcutaneous fats were different in linear combinations of fatty acids and indeed on some of the specific fatty acids quantified due to the feed additives treatment used. Therefore, the statistical analyses affirmed that the alternative hypothesis was probable, so the null hypothesis was rejected.

Table 4.4.2 Descriptive statistics and multiple comparisons of fatty acids gravimetric content

Fatty acids	Control			Moringa			Neem			Monensin			
	M		*SD*	*M*		*SD*	*M*		*SD*	*M*		*SD*	*P*
C14:0	7.41	±	0.38	5.70	±	1.28	7.56	±	2.70	8.91	±	2.77	0.09
C16:0	59.18	±	5.78	53.92	±	10.72	64.83	±	15.77	66.63	±	16.77	0.13
C17:0	9.34	±	0.82	9.26	±	1.03	8.35	±	1.22	8.05	±	1.62	0.18
C18:0	37.66	±	13.72	32.51	±	6.35	41.62	±	12.11	49.96	±	23.38	0.07
C20:0	0.20	±	0.08	0.23	±	0.06	0.26	±	0.09	0.34	±	0.16	0.15
C21:0	0.95	±	0.32	0.97	±	0.36	0.94	±	0.24	1.33	±	0.66	0.35
C22:0	0.05	±	0.01	0.06	±	0.01	0.07	±	0.01	0.10	±	0.05	0.05
C23:0	0.09^{b}	±	0.05	0.13^{ab}	±	0.07	0.17^{ab}	±	0.08	0.22^{a}	±	0.10	0.01
C14:1	0.43	±	0.11	0.36	±	0.05	0.40	±	0.19	0.35	±	0.10	0.63
C15:1	0.05	±	0.04	0.07	±	0.05	0.05	±	0.04	0.06	±	0.04	0.78
C16:1	2.35	±	0.60	2.21	±	0.52	2.55	±	1.07	2.34	±	0.58	0.94
C20:1	0.26	±	0.06	0.28	±	0.11	0.19	±	0.03	0.23	±	0.06	0.14
C18:1n9c	82.37	±	19.20	92.80	±	11.28	98.86	±	20.42	99.83	±	18.72	0.33
C18:1n9t	1.49	±	1.13	1.62	±	1.13	1.43	±	0.72	2.19	±	1.27	0.70
C20:2	0.14	±	0.02	0.16	±	0.01	0.17	±	0.04	0.20	±	0.05	0.06
C18:2n6c	3.85^{b}	±	1.11	4.48^{ab}	±	1.04	4.99^{ab}	±	2.04	6.81^{a}	±	2.05	0.03
C18:3n3	0.69^{b}	±	0.24	0.81^{ab}	±	0.16	0.91^{ab}	±	0.30	1.29^{a}	±	0.43	0.01
C18:3n6	0.06	±	0.02	0.06	±	0.01	0.07	±	0.02	0.09	±	0.03	0.12
C20:3n6	0.06	±	0.02	0.06	±	0.02	0.07	±	0.02	0.07	±	0.02	0.75
SFA	93.98	±	27.00	102.78	±	16.71	123.79	±	28.39	134.16	±	36.49	0.08
MUFA	86.95	±	19.64	97.34	±	11.68	103.47	±	21.75	105.00	±	18.71	0.34
PUFA	4.75^{b}	±	1.43	5.51^{ab}	±	1.24	6.17^{ab}	±	2.43	8.44^{a}	±	2.49	0.02

$^{a\ b}$ means different superscripts in rows are significantly different ($p < 0.05$)

M = Mean

SD = Standard deviation

SFAs means saturated fatty acids

MUFAs means monounsaturated fatty acids

PUFAs polyunsaturated fatty acids

P means p-value

CHAPTER 5: DISCUSSION

5.1 Discussion approach

The public perception concerning the use of antibiotics in ruminant animal feed and its intrinsic consequence on (human) health and animal welfare continues to generate debates about the most acceptable practices for livestock production (Fajt, 2007; Durmic & Blache, 2012). In recent time, the aftermath of the European Union ban (in January 1st, 2006) on growth promotants, has led to a significant breakthrough in animal research, on the utilization of medicinal plants (such as Moringa and Neem leaf extracts) as a potential substitute for conventional methane mitigation additive (Lim, 2014; Fahey, 2017).

These medicinal plants contain phytochemicals that have been researched and described as possible mitigants for ruminant greenhouse gas emissions, as well as natural antibiotics and antioxidants, which could improve animal welfare (Benchaar *et al.*, 2006; Durmic & Blache, 2012; Lim, 2014; Fahey, 2017). However, there are questions about the effect of these bioactive plant extracts on animal food-product quality and therefore, this study seeks to investigate some aspects related to meat quality. The current study focuses on the possible effect of medicinal plant extracts in SA Mutton Merino sheep carcass fat, and fatty acids composition.

5.2.1 Differences in fat-soluble pigment absorbance relative to the additive treatments used along with days-on-trial

As can be seen in Table 4.2.1 the respective (fat-soluble) pigment absorbance quantitative average was relatively similar across the groups, except for the slight numerical ($p > 0.05$) increase in the negative-control group. Following the test of homogeneity of regression assumption, the interaction effect between days-on-trial and feed additive treatments was confirmed ($p < 0.05$) as suspected. According to Parker (1996) and Yang *et al.* (2004), the deposition of carotenoids in animal adipose tissue is considered a species-specific occurrence. Yang *et al.* (2004) also revealed that a very low concentration of carotenoid lutein in sheep body fat is responsible for the adipose tissue white colour, and the physical characteristics of carotenoids are marked by the ability to absorb and transmit radiant energy (Krinsky, 1994).

As can be seen in Table 4.2.2, the estimated means of the low number of days-on-trial absorbance were not significant for Merino sheep drenched with *Azadirachta indica* (A. Juss) extract and those fed monensin additive treatment. This was also similar with negative-control and Moringa group, even as the estimated means of negative-control and Moringa group were lower in comparison to the overall average of tested samples within 56.19 days-on-trial (approximately 0.67).

Furthermore, the estimated means of the fat-soluble pigment absorbance within each level of days-on-trial revealed the positive and negative interactions concerning the carotenoid deposition and the number of days the SA Mutton Merino sheep spent on the trial. This implies that in comparison with the monensin and Neem group, the pair (i.e., negative-control and Moringa) have a higher prevalence of carotenoid deposition to the extent that SA Mutton Merino sheep have had a higher number of days-on-trial. This was affirmed by the statistically significant difference ($p < 0.05$) between the feed additive treatments at 125.47 days-on-trial level as seen in Table 4.2.2.

The interpretation of the results could be better understood bearing in mind the absorption, metabolism, and actions of carotenoids in animal tissue. According to Parker (1996), the extent of carotenoid biotransformation to retinoids (or retinyl ester) in the intestinal lumen is largely reliant on the animal species. Also, Parker (1996) suggested that the incorporation of carotenoids into the micellar lipid composition is a rate-limiting factor to the intestinal uptake, and flow of carotenoids into the circulatory system. This inference indirectly suggests that the statistical difference ($p < 0.05$) between the negative-control, Neem, as well as monensin group, was due to the share proportion of unmetabolized carotenoids and unesterified retinol assimilated into the micellar composition, and absorbed by the lymphatics. Also, Parker (1996) reported that unmetabolized carotenoid (i.e., β-carotene) and retinyl ester constitute the greater proportion of lymphatic products absorbed into the animal mesenteric lymph.

Meanwhile, carotenoids are transported in the blood lipoprotein (Parker, 1996; Yang *et al.*, 2004), and the uptake is similar to cholesterol and free fatty acids (FFAs) absorption (i.e., passive diffusion) (Parker, 1996; Bhagavan & Ha, 2011). However, exogenous sources of cholesterol are improbable in ruminant animals, owing to the nature of their diet (i.e., plant-based). As a result, the more SA Mutton Merino sheep utilize plant-based resources from the start to the high days-on-trial level, so the rate of dietary carotenoid deposition is seen in the negative control group.

The key phytochemical in *Azadirachta indica* (A. Juss) extract is limonoids (Lim, 2014), they are also referred to as isoprenoid volatile aromatic compound (or essential oils, which include diterpenoid, triterpenoid, and tetranortriterpenoid). It is important to note that fat-soluble vitamins (i.e., A, D, E, and K) are put together from isoprenoid units such as cholesterol (Bhagavan & Ha, 2011). According to Holstein (2011), there is a theory that is now known as the isoprene rule, this isoprene rule affirms that all terpenes may well be derived from any precursor composed of isoprene units (Holstein, 2011). In this way, terpene-rich *Azadirachta indica* extract additive could increase the cholesterol biosynthesis in the liver of SA Mutton Merino sheep. Similarly, the increased acetate supply suggested in the previous discussion

for the Neem group would lead to the improved formation of hydroxymethylglutaryl coenzyme A (HMG-CoA). Moreover, HMG-CoA is a rate-limiting enzyme for the mevalonate pathway and indeed the synthesis of cholesterol (Bhagavan & Ha, 2011; Holstein, 2011).

Since the chylomicron conveys the micellar lipid constituents via mesenteric lymph into the bloodstream (Parker, 1996). Therefore, the final assemblage of unmetabolized lutein into the lipoprotein composition would be limited by a potential increase in hepatic cholesterol synthesis. Given that both carotenoid and cholesterol are largely associated with the lipid membrane (Krinsky, 1994; Bhagavan & Ha, 2011). Similarly, the activity of sheep lipoprotein lipase is known to be higher in the subcutaneous fat as compared to any other animal fat depot (Vernon, 1980). Thus, the significant difference between the negative control group and the Neem group is justified as can be seen (in Table 4.2.2) by sample absorbance values throughout the study period.

Moringa group, on the other hand, was neither different from the negative control nor monensin and Neem group as shown in Table 4.2.2. This was perhaps a direct consequence of *Moringa oleifera* hypolipidemic phytochemicals such as β-sitosterol. The supply of dietary sterol (i.e., cholesterol and phytosterol) as stated by Bhagavan & Ha (2011) make possible steroid homeostasis in animals, especially hepatic cholesterol production. This implies Moringa phytosterol somewhat interferes with the micellar incorporation of carotenoid, at the same time constrain hepatic cholesterol synthesis during the trial period. The monensin group concurrently demonstrates a statistically significant difference ($p < 0.05$) as compared to the negative control group (at a high days-on-trial level). Unlike the Neem treatment, the tissue fat effect of monensin treatment on carotenoid deposition (as well as metabolism) was not expected. Therefore, less comprehensible at the very least.

However, the possible explanation for the low absorbance values in comparison to other additive treatment groups (throughout the days-on-trial) is associated with the rate-limiting step of HMG-CoA in cholesterol biosynthesis (i.e., conversion to mevalonate). According to Bhagavan & Ha (2011) HMG-CoA reductase is controlled by the rate of gene transcription and the cellular energy level (i.e., phosphorylation & dephosphorylation). This explains why cholesterol biosynthesis is species-dependent, and the same is true for the utilization of carotenoids. Similarly, improved energy efficiency as a result of monensin treatment will equally reduce adenosine monophosphate to adenosine triphosphate ratio (AMP/ATP) in the animal cell. This point suggests potential dephosphorylation of HMG-CoA reductase following the reduction of cytosolic HMG-CoA via the improved supply of NADPH. Therefore, monensin treatment may possess to a certain extent a similar tendency to improve cholesterol synthesis as *Azadirachta indica* (A. Juss) additive treatment.

5.2.2 The implication on the carotenoid antioxidative capability

Furthermore, a well-known activity of carotenoid is chain-breaking antioxidation of lipid membrane via carotenoid oxidation (Krinsky, 1994). El-Agamey *et al.* (2004) reported that carotenoids can function as pro-oxidant at high concentrations, and to a greater extent at high oxygen concentrations. The evidence put forward by El-Agamey *et al.* (2004) suggests a high concentration of carotenoid modifies the lipid membrane characteristics, and this may result in membrane permeability to toxins and reactive oxygen species (ROS). Additional interaction with ROS according to El-Agamey *et al.* (2004) brings about the proliferation of carotenoid peroxyl radicals (i.e., highly reactive species), which consequently propagate lipoperoxidation in itself. It seems that the negative control treatment appears to be the most appropriate case scenario in line with El-Agamey *et al.* (2004) submission. Ultimately, the possibility of lipid peroxidation and carotenoid protection would be examined in the fatty acid composition of subcutaneous adipose tissue.

5.2.3 Body weight and fat component (*T*) score

In general, change in animal body weight as discussed by Forbes (2006) under the normal physiological condition is largely due to relative accretion of both fat-free body mass (FFM) and body fat. However, the input of FFM and body fat to the total weight loss or gain is mediated by the initial body weight (BW). Also, Forbes (2006) observed that a thin individual's lean body mass (LBM) constitutes half of the combined lean and body fat weight difference. Whereas, in high-body condition individuals, body fat makes up 75% of the lean muscle and fat weight difference. Hence, Forbes (2006) was convinced that the lean body mass of an individual has a logarithmic or curvilinear relationship function with the body fat.

It is worth noting that the findings of this study are in agreement with the Forbes (2006) viewpoints to a certain extent. Similarly, the descriptive statistics in Table 4.1 also support the review on the rate of change in body fat. Likewise of equal importance was the significant ($p \leq 0.05$) positive relationship between intramuscular fat (%) and days-on-trial. Moreover, in contrast to Forbes (2006) research study, this study has more than a single source of variation in its experimental design, such that a direct comparison of the two studies is very much limited by treatment effect (i.e., between-the-group variation). Notwithstanding, the increase in body weight gain over time is primarily due to an increase in muscle together with body fat.

Considering the 60 – 65kg final body weight pre-condition for selection of animal, it could be suggested or expected that the leaner SA Mutton Merino sheep would give rise to a relatively larger muscle percentage than the heavier SA Mutton Merino sheep. This is because in many situations the relative contribution of muscle and fat is dependent on the initial body weight (Forbes, 2006). Since the comparative contribution of lean to the total body weight gain is

inversely proportional to the body fat as stated by Forbes (2006). Therefore, the important physiological factor shaping this variability in the animal fat (i.e., SA Mutton Merino sheep) was indeed the initial body weight. This explains the rationale for using initial body weight as the predictor variable or covariate in the statistical analysis.

According to Webb & O'Neill (2008) the lipid content of ruminant animal intramuscular fat (i.e., *M. longissimus*) especially triacylglycerol proportion, usually increases with the increment in the carcass fat content. Interestingly, the data obtained from this study coincides with the statement, as the corresponding significant ($p < 0.05$) increase between rib-cut fat (%) and intramuscular fat (%) can be seen in Table 4.1. Furthermore, the high lipid yield of a hot Soxhlet extraction method for high lipid samples (i.e., intramuscular fat) as pointed out by Manirakiza *et al.* (2001) could be a result of co-extracts (such as pigments, carotene, and waxes) present in the lipid residue. These co-extracts are soluble under the same condition as the primary lipid residue.

Therefore, the principal component factor analysis used in this study allows circumventing the impact of inexplicit and latent co-extracts present in the lipid residue. In this way, the retained factor of 1.405 eigenvalue between intramuscular fat (%) and rib-cut fat (%) represents the underlying variable of interest (i.e., fat content). Hence, the total rib-cut fat (%) and the (*M.* longissimus) intramuscular fat (%) of the 8th, 9th & 10 thoracic rib-cut samples were well defined by a single component factor (based on eigenvalue >1 criterion).

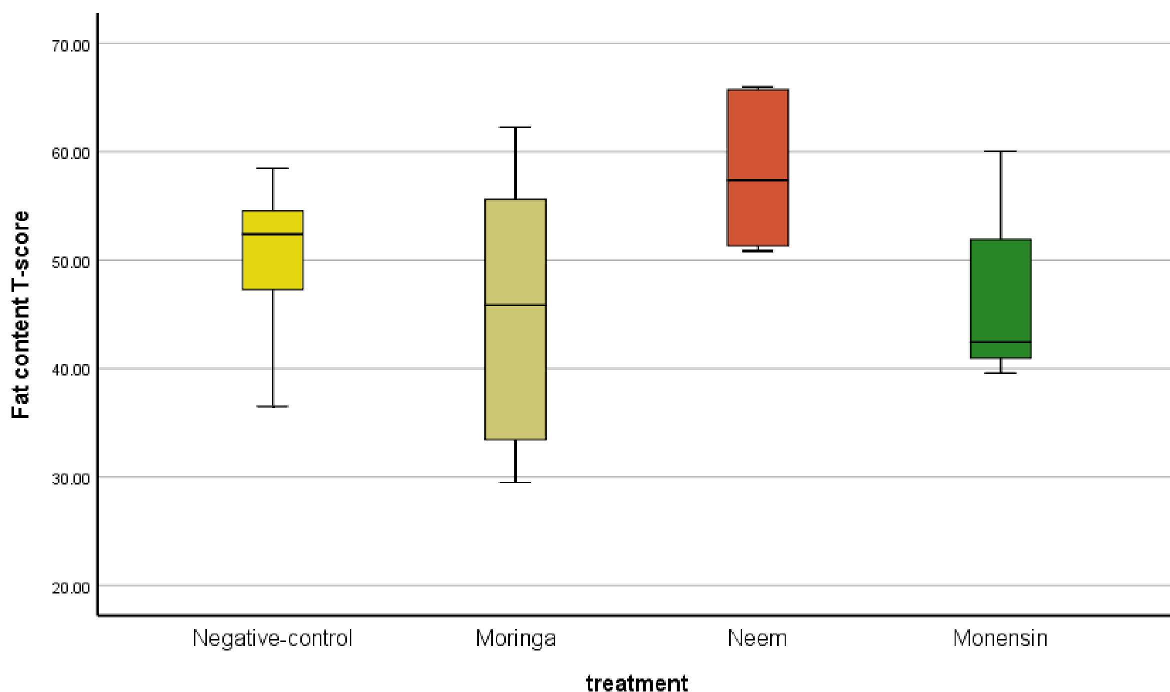

Figure 5.1: The graphical illustration of the feed additives treatment distribution

5.2.4 Potential effect of *Moringa oleifera* and *Azadirachta indica* (A. Juss) on the South African Mutton Merino fatty acid synthesis.

As can be seen in Figure 5.1 the lowest score in the observation (approximately 29) was found in the Moringa treatment. Similarly, the bottom 25% of all the respective feed additive distributions was greater than the 1st quartile of Moringa fat content distribution. This indicates that Moringa treatment corresponds to the minimum fat content in the overall observation scored. However, the dispersion spread in the Moringa group covers the interquartile range of both negative-control and monensin treatment except for the Neem group. This suggests that the variation in Moringa treatment distribution is larger in comparison to other feed additive treatments used. It is also important to note that the standard deviation (*SD*) and the quantitative average (*M*) shown in Table 4.3 supported the graphical illustration in Figure 5.1.

Neem treatment was more consistent by the way of high-fat content scores, as shown with shortened boxplot 1st and 4th quartile as well as the relatively compressed interquartile range (in Figure 5.1). This implies that Neem treatment typified the maximum fat content in the overall distribution scored. Furthermore, the 50th percentile of monensin treatment was the least, meanwhile, the treatment distribution varied more at the upper level (i.e., 4th quartile). Whereas, negative-control treatment distribution varied further to the lower level (i.e., 1st quartile). This point suggests monensin treatment occasionally has high score cases in its upper distribution while the negative-control has low scores in its lower distribution.

Following the estimation of marginal means (via adjustment for the continuous predictor variable as can be seen in Figure 4.6), Neem treatment was shown to be statistically ($p < 0.05$) different from both Moringa and monensin treatment. Whereas, the negative-control treatment was not different statistically in comparison with other feed additives treatments. The supposed explanation for the difference in feed additives treatment could be clarified by taking into account the metabolic pathways of lipid in the ruminant animal. Many studies (Bell, 1979; Vernon, 1980; Christie, 2014) have described acetate as the principal precursor of fatty acid synthesis in the ruminant adipose tissue. The preference for acetate carbon utilization as stated by Bell (1979) and Vernon (1980) was perhaps due to the derivation of nearly all blood glucose from the gluconeogenic pathway, despite the normal physiological state.

Essentially, the *Azadirachta indica* leaf was described to be rich in isoprenoids (such as volatile oils) and limonoids (specifically meliacins, responsible for the bitter taste) (Tarbell, 1957; Lim, 2014). Few non-isoprenoids (such as flavonoids) were isolated from the leaf and this hinted at Neem extracts' potential wide range of antimicrobial capabilities against ruminal microflora (i.e., Gram +ve and Gram -ve bacteria). On the contrary, less varied and steadily high-fat content scores seen in Figure 5.1 suggest that the potential effect of Neem additive

treatment was more predictable *in vivo*. Besides, Hart *et al.* (2008) discussed the effect of adding a blend of essential oil (BEO 5mg/day) into a mixture of continuous fermenters, and the result shows that BEO stimulates increase acetate formation. This calls to attention a potential elevated supply of acetate or acetogenic SCFAs as the theoretical justification for the high-fat content scores seen in the Neem treatment.

Consequently, this would lead to increased adipose tissue *de novo* synthesis. It is also significant to highlight the study described by Vernon (1980) which put adipose tissue lipogenic activity in non-lactating sheep to approximately 90% as compared to hepatic fatty acid synthesis.

Furthermore, there are unique phytochemical agents in Moringa leaf with a wide range of ethnopharmacological benefits namely, glucosinolate, carbamates, and nitriles (Gopalakrishnan *et al.*, 2016; Fahey, 2017). The larger variation observed in the Moringa treatment as seen in Table 4.3 as well as Figure 5.1 suggests a less predictable potential effect of *Moringa oleifera* leaf extract (on SA Mutton Merino sheep tissue fat). This indicates the likelihood of a broader effect of Moringa treatment on ruminant lipid metabolism. According to Fahey (2017) aqueous and ethanolic extract preparations from Moringa leaf were believed to contain some alkaloids with a hypotensive effect on guinea pig and frog heart.

Similar studies reported by Faizi *et al.* (1995); Gopalakrishnan *et al.* (2016); and Fahey (2017) considered glucosinolate (specifically rhamnosylated & isothiocyanate), and carbamates (i.e., thiocarbamate glycosides & acetylated carbamate) from Moringa leaf to be hypotensive. As a result, Moringa leaf extract was referred to as hypolipidemic (Kumar *et al.*, 2012). This suggests Moringa leaf phytochemicals may reduce the accretion of hyperlipidaemic fatty acids (such as palmitic and myristic acids).

Speaking of hypolipidaemia, beta-sitosterol had also been reported (Kumar *et al.*, 2012) in Moringa plant parts, and this phytosterol is known to reduce low-density lipoprotein (LDL) cholesterol. Also, Fahey (2017) discussed the characteristics of Moringa glucosinolate derived glycosylated compound, which was said to contain six-carbon (C_6) rhamnose sugar. Sugar and starch alike are renowned by ruminant nutritionists as precursors of propionic acid in ruminal fermentation (Ipharraguerre & Clark, 2003), suggesting increased propionate proportion. Similarly, Moringa glucosinolate and its derivatives are potent antimicrobial agents, and potential propionate precursors at the same time. How this will affect the rumen microflora leaves much to be desired.

The potential effect of monensin treatment, on the other hand, can be put next to the Moringa treatment effect as can be seen in Figure 5.1 (and in Table 4.3). It is well-known that monensin selectively inhibits Gram +ve bacteria, and consequently reduces methane emission with a

cumulative increase in ruminal propionate proportion. Similarly, the supply of short-chain fatty acids profile in favour of glucogenic products due to monensin additive used could lead to moderate utilization of acetate carbon aimed at adipose tissue *de novo* synthesis (Bell, 1979; Vernon, 1980; Jambrenghi *et al.*, 2007). In this way, the deposition of fatty acids and the reduction in acetate carbon utilized by the adipose tissue may have been responsible for the low-fat content score seen in monensin treatment.

5.2.5 The implication on meat quality

According to Webb & O'Neill (2008) increase in carcass fat content oftentimes initiates a reduction in the shared proportion of polyunsaturated and saturated fatty acids. As a result, more saturated (often hyperlipidemic) fatty acids would be accumulated in the adipose tissue. This might be the case as seen in the Neem treatment high-fat content and to some extent in negative-control treatment. Similarly, the foremost important impetus for consumers' meat preference as described by Webb & O'Neill (2008) is intramuscular fat. Although, the use of marbling fat in meat quality assessment is only limited to a few countries such as the United States of America (USA)

5.3 Effect of feed additives treatment on SA Mutton Merino sheep subcutaneous fatty acid composition

The key fatty acids synthesized from acetyl CoA in sheep adipose tissue are palmitic – C16:0, oleic – C18:1n9c, and stearic – C18:0 acids. Although the percentage composition of these fatty acids varied with the nature of adipose tissue, stage of development, and notably the concentration of acetate utilized by the adipose tissue (Vernon, 1980).

The quantitative average of palmitic – C16:0 acid (i.e., second most abundant fatty acid) as can be seen in Table 4.4.1 corresponds to the viewpoints earlier mentioned in the potential effects of plant extracts treatment on fatty acid synthesis. As monensin and Moringa feed additive group accruing lower molar percentage on palmitic acid while negative control and Neem group average greater molar proportion. On the other hand, the combination of saturated fatty acids (SFA) (molar percentage and gravimetric content respectively) between feed additive groups was found to be statistically nonsignificant ($p > 0.05$). Despite the results statistics, tricosanoic – C23:0 acid was found to be significantly different ($p < 0.05$) between the monensin group and the negative control group (as can be seen in Table 4.4.1). A similar statistically significant difference was also seen in Table 4.4.2 for tricosanoic acid. These results surely implied that indeed the SFA composition in SA Merino subcutaneous fat was similar among the feed additive groups. Nonetheless, the accretion of exogenous SFA in the monensin group improved with the increasing chain length of SFA, and notably significant on tricosanoic – C23:0 acid against the negative control group.

Moreover, lamb adipose tissue compositional analyses reported by Vernon (1980) and Webb & O'Neill (2008) revealed that oleic – C18:1n9c acid is the most abundant fatty acid in the subcutaneous adipose tissue. Similarly, oleic – C18:1n9c acid proportion was the largest on the SA Mutton Merino sheep subcutaneous fatty acid composition (as seen in Table 4.4.1 and Table 4.4.2). According to Bell (1979) and Vernon (1980) the fatty acid desaturase enzyme activity has a preference for stearic – C18:0 acid over palmitic acid, and the rate of desaturation of stearic to oleic acid is greater in sheep subcutaneous adipose tissue. It is also important to highlight the submission by Vernon (1980) which states that a good number of subcutaneous fatty acids are synthesized *de novo*, which caused elevated oleic to stearic ratio since endogenous fatty acids are readily desaturated than exogenous sources. This explained why the Moringa feed additive group which had the least score on stearic acid, was significantly different ($p < 0.05$) from the monensin group on oleic acid. Meanwhile, the monensin group had a lower oleic to stearic ratio while the Moringa group had the highest oleic to stearic ratio.

According to Lough *et al.* (1992) oleic acid like other monounsaturated fatty acids (MUFA) is known as hypolipidemic fatty acid by the virtue of being able to reduce low-density lipoprotein (LDL) triacylglycerol and cholesterol in the blood. It is no surprise that the Moringa feed additive group mean on oleic acid was higher than the corresponding groups (as seen in Table 4.4.1). Likewise, the significant difference ($p < 0.05$) on MUFA between Moringa and monensin feeding additive groups further reinforced the aforementioned probable effect of Moringa phytochemicals (such as glucosinolate & β-sitosterol). These Moringa phytochemicals in solvent extract preparation have indeed influenced the molar percentage of MUFA via stearic acid *de novo* synthesis with subsequent desaturation and elongation. However, odd-chain and trans-unsaturated fatty acids (such as C15:1, & C18:1n9t) are of dietary origin.

Nevertheless, based on fatty acid content per sample weight average, the monensin group appears to be numerically higher than the Moringa group (on MUFA), followed by the Neem feed additive group. However, the differences between the groups did not have statistical significance as can be seen in Table 4.4.2. This point suggests that the increase in the means of monensin and Neem additive group on MUFA was due to the relatively greater LCFAs deposition in the two treatment groups as compared to other groups, without impacting their respective individual fatty acid molar proportion. Also, the level of LCFAs deposition was apparent in the total fatty acids (gravimetric) content of each feed additive group (i.e., monensin > Neem > Moringa > negative-control group) as seen in Table 4.4.2.

Linoleic – C18:2n6cis and α-linolenic – C18:3n3 acids are 18 carbon essential fatty acid molecules important to the formation of 20 carbon essential fatty acids such as arachidonic

acid (20:4n-6), and eicosapentaenoic acid (20:5n-3) respectively (Webb & O'Neill, 2008). As can be seen in Table 4.4.1 the monensin group was significantly different from all other groups (except the Moringa group) on the combination of polyunsaturated fatty acids (PUFA). This implied that SA Mutton Merino sheep that were given monensin feed additive treatment, and (to a lesser extent) those given Moringa plant extract experienced an increase in the molar percentage of PUFA deposited as compared to Neem and negative control group. The significant difference was also similar to the results statistics of the Linoleic acid molar percentage.

However, the Neem group was not significantly different from monensin on gravimetric PUFA content (as seen in Table 4.4.2). More specifically, linoleic and α-linolenic acid was significantly different between monensin, which had the highest quantitative average, and the negative control group on the respective essential fatty acid (gravimetric) content. Hence, the monensin additive treatment without question has improved essential fatty acid deposition on the subcutaneous adipose than any other feed additive treatment used. According to Webb & O'Neill (2008) the fatty acid composition defines the quality of animal fat, particularly the extent of saturated fatty acid ratio. The increase in polyunsaturated-to-saturated fatty acid (P:S) ratio indicates vital disease prevention benefits in humans, as can be seen in Table 4.4.1 monensin group had a fairly higher P:S ratio (i.e., 0.06) in comparison to other feed additive group. Although the P:S ratio approximates an order of 0.1 in all feed additive groups, which implied an imbalanced fatty acid ratio considering the recommendation of over 0.4 as stated by Webb & O'Neill (2008).

5.3.1 Interaction between carcass fat and polyunsaturated fatty acid ratio (PUFA n-6/n-3)

Similarly, the PUFA n-6 to n-3 ratio has been used in recent times to indicate the risk of coronary heart disease or cancer in humans. The PUFA n-6/n-3 ratio as seen in Table 4.4.1 appears to be relatively similar between the feed additive groups and above the less than four (4) recommendations reported by Webb & O'Neill (2008). Nevertheless, Figure 5.2 scattered plot depicts the interaction effect of PUFA n-6/n-3 ratio on carcass fat content (*T*) score as a function of feed additive treatment used. As can be seen in Figure 5.2 a negative relationship exists between PUFA n-6/n-3 ratio and carcass fat on the Neem (r = - 0.64) and negative control (r = - 0.33) treatment groups, with reasonably strong and moderate correlation coefficient respectively. The relationship trend suggests an increase in SA Mutton Merino sheep carcass fat in the Neem, and the negative control group appears to bring about a reduction in adipose tissue PUFA deposition, and in most cases linoleic and α-linolenic acid.

This is because linoleic and α-linolenic acid represents at least 80%, and 14% of the total PUFA in the composition of subcutaneous fatty acids respectively (as can be seen in Table 4.4.1 and Table 4.4.2). Similarly, ruminant dietary PUFA (mainly linoleic and α-linolenic acid) are generally absorbed from the lower gastrointestinal tract as stearic acid, owing to the extensive biohydrogenation by ruminal bacterial (Bell, 1979; Hobson *et al.*, 1997; Bessa *et al.*, 2007). As can be seen in Figure 5.2 the extent of reduced PUFA deposition seems to be greater in the Neem group, as 41% of variance explained by the PUFA ratio interaction effect on carcass fat, was as a result of *Azadirachta indica* (A. Juss) extract used. Therefore, the negative control and Neem additive treatment group have proven the submission by Webb & O'Neill (2008) which states that more saturated fatty acid is accumulated when there is an increase in carcass fat content, resulting in a lower polyunsaturated fatty acid ratio.

Furthermore, there was no relationship between PUFA ratio and carcass fat in the monensin group, at the same time, the Moringa group was positively correlated among the two variables (as can be seen in Figure 5.2). This suggests a relatively constant accretion of PUFA ratio in the monensin additive treatment group irrespective of the increase or decrease in carcass fat content. While in the Moringa treatment group increase in carcass fat content is shown to be accompanied by a rise in PUFA ratio. Monensin like many ionophores selectively inhibit Gram +ve bacterial as aforementioned, and this was expected to facilitate the escape of PUFA from the extensive ruminal biohydrogenation. Hence, the decrease in PUFA n-6/n-3 ratio as seen in negative control and Neem group with a corresponding increase in carcass fat content, suggests extensive lipid metabolism in the rumen of the respective SA Mutton Merino sheep group. In this way, the supplementation of *Azadirachta indica* additive leans towards steadily increasing *de novo* fatty acid synthesis while monensin additive treatment boosted the escape and deposition of dietary unsaturated fatty acids.

5.3.2 The implications for consuming public

Non-essential fatty acids or SFA (aside from stearic acid) are described as the major cause of coronary heart disease and cancer in humans (Webb & O'Neill, 2008). Meanwhile, essential fatty acids (i.e., n-6 & n-3) are important in human and animal nutrition, due to PUFA support for the immune response against diseases. Taking into account Figure 5.2 scattered plot interpretation, Moringa, and monensin feed additive treatment is expected to lower the risk of coronary heart disease or cancer depending on the intake of sheep fat. Whereas, there is a

higher risk of coronary heart disease in SA Merino mutton product from negative-control and Neem treatment group.

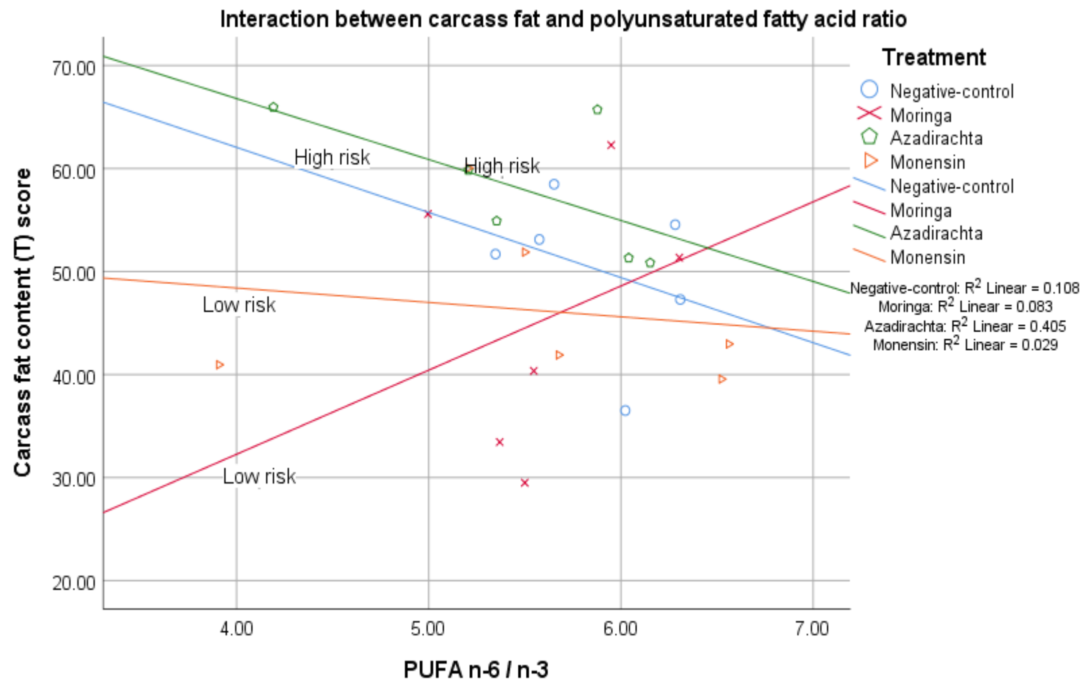

Figure 5.2: The interaction effect of PUFA ratio on carcass fat due to feed additive treatment

5.3.3 Overview of feed additive treatment main effect on physiological parameters

A two-dimension biplot shown in Figure 5.3 gives an overview of the feed additives treatment's main effect on SA Mutton Merino sheep physiological characteristics regarding attributes complexities (i.e., similarities and differences) and performance. As can be seen in Figure 5.3 PC1 (i.e., fatty acid composition component) is found on the x-axis while PC2 (i.e., carcass fat content component) is found on the y-axis (together with variable data projections), and the coloured shapes denote different feed additive treatment.

The carcass fat content (t-score), rib-cut fat % (rib-fat), intramuscular fat % (IM fat), and days-on-trial showed a strong positive correlation with PC2 or fat content component (as shown by the sharp orthogonal angle between variable projection on the positive axis). However, the relationships between the positively correlated attributes on fat content component revealed the changing magnitude of either one or the other respective association. Accordingly, the increase in rib-cut fat % (consisting of subcutaneous and intermuscular fats) was slightly associated with the increase in the number of days SA Mutton Merino sheep spent on the trial. More importantly, the intramuscular fat % increase very much with the increase in SA Mutton Merino sheep days-on-trial, and the same is true considering the carcass fat content *t*-score attribute.

Considering the direction of the PCA-plot projections, it is therefore evident that Neem additive treatment outstrips all other feed additive treatment groups on a fat content component, except for negatively correlated attributes. Meanwhile, the univariate ANOVA discussed earlier also established a significant ($p = 0.05$) increase in the carcass fat content of the Neem treatment group as compared to the Moringa and monensin treatment group. Speaking of negatively correlated attributes, the polyunsaturated fatty acids (n-6/n-3) ratio, unsaturated-to-saturated fatty acids ratio (U:S), the combination of monounsaturated fatty acids (MUFA), and fat-soluble pigment absorbance all point closely in the same direction, and this signifies the opposite end of a two-dimensionality projection.

Moreover, the combination of polyunsaturated fatty acids (PUFA), combination of saturated fatty acids (SFA), and polyunsaturated-to-saturated fatty acid ratio (P:S) showed the largest positive correlation with the fatty acid composition component or PC1. In this way, an increase in PUFA, SFA, and P:S will more likely give rise to a consequential decrease in PUFA (n-6/n-3) ratio, U:S ratio, MUFA, and fat-soluble pigment absorbance respectively. Similarly, following the later attribute projections (i.e., PUFA n-6/n-3ratio, U:S ratio, MUFA, and fat-soluble pigment), negative control treatment outperforms both monensin and Neem additive treatment. However, the performance of the negative control and Moringa group were similar (thus, relatively closer on the PCA-plot). This was due primarily to the significant ($p = 0.02$) increase in the most abundant subcutaneous adipose tissue fatty acid (i.e., oleic acid) on the Moringa group and to some extent negative control group as mentioned earlier.

Hence, the higher the carcass fat content characteristics as well as the number of days SA Mutton Merino sheep spent on trial, the lower the rib-cut lean muscle %, and to some extent P:S ratio, PUFA (n-6 / n-3) ratio, and PUFA composition and vice versa. The multivariate analysis on the subcutaneous fatty acids as aforementioned was in support of the biplot laid out results, as it also revealed a significant rise ($p < 0.01$) in PUFA of the monensin treatment group.

Furthermore, monensin additive treatment performed best on the PUFA, P:S ratio, rib-cut lean muscle (LM) %, and rib-cut bone. On the other hand, monensin and Neem treatment groups are considered to be different from one another on key component attributes however, the projection of the SFA attribute as can be seen on the PCA-plot, is perpendicular to the direction of the distance between the two feed additive groups. This implies that both monensin and Neem groups are similar in SFA properties. Meanwhile, monensin and Moringa group are similar on *LM* % and rib-cut bone %, while negative control and Neem group are comparable on rib-cut fat % as well as carcass fat *t*-score owing to the perpendicular projection of the attributes in the direction of the distance between the respective pairs of feed additive group.

Overall, *Azadirachta indica* (A Juss) additive treatment largely affected the carcass fat content, with respect to the quantity of animal fat as established by both descriptive and inferential statistics. While *Moringa* additive treatment impacts the fatty acid composition positively in terms of SA Mutton Merino sheep fat quality, with respect to MUFA and U:S ratio. Nevertheless, Monensin treatment had the most positive impact on the SA Mutton Merino sheep fat quality while the negative control treatment was more similar to the Neem treatment group in nearly all fat content component attributes.

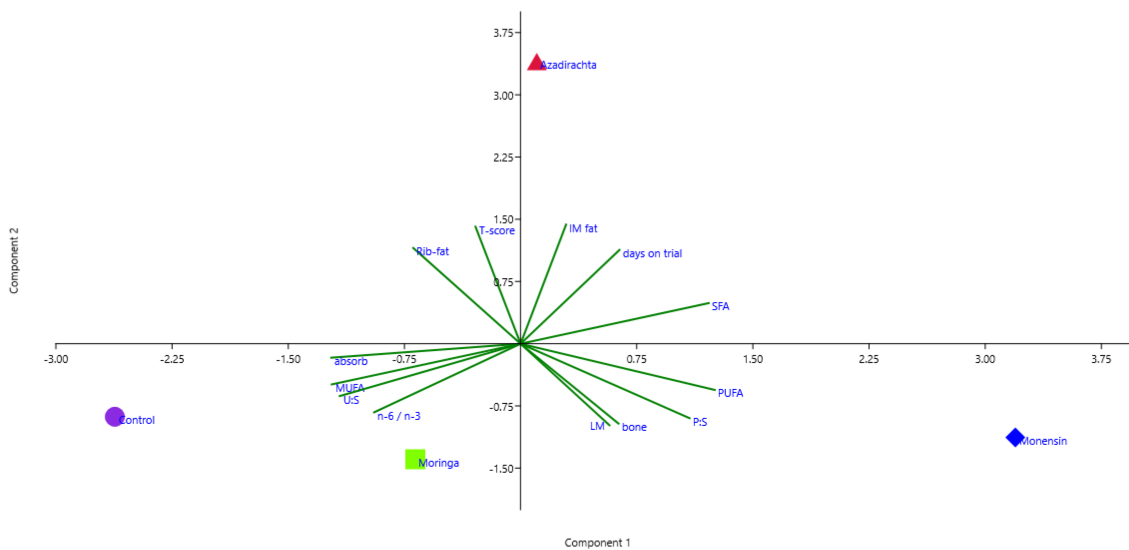

Figure 5.3: The feed additive treatment main effect on two-dimension PCA-plot

CHAPTER 6: CONCLUSION AND RECOMMENDATIONS

In this study, the effect of *Azadirachta indica* (A. Juss) and *Moringa oleifera* phytochemicals in leaf extract have shown indications of predictable outcomes on the SA Mutton Merino sheep tissue fat content, and fatty acid composition. The phytochemicals bioactivities or synergistic mechanisms are unique to each medicinal plant extract used. This was realised concerning the potential effect on the rumen microflora. *Moringa oleifera* could be very much considered a potential substitute for monensin as the two treatment additives were comparable in the reduction of carcass fat content and increase in the unsaturated fatty acid composition of SA Mutton Merino sheep. However, such desirable effect cannot be oversimplified as discussed concerning *Azadirachta indica* (A Juss) additive treatment, which typified an acute negative-control group scenario.

Similarly, the results of soluble fat pigment absorbance demonstrated the presence of carotenoids in the SA Mutton Merino sheep subcutaneous adipose tissue. Whilst bearing in mind a very high concentration of carotenoid does not necessarily mean better lipid molecule protection, as carotenoid may act as a prooxidant at higher concentration according to El-Agamey *et al.* (2004). Noticeably, the significant rise ($p < 0.05$) in the Moringa group on oleic acid, which could be the resultant effect of stearic to oleic acid desaturation, may be linked to the moderate carotenoid deposition in the Moringa group (i.e., 0.862 absorbance value). Hence, the null hypotheses regarding the effect of medicinal plant extract on tissue fat content and fatty acid composition were rejected.

Ultimately, the animal food product quality is defined by the standard practice of livestock production, notwithstanding the growing demand for a quality product with a considerable decrease in the use of livestock synthetic chemicals. The utilization of medicinal plants and plant extracts as a substitute for conventional antibiotic therapy has continued to gain momentum in recent times. This is why studying the effect of these medicinal plants on the quality of animal fat is important. Further investigation should be carried out on medicinal plant test ingredients with similar phytochemicals, and perhaps using a larger experimental animal sample size.

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APPENDICES

Appendix I. Subcutaneous fat pigment characteristics before extraction

Days-on-trial	Negative-control	Moringa	Neem	Monensin
	Sample number & subcutaneous fat color			
64	446(PW)	441(PW)	431(FW)	456(FW)
64	419(AW)	433(PW)	440(BA)	448(AW)
78	451(BA)	422(PW)	401(FW)	420(FW)
78	437(PW)	463(PW)	**	458(PW)
78	450(PW)	**	**	**
99	**	**	447(FW)	424(PW)
99	**	421(AW)	408(FW)	**
162	439(PW)	445(FW)	461(FW)	444(AW)

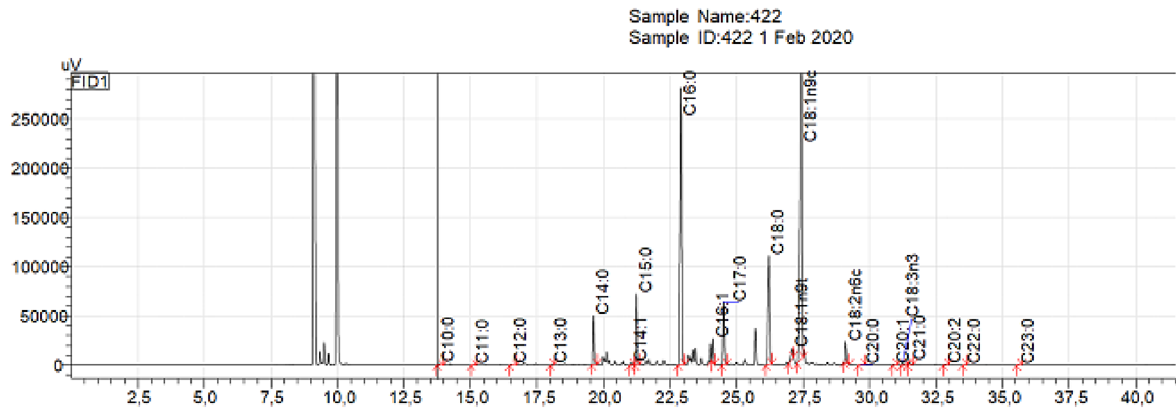
	Red	Green	Blue
Floral white (FW)	255	250	240
Antique white (AW)	250	235	215
Papaya Whip (PW)	255	239	213
Blanched Almond (BA)	255	235	205

Appendix II. Subcutaneous fat pigment characteristics after extraction

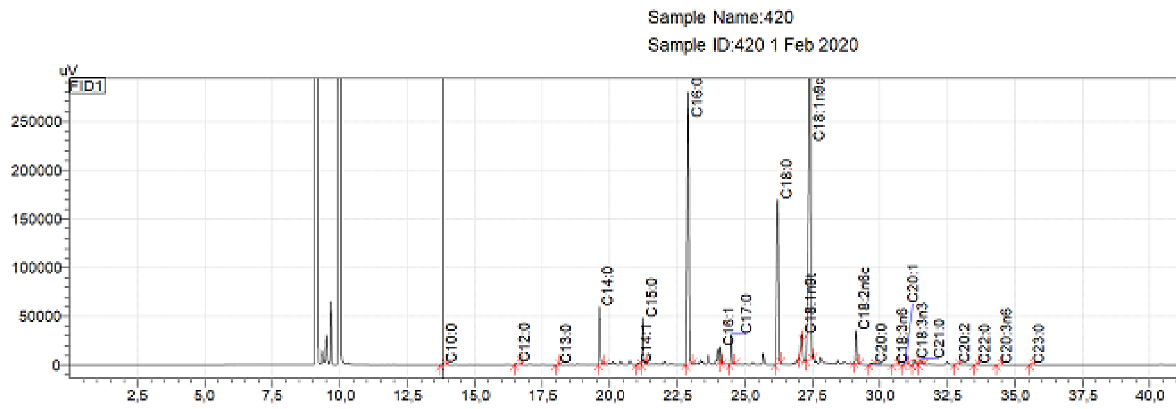
Days-on-trial	Negative-control	Moringa	Neem	Monensin
	Sample number & fat color after extraction			
64	446	441	431	456
64	419	433	440	448
78	451	422	401	420
78	437	463	**	458
78	450	**	**	**
99	**	**	447	424
99	**	421	408	**
162	439	445	461	444

	Red	Green	Blue
Gold 1	255	215	0
Yellow2	238	238	0
Yellow3	205	205	0

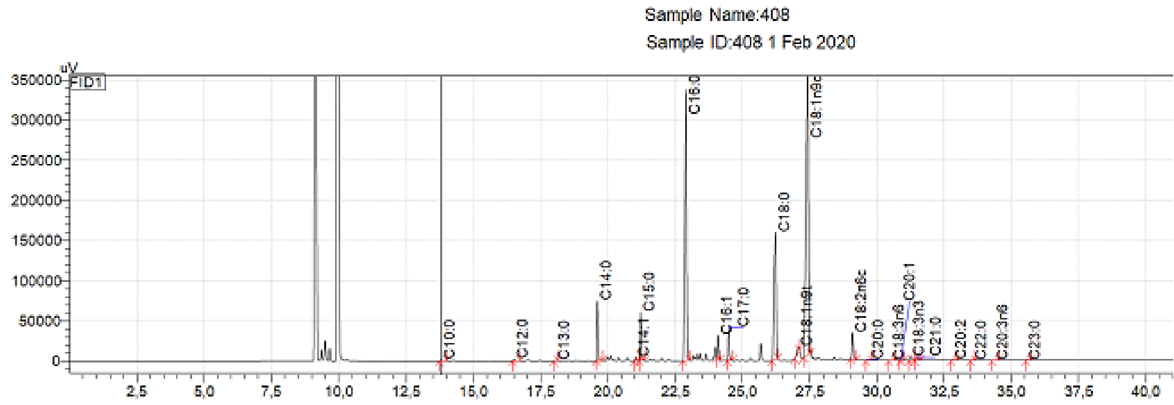
Appendix III. Subcutaneous fatty acids composition of a Moringa group sample



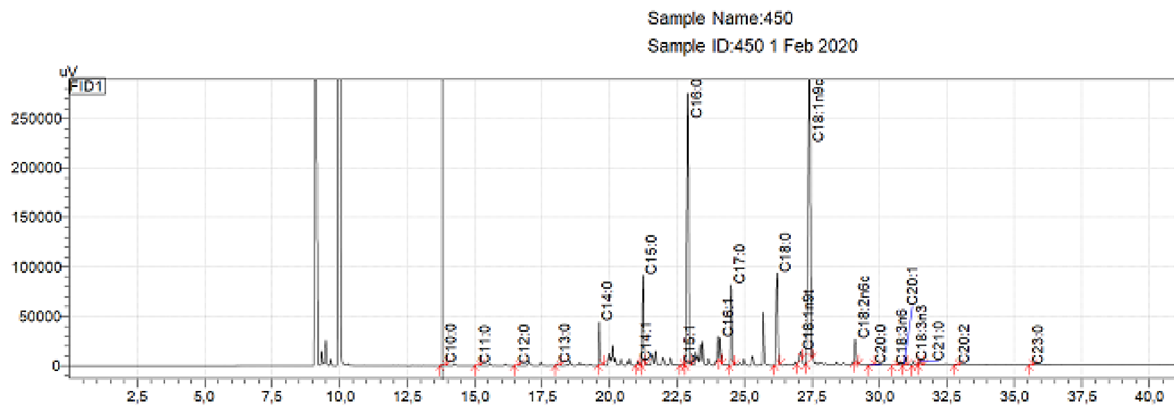
Appendix IV. Subcutaneous fatty acids composition of a monensin group sample



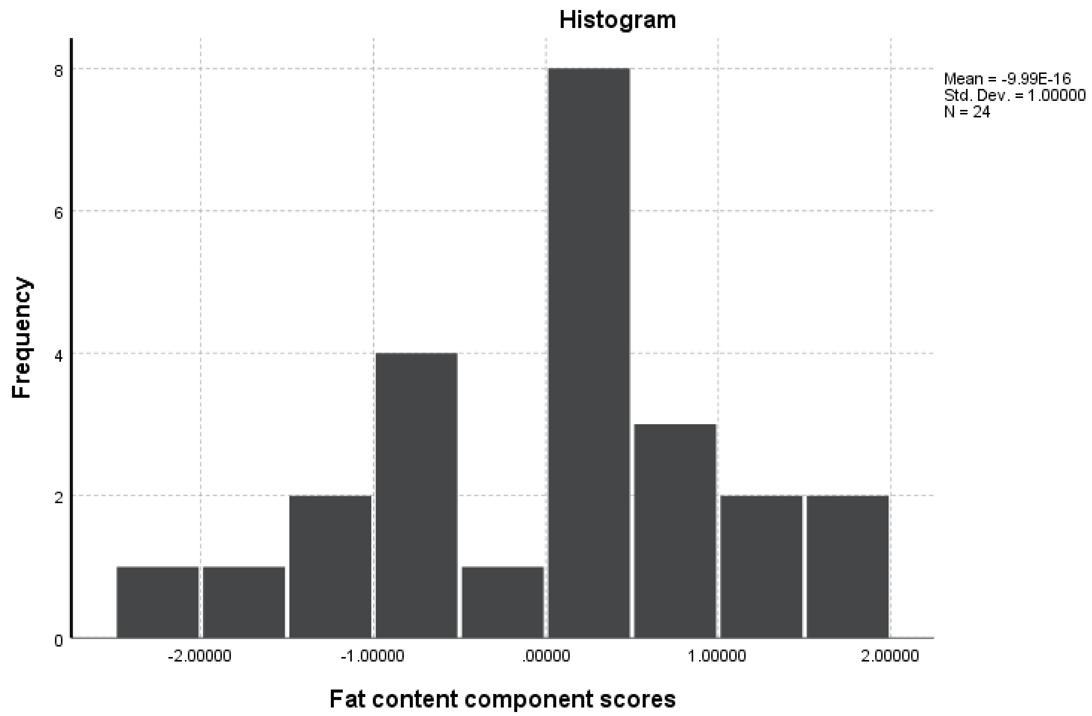
Appendix V. Subcutaneous fatty acids composition of a Neem group sample



Appendix VI Subcutaneous fatty acids composition of a negative control sample



Appendix VII. Fat content component scores or z-scores distribution



Appendix VIII. Fat content component *T*-scores distribution