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Use of lipid encapsulated tannin to replace ionophore in mitigating enteric methane emission and manipulating dietary protein bypass in SA Mutton Merino sheep

A Thesis

Presented to the Faculty of Natural and Agricultural Sciences, University of Pretoria, for the Degree of

DOCTOR OF PHILOSOPHY

In

ANIMAL SCIENCE

By

Shehu Lurwanu Ibrahim

Supervisor: Professor Abubeker Hassen

February 2024

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… Over every possessor of knowledge is one more knowing.

Qur'an (12: 76)

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DECLARATION

I, **Shehu Lurwanu Ibrahim**, declare that this thesis, which I hereby presented for the degree of Doctor of Philosophy in Animal Science at the University of Pretoria, is an original work carried out by me and has not been submitted before by me or another person for a degree at this or any other University or Institution of higher learning.

Shehu Lurwanu Ibrahim

February 2024

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DEDICATION

I dedicate this work to:

My late father, **Malam Lurwanu Ibrahim** (May Almighty Allah have mercy on his gentle soul, amen), and my mother, **Malama Sa'adatu Jafar**.

My children, **Mubarak, Musaddiq, Musayyib, Muhayrah** and **Muhassan**.

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"Slow and steady wins the race."

____ Robert Lloyd

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Preface

This PhD thesis is based on the research I carried out under Prof. A. Hassen's guidance. The thesis has been well arranged into series of chapters, which have been published, submitted for publication or are ready to be submitted for publication in peer-reviewed scientific journals, and preceded by a general introduction and followed by literature review chapter.

1. Ibrahim, S.L. and Hassen, A. (2022). Effects of graded levels of mimosa (*Acacia mearnsii*) tannin purified with organic solvents on gas, methane and *in vitro* organic matter digestibility of *Eragrostis curvula* hay. Published in: Animals, 12, 562. https://doi.org/10.3390/ani120505. (Chapter 2).
2. Ibrahim, S.L. and Hassen, A. (2021). Characterization, density and *in vitro* controlled release properties of mimosa (*Acacia mearnsii*) tannin encapsulated in palm and sunflower oils. Published in: Animals, 11, 2919. https://doi.org/10.3390/ani11102919. (Chapter 3).
3. Ibrahim, S.L. and Hassen, A. (2022). *In vitro* bovine serum albumin protein binding and release capacity of encapsulated mimosa (*Acacia mearnsii*) tannins in ruminal, abomasal and small intestinal tract pH conditions. To be submitted for publication in South African Journal of Animal Science. (Chapter 4).
4. Ibrahim, S.L. and Hassen, A. (2022). Influence of sunflower oil based encapsulated mimosa (*Acacia mearnsii*) tannin on methane, rumen fermentation and *in vitro* organic matter digestibility. Submitted for publication in PLOS ONE. (Chapter 5).
5. Ibrahim, S.L. and Hassen, A. (2022). Effect of non-encapsulated and encapsulated mimosa (*Acacia mearnsii*) tannins on growth performance, nutrient digestibility, methane and rumen fermentation of South African mutton Merino ram lambs. Published in: Animal Feed Science and Technology, 294, 115502. https://doi.org/10.1016/j.anifeedsci.2022.115502. (Chapter 6).

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LIST OF ABBREVIATIONS

3NP	=	3-Nitrooxypropanol
ADF	=	Acid detergent fibre
ADG	=	Average daily gain
ADL	=	Acid detergent lignin
ANOVA	=	Analysis of variance
AOAC	=	Association of Official Analytical Chemists
b	=	Fibre fraction from the insoluble, but slowly fermentable part
BW	=	Body weight
c	=	Rate of fermentation of b
CCC	=	Countercurrent chromatography
CH_4	=	Methane
CO_2	=	Carbon dioxide
CP	=	Crude protein
CT	=	Condensed tannin
DCM	=	Dichloromethane
DM	=	Dry matter
DMI	=	Dry matter intake
E3NP	=	Ethyl-3-nitrooxy propionate
CCC	=	Countercurrent chromatography
EMT^P	=	Encapsulated mimosa tannins in palm oil
ECH	=	*Eragrostis curvula* hay
Ee	=	Encapsulation efficiency
EMT^S	=	Encapsulated mimosa tannins in sunflower oil
EU	=	European Union
FAD	=	Flavin adenine dinucleotide
FAD	=	Flavin adenine dinucleotide + hydrogen
FCR	=	Feed conversion ratio
FE	=	Iron
g	=	Gram
GC	=	Gas chromatography

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GHGs	=	greenhouse gases
GIT	=	Gastrointestinal tract
h	=	Hour
H_2	=	Hydrogen
HT	=	Hydrolysable tannin
IPCC	=	Intergovernmental Panel on Climate Change
IVOMD	=	*In vitro* organic matter digestibility
k	=	Rate of gas production
kg	=	Kilogram
KJ	=	Kilojoule
l	=	Discrete lag time
LCA	=	Life cycle assessment
LSD	=	Least significant difference
ME	=	Metabolizable energy
mg	=	Milligram
MJ	=	Mega-Joule
mL	=	Milli-Liter
mm	=	Milli-meter
mmol	=	Milli mole
mol	=	Mole
N	=	Nitrogen
N_2O	=	Nitrous oxide
NAD	=	Nicotinamide adenine dinucleotide;
NADH	=	Nicotinamide adenine dinucleotide + Hydrogen
NADPH	=	Nicotinamide adenine dinucleotide phosphate
NDF	=	Neutral detergent fibre
NH_3N	=	Ammonia nitrogen
NO_3	=	Nitrate
NRC	=	National Research Council
NTP	=	Non-tannin phenol
^oC	=	Degrees celsius
OM	=	Organic matter

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pH	=	Potency of Hydrogen
psi	=	Per square inch
RBC	=	Red blood cell
RCBD	=	Randomised complete block design
rpm	=	Revolution per minute
S/O/W	=	Solid-in-oil-water
SAS	=	Statistical Analyses Software
SEM	=	Standard error of the mean
t	=	Time
TA	=	Tannic acid
TMR	=	Total mixed ration
TP	=	Total phenols
TT	=	Total tannins
TVFA	=	Total volatile fatty acid
µg	=	Microgram
UMT	=	Unencapsulated mimosa tannin
V	=	Asymptotic gas volume
VFAs	=	Volatile fatty acids

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Use of lipid encapsulated tannin to replace ionophore in mitigating enteric methane emission and manipulating dietary protein bypass in SA Mutton Merino sheep

By

Shehu Lurwanu Ibrahim

Supervisor: Prof. A. Hassen

Department: Animal Science, University of Pretoria

Degree: PhD in Animal Science

ABSTRACT

The significant contribution of the livestock subsector to global warming and dietary energy loss as a result of enteric methane emission has drawn the attention of contemporary ruminant nutritionists to engage in more research to reduce methane production while ensuring viable livestock production. Tannins have been documented to suppress methanogenesis and facilitate dietary protein utilization, though depending on the source of tannin and level of its inclusion in the diet may adversely affect feed consumption and nutrient digestion. The objectives of this study are: to assess the effect of pentanol and ethyl acetate purified *Acacia mearnsii* tannins on methane, gas and *in vitro* organic matter digestibility of *Eragrostis curvula* hay; to evaluate the morphology, density, encapsulation efficiency and tannin release rate of *A. mearnsii* tannin encapsulated in sunflower and palm oils; to evaluate the capacity of palm and sunflower oils based encapsulated *A. mearnsii* tannins to bind and release protein in buffers simulating gastrointestinal tract; to determine the effect of various doses of *A. mearnsii* tannin encapsulated in sunflower oil on methane, gas, *in vitro* organic matter digestibility, ammonia nitrogen and volatile fatty acids concentrations of total mixed ration and *Eragrostis curvula* hay; and lastly to evaluate the influence of encapsulated *A. mearnsii* tannin in sunflower oil on methane, nutrient utilization, growth changes and rumen fermentation characteristics of South African mutton Merino ram lambs. A series of *in vitro* and/or *in vivo* experiments were carried out at the Department of Animal Science Nutrition Laboratory and University of Pretoria Experimental Farm, Hatfield, Pretoria, Republic of South Africa to examine the possibility of enhancing dietary tannin utilization as a feed additive in ruminant nutrition by adopting Sheep

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as a model experimental animal.

Tannin purification with pentanol and ethyl acetate improved the proportion of condensed tannin and decreased gas and methane production with slight reduction on digestibility at dose of 30 g/kg feed DM. Moreover, lesser dosage of purified tannin extracts showed similar impact on gas and methane comparable with higher inclusion level of non-purified tannin. Tannin encapsulation with sunflower oil and palm oil exhibited excellent encapsulation efficiencies, smaller particles sizes, lighter in density and delayed tannin release in the gastrointestinal tract simulated buffers compared to unprotected tannin. However, non-encapsulated tannin bound and released more protein in the buffers simulating rumen and abomasum. Whereas, the encapsulated tannin in sunflower and palm oils released more protein in small intestine simulated buffer. Among the two oils, sunflower oil microparticles bound and released more protein than palm oil microcapsules. Addition of encapsulated and non-encapsulated acacia tannin at dose of 20 g/kg feed DM decreased methane and total gas yield without affecting ammonia nitrogen, volatile fatty acids and *in vitro* organic matter digestibility. Moreover, encapsulated tannin reduced more methane yield compared to equivalent dosage of free tannin. Nevertheless, 30 g/kg feed DM inclusion of encapsulated tannin reduced feed digestibility. *In vivo* results showed that *A. mearnsii* tannin encapsulated in sunflower oil at a dose of 20 g/kg feed DM reduced methane emitted by sheep while increasing the intake of dry matter, organic matter and fibre without decreasing the digestibility of dry matter, organic matter and crude protein compared to the unencapsulated tannin and monensin treatments. Feed conversion ratio was unaffected by inclusion of tannins and was comparable to those animal on monensin additive. Thus, supplementation of encapsulated acacia tannin at the dose of 20 g/kg feed DM could be adopted as safer natural alternative and eco-friendly approach of ruminant animal production to replace the use of ionophore.

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GENERAL INTRODUCTION

Animal agriculture plays a significant part in global food security, and has constituted a major source of livelihood to people. The significance of livestock in human food supply, revenues and employment is well documented (Perry and Sones, 2007; Herrero *et al*., 2009). According to Steinfeld *et al*. (2006), the animal industry contributes almost 40% of the global agricultural subsector's gross domestic product, provide job opportunities to around 1.3 billion persons and sustains the lives of more than a billion poor populace. The world population is anticipated to expand to 9.6 billion by the year 2050 (United Nations, 2013), thus, the overall human demand for animal products projected to rise by 30% for milk and by 60% for meat (Alexandratos and Bruinsma, 2012; Sejian *et al*., 2016), which will be achieved either by increasing the animal population or through enhancing the output of current stock (Sejian *et al*., 2016).

The intensification of animal production, on the other hand, is a substantial contributor to the emission of greenhouse gases (GHGs), which are the primary drivers of global warming (Herrero *et al*., 2011; Rojas-downing *et al*., 2017). Carbon dioxide (CO_2), methane (CH_4) and nitrous oxide (N_2O) form the leading GHGs emitted by livestock (Moss *et al*., 2000; Gerber *et al*., 2013; Rojas-downing *et al*., 2017). With the aid of a life cycle assessment (LCA) method, Steinfeld *et al*. (2006), noted that animal agriculture contributes for 7.1 billion tonnes (18% of CO_2-equivalent) of the total GHGs yields from the fermentation of ruminants and their dung as well as through land utilization and land-use revolution, on-farm fossil fuel, feed production as well as processing and transportation. The Intergovernmental Panel on Climate Change assessment (IPCC, 2007) placed the total GHG discharges from animal agriculture at 10 – 12% of CO_2-equivalent per annum in 2005 and surpassed 30% after the inclusion of land-use modification (Smith *et al*., 2007). Therefore, the ruminant animal sub-sector will serve as the main actor in the moderation of GHGs releases as well as enhancing food security worldwide.

In ruminant production systems, CH_4 is the most significant of the three GHGs (Ogino *et al*., 2007; Vargas-Ortiz *et al*., 2022a), because of its higher global warming potential which is between 21 – 28 times that of CO_2 (Eckard *et al*., 2010; Sun *et al*., 2021). IPCC (2007), estimated that CH_4 contributes between 20 – 30% of global warming within the period of 100 years. Apart from its contribution to climate change, enteric CH_4 emission also resulted in energy and nitrogen losses (Eckard *et al*., 2010; Gerber *et al*., 2013). Takahashi (2006), reported that about 893 kilojoule, KJ/mole of energy is lost due to CH_4 production during rumen

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fermentation, which represents nearly 12% gross energy lost (Piñeiro-Vázquez *et al*., 2015). Thus, several enteric CH_4 abatement approaches were adopted either through animals' manipulation which include animal breeding and improved livestock management or rumen modulation such as biological control, vaccination and chemical defaunation or diets modification comprising of forage quality enhancement, plant breeding, diets supplements like ionophores particularly Monensin and use of polyphenolic compounds specifically tannins and saponins (Eckard *et al*., 2010). Monensin is reported to have residual health concerns on humans and thus, its usage as a dietary additive is prohibited in 2006 by the European Union, EU (Millet and Maertens, 2011). Hence, the use of tannin extracts as dietary supplements gained recent interest, owing to their abundance, effectiveness and health benefits (Soltan *et al*., 2013).

Tannins are well documented to mitigate CH_4 emissions either by direct inhibition of the methane-producing bacteria or indirect prevention of protozoa activities and reduction in dry matter digestion (Goel *et al*., 2011; Jayanegara *et al*., 2012; Hristov *et al*., 2013). Additionally, tannin additions can bind dietary proteins at the usual rumen pH range of 5.5 to 7.5, which reduces the rate of digestion of dietary proteins by ruminal microorganisms and enables protein bypass to the small intestine, maximizing amino acid absorption (Hassanpour *et al*., 2011; Hassanpour and Mehmandar, 2012). For instance, after tannin inclusion in the ruminants diets, CH_4 productions were reduced by up to 58% in goats (Animut *et al*., 2008), 29% in cattle (Grainger *et al*., 2009) and 26% in sheep (Moreira *et al*., 2013). In addition, up to 59% decrease in urinal nitrogen as against faecal nitrogen was observed in dairy cows after mimosa tannin supplementation (Grainger *et al*., 2009).

However, feeding of tannins orally suffers a drawback concerning voluntary intake, caused by tannins' astringency when interacting with proteins secreted in the saliva (Frutos *et al*., 2004b). Also, intake and digestibility appeared to be affected when tannin extracts were supplemented beyond 5% of feed DM (Beauchemin *et al*., 2008; Grainger *et al*., 2009). Hence, it is imperative to come up with microencapsulation technology that will guarantee proper neutralization of the tannin's unpleasant taste and influence its release in a controlled manner across the gastrointestinal tract and thus, decrease CH_4 yield while increasing dietary protein utilization without reduction in feed intake and digestibility. Recent investigations have shown that encapsulation reduced bitter taste, quick tannin solubility and CH_4 volume (Adejoro *et al*., 2018; Adejoro *et al*., 2019a). The review of related literature was prepared to comprehend the

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enteric CH_4 synthesis, role of tannins in CH_4 mitigation and protein bypass as well as explore knowledge gaps and offer novel approaches to the existing strategies.

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CHAPTER ONE

Literature Review

1.1 Enteric Fermentation of Ruminant Animals

Ruminants are known to possess the most complex and distinguished digestive system among mammals (Jouany and Morgavi, 2007). Van Soest (1994), noted that the structural adaptations of ruminant animals' gastrointestinal tract (GIT) offer a distinct approach to the digestion process, which permit greater access to the carbohydrates reserve of plant materials compared with non-ruminants. This is due to the presence of four compartment-stomach: *rumen, reticulum, omasum,* and *abomasum,* thereby making ruminants to be more dependent on pre-gastric retention of digesta accompanied by microbial breakdown of fibrous feeds (Van Soest, 1994; Niwinska, 2013; Stover *et al*., 2016). The first compartment, rumen has been identified as a fermentation vat with a vast and diverse microbial community existing in a symbiotic relationship, and thus, plays the most significant role in the digestion of fibrous feeds by the ruminants (Wolin *et al*., 1997; McDonald *et al*., 2011). It has also been shown that the rumen, which is the major site of methane production, has a stable and dynamic environment fully developed for converting complex carbohydrates into volatile fatty acids, VFAs (Kamra, 2005; Belanche *et al*., 2021; Leahy *et al*., 2022). Hence, the effective utilization of a wide variety of fibrous feeds by ruminants relies on the capacity of the rumen to accommodate various types of fermentative microbes (Niwinska, 2013).

Among various microorganisms living in the rumen, bacteria are the most dominant species (Newbold and Ramos-Morales, 2020). The three main bacterial species that break down fibrous feeds comprise of *Fibrobacter succinogenes, Ruminococcus flavefaciens* and *Ruminococcus albus* (Russell, 2002; Choudhury *et al*., 2015). Other species of bacteria like *Butyrivibro fibrisolvens* and *Eubacterium cellulosolvens* were known to have little influence on cellulolytic activity in the rumen (Shreck, 2013). Methane (CH_4) producing bacterial species (*Methanogenic archeae*) such as *Methanobacterium formicicum, Methanomicrobium mobile* and *Methanobrevibacter ruminatium* are also identified in the rumen (Conklin, 1995). In the rumen environment, protozoa and *Methanogenic archeae* were also reported to have a synergistic relationship (Rahman, 2007; Patra and Saxena, 2010). The fungi species specifically of Neocallimastix, Caecomyces and Piromyces genera are well documented to promote the affinity of bacteria to fibrous diets (Nagpal *et al*., 2009). The interaction of these

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microorganisms in the rumen either directly or indirectly enhanced fibre digestion.

The synergistic and antagonistic relationship among the rumen microorganisms has been described as highly complicated and diverse (Niwinska, 2013). The outcome of this is the digestion of feed material into a number of fermentation byproducts useful to ruminants, particularly ammonia nitrogen (NH_3N) and VFAs. According to Soltan and Patra (2021), the ruminal degradation of carbohydrates and protein resulted in the production of simple sugars and amino acids as intermediate products, utilize by the microbes to produce microbial biomass, VFAs, NH_3N, CH_4 and CO_2. The acetic, propionic and butyric acids are the principal VFAs formed by the rumen microorganisms. Others are lactic acid, valeric acids and branched chain VFAs which include isobutyric and isovaleric. Niwinska (2013), noted that VFAs are absorbed via the rumen walls, whereas feed residues and microbial biomass pass to the lower gut and supply ruminants with protein, fats and oils as well as vitamins. The growth performance and yields of animal products is determined by the availability of these fermentation products to the ruminants (Soltan and Patra, 2021).

1.2 Enteric Methane Formation and Associated Dietary Energy Loss

In the course of rumen fermentation, anaerobic microorganisms break down dietary carbohydrates to form energy, VFAs, CO_2 and CH_4 as well as heat. The microbes utilized the energy and VFAs for their growth and proliferation while, CO_2 and CH_4 are removed through eructation and belching, resulting in dietary energy loss (Murray *et al*., 1976). The substantial amount of heat generated is used in regulating the temperature of the body and the excess heat energy is eliminated via dissipation. Hydrogen (H_2) is produced as among the key intermediate products of rumen microbial feed digestion, and it is instantly utilized by *Methanogenic archaea* present in the rumen environment (Moss *et al*., 2000; Leahy *et al*., 2022). This symbiotic relationship between fermenting microbial species (bacteria and fibrolytic fungi) and the methanogens (H_2-utilizing species) is described as 'interspecies hydrogen transfer' (Miller, 1995), which permits reduced co-factors particularly flavin adenine dinucleotide (FADH), nicotinamide adenine dinucleotide (NADH) and nicotinamide adenine dinucleotide phosphate (NADPH) in the H_2-forming microbial species to be oxidized and reused. Immediately, reduced co-factors are formed, methanogens utilize H_2 to reduce CO_2 and yield CH_4 as presented by Moss *et al*. (2000) in equation (1.1) below:

$$CO_2 + 4H_2 = CH_4 + 2H_2O \qquad (1.1)$$

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The feat of microbial feed digestion is determined by the constant production of FAD^+, NAD^+ and $NADP^+$ and elimination of H_2 (Nolan *et al.*, 2010; Knapp *et al.*, 2014). Thus, methanogenesis is vital for optimum rumen function, because it inhibits hydrogen accrual thereby prevention of dehydrogens associated with reduced co-factors oxidation process (Patra *et al.*, 2017).

However, CH_4 synthesis led to a substantial loss of dietary energy (Waghorn *et al.*, 2002; Bayat *et al.*, 2012). According to Johnson and Ward (1996), whenever CH_4 is lost to the atmosphere, the ruminants undergo partial loss of utilizable dietary energy. It is well documented that enteric CH_4 emission represents between 7 – 17% of animal's energy intake (Woodward *et al.*, 2001; Robertson and Waghorn, 2002; Hristov *et al.*, 2013). This substantial proportion is lost and therefore, reduce the overall energy needed by the ruminants for metabolism (Takahashi, 2006). The amount of gross energy lost by the ruminants due to enteric CH_4 synthesis could even be higher in the tropical environment due to the deficiency of qualitative feeds required for optimum rumen microbial growth and multiplication (Hristov *et al.*, 2013).

Hence, enteric CH_4 abatement strategies are typically targeted at hindering the multiplication of methanogens which utilize H_2 to produce CH_4 and/or providing another pathway for hydrogen sinks. According to Janssen (2010), the quality of feed and the population of diverse microorganisms determine the concentration of H_2 generated in the rumen. Literature has also established that the more the carbohydrate degradation, the more the H_2 accumulation, which has to be removed for optimum rumen microbial function thereby facilitating the perpetual digestion of diet (Sharp *et al.*, 1998; Hook *et al.*, 2010; Leahy *et al.*, 2022). Thus, the elimination of H_2 by CH_4 producing bacteria has a significant influence on the degree of fibre degradation in addition to the ruminal digestion end products (Attwood and McSweeney, 2008). Wolin *et al.* (1997), noted that methanogenesis is the key H_2-reduction pathway identified in the rumen. Other important H_2-reduction pathways in the rumen environment comprised of the acetogens, sulphate, nitrate, fumarate in addition to hydrogenation of unsaturated fatty acids. Thus, understanding the trends of VFAs production could facilitate the development of a practical method to be adopted in modifying the rumen environment thereby decreasing enteric CH_4 production while improving the synthesis of beneficial end products (Ungerfeld and Kohn, 2008).

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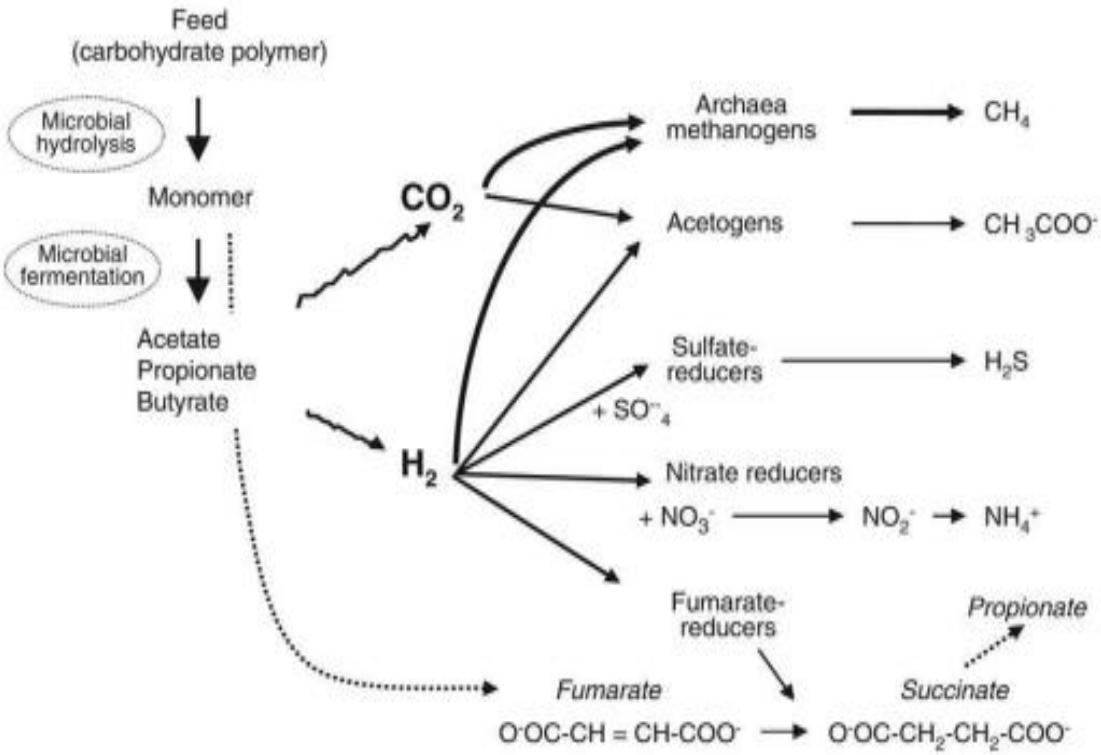

Figure 1.1 Rumen microbial carbohydrate fermentation and H_2-reduction pathways. Adopted from Morgavi *et al.* (2010).

1.3 Additive-Based Enteric Methane Abatement Strategies

Modern ruminant nutritionists have been motivated to do additional research to reduce enteric CH_4 emission while assuring sustainable livestock production as a result of the considerable contribution of the livestock subsector to global warming. It is documented that the volume of enteric CH_4 produced by the ruminants relies on the feed's quality and quantity (Beauchemin *et al.*, 2008), the stage of harvesting of the feed (Arthington and Brown, 2005), the type of feed material (Santoso *et al*., 2003), the quantity of fibre in the diet (Hindrichsen *et al.*, 2003), as well as the ratio of acetic to propionic acids of digested feed material (McAllister *et al.*, 1996). Therefore, numerous strategies were employed to reduce CH_4 production by the ruminants through animals' manipulation which include animal breeding and improved livestock management; the rumen modulation such as biological control, vaccination and chemical defaunation as well as the diets modification like plant breeding and enhancing the quality of the forage, diets supplements and use of plant secondary substances especially saponins, essential oils and tannins (Eckard *et al*., 2010; Llonch *et al.*, 2017; Islam and Lee, 2019; Mijena and Getiso, 2021).

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The current review will be limited to the dietary supplements or additives used in mitigating CH_4 produced by ruminants. Hook *et al*. (2010), categorized dietary additives into either those that reduce CH_4 yield via direct disturbance of the growth and activities of *Methanogenic archaea* or those that decrease CH_4 emission through indirect means by passing of H_2 away from CH_4 synthesis using an alternative pathway for H_2 metabolism. Several studies revealed the potential of supplementing polyphenolic substances in the direct prevention of methanogens' growth and multiplication in the rumen as well as strategies aimed at indirect inhibition of *Methanogenic archaea* activities via redirection of H_2 away from methanogenesis which involved addition of tannins, ionophores and organic acids, bacteriocins and bacteriophages, lipids, nitrates and sulphates (Cottle *et al*., 2011; Hristov *et al.*, 2013; Piñeiro-Vázquez *et al*., 2015; Llonch *et al.*, 2017; Leahy *et al*., 2022). Tannin additives have also been reported to serve as H_2 sink (Min *et al*., 2019; Pereira *et al.*, 2022). Specifically, the present literature review will give more details on the addition of tannins in enteric CH_4 mitigation due to their abundance, safety and efficacy with a view to identifying research gaps while addition of other supplements will be discussed in brief as follows:

1.3.1 Addition of ionophores and organic acids

Ionophores such as Monensin, salinomycin and lasalocid are described as antimicrobial compounds that interact with cations in a reversible form which is soluble in lipid, and thus, enable specific transfer of ions across membrane (Novilla, 2018). The most extensively used ionophore is Monensin, a polyether antibiotic widely included as premix in the animal feeds to decrease CH_4, acidosis and bloat while enhancing voluntary feed intake and efficient utilization of feeds (Llonch *et al.*, 2017; Haque, 2018; Islam and Lee, 2019). Monensin raise the ratio of acetate: propionate via expanding the reducing equivalents which are responsible for propionate formation (Beauchemin *et al.*, 2008), and may also reduce rumen protozoan population (Haque, 2018). Recent studies have reported that Monensin reduced CH_4 output by 25% in dairy cows (Gerber *et al.*, 2013; Hristov *et al.*, 2013) and 30% CH_4 reduction in beef cattle (Llonch *et al.*, 2017). However, the inhibitory effect of Monensin on enteric CH_4 emission for long-term use is not guaranteed because of the rumen microbial adaptation to Monensin (Johnson and Johnson, 1995; Beauchemin *et al.*, 2008). In addition, the utilization of Monensin as a feed additive for ruminants is banned by the regulation of the EU in 2006, because of its health concerns on human (Millet and Maertens, 2011; Llonch *et al.*, 2017).

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Thus, the utilization of Monensin in controlling ruminants' CH_4 production remains contentious because of safety issues (Gerber *et al.*, 2013).

Organic acids like acrylate, fumarate and malate are widely utilized as dietary supplements in reducing enteric CH_4 emission (Wood *et al.*, 2009; Li *et al.*, 2018). Organic acids have been identified as the precursors of propionic acid via succinate-propionate pathway which prevent methanogenesis through provision of another H_2 sink (Eckard *et al.*, 2010). Newbold *et al.* (2005), noted that fumarate is more efficient in reducing CH_4 volume. However, its use is not encouraging because, large quantity of fumarate (4 moles) is needed to hinder the production of single mole of CH_4 (Gomez *et al.*, 2005). Moreover, Castillo *et al.* (2004), reported that the use of fumarate is not favorable in intensive livestock production due to its action in the reduction of rumen pH. Nevertheless, up to 75% reduction in CH_4 was recorded after adding 100 g/kg DM of encapsulated fumaric acid devoid of any adverse effect on the lambs' performance (Wallace *et al.*, 2006).

1.3.2 Addition of bacteriocins and bacteriophages

Bacteriocins are a group of anti-bacterial proteins or peptides produced by bacteria in the rumen which are antagonistic to other species of microorganisms for niches within the rumen environment (Patra, 2012). Bacteriocins are known to be efficient in hindering the activities of methane-producing bacteria and bypassing H_2 to other pathways (Martin *et al.*, 2010; Patra, 2012). They are also reported to prevent lactic acidosis and bloat (Teather and Forster, 1998). Nicin, which is among the widely used bacteriocin synthesized by *Lactococcus lactis*, is shown to increase propionic acid production and reduce enteric CH_4 yield by indirect action on H_2 producing microorganisms (Callaway *et al.*, 1997; Patra, 2012). Santoso *et al.* (2004), recorded around 10% decrease in CH_4 produced by sheep supplemented with bacteriocin. In addition, a significant decline in CH_4 volume was obtained after feeding a mixture of nicin and nitrate (NO_3^-) to sheep (Sar *et al.*, 2005). Another bacteriocins, Bovicin HC5 produced by *Streptococcus bovis* is reported to reduce CH_4 volume by about 50% (Lee *et al.*, 2002; Kobayashi, 2010). However, the cross-adaptation of rumen microorganisms to bacteriocins limits its usage as an anti-methanogenic supplement (Martin *et al.*, 2010).

Bacteriophages are identified as microbial viruses that prevent methanogenesis by poisoning the methanogens and the bacteria (Patra, 2012). Bacteriophages are considered as one of the significant tools for CH_4 mitigation strategies because of their genes and lytic potential (Kumar

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et al., 2013). Patra (2012), recorded a large number (10^9 per mL) of bacteriophages in the rumen fluid. Likewise, Kumar *et al.* (2013) indicated that from the six archaeal bacteriophages sequenced and described, only three bacteriophages belong to *Methanothermobacter* phage psi M100 and *Methanobacterium* phage psi M1, M2, M100. Furthermore, some bacteriophages capable of infecting *Methanococcus spp.*, *Methanobrevibacter spp.* and *Methanobacter spp.*, were observed (Mcallister and Newbold, 2008). However, these siphophages are yet to be isolated from rumen fluid (Patra, 2012; Kumar *et al.*, 2013).

1.3.3 Addition of fats and oils

Dietary lipids sourced from either vegetables or animals have been shown to decrease enteric CH_4 yield with no negative impact on rumen fermentation and animal growth or development (Llonch *et al.*, 2017; Haque, 2018; Mijena and Getiso, 2021). The efficiency of fats or oils supplementation on CH_4 mitigation differs based on the fatty acids' level of unsaturation, chain length and concentration as well as the interconnections between dietary composition and the fatty acids (Ribeiro *et al.*, 2015; Patra *et al.*, 2017; Islam and Lee, 2019).

Earlier investigations have shown that lauric and myristic acids, which are the rich sources of medium chain fatty acids, as the most potent inhibitors of CH_4 production compared to short or long chain fatty acids (Dohme *et al*., 2001; Jordan *et al*., 2006; Ding *et al*., 2012). Furthermore, Zhou *et al*. (2013), detected an appreciable reduction in the cell viability of methanogen, *Methanobrevibacter ruminantium* after inclusion of lauric acid. Similarly, Eckard *et al*. (2010) also noted that coconut oil, which is a medium chain fatty acids, reduced more CH_4 yield (–7.3%); compared to linseed, a linolenic acid (–4.8%); soybean and sunflower, the linoleic acids (–4.1%); tallow, a saturated fat (–3.5%) and rapeseed, a monounsaturated fatty acid (–2.5%). The reduction in enteric CH_4 due to the addition of fats or oils is induced by the decrease in the concentration of organic matter and fibre digested as well as their direct inhibitory influence on *Methanogenic archaea* (Eugène *et al*., 2008; Haque, 2018). However, supplementation of fats and oils at higher concentrations (> 50 g/kg dry matter intake, DMI) was found to affect the intake and digestibility of feed leading to a decline in daily weight gain and milk volume (Patra, 2012; Kumar *et al.*, 2013).

1.3.4 Addition of nitrogenous compounds

Nitrogenous compounds are added in ruminant diets in the form of ammonium nitrogen supplements (Sun *et al.*, 2021). Nitrate is one of the nitrogen-containing compounds described

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as an effective inhibitor of enteric CH_4 emission due to its capacity to prevent methanogens from utilizing H_2 in addition to its nitrogen (N) supply which enhance rumen microbial biomass production (Adejoro and Hassen, 2017; Haque, 2018; Alvarez-Hess *et al.*, 2019; Wu *et al.*, 2019). Sophal *et al*. (2013), reported approximately 43% drop in CH_4 volume and about 28% increase in nitrogen retention after the inclusion of NO_3 at 5% DM. Nitrate supplementation is reported to reduce up to 50% CH_4 production in dairy cattle (van Zijderveld *et al*., 2011; Hulshof *et al*., 2012), and small ruminants (Nolan *et al*., 2010; van Zijderveld *et al*., 2010). However, the use of large doses of NO_3 as an additive in ruminant diet received a lot of criticism because of its intoxication (methemoglobinemia) (Leng, 2008; Jeyanathan *et al*., 2014).

Recently, new nitrogenous compounds, popularly known as 3-nitrooxypropanol, 3NP and ethyl-3-nitrooxy propionate, E3NP have been shown to reduce CH_4 emission in a sustained manner devoid of negative impact on the animal productivity (Romero-Pérez *et al*., 2016; Sun *et al.*, 2021). Both E3NP and 3NP are reported to hinder the activities of the methanogens, modify the rumen microbial composition, control the activities of enzymes participated in CH_4 synthesis, and modulate the distributions of VFAs, resulting in the depletion of H_2 and thus, decrease ruminal CH_4 production (Zhang *et al*., 2020; Sun *et al.*, 2021).

1.3.5 Addition of plants secondary metabolites

The utilization of plant secondary compounds in enteric CH_4 mitigation gained recent recognition (Haque, 2018). The anti-methanogenic properties of plant secondary metabolites is linked to anti- bacterial, anti-protozoal and anti-fungal effects (Bodas *et al.*, 2012; Haque, 2018). The most widely used secondary metabolites of plant origin include tannins, essential oils and saponins.

Saponins are naturally occurring glycosides present in numerous plants that decrease enteric CH_4 emission via direct influence on rumen microorganisms (Martin *et al*., 2010; Haque, 2018). Some of the most commonly used saponins include: triterpenoid saponin, tea saponin, methanol extract saponin. Previous investigations revealed that, saponins inhibit the growth and activities of protozoal, bacterial and fungal species during rumen fermentation and thus, limit the accessibility of H_2 for CH_4 synthesis (Bodas *et al.*, 2012; Goel and Makkar, 2012; Haque, 2018). Tea saponin has been shown to decrease the protozoan population by 50%, while the concentration of CH_4-producing bacteria reduced by 8% *in vitro* (Guo *et al*., 2008). Recent

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investigations revealed that, inclusion of ivy fruit saponin reduced CH_4 production by about 40% (Belanche *et al*., 2016). Patra and Saxena (2009), recorded around 50% drop in CH_4 yield after inclusion of saponin extracts. However, inclusion of saponins (from Quillaja) did not influence the population *Methanogenic archaea* or reduce CH_4 production (Patra and Yu, 2014, 2015). Hence, the CH_4 suppressing effects of saponins differs based on the plant sources and the extraction solvents (Patra, 2012), as well as level of inclusion of the saponin extract (Islam and Lee, 2019).

Essential oils such as garlic oil, cinnamon oil and thyme oil are identified as aromatic lipophilic compounds and steam volatile extracts which have strong antimicrobial properties capable of modulating rumen fermentation by interaction with the microbial cell membrane (Greathead, 2003; McIntosh *et al.*, 2003; Benchaar *et al.*, 2008; Kamra *et al.*, 2012). Essential oils are known to be effective in hindering CH_4 synthesis while enhancing propionic acid production (Macheboeuf *et al*., 2008). Nevertheless, essential oils differ in terms of functional groups and chemical properties, with consequent variation in antimicrobial properties (Lin *et al*., 2013; Haque, 2018). For instance, the bioactive molecules present in essential oils like garlic oil particularly, allicin and diallyl sulfide are reported to moderate *in vitro* CH_4 yield through toxicity on methanogens (Busquet *et al*., 2005; Macheboeuf *et al*., 2006). According to Calsamiglia *et al*. (2007), further investigation is necessary to detect the likely adverse effects of these compounds, to establish the best inclusion level of the active molecule, their residual effects and to study the possible adaptation of rumen microbes. In addition, the palatable characteristics of these molecules could present a practical concern (Martin *et al*., 2010).

Tannins are defined as high molecular weight (500 – 20, 000 g/mol) polyphenolic secondary metabolites, that are soluble in water, available in all plants part such as bark, leaves, fruit, roots and wood, and are well known to possess defense reaction against herbivores, birds and insects (Hassanpour *et al.*, 2011). Plant tannins are classified as hydrolysable tannin (HT) and condensed tannin (CT) according to the variation in chemical properties (ChaichiSemsari *et al*., 2011; Maheri-Sis *et al*., 2011), as illustrated in figure 1.2 below:

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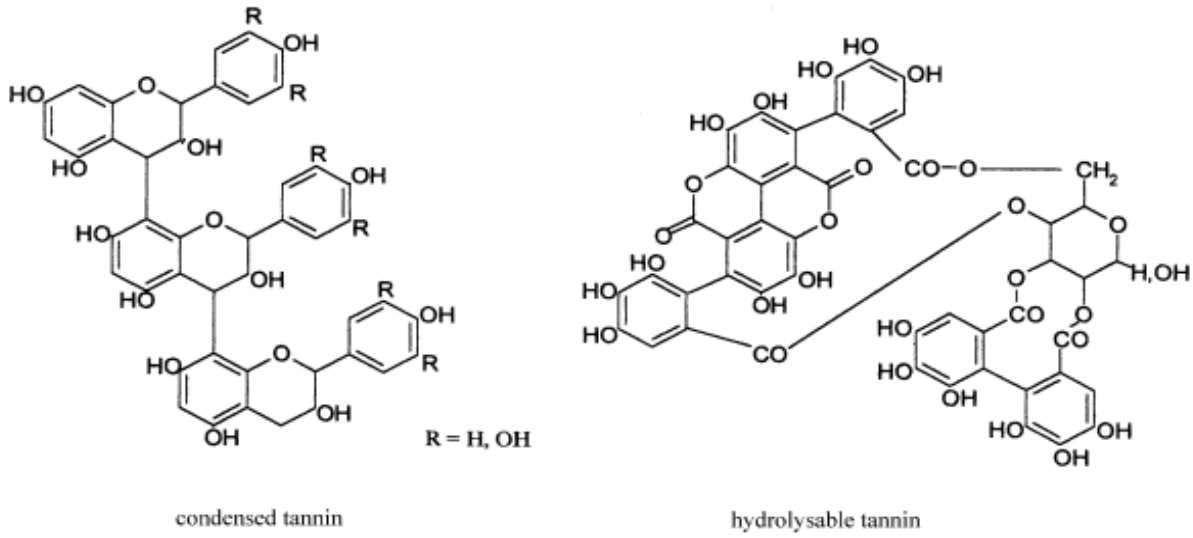

(a) (b)

Figure 1.2 Structural composition of (a) CT and (b) HT. Source: Hassanpour *et al.* (2011).

Hydrolysable tannins refer to polyphenolic compounds which possess a carbohydrate core and their hydroxyl fractions are esterified with phenolic acids (gallotannin and ellagitannin) in their external rings (Min and Hart, 2003). Hassanpour *et al.* (2011), noted that HTs are generally obtained in lower quantity in plants, and are divided into caffetannins made up of caffeic and quinic acid, and taragallotannins which constitute gallic and quinic acid. Hydrolysable tannins are known to be easily hydrolyzed by the rumen microorganisms and mild acids or mild bases to produce phenolic acid and carbohydrate (McSweeney *et al.*, 2001). They can also be hydrolyzed by tanninase enzymes and yield molecules principally pyrogallol that is considered toxic to ruminant animals (Hassanpour *et al.*, 2011). Hydrolysable tannins also had negative effects in non-ruminants by decreasing growth performance and protein utilization, and causing damage to the lining of the digestive tract, which results in higher nitrogen excretion (Hassanpour *et al.*, 2011). Whereas, CTs (proanthocyanidins) are described as the widely distributed tannin types obtained in legume pastures, stems and trees (Barry and McNabb, 1999). Denninger *et al.* (2020), revealed that CTs possess higher molecular weight (up to 20,000 g/mol) compare to HT (500 – 3,000 g/mol), and are made up of flavanoid units connected by carbon-carbon links (Hassanpour *et al.*, 2011). Hence, their complexity depend on the variations among the flavanoid constituents. According to Piñeiro-Vázquez *et al.* (2015), CTs exhibit a greater affinity for other compounds and are capable of precipitating proteins, carbohydrates and alkaloids. Condensed tannins are the most abundant, non-toxic and extensively utilized in reducing CH_4 production and rumen microbial degradation of feed

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protein compared to HTs. The subsequent section will discuss the tannins in detail.

1.3.6 Dietary Tannins

Influence of dietary tannins on rumen microorganisms

Dietary tannins are shown to discourage the proliferation of rumen microorganisms using three mechanisms of action which include: disturbing the activities of enzymes, substrate scarcity and direct bioactive effect on the cell membrane as well as shortage of metallic ions (McSweeney *et al.*, 2001; Piñeiro-Vázquez *et al*., 2015). These mechanisms interfere with the multiplication and activities of microbes (Bodas *et al.*, 2012). For example, the activities of enzymes amylase, carboxymethyl cellulose, β-glucosidase and protease were reported to be reduced by the inclusion of *Leucaena leucocephala* CTs (Mahanani *et al.*, 2020). While, HTs extracts from tara (*Caesalpinia spinosa*) and chestnut (*Castanea sativa*) were found to decrease amylase activity in the rumen (Salami *et al*., 2018). Addition of *Samanea samon* pod extract also inhibited the ruminal amylolytic activities (Anantasook *et al*., 2013).

An investigation reported bactericidal and bacteriostatic influences of Bird's-foot-trefoil (*Lotus pedunculatus*) on *Methanobrevibacter ruminantium* (Tavendale *et al.*, 2005), which is the most prevalent species of *Methanogenic archaea* in the rumen environment. Also, 37% reduction in the population of *Methanobacteriaceae species* and 33% drop in *Methanosphaera species* were recorded after supplementing goats with the pine cortex of tannin at 30% of DM (Min *et al*., 2014). Furthermore, inclusion of 66 mg of fenugreek (*Trigonella foenun-graecum*) and sesbania (*Sesbania sesban*) CTs extracts were found to reduce the population of rumen microbes by 47% and 44%, respectively (Goel *et al*., 2008). Moreover, Galindo *et al*. (2008), noted that the ruminal concentrations of bacteria and protozoa dropped by 44% and 39%, respectively, when river tamarind (*Leucaena leucocephala*) CTs was added at 30%. Similarly, up to 54% decline in the concentrations of protozoa was observed when tannin extracts were incubated *in vitro* (Bhatta *et al*., 2013a). Cieslak *et al.* (2012), also recorded a reduction in the concentrations of rumen protozoa by around 23% after supplementing cows with CT at 2% DM.

However, some investigations revealed that there are certain bacterial species that could produce enzymatic mechanisms to evade the bioactive properties of tannins or are capable of degrading tannins (Goel *et al*., 2007). Jones *et al*. (1994), revealed that some strains of

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Prevotella ruminicola were tolerant to CTs from sainfoin (*Onobrychis viciifolia*) when added at lower concentrations (600 ug/ml). Similarly, McAllister *et al*. (1994), indicated that the population of *Neocallimastix patriciarum* fungus species was not moderated by the addition of *Lotus corniculatus* CTs at 100 ug/ml. Thus, Wischer *et al.* (2014), suggested that microorganism may tolerate the existence of tannins in the feed or their depressing effects on the bacteria, protozoa and fungi can only be shown for a short duration before their adaptation.

Influence of dietary tannin on volatile fatty acids production

The capacity of tannins to modify rumen microbial fermentation and degradation of feed material which in turn may alter the concentrations VFAs, particularly the ratio of acetate to propionate, is well documented (Jayanegara *et al*., 2012; Piñeiro-Vázquez *et al.*, 2015). The influence of tannins on the proportion of VFAs is found to be dose-dependent (Tiemann *et al*., 2008b; Rira *et al.*, 2019). Thus, the more the increase in the amount of tannin extracts, the less the molar proportion VFAs productions. Rira *et al.* (2019), reported 29% and 11% reductions in total volatile fatty acid (TVFA) concentration after the addition of tannins from pure *Acacia nilotica* pods and leaves, respectively.

Moreover, linear reductions in acetate: propionate ratio as well as TVFA were recorded when quebracho, mimosa and pine bark tannins were incubated *in vitro* (Min *et al*., 2015). Hassanat and Benchaar (2013), also reported declines in the concentration of TVFA production, branched chain VFA, butyric acid, valeric acid and acetate: propionate ratio while, the molar concentration of propionate increased after inclusion of tannins extracts from two CT sources, quebracho (*Schinopsis balansae*) and black wattle (*Acacia mearnsii*) as well as two HTs rich extracts, chestnut (*Castanea sativa*) and Oak (*Quercus aegilops*). However, Soltan *et al.* (2012), observed insignificant effect of four CT sources on the acetic acid concentration, while two of the tannin sources, *Prosopis juliflora* and *Leucaena leucocephala* increased the concentration of propionic acid with a decline in the ratio of acetate to propionate. In addition, tannin supplementation at a lower dosage (< 5% DM) did not influence TVFA production in sheep (Aboagye *et al*., 2018; Adejoro *et al*., 2020). Rira *et al.* (2019), suggesting that the effect of tannin on the ratio of acetate to propionate as well as the molar concentrations of VFAs depend on the source of tannin, structure and inclusion level.

Influence of dietary tannins on ruminal gas and methane production

Tannins have been shown to interferes with the CH_4 synthesis in the rumen via three

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mechanisms of actions as follows: (1) Direct influence of tannin on CH_4 producing bacteria either by poisoning or by attaching to the microbial cell membrane thereby preventing the proliferation of *Methanogenic archaea* with consequent ineffective exchange of H_2 between methanogens and protozoa; (2) Occurrence of tannins in the rumen serve as H_2 sinks by making it unavailable for the CH_4 synthesis; (3) The tannin-protein complexes or tannin-carbohydrate bonds, which in turns reduce substrate for microbial degradation (Hristov *et al.*, 2013; Boussaada *et al*., 2018; Ku-vera *et al.*, 2020; Tedeschi *et al.*, 2021; Vargas-ortiz *et al.*, 2022b). It has also been shown that the responses of CH_4 producing bacteria to tannins rely on the tannin source and inclusion level. Mahanani *et al*. (2020), reported approximately 13% and 21% decline in potential gas production after inclusion of tannin extracts from *Leucaena leucocephala* at 10% and 25% doses, respectively. Appreciable reduction in total gas (-19%) and CH_4 (-23%) were observed when mimosa CT was added at 2.63% DM of substrates (Adejoro *et al*., 2018). The proportion of *in vitro* gas and CH_4 dropped after addition of valonea (-17% vs -36%) and chest nut (-15% vs -40%) HT sources as well as mimosa (-19% vs -38%) and quebracho (-24% vs -40%) CT extracts at 20% of substrate DM (Hassanat and Benchaar, 2013). Incubation of quebracho (*Schinopsis balansae*) tannin extract at 30 g/kg dose of feed DM has been found to decrease CH_4 volume by 41% (Pineiro-Vazquez *et al*., 2018). Waghorn *et al*. (2002), recorded a 16% drop in CH_4/kg intake in lambs supplemented with bird's-foot-trefoil (*Lotus pedunculatus*) CTs. Likewise, a 13% decline in enteric CH_4 yield was obtained after addition of mimosa CT at 25 g/kg of diet DM (Carulla *et al.*, 2005).

A negative correlation between the tannin inclusion level and CH_4/kg digested organic matter (OM) was observed by Jayanegara *et al*. (2009). In another research, by Huang *et al.* (2010), Leucaena-hybrid Bahru CT extracts revealed a decreasing trend in CH_4 production with the increase in the dosage. Total gas was found to reduce linearly by 43% and CH_4 by 63% when *Leucaena leucocephala* CTs were mixed with the substrate (Tan *et al.*, 2011). In addition, Jayanegara *et al.* (2011), revealed that CT rich plants, *Swietenia mahagoni*, *Acacia magnium* and *Leucaena leucocephala* were capable of decreasing CH_4 emission by 78%, 65% and 43%, respectively. Generally, literature have shown that tannins vary in their anti-methanogenic effect, which could be linked to the source, their chemical structure, molecular weight and the degree of polymerization as well as tannin-protein complexes (Huang *et al.*, 2011; Haque, 2018; Vargas-ortiz *et al.*, 2022a).

Influence of dietary tannins on feed intake and digestibility

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Tannins are well documented to exhibit anti-nutritional effects when fed to animals which include: reduced feed palatability, reduced dietary feed intake, feed conversion efficiency and nutrients digestibility (Frutos *et al*., 2004b; Martin *et al*., 2010; Piñeiro-Vázquez *et al.*, 2015; Naumann *et al.*, 2017). Depressed palatability is linked to astringency and bitterness as a results of tannin-protein interaction in the saliva (Mueller-Harvey, 2006). Breslin *et al*. (1993), defined astringency as mouth sensation of dryness and puckering whereas bitterness involved a sense of taste identified by nervous signals. According to Naumann *et al.* (2017), the higher the tannin's astringency, the more the proportion of protein bound and the lesser the palatability. Häring *et al*. (2008), attributed higher consumption of Sainfoin (*Onobrychus viciifolia*) compared to Bird's-foot trefoil (*Lotus corniculatus*) by the sheep to its increased palatability due to greater concentrations of CT (up to 10%). However, tannins varies in their potential to bind feed protein. CT rich Panicled tick-trefoil (*Desmodium paniculatum*) was found to exhibit lesser interactions with protein compared with lower CT source Chinese bush clover (*Lespedeza cuneata*) (Naumann *et al*., 2014).

Feed intake and digestibility of feed in ruminant animals are also strongly affected by the nature and concentrations of tannins added in the feed (Waghorn, 2008; Cabral Filho *et al*., 2013; Pathak *et al*., 2016; Méndez-Ortiz *et al*., 2018). Reduction in nutrient intake and digestibility principally, dietary protein, with the increase in dosage of tannin extracts is well captured (Hristov *et al.*, 2013; Haque, 2018; Besharati *et al*., 2022). For instance, inclusions of tannin above 5% of feed DM was reported to reduce diet ingestion by sheep (Barry and McNabb, 1999) and cows (McNabb *et al*., 1996). Furthermore, Frutos *et al*. (2004a), obtained a considerable decline in feed intake (from 18 g to 2.5 g DM/kg body weight, BW) of sheep after supplementing HT rich tannic acid at 8 g/kg BW. Significant reduction in diet intake of sheep was also reported after inclusion of HT rich shrub, *Clidemia hirta* (McSweeney *et al.*, 2001). Dschaak *et al.* (2011), also recorded a decline in the nutrient intake of dairy cows after supplementing tannin extract. The reduction in intake and digestibility has been linked to tannins' astringency or reduced palatability (Landau *et al*., 2000; Dschaak *et al.*, 2011). Moreover, the prevalence of procyanidins over prodelphinidins in the tannin sources has been shown to affect voluntary feed intake and DM degradation. Lascano *et al*. (2003), recorded reduced diet consumption and digestibility in *Patulul*, procyanidins-rich cultivar of *Calliandra calothyrsus* and higher intake and degradability in *San Ramon*, a prodelphinidins-rich cultivar.

The impact of dietary tannins on voluntary feed intake and digestion has also been identified

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to differ based on species of ruminants (Besharati *et al.*, 2022). Some ruminants are found to possess abundant proline in their saliva that readily complex dietary tannins and cancel their negative influence on diet palatability, intake and digestibility (Narjisse *et al*., 1995; Foley *et al*., 1999; Besharati *et al.*, 2022). According to Reed (1995), goat's saliva possess the highest proline-rich mucoproteins followed by sheep while cattle had the lowest. Some investigations revealed that addition of tannins below 5% DM of feed had no effect on intake and digestibility in ruminants (Frutos *et al*., 2004b; Hassanpour *et al.*, 2011; Besharati *et al.*, 2022). Adejoro *et al*. (2020), noted that inclusion of *A. mearnsii* tannin powder at 42 g/kg DM had no influence on nutrient intake in South African Mutton Merino lambs. Nutrient intake of cows was also found not to be affected by the inclusion of valonea (*Quercus aegilops*) HT as well as wattle (*Acacia mangium*) and bayberry (*Myrica rubra*) CT sources at 3% DM (Zhang *et al.*, 2019). In addition, Beauchemin *et al.* (2007), recorded zero adverse effect on dietary intake and nutrient degradation of beef steers after supplementing Quebracho CT at 2% DM.

Influence of dietary tannins on animal performance

Tannins are shown to exhibit a positive effect on animal performance owing to the fact that dietary tannins prevent rumen microbes from degrading feed protein which in turns increases nitrogen bypass to the distal part of GIT and therefore, maximize protein absorption (Min *et al*., 2006; Soltan *et al.*, 2012; Mergeduš *et al*., 2022). Thus, the higher the dosage of tannins supplemented, the lower the dietary protein degraded by the rumen microorganisms. For instance, a recent study reported a reduction in microbial protein digestion to 117 g/kg from the initial 614 g/kg of DM compared to control, and an increase in rumen undegradable protein to 888 g/kg DM from the initial 386 g/kg when *Leucaene. leucocephala* CT was added at 46 g tannic acid (TA) equivalent/kg DM (Soltan *et al.*, 2012). This mechanism revealed a positive relationship with the increase in faecal N excretion. Soltan *et al.* (2013), observed up to 70% increase in faecal N and around 13% reduction in urine N when 8.8 g of TA equivalent/kg DM of tannin was supplemented. Carulla *et al.* (2005), also recorded approximately 9% decrease in urinary N while faecal N content increased after supplementing sheep with black wattle tannin at 2.5% DM. It has been well established that reduction in urine N excretion is useful to the environment (Patra and Saxena, 2011). This is because, unlike faecal N that is more stable and not quickly released to the surface (Aboagye *et al*., 2018), urea which is the major N component in urine, is not stable and therefore readily escape to the environment as N_2O and NO_3 (Eckard *et al*., 2010). The drop in microbial protein digestion is related to tannin-protein

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bonds formed in the rumen as well as tannin's interference with the activities of proteolytic bacteria or endogenous enzymes (Piñeiro-Vázquez *et al.*, 2015; Sun *et al.*, 2021). The interaction between the tannin and protein generally dissociate in the abomasum when pH rose to about 7 – 7.5, thereby making the dietary proteins more available for absorption in the duodenum (Hassanpour *et al.*, 2011). The degree of tannin-protein binding and dissociation varies among tannin sources, structure and molecular weight (Soltan and Patra, 2021; Sun *et al.*, 2021; Besharati *et al.*, 2022).

Numerous researches reported an improvement in animal weight gains after supplementing tannins depending on the sources and dosages. Supplementing tara (*Caesalpinia spinosa*) and sweet chestnut (*Castanea sativa*) HTs as well as gambir (*Uncaria gambir*) and Australian black wood (*Acacia negra*) CTs at 40 g/kg DM of feed were reported to enhance the performance of lambs (Salami *et al*., 2018). Addition of 900 g of *L. cuneata* tannin was also found to increase weight gain of grazing sheep by 26% per day (Burke *et al.*, 2014). Min *et al.* (2006), obtained approximately 21% increase in weight gain of steers when fed quebracho CT at 2% DM. Inclusion of sulla tannin at 7.2% DM of feed had no influence on live weight changes in lambs (Douglas *et al*., 1999). Furthermore, a number of research indicated that tannin supplementation enhance milk yield and quality. Dey and De (2014), recorded about 25% increase in milk yield when tannin was supplemented at 15 g/kg DM of feed to crossbred cows. Equally, 10% improvement in milk volume of cows supplemented with CT at 88 g/kg of DM dose was also documented (Anantasook *et al.*, 2014). In addition, Harris *et al*. (1998), obtained around 20% increase milk yield after feeding dairy cows *L. corniculatus* CT at 27 g/kg DM dosage. Addition of *L. corniculatus* CT also enhanced the proportion of milk lactose by 12%, protein by 14% and whole milk by 21% (Wang *et al*., 1996).

However, several researchers observed reduction in daily gains of animals when tannin extracts were included even below 5% of diets DM. For instance, Bhatta *et al.* (2013b), reported a significant drop in weight gain of goats supplemented with CT extract at the dosage of 5.7 g/kg DM. Addition of carob pulp (*Ceratonia siliqua*) CT at 25 g/kg DM also resulted in significant drop in lambs' weight (48 g) from the initial of weight of 135 g (Priolo *et al*., 2000). In addition, Grainger *et al.* (2009), observed a drastic decline in milk volume (-29.7%) after supplementing higher dosage (244 g/day) of black wattle CT extract to lactating cows. These negative impacts of tannins on ruminants and their products were attributed to the decrease in dietary intake and digestibility, upsurge in energy loss and decline in N retention especially when supplemented

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at higher concentrations (Hristov *et al.*, 2013; Piñeiro-Vázquez *et al*., 2015; Besharati *et al.*, 2022).

Dietary tannins intoxications

Tannins especially, hydrolysable sources are widely regarded as poisonous to ruminant animals. Numerous information are available on the intoxications of HT compared to CT (Frutos *et al*., 2004a, b; Hassanpour *et al.*, 2011; Jayanegara *et al.*, 2015; Naumann *et al.*, 2017). Jayanegara *et al.* (2015), attributed the greater toxicities of HTs to their superior protein binding ability compared to CT sources. For instance, signs of acute toxicity were observed in cattle fed Pyrenean oak (*Quercus pyrenaica*) leaves, which is rich in HT (Doce *et al*., 2013). Furthermore, when oak leaves tannins were fed to calves at above 4.4 mg/kg BW, a clear poisonous symptoms including methaemoglobinaemia and renal failure were observed (Guitart *et al*., 2010; Pérez *et al*., 2011). Filippich *et al*. (1991), also recorded a hepatotoxic and nephrotoxic effects of punicalagin, a HT source on some ruminants.

The intoxications of tannins has also been linked to their variation in molecular size. Tannins with lower molecular weights can easily be absorbed while higher molecular size tannins were not simply degradable. This could justify the non-toxic property of high molecular sized *Lespedeza cuneata* CT and greater intoxication of low molecular size oak (*Quercus* species) HT in rats (Frutos *et al*., 2004b). It is well known that HTs are hydrolyzed by rumen microorganisms or by tanninase enzymes and by mild alkalis or mild acids (Lochab *et al*., 2014). During rumen fermentation, the ester bonds in HTs are detached to yield molecules like pyrogallol which is poisonous to animals (Hassanpour *et al.*, 2011). Numerous investigations were carried out with regards to tannin toxicity for some rumen bacterial species particularly, *Prevotella ruminicola*, *Butyvibrio fibrosolvens*, *Ruminobacter amylophilis*, *Fibrobacter succinogenes* and *Streptococcus bovis* (Nawab *et al*., 2020). Tannin influenced rumen fermentation via ulceration of the rumen, membranes destruction, mucosal necrosis, substrate deficiency, metal ion deficit and enzyme inactivation (Pérez *et al*. (2011). Example, Reed (1995), reported that inclusion of HT at 20% level in the diet of cattle and sheep resulted in lesions associated with hemorrhagic gastroenteritis, kidney damage, liver necrosis and high mortality. While, Murdiati *et al*. (1992), recorded signs of intoxications in sheep after supplementing gallic acid and tannic acid above 4% of BW.

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Hydrolysable tannin toxicities were also observed in non-ruminant animals which include: gastrointestinal tract mucosal damage and increased N excretion as well as reduced protein utilization and depressed growth (Hassanpour *et al.*, 2011). An investigation by Lee *et al.* (2010), showed a decline in hematocrit and red blood cell (RBC) counts as well as reduction in daily weight gain, after addition of TA to piglets at 0.25, 0.5 and 1g/kg DM, respectively. Higher concentrations (5 – 20 g/kg DM of diet) of TA was also discovered to affect absorption of iron (Fe) in rats (Afsana *et al*., 2004; Karamać, 2009). However, inclusion of chestnut (*Castanea sativa*) at 2 g/kg of TA equivalent had no influence on the growth of fattened pigs (Prevolnik *et al*., 2012). Moreover, feeding chestnut wood tannins to New Zealand boar rabbits was found not to have any signs of toxicity (Liu *et al*., 2011). Reports have shown that tannin intoxication relies significantly on dosage and age of the animals. Hence, aged animals can tolerate higher concentrations of HT (15 – 25 g/kg DM of diets) devoid of any deleterious effects on the ruminants (Krueger *et al*., 2010; Liu *et al*., 2013; Nawab *et al*., 2020). Condensed tannins are not easily digestible by rumen microorganisms like HT and thus, not readily absorbed into the blood stream (McSweeney *et al.*, 2001). Hence, CTs supplementation had no history of toxicity to important internal organs usually damaged by HTs (Terrill *et al*., 1994). Nevertheless, feeding high amount of some CT sources might resulted in the damage of intestines and therefore higher absorption of polyphenols into the bloodstream (Makkar, 2003).

Tannin sources in sub-Saharan Africa

Tannins are extensively distributed in the tropics, in virtually all the trees, shrubs, legumes, vegetables and fruits. *Acacia* is one of the leading tannin-rich tropical plants of the family and subfamily, *Fabaceae* and *Mimosoideae*, respectively (Hassanpour and Mehmandar, 2012). *Acacia* composed of about 1300 species, among which *Acacia mearnsii* tannin also called Black wattle or mimosa (Kardel *et al*., 2013), is the most abundant species and well distributed alien plants covering a land area of approximately 2.5 million hectares in the Republic of South Africa (de Wit *et al*., 2001; Galatowitsch and Richardson, 2005). Moreover, a commercial establishment of 300, 000 hectares of land for Black wattle tannin has also been reported (Ogawa and Yazaki, 2018). *A. mearnsii* tree is considered as the most invasive species imported from Australia to South Africa in the 19th century (Hassanpour and Mehmandar, 2012).

The bark of mimosa tree constitutes 35 – 45% CT, depending on the growing seasons (Gujrathi and Babu, 2007). Furthermore, *A. mearnsii* is discovered to contain about 30% of non-tannin compounds which include hydrocolloids, sugars, lipids, organic acids and gums in addition to

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an appreciable amount of HT (Missio *et al*., 2017), which is believed to be poisonous to animals. Purification of industrially extracted *A. mearnsii* tannin with organic solvents (hexane, methanol, pentanol, propanol and ethyl acetate) using Soxhlet apparatus was found to reduce the concentration of HT and non-tannin molecules while increasing the CT content (Missio *et al*., 2017). Also, greater proportions of CTs: 82% (Hassanat and Benchaar, 2013); 61.5% (Carulla *et al.*, 2005), and 60.3% (Grainger *et al.*, 2009), were obtained in mimosa tannin after purification of the tannin standard with recent countercurrent chromatography (CCC) technique using Sephadex LH-20. Because, CCC is capital intensive and more technical (Missio *et al*., 2017), hence, not widely utilized by most quality control and analytical laboratories.

Recent investigations revealed that, addition of Black wattle tannin in the ruminants' basal diets influenced rumen microbial fermentation, depressed methanogenesis while enhanced dietary protein bypass and amino acids utilization. *A. mearnsii* tannin inclusion at 42 g/kg DM level, increased propionate concentration by 13.7% and reduced acetate by 11.8% (Adejoro *et al*., 2020). Moreover, *A. mearnsii* CT addition in the feed of Jersey steers at 2% DM, resulted in 9.1% decline in crude protein digestibility, 2.7% drop in rumen pH, 4.5% decrease in molar concentration of acetic acid and 9.9% increase in TVFA production (Avila *et al.*, 2020). Furthermore, *in vitro* incubation of *A. mearnsii* CT increased molar concentration of propionic acid (Hassanat and Benchaar, 2013). Denninger *et al.* (2020), obtained 16% reduction in CH_4 emitted by cows after supplementing black wattle tannin at 3% DM of feed. In addition, Adejoro *et al*. (2019b), recorded 30% drop in enteric CH_4 emitted by merino wethers after feeding Black wattle tannin at 40 g/kg DM. Also, *in vitro* inclusion of mimosa CT at 2.63% of substrates was reported to reduce CH_4 yield by about 24% (Adejoro *et al*., 2018). Moreover, when animals were supplemented with *A. mearnsii* CT extracts, significant reductions in urine N (-59%) and CH_4 (-13%) were recorded in sheep (Carulla *et al.*, 2005), as well as -29% and -9.3% decrease in CH_4 and urine N, respectively, in cows (Grainger *et al.*, 2009). Adejoro *et al*. (2020), also reported 20% increase in faecal N excretion and 13% drop in urine N excretion following addition of mimosa tannin in the urea containing diet of merino sheep. This showed that mimosa tannin could be utilized as natural alternative to the widely criticized monensin in modifying rumen fermentation.

However, feeding of *A. mearnsii* tannin extract induced negative effects on voluntary intake, microbial activities and DM digestibility especially, when supplemented at higher dose

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(Grainger *et al.*, 2009; Bhatta *et al.*, 2013b; Adejoro *et al*., 2020). This is due to the tannins aversive taste and instability across gastrointestinal tract. For example, *A. mearnsii* tannin addition at 4.2% DM decreased the digestibility of feed DM (-11.1%), OM (-11.6%), CP (-6.8%), NDF (-24.2%) and ADF (-34.4%) in South African mutton merino lambs (Adejoro *et al*., 2020). Biondi *et al.* (2019), also noted that inclusion of *A. mearnsii* tannin at 40 g/kg of feed DM reduced the population of *Pseudomonas species*, *Enterobacteriaceae* and *Eschericia coli* as well as total psychrotrophic bacteria and total mesophilic bacteria in the rumen of goats. Addition of Black wattle tannin at 40 g/kg DM level also reduced digestibility of diet DM by 20.3%, OM by 20.3%, CP%, NDF by 37.8% and ADF by 42.2% in the Merino wethers (Adejoro *et al*., 2019b). *In vitro* incubation of mimosa CT at ≥50 g/kg of feed DM was found to reduce TVFA production (Hassanat and Benchaar, 2013). Moreover, Bhatta *et al.* (2013b), reported 30% reduction in N retention and around 45% increase in energy loss when goats were given Black wattle CT above 20 g/kg of feed DM. Furthermore, Grainger *et al.* (2009), obtained 29.7% reduction in milk yield after drenching *A. mearnsii* tannins extracts at 1.8% of DMI to Holstein–Friesian cows. Hence, it is essential to come up with a novel approach that will mask the undesirable taste of tannin and promote sustained release of tannin extract across GIT thereby facilitating intake and digestibility without any adverse effects on normal rumen function.

Microencapsulation of tannins

Bakry *et al.* (2016), defined microencapsulation as a method of building a functional barrier between the internal phase (core material or fill) and the shell (coating or wall material) to conserve the physical, functional, chemical and biological characteristics of the core material as well as to prevent physico-chemical reactions. Desai and Park (2005), identified seven reasons for microencapsulation process which include: (i) masking the undesirable flavor or taste of the bioactive compound; (ii) ensuring the release of the fill in a sustained manner over time; (iii) safeguarding the core material from quick dissolution; (iv) moderating the evaporation of the bioactive substance; (v) altering the physical properties of the fill for easy handling; (vi) diluting the active ingredient when little quantities are needed; (vii) separating the portions of the mixture that may interact with one another. Recently, feed and pharmaceutical industries have adopted a number microencapsulation techniques to mask the astringency of tannins and ensure their sustained delivery to the specific organ of action (Fang and Bhandari, 2010; Munin and Edwards-lévy, 2011). Some of the microencapsulation

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methods include: centrifugal suspension separation, coacervation, coaxial electrospray system, extrusion, fluidized bed coating, inclusion complexation, *in situ* polymerization, spray-chilling, freeze-drying, spray-cooling, emulsification, spray-drying and superficial fluid technology (Gharsallaoui *et al.*, 2007; Sanchez *et al.*, 2013; Bakry *et al.*, 2016). However, double emulsion technique gained recent acceptance for tannin encapsulation, because of its efficiency in hiding the aversive taste and capability of coating the polyphenolic compound until it reaches the target organ of action (Dickinson, 2011; Adejoro *et al*., 2018). In addition, the technique could regulate tannin particle size, density and improve the stability of tannin microcapsules compared to other methods (Ho *et al*., 2017).

Lipids such as palm oil and lard were successfully utilized as wall materials for acacia tannin microencapsulation (Adejoro *et al*., 2018; Adejoro *et al*., 2019a). They are widely available and inexpensive while possessing all the features required for ideal encapsulant such as excellent film formation, good emulsification, tasteless and low viscosity (Jafari *et al*., 2008; Abedi *et al.*, 2016). Sunflower oil is another lipid that is found in large amount and can be also be adopted for tannin encapsulation because of tasteless (Bakry *et al.*, 2016), good emulsifying properties and low viscous in nature (Flanagan *et al*., 2006). When *Acacia mearnsii* tannin was encapsulated using lard and palm oil, 80% encapsulation efficiency (Ee) was recorded while, tannin dissolution in buffers simulating rumen, abomasum and small intestine reduced to 20%, 34% and 25%, respectively, for lard microcapsules after 24 hours, and 19%, 30% and 22% reductions in similar media and time for palm oil microparticles (Adejoro *et al*., 2018). The same author also reported 16% and 12% reductions in CH_4 for tannin encapsulated with lard and palm oil, respectively, *in vitro*. Furthermore, enteric CH_4 emission was found to reduce by 19% while, faecal N excretion increased by 27% without reductions in feed consumption and DM degradation compared to non-encapsulated acacia tannin (Adejoro *et al*., 2019b). Wood *et al.* (2009), also observed an improvement in voluntary intake and daily gain with 76% declined in CH_4 yield after addition of encapsulated fumaric acid in coconut oil. These could be due to the ability of microencapsulation to mask the tannin astringency thereby reducing their adverse effects on intake and digestibility. Thus, microencapsulation technique has the potential to improve dietary tannin use by ruminants and promote environmentally friendly livestock production by limiting tannin interaction with salivary muco-protein in the mouth and regulating the instability of tannin extract across GIT.

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1.4 Conclusions

It is obvious use of dietary tannin in reducing methane production by the ruminant and maximizing feed protein utilization could be adopted as a better option for eco-friendly livestock production in sub-Saharan Africa. Tannin purification and microencapsulation might be a good step forward in using tannin as natural feed additive. Inadequacies such as the hydrolysable tannin toxicities, crude tannin's bitter taste and instability across gastrointestinal tract, resulted in reduced feed intake and digestibility as well as poor animal performance, and these limitations need to be addressed for wider utilization of tannin as a natural feed additive in organic ruminant animal farming. The following research objectives are borne out of this conclusion.

1.5 Objectives of the study

1.5.1 Major objective of the study

To examine the potential of using encapsulated *Acacia mearnsii* tannin as a natural additive that can be used as a better alternative to ionophore in reducing enteric methane emission and facilitating dietary protein bypass.

1.5.2 Specific objectives of the study

i. To purify *Acacia mearnsii* tannin using pentanol and ethyl acetate, and evaluate the impact of the purified tannins on the *in vitro* organic matter digestibility of *Eragrostis curvula* hay and the production of gas and methane.

ii. To encapsulate *A. mearnsii* tannin using sunflower oil and palm oil, and evaluate the microcapsules' morphology, density, encapsulation efficiency and tannin release rate in buffer solution simulating pH condition of various gastrointestinal tracts.

iii. To evaluate the capacity of palm and sunflower oils based encapsulated *A. mearnsii* tannins to bind bovine serum albumin (BSA) protein in rumen simulated buffer, and subsequently quantify the amount of BSA released in buffer media simulating abomasum and small intestine.

iv. To ascertain the impact of various dosages of *A. mearnsii* tannin encapsulated in sunflower oil on the concentrations of methane, gas, *in vitro* organic matter digestibility, volatile fatty acids and ammonia nitrogen of total mixed ration and *Eragrostis curvula*

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hay.

v. To evaluate the influence of encapsulated *A. mearnsii* tannin in sunflower oil and non-encapsulated tannin on methane, nutrient utilization, growth changes and rumen fermentation characteristics in South African mutton Merino ram lambs.

1.6 Hypothesis of the Study

In order to achieve the aforementioned objectives, a series of studies were designed with the intention to test the following hypothesis:

i. Purified *A. mearnsii* tannins with either pentanol or ethyl acetate vary with the unpurified tannins in their influence on methane, gas and *in vitro* organic matter digestibility of *Eragrostis curvula* hay.

ii. Encapsulated *A. mearnsii* tannins with either sunflower oil or palm oil differ with the non-encapsulated tannin on their morphology, density, encapsulation efficiency and tannin release rate in various media simulating gastrointestinal tract.

iii. The binding of bovine serum albumin protein in rumen simulated buffer, and its release in abomasum and small intestine simulated buffer media vary between unencapsulated and encapsulated *A. mearnsii* tannins with either sunflower oil or palm oil.

iv. The variation in the doses of *A. mearnsii* tannin encapsulated in sunflower oil affect the methane, gas, *in vitro* organic matter digestibility, ammonia nitrogen and volatile fatty acids concentrations of total mixed ration and *Eragrostis curvula* hay.

v. Inclusion of encapsulated *A. mearnsii* tannin in sunflower oil and non-encapsulated tannin cause variation on methane, nutrient utilization, growth changes and rumen fermentation characteristics of South African mutton Merino ram lambs.

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CHAPTER TWO

Effects of graded levels of mimosa (*Acacia mearnsii*) tannin purified with organic solvents on gas, methane, and *in vitro* organic matter digestibility of *Eragrostis curvula* hay

Abstract

The higher contribution of methane (CH_4) to global anthropogenic potential is a cause of concern to livestock producers. Mimosa tannin gained recent acceptance as an additive for enteric CH_4 mitigation. However, rumen fermentation and digestibility are compromised when large quantities of tannins are supplemented due to the presence of hydrolysable tannin and other non-tannin molecules in mimosa extract, which are toxic to animals. Purification could eliminate the toxins, and thus, reduce the CH_4 yield without negative effects on rumen microbial activities and organic matter degradation. The Soxhlet extraction method was used to purify the tannin using organic solvents (ethyl acetate and pentanol). The unpurified, ethyl acetate purified, and pentanol purified tannins at the dosages of 10, 20, 30, and 40 g/kg DM of substrate (*Eragrostis curvula* hay) were evaluated for gas, CH_4, and *in vitro* organic matter digestibility (IVOMD) in comparison with substrate alone. Gas kinetics were tested using a simple exponential model with lag. The results showed that compared with control, gas, CH_4, IVOMD, CH_4/gas, CH_4/IVOMD, gas/IVOMD, asymptotic gas volume (v), and rate of gas production (k) decreased ($p < 0.01$) linearly with the increase in the inclusion levels of all tannin extracts. Also, ethyl acetate purified and pentanol purified tannin extracts reduced gas and CH_4 at lower dosage (30 g/kg DM) compared to unpurified tannin extract at a higher level (40 g/kg). It was concluded that the purification of mimosa tannin with ethyl acetate and pentanol reduced potential gas production and CH_4 without much reduction in substrate digestibility when up to 30 g/kg DM of feed was used. Lower inclusion levels of ethyl acetate and pentanol purified extracts could give a similar result with a higher dosage of unpurified tannin.

Keywords: digestibility; ethyl acetate; inclusion level; methane; mimosa tannin; pentanol; purification; Soxhlet.

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2.1 Introduction

Recent research in ruminant nutrition gave more emphasis to enteric methane (CH_4) abatement strategies, because of its global warming potential, and the high impact it has on the animal industry. Enteric emissions account for about 39% of the agricultural contribution of global CH4 yield per annum (Gerber *et al.*, 2013), and are responsible for the loss of up to 12% of animal energy intake (Piñeiro-Vázquez *et al.*, 2015). Several approaches were adopted to moderate CH_4 production by ruminants, which include animal breeding; improved livestock production; feeding concentrates; antibiotics; and plant secondary compounds, particularly tannins and saponins (Eckard *et al.*, 2010). However, the use of condensed tannins received more recent attention due to their availability, safety, and efficiency (Martin *et al.*, 2010).

Mimosa (*Acacia mearnsii*) is regarded as a highly invasive and widely spread tannin rich legume; extending an area of over 2.5 million hectares (de Wit *et al.*, 2001), in addition to more than 130,000 hectares of commercial plantation (Galatowitsch and Richardson, 2005). Earlier research showed that mimosa tannin has the potential to reduce enteric CH_4 and ammonia nitrogen (NH_3N) production, but with some negative effects on rumen fermentation and digestibility when supplemented above 50 g kg^{-1} DM (Carulla *et al.*, 2005; Grainger *et al.*, 2009; Hassanat and Benchaar, 2013), attributed to the high amount of hydrolysable tannins, which are regarded as toxic to animals. For instance, when mimosa tannin was included at ≥50 g/kg DM of substrate, *in vitro* CH_4 and NH_3N reduced by 12% and 47%, respectively; however, the proportion of total volatile fatty acids (TVFAs) decreased (–6%) compared to the control diet (Hassanat and Benchaar, 2013). Similarly, reductions in enteric CH4 (–29%) and urine nitrogen (–9%) were observed when higher amount of mimosa tannin extract was included in the diet of lactating cows, but milk yield dropped by 30% compared with the cows fed the control diet (Grainger *et al.*, 2009). On the other hand, some studies reported little or no impact of mimosa tannin on rumen fermentation and methane mitigation when added below 50 g/kg DM (Gemeda and Hassen, 2015; Adejoro *et al.*, 2020). This is because mimosa tannin is not pure tannin phenolic (Tondi and Pizzi, 2009); it is composed of other non-tannin molecules (Arbenz and Avérous, 2015; Adejoro *et al.*, 2019a; Adejoro *et al.*, 2019b), in the form of gums, simple sugars, and organic acids (Missio *et al.*, 2017), which influence the bioactive properties of the tannin, making a lower dosage ineffective. Hence, there is the need for the purification of the mimosa tannin to remove the toxins and other non-tannin substances, thereby reducing the methane yield without compromising the normal rumen function.

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Tannin purification is usually achieved with the use of Sephadex LH-20 (Van Der Watt and Pretorius, 2001; Seeram *et al.*, 2005; Zhou *et al.*, 2011). However, the method is very costly and difficult to adopt by most laboratories (Missio *et al*., 2017). Organic solvents, such as ethyl acetate and pentanol, are extensively utilized for extraction processes, coatings, and as carriers in the production of pharmaceuticals, flavorings, inks, adhesives, cosmetics, and antioxidants (Marino, 2005; Cann and Liao, 2010; Pattanaik and Mandalia, 2011). Recently, Missio *et al.* (2017) reported an increase in tannin phenols at the expense of non-tannin compounds when mimosa tannin was purified with ethyl acetate and pentanol, using the simple and less expensive Soxhlet technique. However, to the best of our knowledge, there is little or no information on the effect of ethyl acetate purified or pentanol purified mimosa tannin extracts in reducing enteric CH_4 emission. The objectives of this study, therefore, were to purify the industrially extracted mimosa tannin with ethyl acetate and pentanol using the Soxhlet technique, to characterize the tannin constituents as influenced by the purification with organic solvents, and to evaluate the influence of adding various levels of ethyl acetate and pentanol purified tannin extracts on gas, methane, and *in vitro* organic matter digestibility of *Eragrostis curvula* hay.

2.2 Materials and Methods

Study Area

This investigation was carried out in the Department of Animal Science, University of Pretoria, South Africa. The area lies at 25º44′30″ south and 28º15′30″ east, at an elevation of 1360 m above sea level (van Niekerk *et al.*, 2009). The study was reviewed and approved by the Animal Ethics Committee of the University of Pretoria (Ref No: EC075-17).

Materials

The industrially extracted mimosa (*Acacia mearnsii*) tannin powder used in the current study was obtained from UCL Tannin Company Pty (Ltd), Kwa-Zulu Natal, South Africa. The extract was obtained from the bark of *Acacia mearnsii* tree through a series of hot water extraction processes at a controlled temperature, pressure, and time, followed by vacuum evaporation and subsequent air-drying into fine powder before packaging and storage at −4 ºC (Missio *et al*., 2017). The molecular weight of the mimosa ranged from 500–3000 Daltons (Adejoro *et al*., 2018). The organic solvents used were: ethyl acetate and pentanol, procured from Sigma Aldrich, Johannesburg, South Africa. According to Missio *et al.* (2017), pentanol

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has a polarity index of 0.568, whereas ethyl acetate has a polarity index of 0.228. Tannic acid, Folin–Ciocalteu reagent, butanol, and all other reagents used were of analytical grades supplied from Sigma Aldrich, Johannesburg, South Africa.

Purification Process

The purification of the mimosa tannin was carried out using the Soxhlet extraction technique as described by Missio *et al.* (2017). The samples (15 g) of mimosa tannin powder were weighed into thimbles, and fractionated separately with ethyl acetate and pentanol (200 mL) using a Soxhlet apparatus. The purifications were done at the boiling points of the solvents for 6 h (in triplicates). The ethyl acetate and pentanol purified tannin extracts were collected, oven dried at 70 ^{o}C, and kept in a refrigerator before analysis.

Tannin Characterization

The powdered samples (200 mg) of unpurified and purified mimosa tannins with ethyl acetate and pentanol were weighed separately in 25 mL volume glass beakers. Aqueous acetone (10 mL) was added and suspended in an ultrasonic bath for 20 min. The contents of the beakers were transferred into the centrifuge tubes, and kept in ice for 15 min. The tubes were then centrifuged at 2500 rpm for 15 min, and the supernatants were collected and kept in ice prior to analysis. The Folin–Ciocalteu method was used to determine the concentrations of total phenols, non-tannin phenols, and total tannins as tannic acid equivalent (Makkar, 2000), whereas the proportions of condensed tannins were obtained using the butanol-HCl technique as leucocyanidin equivalent, following the procedure of Porter *et al*. (1986). The concentration of hydrolysable tannin was calculated by differences between total tannins and condensed tannins according to the method of Singh *et al.* (2005).

Chemical Analysis of Substrate

Freshly harvested *Eragrostis curvula* hay was procured from the Experimental Farm, University of Pretoria, and used as substrate. The sample was milled through a 2 mm screen, and evaluated for chemical composition. Dry matter (DM) and ash were determined according to the method of the Association of Official Analytical Chemists (AOAC, 2002). Nitrogen Analyzer (FP-2000, Leco Instrumente GmbH, Kirchheim, Germany) was used to analyze nitrogen content of the hay, and the value of nitrogen recorded was multiplied by 6.25 to get crude protein (CP). Fibre Analyzer (ANKOM 200/220, ANKOM Technology, New York, NY, USA) was utilized to determine the composition of neutral detergent fiber (NDF), acid

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detergent fiber (ADF), and acid detergent lignin (ADL) of the substrates in sequence using the procedure of Van Soest *et al*. (1991).

In Vitro Incubation

Buffer was formulated in a three-litres volumetric flask, placed in a water bath, and constantly flushed with saturated carbon dioxide (CO_2) at 39 ^oC for about 45 min, as described by Menke and Steingass (1988), and modified by Mould *et al.* (2005). Rumen fluid was collected within the above 45 min period from three rumen fistulated Pinzyl steers fed with *Eragrostis curvula* hay and supplemented with *Medicago sativa* hay. The rumen fluid was squeezed through four folds of cheese cloth, taken to the laboratory without delay in a warmed thermos flask, and continuously flushed with CO_2. Before incubation, the buffer solution was mixed with the rumen fluid at a ratio of 5:3 into a volumetric flask continuously flushed with CO_2, and heated at 39 ^oC in a water bath. The inoculum (40 mL) was then poured into150 mL serum bottles, which already contained 400 mg each of the substrate (*Eragrostis curvula* hay) mixed with 4 mL extracts of unpurified, and purified mimosa tannins with ethyl acetate and pentanol each of them at four different dosages (10, 20, 30, and 40 g/kg DM). The serum bottles were immediately closed tightly using blue rubber stoppers with needles attached to three-way stopcocks, and then incubated at 39 ^oC, and shook at 120 revolutions per minute (rpm). All the treatments and control were replicated four times, and three blank bottles were included for correction in each incubation run, and four separate runs were conducted in a randomized complete block design (RCBD).

Gas Estimation

The *in vitro* gas volume produced during the fermentation process was calculated from pressure recorded in per square inch (psi) using a semi-automated pressure Transducer (PX4200-015GI; Omega Engineering Inc., Laval, QC, Canada) connected to a digital data logger (220 series indicators; Omega Engineering Inc.) (Theodorou *et al.*, 1994). The pressures built-up in the serum bottles at 3, 6, 12, 24, and 48 h of incubation were recorded by fitting the transducer into the upper tap of a three-way stopcock connected to the needle with a blue rubber stopper closing the fermentation bottle. The gas readings obtained from the bottles at each period (psi) were corrected by the subtraction of ambient pressure (psi) recorded by the data logger, and then converted to mL using the ANKOM technology equation (Pressure to Gas Production Conversion, 2014), shown in Equation (2.1) below:

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$$V_x = V_j P_{psi} X\ 0.068004084 \quad (2.1)$$

where, V_x = volume of gas in mL, V_j = headspace of serum bottle in mL, and P_{psi} = corrected pressure recorded in psi by the data logger.

Methane Determination

Methane analysis was performed according to the method of Tavendale *et al*. (2005). Immediately after reading the pressure built-up in the bottles at 3, 6, 12, 24, and 48 h of incubation periods, gas samples were taken in 10 mL luer-locked syringes, and injected manually into the gas chromatography (SRI GC 8610C BTU Gas Analyzer System, Bad Honnef, Germany) fitted with a flame ionization sensor, and standardized with methane and carbon dioxide following the procedure of Gemeda and Hassen (2015). Methane concentrations were estimated from the area covered by the gas samples in the SRI GC using a standard methane curve.

In Vitro Organic Matter Digestibility Determination (IVOMD)

The IVOMD of *Eragrostis curvula* hay after inclusion of unpurified mimosa tannin, and mimosa tannins purified with ethyl acetate and pentanol at 10, 20, 30, and 40 g/kg DM was assessed using two-stage digestion processes, as described by Tilley and Terry (1963), with the modifications of Engels and Van der Merwe (1967). Artificial saliva was prepared in a two-litre volumetric flask, kept in a water bath heated at 39 ^{o}C, and flushed with saturated CO_2 continuously. Rumen fluid was obtained from three donor cannulated Pinzyl steers fed with *Eragrostis curvula* hay and supplemented with *Medicago sativa* hay. The rumen fluid was mixed with artificial saliva at a ratio of 1:3, and heated at 39 ^{o}C in a water bath with continuous flushing with CO_2. In stage one, the saliva and rumen fluid mixtures (20 mL) were splashed into digestion tubes, which already contained 200 mg each of the substrate and 2 mL each of urea and tannin extracts (unpurified, ethyl acetate purified, or pentanol purified) at four different concentrations (10, 20, 30, and 40 g/kg DM of feed). The tubes were immediately closed tightly using stoppers fitted with marbles, and digested for 48 h at 39 ^{o}C, and shook at 100 rpm. After 48 h, all the tubes were centrifuged at 2500 rpm for 15 min, and the supernatants were carefully removed. In stage two, acid-pepsin solutions (20 mL) were added into the tubes, and digested for another 48 h at 39 ^{o}C and 100 rpm.

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The tubes were removed and centrifuged again at 2500 rpm for 15 min. The supernatants obtained were oven dried at 105 ^{o}C for 18 h. The dried supernatants were weighed and ashed at 550 ^{o}C in a muffle furnace for three hours. The IVOMD of the substrate was evaluated from the weights of the initial samples, oven dried, and the ash left over. All the treatments and control were in triplicates, three blank tubes were added for correction within each run, and four separate digestion runs were done in RCBD.

Statistical Analysis

All statistical analyses were performed using the general linear model procedure of SAS 9.4 (SAS Institute Inc., Cary, NC, USA). Data on mimosa tannin characterization were analyzed using a one-way ANOVA. Where significant differences existed, means were separated using LSD. Gas production kinetics were evaluated using the Schofield (2000) simple exponential Equation (2.2).

$$V_t = v * (1 - e^{(-k*(t-l))}) \quad (2.2)$$

where V_t = volume of gas at t = time; v = asymptotic gas volume corresponding to complete substrate digestion; k = rate constant; l = discrete lag time before gas production commences. For gas, methane, and in vitro organic matter digestibility, and their ratios and gas kinetics (v, k, and l), the experimental design was a randomized complete block design. The statistical model adopted for the analysis is stated in Equation (2.3) below:

$$y_{ijk} = \mu + Block + T_i + L_j + (TL)_{ij} + \varepsilon_{ijk} \quad (2.3)$$

where y_{ijk} = observation k for various mimosa tannin extracts, T (i; unpurified, ethyl acetate purified and pentanol purified), and level of inclusion, L (j; 10, 20, 30, and 40 g/kg DM), of the extracts, μ = overall mean, $Block$ = blocking effect (incubation runs), T_i = effect of mimosa tannin extract, L_j = effect of inclusion level, $(TL)_{ij}$ = effect of interaction between tannin extract and inclusion level, and E_{ijk} = random error. Significantly different means for unpurified and purified tannin extracts were separated using Tukey's test. For each tannin extract, single degrees of freedom orthogonal polynomial contrasts (linear, quadratic, and cubic) were used to test the effect of the inclusion level of tannin extracts.

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2.3 Results

Characterization of Unpurified and Purified Mimosa Tannins

Figure 2.1 shows tannin characterization for unpurified and purified mimosa tannins. The results indicated that the purification of mimosa tannin with ethyl acetate and pentanol did not influence ($p > 0.05$) the concentration of total phenol (TP), non-tannin phenol (NTP), total tannin (TT), and hydrolysable tannin (HT). However, ethyl acetate purified and pentanol purified extracts had ($p < 0.05$) higher proportion (278.1 g/kg vs. 261.5 g/kg DM) of condensed tannin (CT) compared with purified tannin (221.7 g/kg DM). In general, the Soxhlet purification of the mimosa tannin with organic solvents did not affect any of the parameters measured except condensed tannins, which increased by 26% after purification with ethyl acetate and 18% with pentanol.

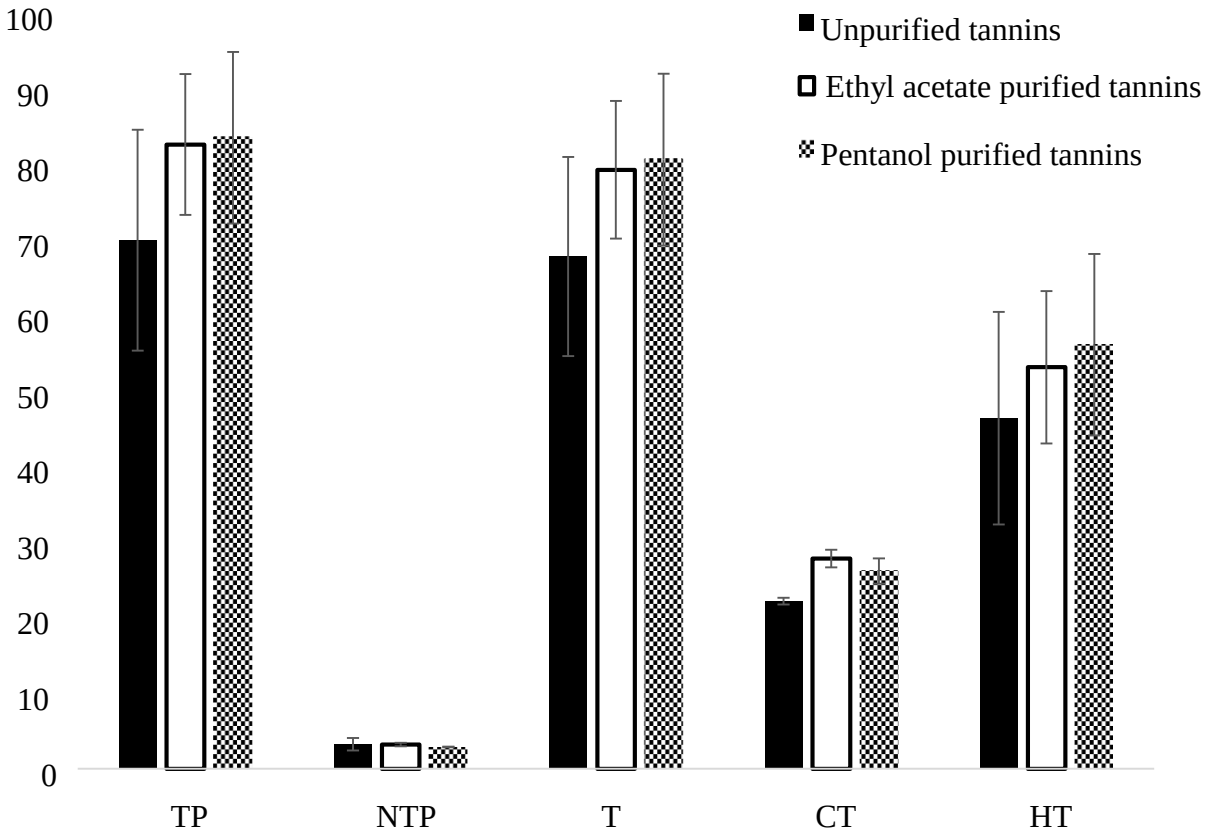

Figure 2.1. Characterization of unpurified and purified mimosa tannin with ethyl acetate and pentanol.

TP = total phenols, NTP = non-tannin phenols, TT = total tannin, CT = condensed tannin, and HT = hydrolysable tannin.

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Gas, Methane and In Vitro Organic Matter Digestibility

The substrate (*Eragrostis curvula* hay) used in the current study constituted the following: DM (912.1 g/kg), ash (50 g/kg), CP (78.5 g/kg), NDF (698.4 g/kg), ADF (396.1 g/kg), and ADL (59.3 g/kg). The chemical composition showed that the substrate had high fiber and low crude protein, which favors a higher production of hydrogen gas (H_2), utilized by methanogens to reduce CO_2 to CH_4. The current study aimed at examining the effects of unpurified, ethyl acetate purified, and pentanol purified mimosa tannins on suppressing enteric methane production in relation to dry matter digestibility of the substrate.

Table 2.1 present the summary of gas, methane, and *in vitro* organic matter digestibility from *Eragrostis curvula* hay after the inclusion of various levels of unpurified, ethyl acetate purified, and pentanol purified mimosa tannin extracts. The results indicated that gas, CH_4, and IVOMD reduced at a decreasing rate (linear $p < 0.01$) with increasing levels of both purified and non-purified mimosa tannins compared to substrate alone. Compared to control, unpurified and purified tannin extracts reduced gas ($p < 0.01$) at 20–40 g/kg dosages, whereas a significant decrease in CH_4 was observed only at the 40 g/kg inclusion level. However, the IVOMD of *Eragrostis curvula* hay was affected by all the dosages of both unpurified and purified mimosa tannin extracts. The simple effect of tannin type revealed significant effects of unpurified, ethyl acetate purified, and pentanol purified mimosa tannin extracts on CH_4 and IVOMD. However, gas volume was not influenced by tannin type ($p > 0.05$). Moreover, inclusion levels reduced gas, CH_4, and IVOMD ($p < 0.01$) for all the tannin extracts. However, there were no interaction effects ($p > 0.05$) between tannin extracts and inclusion level on gas and CH_4, whereas IVOMD showed a tendency for tannin extract and dosage interaction.

Generally, the inclusion of 40 g/kg DM of ethyl acetate purified and pentanol purified tannins reduced CH_4 by approximately 23% each, whereas unpurified extract reduced CH_4 by 21% at a similar dosage, with little effect on substrate digestibility.

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Table 2.1. Gas, methane (CH_4) and *in vitro* organic matter digestibility (IVOMD) from *Eragrostis curvula* hay incubated with various levels of unpurified, ethyl acetate purified, and pentanol purified mimosa tannin extracts.

Tannin Extracts		**Parameters**		
	Level (g/kg DM)	**Gas (mL/g DM)**	**CH_4 (mL/g DM)**	**IVOMD (g/kg)**
Unpurified Tannin	0	155.2 ^{a}	7.9 ^{a}	600.7 ^{a}
	10	149.8 ^{ab}	7.4 ^{ab}	592.5 ^{bc}
	20	145.0 ^{bc}	7.1 ^{ab}	592.9 ^{b}
	30	142.5 ^{c}	6.8 ^{ab}	589.0 ^{bc}
	40	138.2 ^{c}	6.2 ^{b}	582.5 ^{def}
	SEM	1.59	0.33	14.50
	Linear	<0.0.01	0.01	<0.01
	Quadratic	0.45	0.95	0.99
	Cubic	0.65	0.65	0.81
Ethyl acetate purified Tannin	0	155.2 ^{a}	7.9 ^{a}	600.7 ^{a}
	10	150.1 ^{ab}	7.3 ^{ab}	592.9 ^{b}
	20	144.5 ^{bc}	6.9 ^{ab}	584.7 ^{cde}
	30	139.7 ^{cd}	6.3 ^{ab}	582.4 ^{def}
	40	137.3 ^{d}	6.1 ^{b}	576.3 ^{f}
	SEM	1.63	0.41	13.73
	Linear	<0.01	0.01	<0.01

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	Quadratic	0.33	0.73	0.86
	Cubic	0.58	0.94	0.94
	0	155.2 [a]	7.9 [a]	600.7 [a]
	10	149.4 [ab]	7.1 [ab]	592.5 [bc]
	20	143.7 [bc]	6.6 [ab]	585.4 [cde]
	30	141.5 [c]	6.40 [ab]	582.0 [def]
Pentanol purified Tannin	40	139.6 [c]	6.1 [b]	578.9 [ef]
	SEM	1.43	0.39	13.84
	Linear	<0.0.01	0.01	<0.01
	Quadratic	0.05	0.42	0.79
	Cubic	0.96	0.77	0.98
SEM		0.414	0.387	0.696
p values				
T		0.25	<0.01	<0.01
L		<0.01	<0.01	<0.01
T * L		0.25	0.10	0.05

For each tannin extract means with different superscripts ([a], [b], [c], [d], [e], [f]) within a column differ significantly at $p < 0.05$.
SEM = standard error of mean,
T = tannin extract,
L= level of inclusion,
T * L = interaction effect between tannin extracts and inclusion level.

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The ratios of gas, methane, and *in vitro* organic matter digestibility from *Eragrostis curvula* hay supplemented with different levels of unpurified and purified mimosa tannin extracts is summarized in Table 2.2. The results showed that increases in dosages of purified and non-purified mimosa tannin extracts decreased CH_4 per unit gas, CH_4 per unit IVOMD, and gas per unit IVOMD linearly ($p > 0.01$) compared to substrate alone. When compared with the control, unpurified tannin extract mainly reduced ($p < 0.01$) CH_4/gas and CH_4/IVOMD at a 40 g/kg DM concentration, whereas ethyl acetate purified extract had a significant effect on CH_4/gas and CH_4/IVOMD at a 30–40 g/kg DM inclusion level, and CH_4/gas and CH_4/IVOMD were influenced ($p < 0.01$) by the addition of 20–40 g/kg DM of pentanol purified tannin. For gas/IVOMD, both unpurified and purified mimosa tannins had significant effects at 20–40 g/kg DM dosages compared with the control.

The main effect of tannin extract revealed a significant effect of unpurified and purified mimosa tannin extracts on CH_4/gas and CH_4/IVOMD. However, gas/IVOMD was not affected ($p > 0.05$) by the purification. Furthermore, a simple effect of tannin concentrations showed that levels of inclusion influenced CH_4/gas, CH_4/IVOMD, and gas/IVOMD significantly. There was no significant interaction effect between mimosa tannin type and level of inclusion for CH_4 per unit gas, CH_4 per unit IVOMD, and gas per unit IVOMD of the substrate.

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Table 2.2. The ratios of gas, CH_4, and IVOMD from *Eragrostis curvula* hay incubated with various levels of unpurified, ethyl acetate purified, and pentanol purified mimosa tannin extracts.

Tannin Extracts	Parameters			
	Level (g/kg DM)	**CH_4/Gas**	**CH_4/IVOMD (mL/kg)**	**Gas/IVOMD (mL/kg)**
Unpurified tannin	0	0.051 ^{a}	0.013 ^{a}	0.259 ^{a}
	10	0.050 ^{ab}	0.013 ^{a}	0.254 ^{ab}
	20	0.049 ^{abc}	0.012 ^{ab}	0.245 $^{b-e}$
	30	0.048 $^{a-d}$	0.012 ^{ab}	0.243 ^{de}
	40	0.045 ^{de}	0.011 ^{b}	0.238 ^{e}
	SEM	0.002	0.005	0.008
	Linear	<0.01	0.003	<0.01
	Quadratic	0.724	0.793	0.832
	Cubic	0.732	0.877	0.969
Ethyl acetate purified tannin	0	0.051 ^{a}	0.013 ^{a}	0.259 ^{a}
	10	0.049 ^{abc}	0.012 ^{ab}	0.254 ^{ab}
	20	0.048 $^{a-d}$	0.012 ^{ab}	0.248 ^{bcd}
	30	0.045 ^{de}	0.011 ^{b}	0.240 ^{de}
	40	0.044 ^{e}	0.011 ^{b}	0.239 ^{e}
	SEM	0.003	0.001	0.008
	Linear	<0.01	0.010	<0.01

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	Quadratic	0.959	0.837	0.864
	Cubic	0.951	0.808	0.784
	0	0.051 [a]	0.013 [a]	0.259 [a]
	10	0.048 [a-d]	0.012 [ab]	0.253 [abc]
	20	0.046 [cde]	0.011 [bc]	0.246 [b-e]
	30	0.045 [de]	0.011 [bc]	0.244 [cde]
Pentanol purified tannin	40	0.044 [e]	0.010 [c]	0.242 [e]
	SEM	0.003	0.001	0.008
	Linear	<0.01	0.007	<0.01
	Quadratic	0.669	0.688	0.700
	Cubic	0.822	0.721	0.955
SEM		0.0003	0.0001	0.0008
p-values				
T		<0.01	0.01	0.48
L		<0.01	<0.01	<0.01
T * L		0.59	0.40	0.46

For each tannin extract means with different superscripts ([a], [b], [c], [d], [e]) within a column differ significantly at $p < 0.05$.
SEM = standard error of mean,
T = tannin extract, L= level of inclusion,
T * L = interaction effect between tannin extracts and inclusion level.

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Table 2.3 presents the effect of various inclusion levels of unpurified, ethyl acetate purified, and pentanol purified mimosa tannin extracts on gas production indices. The results revealed that the asymptotic gas volume (v) and rate of gas production (k) reduced at a decreasing rate (linear, $p > 0.01$) with increasing levels of unpurified, ethyl acetate purified, and pentanol purified mimosa tannin extracts compared to control. However, compared with control, the lag term (l) was not affected by all the mimosa tannin inclusion levels. Compared with control, though the inclusion of unpurified tannin resulted in lower 'v' and 'k' at a 40 g/kg dosage, ethyl acetate purified and pentanol purified extract affected 'v' and 'k' at 30–40 g/kg levels. The simple effect of tannin extracts showed a significant effect of purification on 'v'. However, 'k' and 'l' were not affected by tannin types ($p > 0.05$). Likewise, inclusion levels reduced 'v' and 'k' ($p < 0.01$) with the exception of 'l'. Though, there was no interaction effect ($p > 0.05$) between tannin extracts and dosages on 'k' and 'l', mimosa extracts and inclusion levels interaction affected 'v' significantly.

Generally, potential gas production, as a result of complete substrate digestion and rate of gas production, decreased with the increase in the concentrations of mimosa tannin extracts. Moreover, a similar concentration of purified tannins resulted in lower 'v' and 'k' compared to the unpurified tannin extract.

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Table 2.3. The gas production kinetics from *Eragrostis curvula* hay incubated with various levels of unpurified, ethyl acetate purified, and pentanol purified mimosa tannin extracts.

Tannin Extracts	**Parameters**			
	Level (g/kg DM)	**v[a] (mL/g DM)**	**k[b] (mL/h)**	**l[c] (h)**
Unpurified tannin	0	195.7 [a]	0.034 [a]	0.017
	10	193.6 [a]	0.032 [ab]	0.079
	20	188.6 [ab]	0.031 [ab]	0.221
	30	187.9 [ab]	0.031 [ab]	0.249
	40	180.7 [b]	0.030 [b]	0.086
	SEM	3.566	0.002	0.171
	Linear	0.007	0.022	0.578
	Quadratic	0.659	0.495	0.394
	Cubic	0.749	1.000	0.626
Ethyl acetate purified tannin	0	195.7 [a]	0.034 [a]	0.017
	10	192.5 [ab]	0.032 [ab]	0.150
	20	186.7 [ab]	0.031 [ab]	0.238
	30	182.7 [b]	0.030 [b]	0.177
	40	180.4 [b]	0.030 [b]	0.144
	SEM	2.881	0.001	0.158
	Linear	0.001	0.02	0.585

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	Quadratic	0.748	0.640	0.429
	Cubic	0.636	0.911	0.885
	0	195.7 [a]	0.034 [a]	0.017
	10	192.1 [ab]	0.032 [ab]	0.255
	20	187.6 [ab]	0.031 [ab]	0.132
	30	187.4 [b]	0.030 [b]	0.087
Pentanol purified tannin	40	186.7 [b]	0.029 [b]	0.083
	SEM	2.700	0.001	0.191
	Linear	0.024	0.022	0.551
	Quadratic	0.220	0.813	0.318
	Cubic	0.769	0.779	0.701
SEM		0.703	0.0003	0.039
p-values				
T		0.02	0.43	0.32
L		<0.01	0.01	0.11
T * L		0.03	0.73	0.26

[a] v = asymptotic gas volume,
[b] k = rate constant,
[c] l = discrete lag term.
For each tannin extract means with different superscripts ([a], [b]) within a column differ significantly at $p < 0.05$. SEM = standard error of mean,
T = tannin extract,
L = level of inclusion,
T * L = interaction effect between tannin extracts and inclusion level.

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2.4 Discussion

Characterization of Unpurified and Purified Mimosa Tannins

The higher concentration of condensed tannins in ethyl acetate purified extract suggests that ethyl acetate being relatively polar solvent, with a lower boiling temperature has stronger affinity to the CT constituent of the mimosa tannin. The concentration of CT has been shown to decrease with an increase in the polarity of solvents (Missio *et al*., 2017). The proportion of CT recorded in this study for both ethyl acetate purified and pentanol purified mimosa extracts were higher than the 150 g of CT kg^{-1} DM reported by Minho *et al.* (2008), and Bhatta *et al.* (2013b), as well as the 235 g/kg DM obtained by Kardel *et al*. (2013), for unpurified mimosa tannin. However, Carulla *et al.* (2005); Grainger *et al.* (2009); Hassanat and Benchaar (2013) reported higher concentrations of CT (603, 615, and 820 g kg^{-1} DM, respectively) after purification of mimosa tannin with a more advanced Sephadex LH-20. Missio *et al.* (2017) suggested that Sephadex LH-20 involves complex analytical procedures and an expensive apparatus; thus, it is difficult to adopt in most laboratories.

Gas, Methane and In Vitro Organic Matter Digestibility

The effect of unpurified and purified tannin extracts recorded in this study could be connected to their interference with the proliferation and activities of methanogens. It has been well documented that condensed tannins reduced enteric methane either directly by hindering the activities of methanogenic bacteria, or indirectly by reducing organic matter digestibility (Animut *et al.*, 2008; Patra and Saxena, 2010; Jayanegara *et al*., 2012; Sirohi *et al*., 2012; Hristov *et al.*, 2013). This finding is in agreement with that of Hassanat and Benchaar (2013), who reported more reduction in gas and CH_4 with an increase in the concentration of *A. mearnsii* tannin extracts. Tan *et al.* (2011) also reported that increasing levels of *Leucaena leucocephala* CTs in the range of 20 to 60 g/kg DM decreased gas, CH_4, and IVOMD.

Ethyl acetate purified and pentanol purified tannin extracts reduced more CH_4 yield than unpurified tannin extract at a similar inclusion level. This could be attributed to the purification effects, which showed a significant increase in condensed tannin concentration for ethyl acetate purified extract. In corroboration with our finding, Tan *et al.* (2011) reported CH_4 reduction (−33%) at lower concentrations (20 g kg^{-1} DM) of *L. leucocephala* extracts purified using Sephadex LH-20. However, Hassanat and Benchaar (2013) reported 9% and 12 % reductions in gas and CH_4, respectively, for unpurified mimosa extract at 50 g/kg dosage, which are lower

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than the values obtained in this study for ethyl acetate and pentanol purified tannins at a 40 g/kg DM inclusion level.

Similarly, Adejoro *et al*. (2018) obtained about a 20% decrease in gas and 24% decrease in CH_4 after the inclusion of 42 g/kg DM of mimosa tannin extract, which is similar with the percentage reduction recorded in the present study for ethyl acetate purified tannin at a 40 g/kg DM concentration. Carulla *et al.* (2005) also reported around a 13% decrease in CH_4 at a 25 g/kg DM inclusion level of unpurified mimosa tannin, which is similar to the proportion obtained for pentanol purified tannin, but below that of ethyl acetate purified extract at 20 g/kg DM.

In general, the volume of CH_4 per unit gas, CH_4 per unit IVOMD, and gas per unit IVOMD decreased with the increase in the concentrations of unpurified and purifed tannin extracts. Moreover, similar inclusion levels of ethyl acetate purified and pentanol purified mimosa extracts had lower CH_4/gas, CH_4/IVOMD, and gas/IVOMD compared to unpurified tannin. Pentanol purified tannin was more effective at lower dosage in terms of CH_4/gas, CH_4/IVOMD. This suggested that the anti-methanogenic effect of purified tannin extracts was more prominent than their relative effect on organic matter digestibility. This could be linked to the relative increases in CT concentrations due to the purification effects. Condensed tannins have been shown to slow down the activities of microorganisms, as well as the rate of fiber and organic matter degradation, which, in turn, reduces the volume of hydrogen gas (H_2) required by methanogens to produce CH_4 (Piñeiro-Vázquez *et al*., 2015). In concurrence with our results, previous *in vitro* studies also reported a significant reduction in CH_4/gas, CH_4/IVOMD, and gas/IVOMD when some tannin rich browse plants (Gemeda and Hassen, 2015), and medicinal plant extracts were incubated together with *Eragrostis curvula* hay (Akanmu and Hassen, 2017; Akanmu *et al*., 2020).

Lower asymptotic gas production from *in vitro* fermentation is linked to a slow rate of organic matter degradation in the rumen (Schofield *et al*., 1994). A slow rate of gas production has been attributed to the higher concentration of hydrolysable tannins in mimosa extract, which affect the fermentation of lower quality feeds such as Eragrostis hay (Beauchemin *et al.*, 2007). However, the anti-methanogenic effect of tannins involves a mixture of direct toxicity on the methanogens, or an indirect reduction in fiber and organic matter degradation (Beauchemin *et al.*, 2007; Patra and Saxena, 2010). The asymptotic gas volume (mL/g) and rate of gas production (h^{-1}) obtained in the current study were lower than the 20.2 mL vs. 24.7 mL, and

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10.9% vs. 12% per 100 mg DM, reported by Schofield and Pell (1995), and (Mir *et al*., 1997), for alfalfa and fenugreek, respectively. In addition, longer lag time was recorded by Mir *et al.* (1997) compared to the values obtained in this study. These could be attributed to the variation in chemical composition, particularly the differences in fiber and protein content.

2.5 Conclusion

The findings of this study showed that the Soxhlet purification of mimosa tannin with ethyl acetate and pentanol increased the condensed tannin concentration. However, the highest increase in condensed tannin was achieved by using ethyl acetate. Gas, methane, and *in vitro* organic matter digestibility of *Eragrostis curvula* hay, and their ratios, decreased with the increase in the inclusion levels of unpurified, ethyl acetate purified, and pentanol purified mimosa tannin extracts. Pentanol purified tannin was more effective at lower dosage in terms of CH_4/gas, CH_4/IVOMD. Similarly, asymptotic gas volume and rate of gas production reduced with the increase in the concentration of tannin extracts. Ethyl acetate and pentanol purified mimosa extracts reduced gas and methane volume at a lower dosage (30 g/kg DM) compared to unpurified tannin, which has similar efficacy at a higher level (40 g/kg). It was concluded that the purification of mimosa tannin with ethyl acetate and pentanol reduced potential gas production and CH_4 with little impact on digestibility when up to 30 g/kg DM of feed was used. Lower inclusion levels of ethyl acetate purified and pentanol purified extracts could give the same effect with higher concentrations of unpurified tannin extract.

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CHAPTER THREE

Characterization, density and *in vitro* controlled release properties of mimosa (*Acacia mearnsii*) tannin encapsulated in palm and sunflower oils

Abstract

Tannin has gained wider acceptance as a dietary supplement in contemporary animal nutrition investigations because of its potential to reduce enteric methane emission. However, a major drawback to dietary tannin intake is the bitter taste and instability in the gastrointestinal tract (GIT). The utilization of fats as coating materials will ensure appropriate masking of the tannin's aversive taste and its delivery to the target site. The aims of this study were to encapsulate mimosa tannin with palm oil or sunflower oil, and to assess the microcapsules in terms of encapsulation efficiency, morphology, density, and *in vitro* release of tannin in media simulating the rumen (pH 5.6), abomasum (pH 2.9) and small intestine (pH 7.4). The microencapsulation of mimosa tannin in palm or sunflower oils was accomplished using a double emulsion technique. The results revealed that encapsulated mimosa tannins in palm oil (EMT^P) and sunflower oil (EMT^S) had high yields (59% vs. 58%) and encapsulation efficiencies (70% vs. 68%), respectively. Compared to unencapsulated mimosa tannin (UMT), the morphology showed that the encapsulated tannins were smaller in size and spherical in shape. The UMT had ($p < 0.01$) higher particle density (1.44 g/cm^3) compared to 1.22 g/cm^3 and 1.21 g/cm^3 for the EMT^S and EMT^P, respectively. The proportion of tannins released by the UMT after 24 h in the rumen (94%), abomasum (92%) and small intestine (96%) simulated buffers, reduced ($p < 0.01$) to 24%, 21% and 19% for the EMT^S and 18%, 20% and 16% for the EMT^P in similar media and time-frame. The release kinetics for the encapsulated tannins was slow and steady, thus, best fitted by the Higuchi model while the UMT dissolved quickly, hence, only fitted to a First order model. Sequential tannin release also indicated that the EMT^S and EMT^P were stable across the GIT. It was concluded that the microencapsulation of mimosa tannin in palm or sunflower oils stabilized tannins release in the GIT simulated buffers with the potential to modify rumen fermentation. Further studies should be conducted on the palm and sunflower oils microcapsules' lipid stability, fatty acid transfer rate in the GIT and antioxidant properties of the encapsulated tannins.

Keywords: particle density; gastrointestinal tract; microencapsulation; mimosa tannin; morphology; palm oil; sunflower oil; release kinetics

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3.1 Introduction

The utilization of tannins as feed supplements in recent ruminant nutrition studies is linked to their positive roles in modulating rumen fermentation, methane (CH_4) emission and protein metabolism (Patra *et al*., 2006). Enteric CH_4 is one of the by-products of anaerobic fermentation of structural carbohydrates in the rumen which has a potential global warming impact twenty-five times greater than that of carbon dioxide (Piñeiro-Vázquez *et al*., 2015). Several studies have showed that condensed tannins are capable of mitigating CH_4 emissions either directly by interfering with the proliferation and activities of methanogens or indirectly through the reduction in fiber degradation and hindrance of protozoa activities (Animut *et al.*, 2008; Goel *et al*., 2008; Hristov *et al.*, 2013). In addition, condensed tannins have the ability to bind dietary proteins at the normal rumen pH, thereby increasing amino acid absorption in the small intestine (Hassanpour, 2011; Hassanpour and Mehmandar, 2012).

Among the condensed tannins of the rich leguminous trees mostly consumed by ruminants in the tropics (Vasta *et al.*, 2008), *Acacia mearnsii*, popularly known as mimosa tannin (Kardel *et al*., 2013), is considered as the most widely spread and highly invasive alien species in South Africa, covering an area of over 2.5 million hectares (Galatowitsch and Richardson, 2005), and more than 130,000 ha of commercial plantations (de Wit *et al*., 2001). Numerous studies have revealed that Mimosa tannin has the potential to reduce enteric CH_4 and ammonia nitrogen (NH_4-N) production while enhancing dietary protein by-pass. For instance, when an extract of mimosa tannin was fed to cows, CH_4 and urine nitrogen were reduced by up to 29% and 9.3%, respectively (Grainger *et al.*, 2009). Likewise, in sheep, urine nitrogen dropped by 59% and CH_4 by 13% (Carulla *et al.*, 2005). Additionally, *A. mearnsii* tannins increased propionate levels beyond 100 g/kg of dry matter (DM) against the acetate (Hassanat and Benchaar, 2013).

However, the oral administration of tannins suffers some drawbacks in terms of dietary intake and digestibility, due to the bitter taste largely attributed to the tannins' negative reaction with salivary proteins (Frutos *et al*., 2004b), as well as their instability and binding nature in the gastrointestinal tract (GIT) (Dschaak *et al.*, 2011). Furthermore, intake and digestibility of the tannins have been found to reduce when condensed tannins are fed above 50 g/kg of DM (Min *et al.*, 2003; Beauchemin *et al*., 2008). Some studies such as that of Priolo *et al.* (2000) reported a 48 g reduction in average daily gain in sheep while Bhatta *et al.* (2013b) observed a decrease of 30% in nitrogen retention and an increase in energy loss up to 45% in goats supplemented with high amounts of condensed tannins. In addition, Grainger *et al.* (2009) recorded a 29.7%

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reduction in milk yield when a large quantity of mimosa tannin extract was fed to cows. Therefore, there is a need for the development of a technique that will ensure the appropriate masking of tannins' aversive taste and their sustained release into the GIT without compromising normal rumen function.

Various encapsulation technologies have been developed by feed and pharmaceutical industries to conceal the aversive tastes of many bioactive compounds, to improve their stability and control their release to target site of digestion without any adverse effects (Fang and Bhandari, 2010; Munin and Edwards-lévy, 2011). Bakry *et al.* (2016), defined encapsulation as a method of building an efficient barrier between the active ingredients and coating material to prevent any form of chemical and physical reactions, and to sustain the biological, functional, and physicochemical characteristics of the active ingredient. Numerous microencapsulation methods were adopted such as spray-drying, spray-cooling, spray-chilling, freeze-drying, centrifugal suspension separation, inclusion complexation and coacervation using various wall materials (Gharsallaoui *et al*., 2007). The best coating material for use in masking the bitter taste of tannins should be inexpensive, tasteless, have low viscosity, be a good film former, have good emulsifying properties and be able to safeguard the bioactive compound up to the target site (Jafari *et al*., 2008; Abedi *et al.*, 2016).

However, the feed industry is faced with the challenges of selecting appropriate wall materials that are available, inexpensive, and safe (Krishnan *et al*., 2005), which affect the practicability of encapsulation technology in animal nutrition. In southern Africa, sunflower oil and palm oil are found in abundance and could serve as suitable wall materials for tannin encapsulation due to their desirable aroma (Bakry *et al.*, 2016), good emulsifying properties and low viscosity (Flanagan *et al*., 2006). A review by Eckard *et al*. (2010) reported a significant reduction in CH_4 when sunflower oil was supplemented in the diets of ruminants. In addition, *in vitro* CH_4 volume dropped when palm oil was adopted as a coating material to encapsulate *A. mearnsii* extract using a solid-in-oil-water (S/O/W) technique (Adejoro *et al*., 2018). However, literature on using lipids such as sunflower and palm oils as coating materials to mask the tannins' bitter taste and ensure their controlled release to the target sites of function in the GIT is still scarce. The objectives of the present study, therefore, are to encapsulate mimosa tannin with sunflower and palm oils using the double emulsion method, and to evaluate the sunflower and palm oils' microparticles in terms of their encapsulation efficiency, morphology, density, and in vitro release rate in various media simulating the GIT.

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3.2 Materials and Methods

Study Area

The experiment was conducted in the Department of Animal Science, University of Pretoria, South Africa. The location lies at a latitude 25°44′30″ south and longitude 28°15′30″ east at an altitude of 1360 m above sea level (van Niekerk *et al.*, 2009). This research was approved by the Animal Ethics Committee of the University of Pretoria (Ref No: EC075-17).

Materials

The mimosa (*Acacia mearnsii*) tannin extract used in this study was gifted by the UCL Company Pty (Ltd), Dalton, South Africa. The extract was reported to have been obtained from the bark of the *A. mearnsii* tree following hot water extraction processes at specific temperature, pressure, and time. The water was removed using a vacuum evaporator and the extract was air-dried into fine powder, packaged, and refrigerated before use (Missio *et al*., 2017). The palm and sunflower oils were purchased from Pick 'n' Pay grocery, Hatfield, Pretoria. The emulsifiers (Span80, Tween80) and dichloromethane (DCM) were procured from Sigma Aldrich, St. Louis, MO, USA. Filter bags (F57 fiber) were purchased from ANKOM Technology, New York, NY, USA. All other reagents utilized were of analytical grade and sourced from Sigma-Aldrich, Johannesburg, South Africa.

Characterization of Mimosa Tannin

Mimosa tannin powder (0.2 g) was added in 25 mL volume glass beakers containing aqueous acetone (10 mL) and dissolved for 20 min in an ultrasonic bath. The solution was transferred into tubes and centrifuged in a refrigerated centrifuge at 2500 rpm for 15 min. and the supernatant collected and kept in ice blocks. The concentrations of total phenol, non-tannin phenols and total tannins in the extracts were determined using the Folin– Ciocalteu method (Makkar, 2000), as a tannic acid equivalent, while condensed tannin content was analyzed as a leucocyanidin equivalent using the Butanol–HCl method (Porter *et al*., 1986). The proportion of hydrolysable tannin was estimated by the differences between the total tannins and condensed tannins (Singh *et al.*, 2005).

Microencapsulation of Mimosa Tannin

Mimosa tannin was encapsulated with either palm oil or sunflower oil using the S/O/W technique described by Adejoro *et al*. (2018) with little modification. The water (W) solution was first prepared in a 500 mL beaker by adding 300 mL distilled water comprising 1% (*w*/*v*)

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Tween80 emulsifier. The mixture was then homogenized using an iron rod homogenizer (PRO400DS, Pro Scientific Inc., Oxford, CT 06478, USA) set at 20,000 revolutions per minute (rpm) for 3 min until the solution foamed. The solid-in-oil (S/O) solution was simultaneously prepared by adding 8.5 g of mimosa tannin powder into a 100 mL beaker containing 30 mL palm or sunflower oil solution in DCM (50 mg/mL) mixed with 0.5% (*w*/*v*) Span80 as a surfactant then stirred thoroughly for 2 min using a magnetic stirrer set at 400 rpm. The S/O solution was subsequently added to the W solution and homogenized for 3 min at 20,000 rpm to form the final S/O/W mixture. The mixture was stirred for three hours using a magnetic stirring plate set at 800 rpm to completely evaporate the DCM. The palm and sunflower oil microcapsules produced were squeezed through a four-fold layer of cleaned cheese cloth, rinsed with about 100 mL distilled water, transferred into the aluminum container, and freeze-dried for 5 days. The encapsulated mimosa tannins in palm oil (EMT^P) or sunflower oil (EMT^S) were collected, ground to powder and refrigerated before analysis.

Optimization of Mimosa Tannin Microcapsules

Encapsulation efficiency and tannin yield

The encapsulation efficiency (Ee) and tannin yield of sunflower and palm oil microcapsules were determined according to the procedure of Adejoro *et al*. (2018), with slight changes. The powdered samples (0.1 g) of EMT^P and EMT^S were separately weighed into a beaker containing DCM (20 mL), and heated in an ultrasonic bath for 10 min to disband the lipid coatings. The solutions were centrifuged at 2500 rpm for 10 min and the tannin pellets were collected and dissolved in 70% aqueous acetone (20 mL) to reconstitute the extracts. The concentration of the actual loaded tannin (LTa) was determined using a spectrophotometer absorbance at 725 nm. The Ee of the EMT^P and EMT^S were estimated from the Equation (3.1) below:

$$\text{Ee}\ (\%) = \frac{\text{LT}_\text{a}}{\text{LT}_\text{t}} \times 100 \qquad (3.1)$$

where Ee = encapsulation efficiency, LT_a = actual loaded tannin (%, *w*/*w*) in the oil microcapsules and LT_t = theoretical loaded tannin (i.e., the amount of mimosa tannin added during encapsulation).

The mimosa tannin encapsulation yield (%) was estimated from the total tannin content obtained in the EMT^P and EMT^S microcapsules and the total amount of tannin initially added as shown in Equation (3.2):

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$$\text{Yield (\%)} = \frac{\text{amount of tannin obtained (g)}}{\text{total tannin added (g)}} \times 100 \qquad (3.2)$$

Scanning electron microscopy

The morphology of the EMT^P and EMT^S compared to the unencapsulated mimosa tannin (UMT) were determined using a scanning electron microscope (SEM) following the procedure of Taylor *et al.* (2009) with slight modifications. The powdered samples (UMT, EMT^P and EMT^S) were prepared separately and smeared with carbon, then mounted to the stubs of the SEM (JEOL, JSM-840 Tokyo, Japan) using adhesive tape and viewed at 20 kV.

Microparticle density

The three mimosa tannin samples (UMT, EMT^P and EMT^S) were evaluated for particle density using a Gas Pycnometer (AccuPyc II 1340 Micromeritics Instr. Corp. Norcross, GA, USA). The powdered samples (52 g) were separately transferred and sealed into the vessel of the Pycnometer. The vessel used an automated pressure determining technique to detect the pressure change resulting from the displacement of Helium gas by the tannin samples. Helium was used as the choice gas medium because it is small enough to penetrate virtually all connected pores within a sample. The volume determined was finally divided into sample weights and the microparticle densities of the UMT, EMT^P and EMT^S were recorded.

In Vitro Release of Tannin from Oil Microcapsules

The *in vitro* release properties of the UMT, EMT^P and EMT^S were evaluated following the procedure of Adejoro *et al*. (2018) with little change. Three different buffer solutions were prepared: an acetate buffer (pH 5.6), citrate buffer (pH 2.9) and phosphate buffer (pH 7.4) simulating the rumen, abomasum, and small intestine pH, respectively (Papas *et al*., 1984; Rossi *et al.*, 2003). Samples (100 mg) from each of the three mimosa tannin treatments (UMT, EMT^P and EMT^S) were separately weighed into 25 µm porosity filter bags (F57; ANKOM) and suspended in three separate bottles containing 50 mL solutions of acetate, citrate and phosphate buffers. The bottles were placed in an incubator shaker and rotated at 50 rpm at 39 °C. The solutions were sampled (2 mL) in triplicates at intervals as follows: 1, 2, 4, 8, and 24 h of incubation. The original volume of the media was maintained by the replacement of a 2 mL fresh buffer immediately after each sampling. The buffer samples collected were frozen immediately for latter analysis. The proportion of mimosa tannins released by the UMT, EMT^P and EMT^S into the three different pH media simulating the GIT at 1, 2, 4, 8, and 24 h were assessed by spectrophotometer absorbance at 725 nm.

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The mimosa tannin release kinetics were assessed using the Zero order, First order and Higuchi model (Equations 3.3 –3.5, respectively) to determine the best model for tannins released by UMT, EMT^P and EMT^S microcapsules in the rumen, abomasum and small intestinal simulated buffer as described by Adejoro *et al*. (2018) and Tolve *et al.* (2021):

$$Q_t = Q_0 + Q_0 t \quad (3.3)$$

$$\log Q_t = \log Q_0 - Q_1 t \quad (3.4)$$

$$Q_t = Q_0 - Q_H t^{1/2} \quad (3.5)$$

where k_0 = Zero-order rate constant; t = time; Q_t = released concentration of mimosa tannin at time t; Q_0 = initial concentration of tannins within solutions (usually $Q_0 = 0$); k_1 = First order rate constant and k_H = Higuchi dissolution constant.

According to Tolve *et al*. (2021), the Zero order kinetic model describes the phenomenon of slow-release, in a shell that does not disintegrate easily and generally applicable to poorly soluble compounds. In First order kinetics, the dissolution of the bioactive compound, which is usually soluble in water and entrapped in a porous shell material, is proportional to its concentration. The Higuchi model refers to release kinetics involving both diffusion and dissolution.

Another experiment was carried out to examine the sequential tannin release at 24 h in a rumen simulated medium (pH 5.6), and at 8 h each in abomasum (pH 2.9) and small intestine (pH 7.4) simulated media. The three mimosa tannin treatments (UMT, EMT^P and EMT^S) were weighed (100 mg) in filter bags and suspended in bottles containing acetate buffer (50 mL). The bottles were placed in shaker incubator and rotated at 50 rpm at 39 °C. The solutions were sampled after 24 h and the filter bags containing the unencapsulated and encapsulated tannin residues were rinsed with water and subsequently transferred to bottles containing citrate buffer inside incubator shaker set at 50 rpm and 39 °C. The solutions were sampled after 8 h incubation and the bags containing residue samples were rinsed with water and then suspended in the bottles containing phosphate buffer. The bottles were similarly incubated at 50 rpm and 39 °C and then sampled after 8 h. Following each incubation period, extracts (2 mL) were sampled in triplicate and stored inside vials at −20 °C before analysis. Three independent incubation cycles was carried out across the three buffer media. The proportion of mimosa tannins released by the UMT, EMT^P and EMT^S sequentially after 24 h of incubation in the respective buffer simulating

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the rumen, abomasum and small intestine were determined from a spectrophotometer absorbance at 725 nm.

Statistical Analysis

All data were coded in a Microsoft Excel (Microsoft Corp. Redmond, WA, USA) spread sheet and analyzed for variance using SAS version 9.4 (SAS Institute Inc., Carry, NC, USA). Data on encapsulation efficiency and tannin yield, tannin microparticle density and tannin release rate for the UMT, EMT^P and EMT^S at various pH were subjected to oneway ANOVA. Significantly different means were separated using the least significant difference (LSD) and differences reported at 5% or a 1% level of probability where applicable.

3.3 Results

Characterization of Mimosa Tannins

Prior to the encapsulation process, mimosa tannin was characterized to ascertain its total phenol, non-tannin phenol, total tannin, condensed tannin and hydrolysable tannin concentrations. The extract comprised of tannin phenol (699.3 g kg^{-1} DM), non-tannin phenol (32.3 g kg^{-1} DM), total tannin (677.6 g kg^{-1} DM), hydrolysable tannin (463.8 g kg^{-1} DM) and condensed tannin (221.7 g kg^{-1} DM).

Optimization of Mimosa Tannins' Microcapsules

The present study did not observe any significant differences between the mimosa tannins encapsulated with palm oil and sunflower oil in terms of tannin yield and encapsulation efficiency. However, a good proportion of tannin yields (59% vs. 58%) and encapsulation efficiencies (70% vs. 68%) were obtained from the EMT^P and EMT^S microcapsules, respectively.

Scanning electron microscopy (Figure 3.1a–c) indicated that EMT^P and EMT^S microparticles were smaller and more uniform in size and spherical in shape with a visible whitish oil color enveloping the tannin particles, while UMT morphology revealed tannin particles with bigger and more heterogeneous sizes, irregular shapes and a consistent dark brown colour.

With respect to tannin particle density, the results showed that the UMT was heavier (1.44 g/cm^3) than the EMT^S (1.22 g/cm^3) and the EMT^P (1.21 g/cm^3) ($p < 0.01$). However, there was no statistical difference between EMT^P and EMT^S microcapsules with regards to density.

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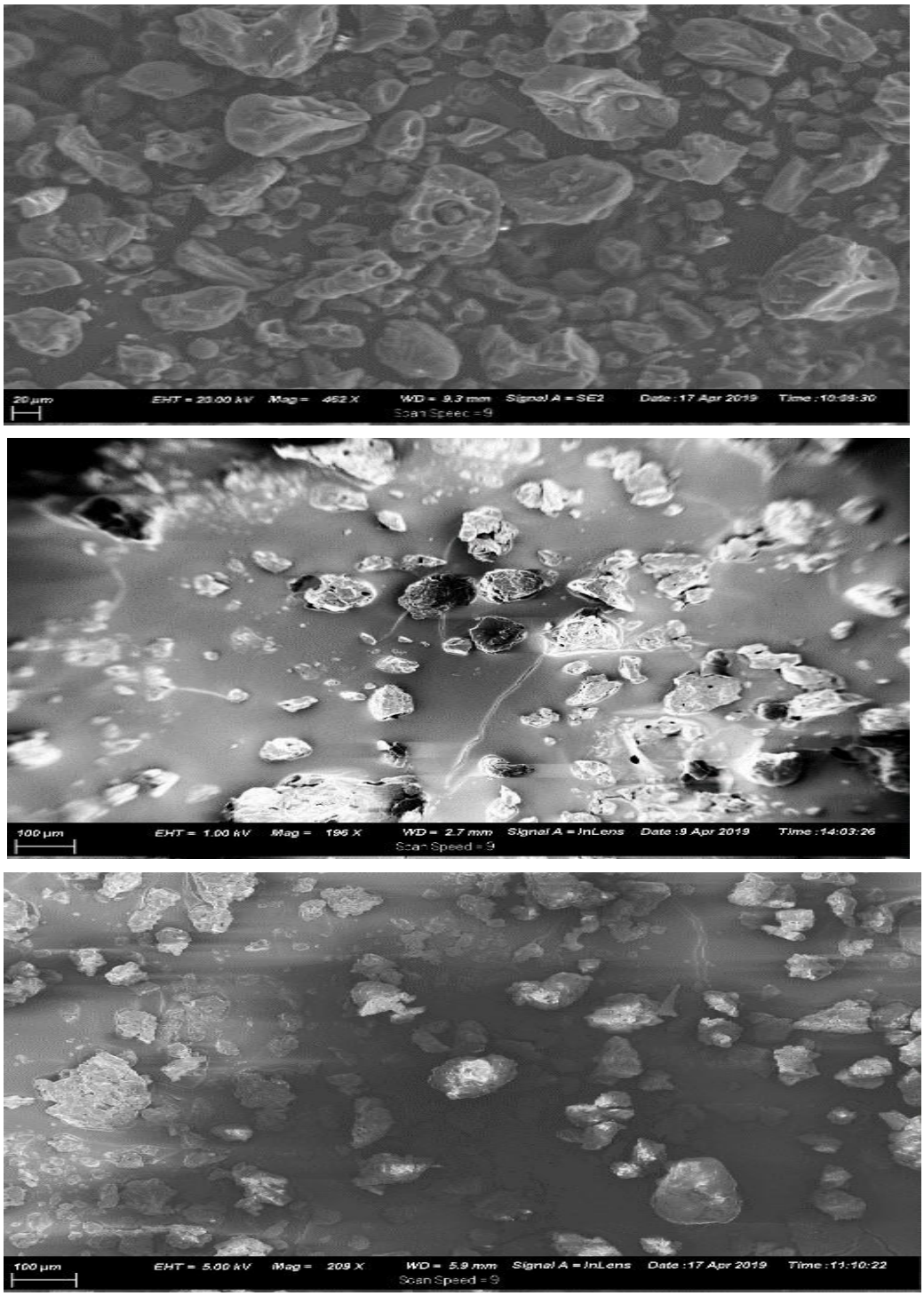

(a)

(b)

(c)

Figure 3.1 Morphology of *A. mearnsii* tannin particles viewed using SEM (a) unencapsulated mimosa tannin, UMT, (b) encapsulated mimosa tannin in palm oil, EMT^P and (c) encapsulated mimosa tannin in sunflower oil, EMT^S.

Mimosa Tannin Release Rate Properties

Table 3.1 presents the summary of *in vitro* tannin release for the UMT, EMT^P and EMT^S at predetermine hours in an acetate buffer (pH 5.6), citrate buffer (pH 2.9) and phosphate buffer

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(pH 7.4), simulating the rumen, abomasum, and small intestine, respectively. In the acetate buffer (pH 5.6), 68, 77, 81, 90 and 94% of UMT was released at 1, 2, 4, 8 and 24 h, respectively, and this was significantly higher ($p < 0.01$) compared to the 6.5, 10.3, 15, 19 and 24% of tannins released from the EMT^S and 5.2, 9.1, 12.3, 16 and 18.3% released from the EMT^P at 1, 2, 4, 8 and 24 h, respectively. There were no statistical effects on the amount of tannin released in a similar medium between the EMT^S and EMT^P at 1 and 2 h of incubation, however, the EMT^S had a higher tannin release rate from 4–24 h periods ($p < 0.01$).

In the citrate buffer (pH 2.9), a significantly higher proportion of mimosa tannins (69, 73, 79.3, 86.4 and 92.2%) was released compared to the 6.4, 9.7, 13.4, 17 and 20.4% of tannins released from the EMT^P, and 6, 10, 14, 20 and 21.4% from EMT^S microcapsules at 1, 2, 4, 8 and 24 periods, respectively. Nevertheless, no differences in tannin release pattern were observed between the EMT^S and EMT^P at across the time periods ($p > 0.05$). In the phosphate buffer (pH 7.4), the UMT released higher proportion of tannins with 66, 71.4, 80.4, 87.4 and 96% released compared to 4.5, 7, 11, 14 and 19% released from the EMT^S microparticles and 4, 6, 9.3, 12 and 16% released from the EMT^P, at 1, 2, 4, 8 and 24 h, respectively ($p < 0.01$). Tannin release rate was significantly different between the EMT^S and EMT^P only at 2 h of incubation across the acetate, citrate and phosphate buffer media.

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Table 3.1 *In vitro* release rate of unencapsulated mimosa tannin or tannins encapsulated with palm and sunflower oils in GIT simulated media.

Tannin Types	Rumen Simulated Buffer (pH 5.6)					Abomasum Simulated Buffer (pH 2.9)					Small Intestine Simulated Buffer (pH 7.4)				
	1 H [e]	**2 H**	**4 H**	**8 H**	**24 H**	**1 H**	**2 H**	**4 H**	**8 H**	**24 H**	**1 H**	**2 H**	**4 H**	**8 H**	**24 H**
UMT [a] (%)	68.0 [a]	76.9 [a]	81.0 [a]	89.6 [a]	94.1 [a]	68.5 [a]	72.8 [a]	79.3 [a]	86.4 [a]	92.2 [a]	65.8 [a]	71.4 [a]	80.4 [a]	87.4 [a]	95.7 [a]
EMT^{P} [b] (%)	5.19 [b]	9.09 [b]	12.3 [c]	15.6 [c]	18.3 [c]	6.42 [b]	9.70 [b]	13.4 [b]	17.0 [b]	20.4 [b]	3.57 [b]	5.71 [c]	9.34 [b]	11.5 [b]	15.6 [b]
EMT^{S} [c] (%)	6.46 [b]	10.3 [b]	14.8 [b]	19.0 [b]	23.7 [b]	6.14 [b]	10.0 [b]	14.3 [b]	20.0 [b]	21.4 [b]	4.47 [b]	6.96 [b]	10.5 [b]	13.6 [b]	18.6 [b]
[d] SEM	0.19	0.39	0.47	0.75	0.93	0.71	0.74	0.92	0.96	0.71	0.49	0.25	0.54	0.54	0.91
p–value	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01

Means with different superscripts across the column ($P < 0.01$).

[a] UMT = unencapsulated mimosa tannin;

[b] EMT^{P} = encapsulated mimosa tannin in palm oil;

[c] EMT^{S} = encapsulated mimosa tannin in sunflower oil;

[d] SEM = standard error of mean;

[e] H = hour.

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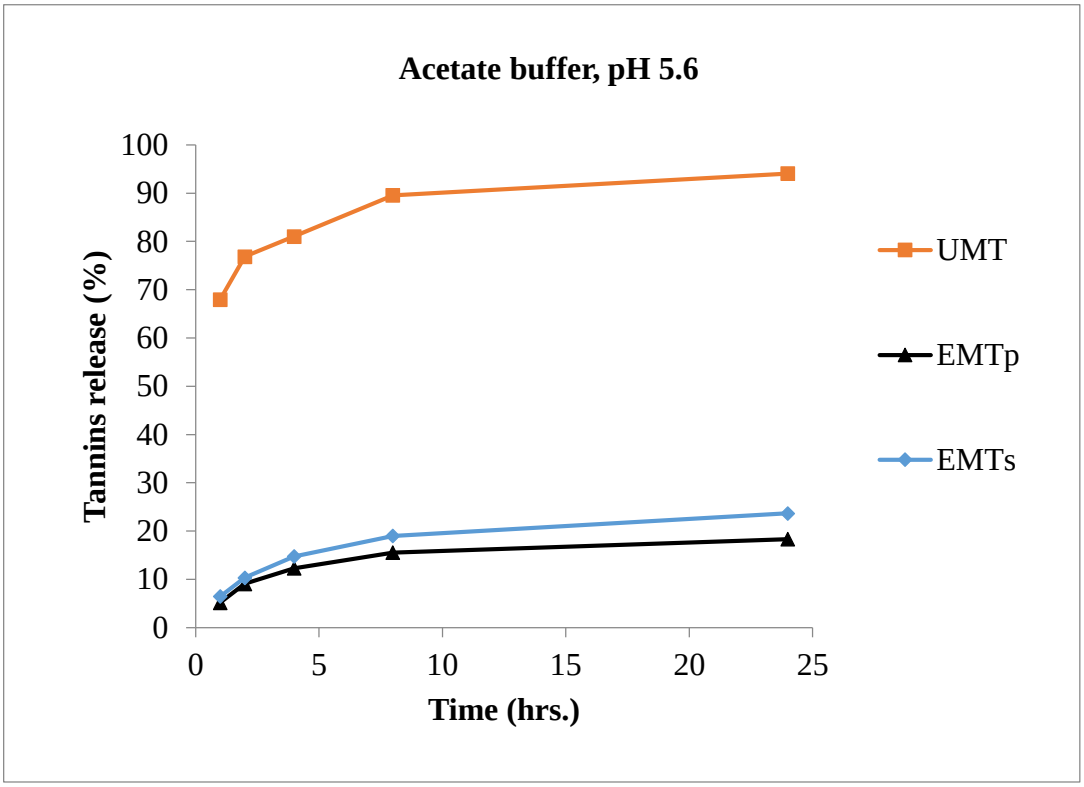
The release profiles of UMT, EMT^{P} and EMT^{S} in the rumen, abomasum and small intestine simulated buffers are displayed in Figure 3.2a–c. The chart shows that UMT burst rapidly in the rumen simulated buffer (pH 5.6) with 77% of the extracts released within 2 h and about 94% dissolved after a 24 h incubation period (Figure 3.2a). However, the EMT^{P} and EMT^{S} microcapsules eroded slowly releasing only 9% and 10% of the extracts, respectively, in the first 2 h in the acetate buffer. After 24 h, 18% of the tannins were dissolved from the EMT^{P} and 24% from the EMT^{S}. Similarly, in the abomasum simulated buffer (pH 2.9), about 73% of the UMT extracts dissolved within 2 h and 92% of tannins were released after a 24 h incubation (Figure 3.2b). In contrast, only 10% of the extracts were released by the lipid microcapsules in the first 2 h, while 20% and 21% of the extracts were released, respectively, after a 24 h period. In the small intestine simulated buffer, 71% of the UMT dissolved in the first 2 h of incubation (Figure 3.2c), and after 24 h, 96% of the extracts were released in the buffer. However, only 6% and 7% were released by the EMT^{P} and EMT^{S}, respectively, at 2 h. After 24 h, only 16% of the extracts were dissolved from the EMT^{P} and 19% from the EMT^{S} in the phosphate buffer. In general, the unencapsulated mimosa dissolved faster, releasing most of the extract before 24 h periods in all the buffers compared to the tannin encapsulated in sunflower oil or palm oil which dissolved slowly releasing the tannin in a sustained manner.



(a)

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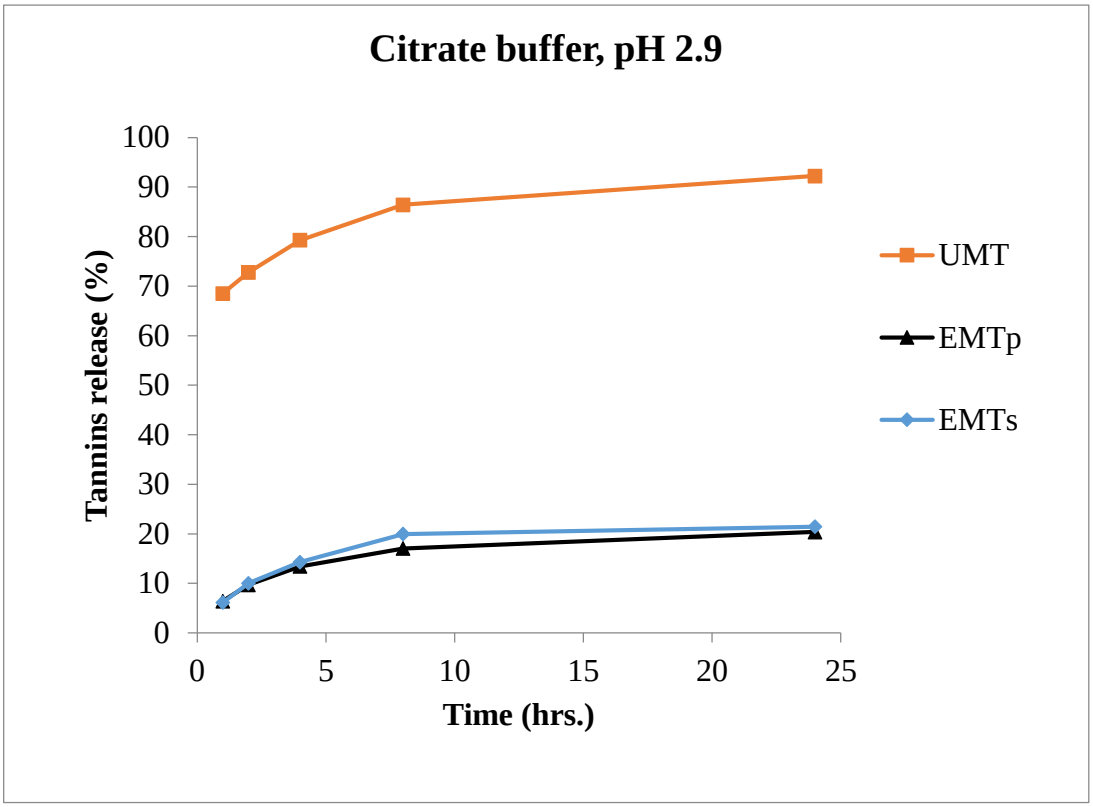
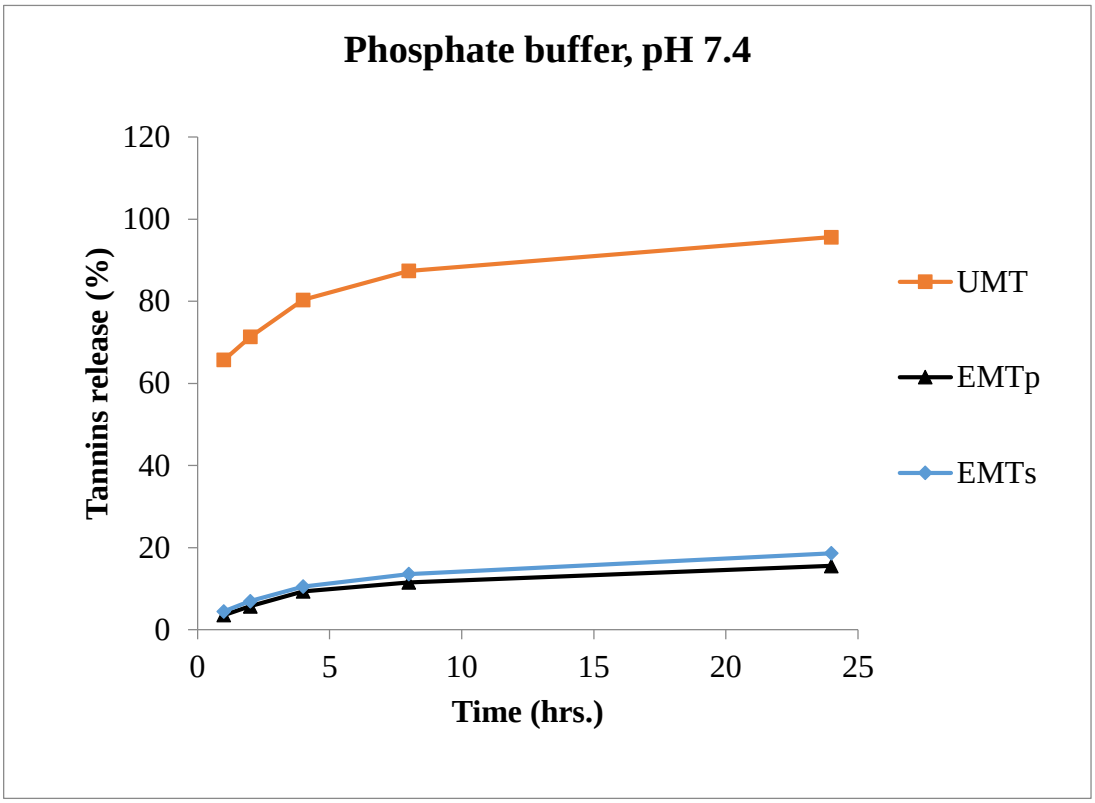


(**b**)



(**c**)

Figure 3.2 Release chart for UMT, EMT^P and EMT^S in (a) acetate buffer (pH 5.6), (b) citrate buffer (pH 2.9) and (c) phosphate buffer (pH 7.4).

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The tannin release rate kinetics for the UMT, EMT^P and EMT^S in the buffers simulating the rumen, abomasum and small intestine are shown in Table 3.2. The results show that the release pattern for the UMT was best fitted to a negative regression model according to the First order kinetics (R^2 = 0.87, 0.91 and 0.96), indicated by its immediate burst into the various elution media and releasing most of the tannin extract within the first 2 h of in vitro incubation. However, the EMT^P and EMT^S microcapsules did not disintegrate quickly and released smaller proportion of tannins over a 24 h period in a controlled manner while retaining most of the tannin and thus, the release of tannin fitted with high accuracy to the square root regression equation of the Higuchi order for EMT^P (R^2 = 0.86, 0.89 and 0.93) and EMT^S (R^2 = 0.91, 0.81 and 0.96).

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Table 3.2. Release rate kinetics of unencapsulated mimosa tannin or encapsulated mimosa in palm and sunflower oils in GIT simulated media.

Tannin	Model	Rumen Simulated Buffer (pH 5.6)		Abomasum Simulated Buffer (pH 2.9)		Small Intestine Simulated Buffer (pH 7.4)	
UMT	Zero	y = 0.9125x + 74.781	R^2 (0.696)	y = 0.9033x + 72.801	R^2 (0.7752)	y = 1.1231x + 71.346	R^2 (0.7791)
	First	y = −0.0288x + 1.412	R^2 (0.868)	y = −0.0249x + 1.4483	R^2 (0.9054)	y = −0.0371x + 1.4941	R^2 (0.9595)
	Higuchi	y = 6.1422x + 66.982	R^2 (0.842)	y = 5.9738x + 65.34	R^2 (0.9042)	y = 7.4188x + 62.091	R^2 (0.9067)
EMT^P	Zero	y = 0.464x + 8.4782	R^2 (0.712)	y = 0.5119x + 9.3987	R^2 (0.7517)	y = 0.4521x + 5.6103	R^2 (0.8142)
	First	y = −0.0023x + 1.9615	R^2 (0.730)	y = −0.0033x + 1.9687	R^2 (0.6809)	y = −0.0026x + 1.9818	R^2 (0.7719)
	Higuchi	y = 3.1161x + 4.5304	R^2 (0.857)	y = 3.4061x + 5.1203	R^2 (0.8877)	y = 2.9567x + 1.9571	R^2 (0.9286)
EMT^S	Zero	y = 0.6368x + 9.8738	R^2 (0.779)	y = 0.5522x + 10.07	R^2 (0.648)	y = 0.5424x + 6.5923	R^2 (0.8496)
	First	y = −0.0033x + 1.955	R^2 (0.802)	y = −0.0036x + 1.9662	R^2 (0.6318)	y = −0.0032x + 1.9785	R^2 (0.7983)
	Higuchi	y = 4.2071x + 4.6243	R^2 (0.907)	y = 3.7714x + 5.2192	R^2 (0.8063)	y = 3.5169x + 2.283	R^2 (0.9526)

UMT = unencapsulated mimosa tannin;

EMT^P = encapsulated mimosa tannin in palm oil;

EMT^S = encapsulated mimosa tannin in sunflower oil;

R^2 = Coefficient of determination

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Figure 3.3 presents the sequential release of tannin extract from the UMT, EMT^P and EMT^S microcapsules. In the acetate buffer, 90.4% of UMT extracts dissolved after 24 h, while in the citrate buffer 5.1% was released within 8 h but nothing was recorded in the phosphate buffer after an 8 h sequential incubation. In contrast, the EMT^S and EMT^P matrixes released 30% and 22% tannins, respectively, in the acetate buffer over the period of 24 h, and 10% each in the citrate buffer within 8 h, while in the phosphate buffer, the EMT^P released 6% extract and EMT^S 7% after an 8 h incubation. Generally, most of the extracts from the UMT were dissolved in the rumen simulated buffer and a small proportion (5%) released in the abomasum simulated buffer, while no tannin was detected in the buffer simulating the small intestine. However, for the EMT^S and EMT^P, less than one third of the extracts were released in the buffer simulating the rumen after 24 h and an appreciable amount were dissolved in the abomasum and small intestine simulated media.

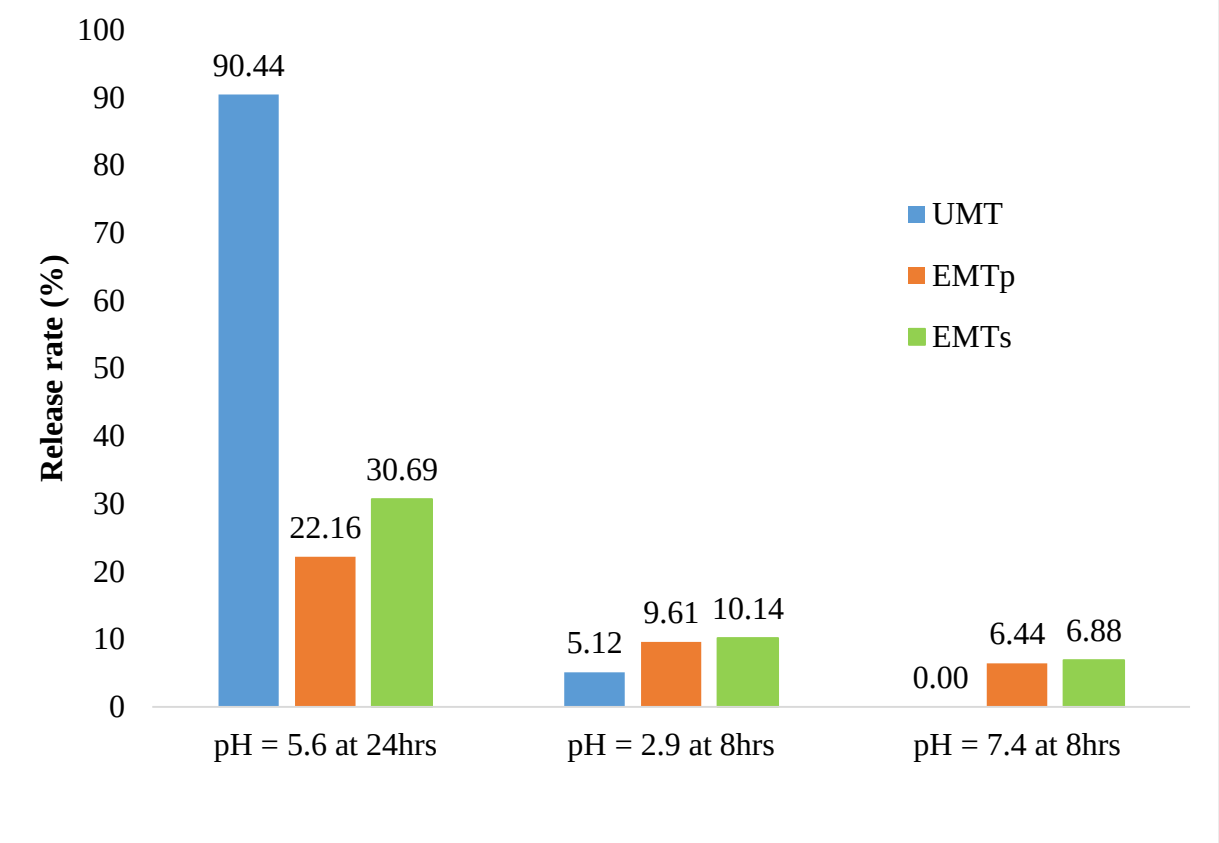

Figure 3.3 Sequential release rate for unencapsulated mimosa tannin (UMT), encapsulated mimosa tannin in sunflower oil (EMT^S) & encapsulated mimosa tannin in palm oil (EMT^P) in acetate, citrate and phosphate buffers.

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3.4 Discussion

Mimosa tannin constituted of different concentrations of tannin phenol, non-tannin phenols, total tannins, condensed tannins and hydrolysable tannins as obtained in this investigation. In corroboration with this finding, several studies reported comparable results (Bhatta *et al.*, 2009; Missio *et al.*, 2017; Adejoro *et al.*, 2018; Adejoro *et al.*, 2019a) for the mimosa tannin characterization.

According to Mehran *et al.* (2020), an effective encapsulation technique ensures a high retention of the core materials and only low concentration is trapped on the surface. Moreover, encapsulation efficiency of lipid microcapsules has been shown to depend on the emulsifying properties and viscosity of the coating materials (Flanagan *et al.*, 2006; Bakry *et al.*, 2016), as well as the tannin particle size (Castellanos *et al.*, 2001). Although, the palm and sunflower microparticles did not differ statistically with regards to their encapsulation efficiency and tannin yield, nevertheless, the slightly higher Ee and yield for the EMT^{P} could be attributed to its superior viscosity compared to the EMT^{S}. Davies (2016) reported that palm oil had a viscosity of between 20 and 39.5 cP while, sunflower oil had between 17 and 29 cP. The higher encapsulation efficiency and tannin yield recorded in the current study compares favorably with the findings of Adejoro *et al.* (2018), who reported tannin yield of 63% vs. 57% for acacia tannin encapsulated with palm oil and lard, and Ee of 80% vs. 69%, respectively. Tolve *et al.* (2021) also obtained a comparable encapsulation efficiency of 76% in Quebracho condensed tannin encapsulated with gum arabic–maltodextrin using a spray dryer. The smaller and more uniform in size and spherical in shape of EMT^{P} and EMT^{S} microparticles observed in this study compared to UMT showed that both palm and sunflower oils are good film formers which could be traced to their good emulsifying properties.

Encapsulated mimosa tannins were lighter in terms of particle density compared to unencapsulated tannin. This showed that the unencapsulated mimosa tannin particles, being heavier, might sink to the bottom of the rumen and thus remain longer compared to the palm and sunflower oil microcapsules that, being lighter, could float in the middle of the rumen and hence quickly pass to the next compartment. Kaske and Engelhardt (1990) reported that coarser and heavier feed particles had longer retention times in the rumen as well as other compartments.

Numerous studies have revealed the significance of feeding an optimum concentration of mimosa tannins to ruminants to enhance dietary protein utilization, reducing enteric methane

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production as well as improving the acetate: propionate ratio (Grainger *et al.*, 2009; Hassanat and Benchaar, 2013; Adejoro *et al*., 2020). The quality of wall materials is associated with their capacity to preserve the bioactive compound and ensure its delivery to the target site of function (Martínez *et al*., 2018; Kar *et al*., 2019). Such quality can be assessed by carrying out a release rate trial using a GIT simulated buffer solution (Adejoro *et al*., 2020).

The significantly higher *in vitro* release rate of tannin from the UMT compared to those of the EMT^S and EMT^P across the incubation periods and buffer media could be linked to the entrapment of the tannin within the lipid wall matrices of the microcapsules. In addition, the EMT^S had a higher release rate than the EMT^P, indicating that the microparticle with a higher Ee released lower tannins and vice versa. This finding is corroborated by the report of Adejoro *et al*. (2018) who obtained a 20%, 34% and 25% tannin release from acacia encapsulated with lard and 19%, 30% and 22% from tannin encapsulated with palm oil in acetate, phosphate and HCl media, respectively, after 24 h incubation.

The most exciting function of microencapsulation technology has been identified as the provision of sustained release of bioactive compounds to the targeted site at the right time which in turns improves their efficiency and bioavailability (Priolo *et al.*, 2000; Augustin *et al*., 2001; Adejoro *et al*., 2019a). The unencapsulated mimosa dissolved faster, releasing most of the extract before 24 h periods in all the buffers compared to the tannin encapsulated in sunflower oil or palm oil which dissolved slowly releasing the tannin in a sustained manner. This could be attributed to the good emulsifying properties of the palm and sunflower oils. Nevertheless, the sunflower oil microcapsule eroded faster than the palm oil microparticle which could be traced to their variations in viscosity.

UMT was best fitted to a negative regression model (First order kinetics), indicated by its immediate burst into the various elution media and releasing most of the tannin extract within the first 2 h of in vitro incubation. However, the EMT^P and EMT^S microcapsules did not disintegrate quickly and released smaller proportion of tannins over a 24 h period in a controlled manner while retaining most of the tannin and thus, the release of tannin fitted with high accuracy to the square root regression equation (Higuchi order). This shows that the unidirectional pattern in which mimosa tannins was released into the buffer solutions followed Fick's diffusion law as noted by (Martínez *et al*., 2018). Similar patterns were observed by Adejoro *et al*. (2018) and Tolve *et al.* (2021).

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The sequential release of tannin depicted that majority of the extracts from the UMT were dissolved in the rumen simulated buffer and very negligible amount released in the abomasum simulated buffer and vice versa for the EMT^S and EMT^P. This further confirmed the emulsifying properties of the lipid microcapsules which reduced the tannin release in the media. Literature on sequential tannin release is scarce, thus it is difficult to compare these findings with previous reports.

3.5 Conclusion

The current investigation showed that palm oil and sunflower oil could be adopted as coating materials in encapsulating Mimosa tannin to mask the tannin's bitter taste and control the release of the extract in the GIT. The encapsulated mimosa tannins demonstrated good encapsulation efficiencies, had smaller particle sizes and were lighter than the unencapsulated tannin. Palm or sunflower oil microcapsules also stabilized the tannins' release in gastrointestinal tract simulated buffers at predetermined periods with the potential to modify rumen fermentation. Further studies should be conducted on the lipid stability of microparticles, fatty acids transfer rate and the antioxidant properties of encapsulated tannins to justify commercial application.

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CHAPTER FOUR

In vitro Bovine serum albumin protein binding and release capacity of encapsulated vs non-encapsulated mimosa (*Acacia mearnsii*) tannins in ruminal, abomasal and small intestinal tract pH conditions

Abstract

The anti-methanogenic and protein bypass benefits of tannins cannot be fully actualized due to their astringency and instability in the digestive tract. Microencapsulation could hide the aversive taste, control tannin solubility as well as facilitate gradual tannin – protein binding and release across gastrointestinal tract. The objectives of the present investigation were to assess the capacity of palm oil and sunflower oil based encapsulated mimosa tannins to form tannin – protein complex, and to determine the amount of protein release from the complex at ruminal, abomasal and intestinal pH conditions. The encapsulation process was achieved using double emulsion technique. Ninhydrin assay was used to determine the amount of bovine serum albumin (BSA) protein bound in acetate buffer (pH 5.6), simulating rumen and released in abomasum simulated citrate buffer (pH 2.9), and small intestine simulated phosphate buffer (pH 7.4), by non-encapsulated mimosa tannin (UMT) and mimosa tannins encapsulated in either sunflower oil (EMT^S) or palm oil (EMT^P). The results indicated that, 1 mg of UMT bound 0.29 mg of BSA while, 1 mg of standard tannic acid bound 2.51 mg of protein. Out of 50 mg BSA incubated with 100 mg equivalents of tannins for 24 h in acetate buffer, UMT bound (p <0.01) higher BSA (27.7 mg) compared to 13.6 mg and 10.1 mg bound by EMT^S and EMT^P, respectively. Similarly, UMT released (p <0.01) greater amount of BSA (17.6 mg) in citrate buffer compared with 7.7 mg released by EMT^S and 4.6 mg by EMT^P within 8-h period. However, EMT^S and EMT^P released (p <0.01) higher BSA (1.0 vs 1.1 mg) compared to 0.65 mg released by UMT after 8 h in phosphate buffer. In conclusion, UMT bound and released higher protein in rumen and abomasum simulated media, respectively and thus may likely compatible for use with diets rich in protein sources with high soluble nitrogen in order to avert reduced nitrogen supply to the rumen microbes. While, EMT^S and EMT^P released more protein in small intestine simulated buffer, hence, suitable for diets with protein sources with low to moderate soluble nitrogen but rich in amino acid profile, as it has a potential to increase intake of tannin supplemented diets and improves protein bypass.

Keywords: Bovine serum albumin; encapsulation; mimosa tannin; palm oil; protein binding; sunflower oil.

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4.1 Introduction

Recent studies in animal production have focused on the use of plant secondary metabolites, particularly tannins, as dietary supplements due to their wide range of biological benefits, which include suppressing methane (CH_4) production, reducing rumen microbial digestion of feed protein, shifting nitrogen (N) excretion from urine to faeces, and having anti-parasitic effects, among other things (Piñeiro-Vázquez *et al*., 2015). However, the most significant and well acknowledged benefit of tannins is their capacity to bind dietary protein and prevent bacteria from breaking it down (McNabbl *et al*., 1993; Frutos *et al*., 2004b). According to Hagerman *et al*. (1992), tannin – protein bonds are firm under rumen pH condition, however the bond detaches once the pH dropped to that of abomasum or increased to that of small intestine. Consequently, the tannin – protein bonds are hydrolyzed in the lower gastrointestinal tract (GIT) thereby releasing the protein for abomasal and intestinal digestion by the host animal (McNabb *et al*., 1998; Hassanpour *et al*., 2011; Soltan *et al*., 2012).

Recent studies revealed effective tannin – protein bonding in condensed tannins rich legumes. McNeill *et al*. (1998), reported >78% increase in nitrogen digestibility in the duodenum of ruminants after inclusion of condensed tannin (CT) from *Leucaena leucocephala* species. Saminathan *et al*. (2014), also discovered greater protein-binding potential from higher molecular-sized CT portions of *L. leucocephala*. In Southern Africa, *Acacia mearnsii* (mimosa) is identified as invasive CT rich species (de Wit *et al*., 2001; Galatowitsch and Richardson, 2005), but poorly utilized as a dietary additive. Previous investigations showed that *A. mearnsii* tannin was successfully utilized in modifying dietary protein bypass (Carulla *et al*., 2005; Grainger *et al*., 2009). For example, when mimosa extract was given to ruminants, the fraction of faeces N increased while urinary N reduced (Carulla *et al*., 2005; Grainger *et al*., 2009). Likewise, Adejoro *et al*. (2020), recorded a decrease in urine N excretion while proportion of faecal N increased after offering Merino sheep with acacia tannin extract additives.

However, some studies reported poor absorption of amino acid in the duodenum, which is likely attributed to either the failure of tannin – protein bonds to detach in the lower gut, or interaction of tannins with intestinal mucosa, or establishment of new tannin – feed protein bond or tannin – digestive enzyme complex (Silanikove *et al*., 2001; Min *et al*., 2003). Furthermore, dietary inclusion of tannins extract have been well documented to reduce feed consumption and digestion, due to the astringency and slowing down of microbial activities

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associated with reduced supply of rumen degradable proteins (Beauchemin *et al*., 2008; Jerónimo *et al.*, 2015). Grainger *et al.* (2009), observed 30% reduction in milk production of Friesian cows after supplementation of *A. mearnsii* tannin extract. Similar findings were made regarding the quantities of total volatile fatty acids (TVFAs), which were observed to decrease *in vitro* when more mimosa tannin was added (Hassanat and Benchaar, 2013).

Hence, it is essential to introduce a technology that will mask the undesirable taste of tannins and moderate tannin – protein binding and release in the GIT thereby increasing nutrients utilization by the animal without any adverse effects on normal rumen function. Recently, microencapsulation technology have been shown to neutralize the tannin's bitter taste, slowdown tannin release and fluctuation of the GIT pH (Fang and Bhandari, 2010; Munin and Edwards-lévy, 2011; Mamvura *et al*., 2014). According to Adejoro *et al*. (2018), mimosa tannin encapsulated in palm oil (20%) and lard (19%) had lower proportion of tannin dissolution after 24 h incubation in the rumen simulated buffer than unencapsulated acacia extract (90%). Similar pattern was equally discovered by Tolve *et al.* (2021), in Quebracho tannins encapsulated with a blend of maltodextrin and gum arabic using spray drying method. However, research on tannin – protein binding and release for lipid encapsulated mimosa tannin in GIT simulated media is scarce. The goals of the current investigation are: to evaluate the capacity of encapsulated mimosa tannin in sunflower oil (EMT^S) and palm oil (EMT^P) to bind bovine serum albumin (BSA) protein in rumen simulated buffer, and subsequently quantify the amount of BSA released in abomasum and small intestine simulated buffer media.

4.2 Materials and Methods

Animal ethics approval

The National Health Research Ethics Council of South Africa's regulations were followed in conducting this inquiry. The Animal Ethics Committee at the University of Pretoria reviewed and approved the research protocol with reference number (EC075-17). The experiment was carried out in the laboratory for animal nutrition of the Department of Animal Science, University of Pretoria in the Republic of South Africa.

Characterization and microencapsulation of mimosa tannin

UCL Tannin Company Pty (Ltd), Dalton, South Africa, provided the *A. mearnsii* tannin used in this investigation. The sunflower oil and palm oil were obtained from Pick 'n' Pay store,

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Hatfield outlet, Pretoria. The bovine serum albumin (BSA) protein, Ninhydrin reagent and Tannic acid standard were procured from Sigma-Aldrich (Ltd), St. Louis, MO, USA.

The amount of total tannin (TT), non-tannin phenol (NTP) and tannin phenol (TP) in the *A. mearnsii* extract (Makkar *et al*., 1993), as well as CT component (Porter *et al*., 1986) and hydrolysable tannin (HT) (Singh *et al.* (2005) were determined. According to Ibrahim and Hassen (2022), the tannin extract was discovered to make up 699.3 g/kg, 677.6 g/kg, 463.8 g/kg, 221.7 g/kg and 32.3 g/kg DM for TP, TT, HT, CT and NTP, respectively.

Using double emulsion microencapsulation technique, the tannin powder was coated with either sunflower oil or palm oil (Ibrahim and Hassen, 2021). Briefly, 300 mL of water and 1% (w/v) Tween80 were added to create the oil in water solution. The mixture was then homogenized at 20,000 rpm for 180 seconds using an iron rod homogenizer (PRO400DS, Pro Scientific Inc., Oxford, CT 06478, USA). *A. mearnsii* tannin (8.5 g) was then added to a beaker containing a solution of dichloromethane (50 mg/mL), Span80 (0.5%, w/v), and sunflower oil (30 mL), and the combination was agitated for 120 seconds using a magnetic stirrer at 400 rpm. The solutions were mixed together and stirred for three hours at 800 rpm to remove dichloromethane through evaporation. The encapsulated tannin was then thoroughly cleaned with water before being freeze-dried for five days. The mimosa tannin that was encapsulated in sunflower oil (EMT^S) and palm oil (EMT^P) was ground using a pestle and mortar before being chilled at 4 ^oC for analysis.

The non-encapsulated mimosa tannin (UMT) as well as EMT^S and EMT^P were optimized in the laboratory in terms of morphology using scanning electron microscope as described by Taylor *et al*. (2009); encapsulation efficiency and tannin release in pH simulating rumen, abomasum and duodenum as well as particle density in comparison with unencapsulated mimosa tannin (UMT), according to the procedure of Ibrahim and Hassen (2021). The morphology depicted that both EMT^P and EMT^S had smaller particle sizes with good encapsulation efficiencies (70% vs 68%). Additionally, compared to UMT's particle density of 1.44 g/cm^3, EMT^S and EMT^P had lower particle densities of 1.21 and 1.22 g/cm^3, respectively. Moreover, after 24-h incubation period, the amount of tannin extract released by UMT in the elution media simulating ruminal (94%), abomasal (92%) and small intestinal (96%) conditions

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were dropped to 18%, 20% & 16% for EMT^P, and 24%, 21% & 19% for EMT^S in the same elution media and time, respectively (Ibrahim and Hassen, 2021).

Bovine serum albumin protein hydrolysis

The hydrolysis of protein was done according to the technique of Makkar *et al*. (1987) with little adjustment. The standard BSA protein (0.2 – 2.0 mg) was dissolved in deionized water (50 to 500 µL) and the various concentrations were dispensed into the centrifuge tubes placed in an oven adjusted to temperature of 100 °C. Sodium hydroxide (13.5 N NaOH) at 0.6 ml was added to the dried BSA and the test tubes oven dried again at 120 ± 2 °C for 20 min to hydrolyse the BSA. After cooling the test tubes, the NaOH was neutralize by the addition of 1 ml glacial acetate. A 0.1 ml aliquot was combined with 1 ml of the 2% Ninhydrin reagent. The tubes were then covered with marbles and boiled for 20 min in a water bath. After cooling, 5 ml of deionized water was then added. The spectrophotometer absorbance was read at 570 nm and the values were plotted against BSA concentration (mg).

Tannin – BSA protein binding in rumen simulated buffer

The tannin – protein bonding was achieved following the method of Hagerman and Butler (1980), with slight changes. Unencapsulated mimosa tannin (UMT) and standard tannic acid (TA) samples, each weighing 120 mg, were separately extracted in 6 ml of 50% aqueous methanol, and the resulting solutions were filtered. BSA solution was prepared (1 mg/mL) in in rumen simulated medium (0.2 M acetate buffer, pH 5.6) comprising 0.17 M NaCl, and kept in centrifuge tubes. A total volume of 3 ml of BSA solution (2 ml) was combined with TA (0.2–1.2 mg) and UMT (0.5–3.5 mg), mixed, and left at room temperature for 15 minutes.

The mixture was centrifuged at 2500 rpm for 15 minutes, collecting the pellets (tannin-protein complexes), and discarding the supernatant. The pellets were centrifuged once more after being properly washed with new acetate buffer. The hydrolysis of BSA protein in the pellet was accomplished according to the technique of Makkar *et al*. (1987), as described above. The protein bound by the TA and UMT were determined from the BSA standard curve generated. All experiments were done in triplicates.

In vitro mimosa tannin – BSA protein binding at predetermined periods

The method of Ibrahim and Hassen (2021), for *in vitro* tannin release was adopted with little changes in this study for determination of tannin – protein binding in the rumen simulated

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buffer at predetermined hours. Separately weighed 100 mg of UMT, EMTP, and EMTS were placed in filter bags and submerged in bottles containing a 50 mL (mg/mL) solution of BSA-acetate buffer, pH 5.6. In a shaker, the bottles were rotated at 50 rpm and 39 °C. The 3 mL samples of the solutions were taken in triplicate at the predefined times of 1 h, 2 h, 4 h, 8 h, and 24 h. Following the collection of each sample, 3 mL of fresh BSA-buffer solution was added to the mixture to maintain its initial volume. The sample tubes were kept in the freezer before analysis.

The sample tubes were defrosted, spun at 2500 rpm to separate the pellets from the supernatant, and the pellets were collected. The tannin – protein complexes were rinsed with fresh media and centrifuged again. The hydrolysis of BSA protein was done in the same manner as above (Makkar *et al*., 1987). The protein bound by the unencapsulated and encapsulated mimosa tannins in the rumen simulated buffer at 1, 2, 4, 8, and 24 h were estimated from the standard curve generated from BSA.

Bovine serum albumin protein in vitro release from the tannin – protein complex

The dissolution of BSA protein in the buffer media simulating lower gastrointestinal tract from the tannin and protein bonds generated in the rumen simulated medium were analysed according to the method of Ibrahim and Hassen (2021), for sequential tannin release. Immediately, after 24 h sampling of the pellets in acetate buffer – BSA media, the remaining solutions from the three tannin sources (UMT, EMT^{P} and EMT^{S}) were collected and subjected to 15 min centrifugation at 2500 rpm. The tannin – protein complexes were collected, washed with distilled water, added into the container of abomasum simulated medium (pH 2.9), and shaken for 8 h at 50 rpm while, the temperature was maintained at 39˚C. In the test tubes, a sample of the solutions (3 mL) was taken and frozen. The residual solutions were collected, centrifuged once more at 2500 rpm for 15 minutes, and the pellets were gathered, washed with water, and distributed into bottles containing small intestine simulated medium (pH 7.4). The mixture was shaken for 8 h at 50 rpm and 39˚C. Thereafter, 3 mL samples were collected in the test tubes and frozen.

All the samples collected were thawed and BSA protein hydrolyses were conducted according to the technique of Makkar *et al*. (1987), as described above. The amount of protein released

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by UMT, EMT^P and EMT^S in the media simulating abomasum and small intestine, sequentially were estimated from the standard curve generated from BSA protein.

Statistical analysis

The proportion of BSA protein bound by TA and UMT were determined using linear regression coefficients. Data on BSA protein bound and released by EMT^S, EMT^P and UMT at predetermined hours were subjected to one–way ANOVA (SAS 9.4; SAS Institute Inc., Carry, NC, USA). LSD was used to separate means with significant differences at a 5% probability level.

4.3 Results

Tannic acid – BSA protein binding

Figure 4.1 depicts the bonding relationship between different proportion of standard tannic acid (TA) and bovine serum albumin (BSA) protein. The finding showed that the higher the concentration of TA, the more the BSA protein bound. The amount of BSA protein bound revealed a linear increase in relation to the proportion of TA (R^2 = 0.97). Tannic acid started binding BSA protein at 0.2 mg level and the concentration of bound protein continue to rise with the increase in TA. In general, each 1 mg of TA incubated bound 2.51 mg protein.

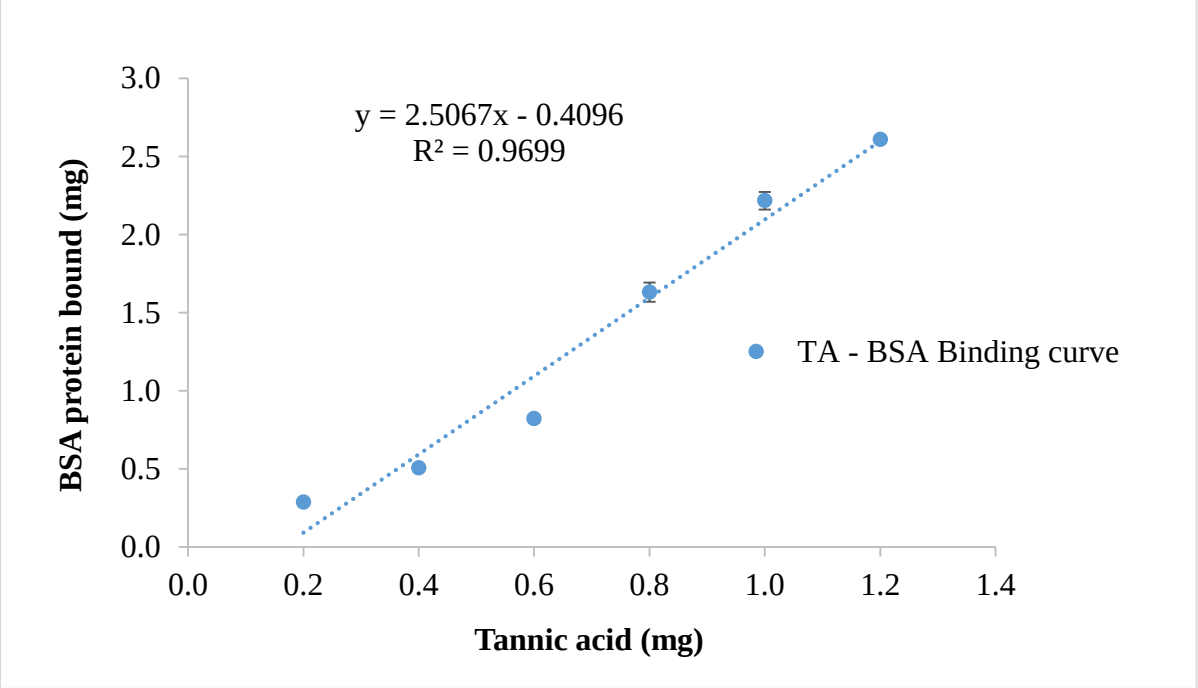

Figure 4.1 A line graph depicting Tannic acid – BSA protein binding

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Unencapsulated mimosa tannin – BSA protein binding

The interaction of various concentration of mimosa tannin and BSA protein is shown in Figure 4.2. The results revealed a linear increase in the proportion of BSA protein bonding with the increase in level of mimosa tannin (R^2 = 0.99). Mimosa tannin extract began to interact with BSA protein at 0.5 mg concentration and the proportion of protein continue to increase with the addition of tannin extracts. Generally, 1 mg of mimosa tannin was discovered to bind 0.29 mg of BSA protein.

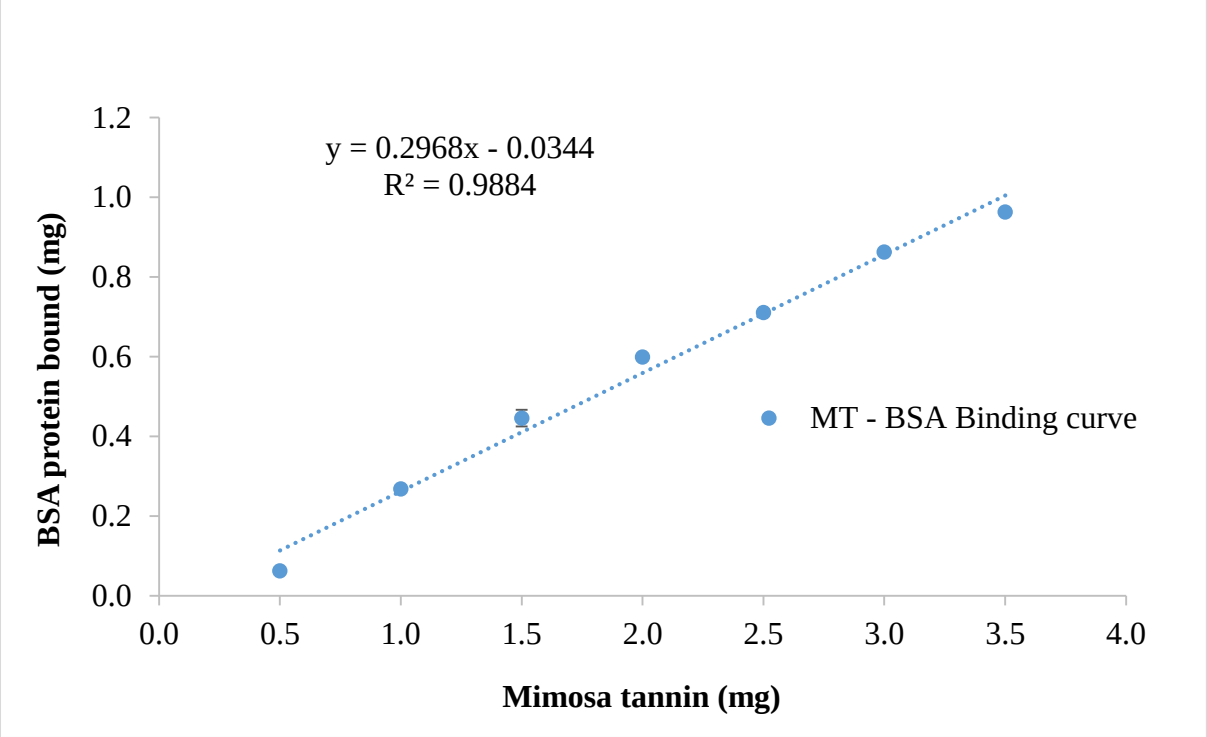

Figure 4.2 A line graph showing unencapsulated Mimosa tannin – BSA protein binding

In vitro mimosa tannin – BSA protein binding in rumen simulated buffer

Table 4.1 summarized the mean BSA protein bound by UMT, EMT^S and EMT^P in acetate buffer (pH 5.6), simulating rumen at predetermined hours. The finding showed that UMT bound (p <0.01) greater BSA protein compared to EMT^P and EMT^S in all the incubation periods. Moreover, EMT^S bound significantly higher BSA than EMT^P. Non-encapsulated mimosa tannin bound 18.3, 21.7, 23.6, 25.1 and 27.7 mg of BSA at 1, 2, 4, 8 and 24 h, respectively. However, the proportions of BSA bound by encapsulated mimosa tannin in similar buffer media and periods were reduced by 82.5, 76.5, 71.6, 64.9 & 63.5% for EMT^P and 77.6, 71.4, 64.0, 57.4 & 50.9% for EMT^S. Generally, out of the 50 mg of BSA protein

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incubated together with 100 mg equivalent of tannin sources in the buffer medium simulating rumen over 24-h period, UMT bound approximately 55.4% of BSA protein while, EMT^S and EMT^P bound 27.2% and 20.2%, respectively.

Table 4.1. Mean BSA protein bound by UMT, EMT^S and EMT^P in the buffer simulating rumen (pH 5.6) at pre-determined hours

	BSA[a] protein bound at predetermined hours				
Treatments	**1 H**	**2 H**	**4 H**	**8 H**	**24 H**
[b]UMT (mg)	18.290[a]	21.736[a]	23.586[a]	25.093[a]	27.722[a]
[c]EMT^P (mg)	3.229[c]	5.117[c]	6.653[c]	8.787[c]	10.078[c]
[d]EMT^S (mg)	4.141[b]	6.219[b]	8.453[b]	10.749[b]	13.645[b]
SEM	0.034	0.067	0.060	0.044	0.193
p – value	< 0.01	< 0.01	< 0.01	< 0.01	< 0.01

[a] BSA: bovine serum albumin protein @ 1 mg/mL of acetate buffer solution.

[b] UMT: unencapsulated mimosa tannin @ 2 mg/mL of BSA – acetate buffer solution.

[c] EMT^P: mimosa tannin encapsulated in palm oil @ 2 mg equivalent per 1 mL of BSA – acetate buffer solution.

[d] EMT^S: mimosa tannin encapsulated in sunflower oil @ 2 mg equivalent per 1 mL of BSA – acetate buffer solution.

Means with uncommon superscripts across a column differed significantly ($p < 0.01$). For all the periods, mean values are based on 3 replicates of each treatment. SEM: Standard error of mean. H: Hour.

The profiles of the mimosa tannin – BSA protein bonding in the rumen simulated media (pH 5.6) by UMT, EMT^P and EMT^S are depicted in Figure 4.3. The graph illustrates that UMT dissolved quickly in the acetate buffer and bound approximately 37%, 44%, 47%, 50% and 55% of BSA protein in 1, 2, 4, 8 and 24 h, respectively. However, lower proportions of protein bonding were recorded by EMT^P & EMT^S microparticles at 1 h (7% vs 8%), 2 h (10% vs 12%), 4 h (13% vs 17%), 8 h (18% vs 22%), and 24 h (20% vs 27%) incubation periods, respectively, in the same elution media.

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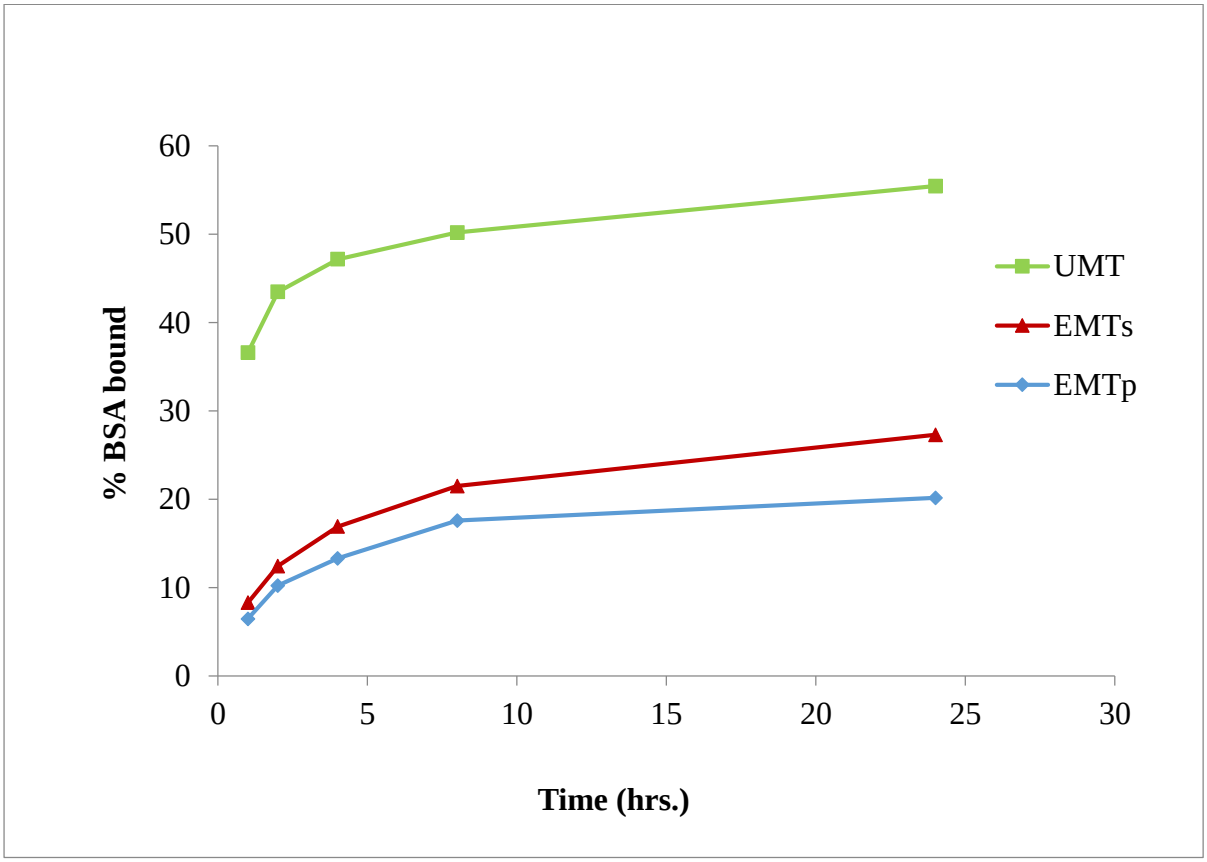

Figure 4.3. BSA binding profile by the UMT, EMT^{P} and EMT^{S} in acetate buffer (pH 5.6)

In vitro release of BSA from the tannin – protein complex

Figure 4.4 depicts the proportion of BSA released from the tannin – protein complexes by UMT or EMT^{P} and EMT^{S} into the abomasum simulated citrate medium (pH 2.9) and small intestine simulated phosphate elution medium (pH 7.4). The results indicated that UMT released (p <0.01) higher BSA protein in citrate buffer compared to EMT^{P} and EMT^{S}. However, EMT^{P} and EMT^{S} released significantly greater amount of protein in phosphate buffer than UMT. Moreover, EMT^{S} released higher (p <0.01) BSA protein than EMT^{P} in both citrate and phosphate buffer media.

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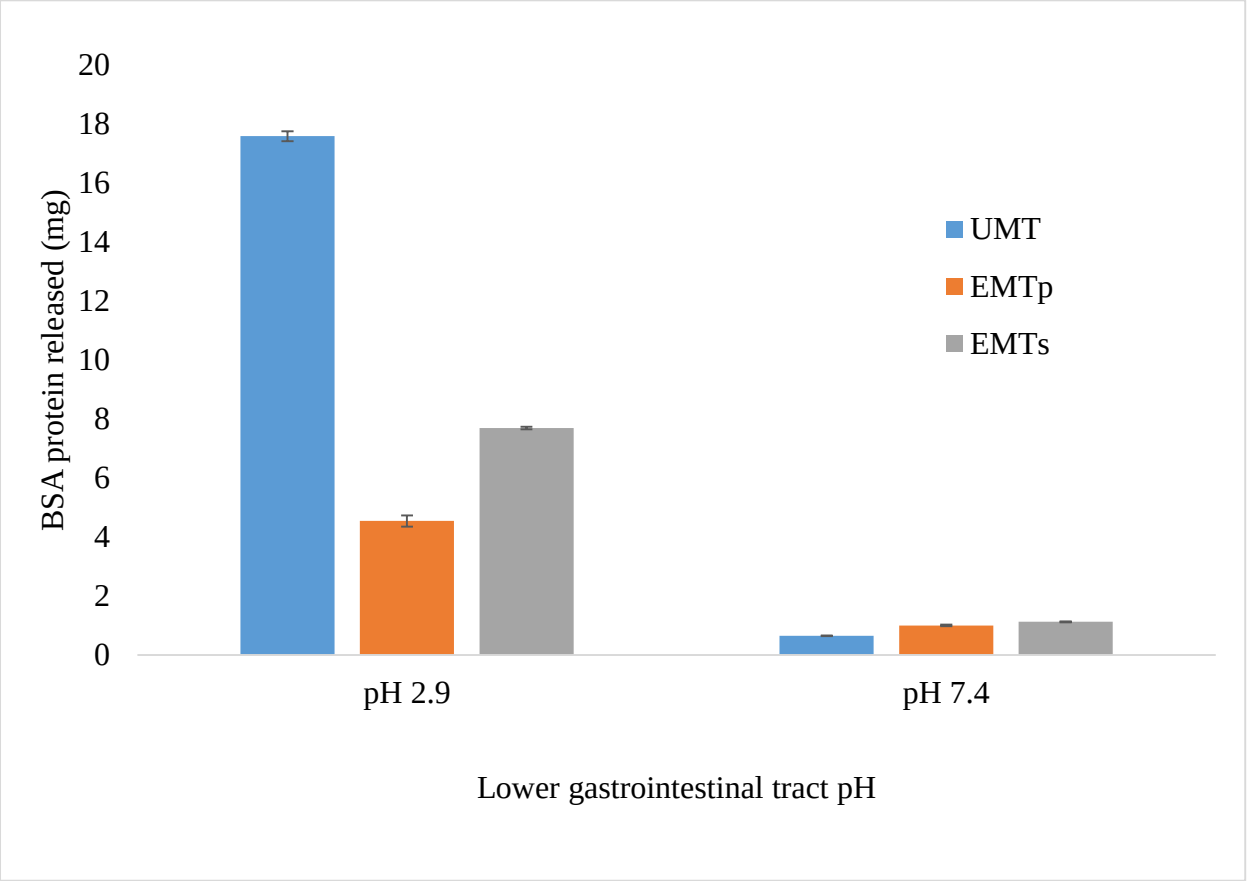

Figure 4.4. BSA protein released by the tannin – protein complex in media simulating abomasum (pH 2.9) and small intestine (pH 7.4), sequentially.

From the 27.7 mg of BSA protein (out of 50 mg of BSA incubated in acetate buffer) bound by UMT in the acetate buffer over 24-h period, 17.6 mg (63.5%) was released into the citrate buffer after 8 h and only 0.65 mg (2.35%) of BSA passed to phosphate buffer within 8-h period. In contrast, out of 10.1 mg of protein bound by EMT^P, 4.6 mg (45.54%) and 1.0 mg (9.90%) of BSA were released into citrate and phosphate media, respectively, within 8 h. Similarly, from the 13.6 mg of BSA protein bound by EMT^S, 7.7 mg (56.62%) was released in citrate buffer and about 1.1 mg (8.09%) in phosphate buffer after 8-h period. In general, UMT released approximately 64% of the bound protein in abomasum simulated buffer while only about 4% was hydrolyzed in small intestine. However, EMT^S and EMT^P released 56.4% and 45% of BSA in abomasum and around 15% and 22% in small intestine simulated buffer, respectively. The unreleased BSA protein amount in both citrate and phosphate buffer represents BSA protein likely excreted in the faeces of the animal and this fraction represents 9.45 mg, 4.5 and 4.8 mg of BSA protein for UMT, EMT^P and EMT^s, respectively.

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4.4 Discussion

Tannic acid – BSA protein binding

According to Frutos *et al*. (2004b), the potential of tannins to bind proteins is associated with their greater amount of phenolic groups, which offer several connection points with the carbonyl groups of proteins. The amount of protein bound by standard tannin acid (TA) obtained in this investigation compares favourably with the results of Makkar *et al*. (1987), and Dawra (1988), who reported that 1 mg of TA bound 2.6 mg BSA. However, the threshold level at which TA begin to bind BSA protein recorded in this study was slightly higher than 0.1 mg and 0.114 mg reported by Makkar *et al*. (1987) and Hagerman and Butler (1980), respectively. This could be linked to the differences in pH of the buffer. It is widely known that changes in the medium's pH have a significant impact on the tannin-protein complex (Kumar and Singh, 1984; Kumar and Horigome, 1986; McNabb *et al*., 1998).s

Unencapsulated mimosa tannin – BSA protein binding

In this study, mimosa tannin bound lower amount of BSA protein compared to standard TA which is pure hydrolysable tannin source. This might be because the mimosa tannin contains certain contaminants and non-tannin phenols. Missio *et al.* (2017), reported that mimosa tannin constituted of about 70% condensed tannins with around 30% of non-tannin phenols in the form of hydrocolloids, gums and other molecules. In concurrence with our finding, Dawra (1988), reported that hydrolysable tannin contributed more towards the protein binding capacity of *Quercus incana*, *Quercus semecarpofolia* and *Quercus dilatata* extracts compared to their condensed tannin portion. However, the threshold level (0.5 mg) at which binding between mimosa tannin and BSA protein commenced was greater than 0.2 mg obtained for TA. These could be traced to differences in chemical composition and structures. It has been established that the molecular weight, polarity, and flexibility of tannins affect their affinity for proteins (Hagerman *et al*., 1998; Soares *et al*., 2007).

In vitro mimosa tannin – BSA protein bindings at predetermined periods

Non-encapsulated mimosa bound more BSA protein in the buffer simulating rumen than encapsulated tannins. These could be attributed to the strong barrier made by lipid microcapsules which delay tannin solubility in the buffer, thus, lower the quantity of protein bound. Adejoro *et al*. (2018), recorded more than 90% dissolution of unencapsulated mimosa tannin in acetate buffer within 24 h, whereas, palm oil and lard microparticles released only

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19% and 20% tannin extract, respectively. Similar trend was also reported by Ibrahim and Hassen (2021), for mimosa encapsulated with palm and sunflower oils. The higher tannin – protein binding capacity exhibited by EMT^S compared to EMT^P in this study, could be traced to the higher tannin release rate of the sunflower oil microcapsule due to its low viscosity. Davies (2016), noted that 29 mPa as the maximum viscosity of sunflower oil and 39.5 mPa for palm oil. According to Naumann *et al.* (2017), the higher the tannin's astringency, the more the proportion of protein bound and the lesser the palatability.

Encapsulated mimosa tannins bound BSA protein gradually in a controlled pattern compared to the free tannin which bound most of the highly soluble protein within the first hour of incubation. This could be linked to the slow tannin release properties of lipid microcapsules as a result of their good encapsulation efficiencies (Ee). Ibrahim and Hassen (2021) reported Ee of 68% & 70%, and tannin release rate of 24% & 18% for EMT^S and EMT^P, respectively. Adejoro *et al*. (2018), also observed higher Ee (79% vs 80%) and lower tannin release (24% vs 18%) in mimosa encapsulated with lard and palm oil, respectively, after 24 h incubation in rumen simulated buffer.

In vitro release of BSA from the tannin – protein complex

Unencapsulated mimosa released more protein in the buffer simulating abomasum than the encapsulated tannin, but released lower protein in small intestine simulated medium. This showed that UMT are more compatible for diet rich in highly soluble nitrogen and may help to reduce risk of ammonia toxicity from such diet while, encapsulated mimosa tannins are more suitable in the diet with low to moderate soluble nitrogen. The scenario could be linked to the excellent emulsification of the lipid microcapsules which delayed tannin dissolution, therefore, ensure tannin – binding and release in a controlled manner (Flanagan *et al*., 2006; Bakry *et al*., 2016), without affecting rumen fermentation negatively. This phenomenon could also be associated to variations in tannin particle size and density. Ibrahim and Hassen (2021), observed UMT as bigger and heavier compared to the encapsulated mimosa, hence, may probably sink in the rumen after bonding and consequently release little protein to the lower gut for absorption and utilization. In concurrence with our finding, Kaske and Engelhardt (1990), who reported longer retention time for coarser and heavier feed particles across gastrointestinal tract of ruminants. Some studies revealed that rumen microorganisms hydrolyze the lipid coating materials to free fatty acids thereby exposing the bioactive

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compound to rumen fermentation (Veneman *et al*., 2015; Bainbridge and Kraft, 2016). Though, literature on tannin – protein binding and release is not readily available for comparison with this result. Nevertheless, Wood *et al.* (2009), found that encapsulated Fumaric acid controlled the release of the bioactive compound in rumen fluid. Despite lower tannin – protein binding and release by the encapsulated tannin, however, the current result describes the ability of palm oil and sunflower oil wall materials to conceal the tannin's aversive taste, slowly release the tannin for protein binding and release in gastrointestinal tract simulated buffer. The higher protein by pass facilitated by UMT could be an advantage but this can be offset by the higher unreleased BSA protein amount in both citrate and phosphate buffer which represents higher proportion of dietary protein likely excreted in the faeces of the animal. The implication is that nitrogen utilization efficiency could be lower considering the two-fold nitrogen amount that will be potentially lost due to the use UMT as compared EMT^{P} and EMT^{S}.

4.5 Conclusions

Conclusively, encapsulating *Acacia mearnsii* tannin with sunflower oil or palm oil ensured controlled tannin-protein binding in the medium simulating rumen, and subsequent release abomasum and small intestine simulated media with potential to modify bypass protein supply in order to optimize amino acid utilization from high quality protein sources. The amount of BSA protein bound increased linearly in relation to the concentration of mimosa tannin extracts. Unencapsulated mimosa tannin bound and released higher protein in rumen and abomasum simulated media, thus, compatible for use in diet rich in highly soluble and greater rumen degradable nitrogen sources, while encapsulated tannins in sunflower and palm oil released more BSA protein in small intestine simulated buffer, thus, suitable for use in diet rich in high quality protein but relatively low to moderate in soluble nitrogen and rumen degradable nitrogen. EMT^{S} bound and released higher BSA than similar concentration of EMT^{P} in all gastrointestinal tract simulated buffer media and could be used to modify rumen by-pass protein supply which in turn can improve nitrogen utilization efficiency provided that adequate amount of energy and other nutrients are available for both the rumen microorganisms and the host animal.

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CHAPTER FIVE

Influence of sunflower oil based encapsulated mimosa (*Acacia mearnsii*) tannin on methane, rumen fermentation and *in vitro* organic matter digestibility

Abstract

Utilization of tannin in reducing enteric CH_4 received wide recognition in contemporary ruminants' nutrition researches, though, feed intake and rumen fermentation were adversely affected because of the astringent and unstable nature of tannin. Encapsulation could neutralize the bitter taste and enhance its delivery across digestive tract. The goal of this research is to evaluate the influence of various inclusion levels of encapsulated mimosa tannins on *in vitro* organic matter digestibility (IVOMD), total gas, methane, volatile fatty acids (VFAs) and ammonia nitrogen (NH_3N). The encapsulation process was done using double emulsification (solid-in-oil-water) technique. The mimosa tannin encapsulated in sunflower oil (EMT^S) at 10 (EMT^{S10}), 20 (EMT^{S20}) and 30 g/kg DM equivalents (EMT^{S30}), and unencapsulated mimosa tannin (UMT) at inclusion level of 20 g/kg DM of feed were evaluated for gas, CH_4, IVOMD, NH_3N and VFAs using two separate substrates: *Eragrostis curvula* hay (ECH) and total mixed rations (TMR). The results indicated that addition of EMT^S and UMT decreased ($p < 0.05$) gas, CH_4, gas/IVOMD, gas/TVFA, CH_4/IVOMD and CH_4/TVFA as well as gas yield from insoluble but slowly fermentable fraction, b and effective gas production, EGP. While, IVOMD was only affected ($p < 0.05$) by EMT^{S30}. Moreover, EMT^{S20} resulted in lower proportion of CH_4, CH_4/IVOMD and CH_4/TVFA compared to UMT. However, UMT and EMT^S did not influenced NH_3N and TVFA ($p > 0.05$). Equally, all dosages of EMT^S and UMT had no effect ($p > 0.05$) on molar concentrations of VFAs. Total gas, CH_4, IVOMD, NH_3N, TVFA, b and EGP were less ($p < 0.05$) in the ECH substrate than the TMR. In conclusion, mimosa tannin encapsulated with sunflower oil and non-encapsulated mimosa tannin decreased appreciable amount of CH_4 without reduction in IVOMD, NH_3N and VFAs when incubated up to 20 g/kg DM dosage. EMT^{S20} produced less CH_4 compared to equivalent inclusion level of UMT.

Keywords: Encapsulation; *in vitro*; methane; mimosa tannin; rumen fermentation; sunflower oil.

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5.1 Introduction

The considerable impact of enteric methane (CH_4) to climate change is a matter of concern (Martin *et al*., 2010). Ruminant animals emit nearly 80 million tonnes of CH_4 per year, representing about 33% of global CH_4 output (Beauchemin *et al*., 2008). In addition, a loss of around 12% dietary energy is attributed to enteric CH_4 emission (Piñeiro-Vázquez *et al*., 2015). Thus, a number of measures were adopted to discourage enteric CH_4 production. However, CH_4 abatement strategy involving tannin supplementation in animal diets gained wider acceptance because of tannins' abundance and safety (Eckard *et al*., 2010). Accordingly, the potential of tannin to mitigate enteric CH_4, in addition to its ability to enhance dietary protein bypass and amino acid utilization, has encouraged more advanced investigations into its usage.

Black wattle (*Acacia mearnsii*) also called mimosa is renowned as the invasive tannin rich plant grown in Southern Africa (Versfeld *et al*., 1998). Numerous studies showed that *A. mearnsii* tannin extract could be exploited in moderating rumen fermentation, enteric CH_4 suppression and dietary protein bypass. Carulla *et al.* (2005), reported more than 12% reduction in enteric CH_4 when mimosa tannin was fed to sheep. In addition, a reduction of around 60% urine nitrogen was recorded in cattle supplemented with *A. mearnsii* tannin extract (Grainger *et al.*, 2009). Also, inclusion of mimosa tannin was found to increase propionic acid level as against the acetic acid (Hassanat and Benchaar, 2013).

However, oral feeding of tannin additives is found to reduce dietary intake and delay dry matter digestibility, because of tannins' astringency and solubility across gastrointestinal tract particularly when supplemented at higher dosage (Frutos *et al*., 2004b). For instance, Bhatta *et al.* (2013b), observed about 45% loss in dietary energy and around 30% decline in nitrogen retention in goats when more than 50 g/kg DM level of black wattle tannin was added to diet. Equally, inclusion of crude *A. mearnsii* tannin extract in the diets of merino sheep at 4.2% DM concentration resulted in 23.4% and 22.9% decrease in total weight gain for nitrate and urea containing diets, respectively (Adejoro *et al*., 2020). Thus, encapsulation could be employed to hide the bitterness of tannins and regulate their dissolution along digestive tract, hence, depress enteric CH_4 and protein degradation by rumen microbes while, enhance nitrogen utilization and animal performance.

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Recently, sunflower oil was successfully used as an encapsulant for mimosa tannin with about 70% encapsulation efficiency and reduced the proportion of tannin solubility to 24%, 21% and 19% from 94%, 92% and 96% released by the unencapsulated *A. mearnsii* tannin in rumen, abomasum and small intestine simulated elution media, respectively, over 24-hours period (Ibrahim and Hassen, 2021). Similarly, Adejoro *et al*. (2018), also recorded approximately 80% encapsulation efficiency for the lard and palm microcapsules and the products slowed down the concentrations of tannin dissolution to 19%, 30% and 22% for mimosa tannin coated with palm oil as well as 20%, 34% and 25% reduction for tannin coated with lard in rumen, abomasum and small intestine simulated buffers, respectively, after 24 hours. This suggested that lipid microcapsules could serve as better alternatives to crude tannin extract drenching. However, there is still no information on the optimum inclusion level of microencapsulated tannins. Hence, this investigation aimed to determine the influence of various doses of mimosa tannin encapsulated with sunflower oil on *in vitro* organic matter digestibility, gas and methane as well as ammonia nitrogen and volatile fatty acids productions of total mixed ration and *Eragrostis curvula* hay.

5.2 Materials and methods

The current study was conducted in compliance with the recommendations of the South African National Health Research Ethics Council. The University of Pretoria's Animal Ethics Committee studied and accepted the experimental protocol (reference number, EC075-17). The University is located at coordinates 28°15′30″ east and 25°44′30″ south at an altitude of 1, 360 meters above sea level (van Niekerk *et al.*, 2009).

The *A. mearnsii* tannin extract was sourced from the UCL tannin company Pty (Ltd), KZN, South Africa. The dichloromethane, Span80, Tween80 and the remaining reagents used in the study were obtained from Sigma-Aldrich, St. Louis, MO, USA. The sunflower oil was procured from Pick 'n' Pay store, Hatfield outlet, Pretoria.

Tannin encapsulation with sunflower oil

Before encapsulation, the tannin powder was analysed to establish its composition of non-tannin phenol, NTP; tannin phenol, TP and total tannin, TT according to the method of Makkar (2000), as well as hydrolysable tannin, HT (Singh *et al.*, 2005) and condensed tannin, CT (Porter *et al*., 1986). The tannin composed of 32.3 g/kg NTP, 221.7 g/kg CT, 463.8 g/kg HT,

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677.6 g/kg TT and 699.3 g/kg TP (Ibrahim and Hassen, 2022). The encapsulation process was done using double emulsion (solid-in-oil-water) technique as described by Ibrahim and Hassen (2021). Briefly, 30 mL of sunflower oil, 50 mg/mL of dichloromethane, and 0.5% (w/v) Span80 were combined with 8.5 g of *A. mearnsii* tannin. With the aid of a magnetic stirrer, the mixture was thoroughly swirled at 400 rpm for 120 seconds. Another combination was made and homogenized for 180 seconds with an iron rod homogenizer (PRO400DS, Pro Scientific Inc., Oxford, CT 06478, USA) set to 20,000 rpm. This mixture contained 300 mL of water and 1% (w/v) Tween80. To remove the dichloromethane, the various solutions were agitated at 800 rpm for three hours with a magnetic stirring plate. The result was transferred into an aluminum container and freeze-dried over the course of five days after being cleaned with distilled water and squeezed in a cheese cloth to remove the liquid fractions. With the help of a pestle and mortar, the freeze-dried, encapsulated *A. mearnsii* tannin was ground to a fine powder and stored in the refrigerator for analysis.

Chemical analysis of the substrates

The substrates (total mixed ration and *Eragrostis curvula* hay) were obtained from the experimental farm's feed store, University of Pretoria. The feeds were milled to pass through 2 mm sieve and determined their chemical constituents. Following the guidelines established by the Association of Official Analytical Chemists (AOAC, 2002), the dry matter (DM) and ash contents were analysed. Dumas method (Nitrogen Analyzer FP-2000, Leco Instrumente GmbH, Kirchheim, Germany) was used to determine the crude protein (CP). Using a Fibre Analyzer (ANKOM 200/220, ANKOM Technology, New York, USA), the proportions of acid detergent fibre (ADF), acid detergent lignin (ADL) and neutral detergent fibre (NDF) of the substrates were analysed (AOAC, 2002).

In vitro gas, gas, methane, ammonia nitrogen and volatile fatty acids analyses

With modifications made by Mould *et al*. (2005), the buffer medium was reconstituted in accordance with Menke and Steingass (1988) instructions before being flushed continuously with carbon dioxide (CO_2) for about 45 min in a water bath set at 39 °C. Three rumen-cannulated Pinzyl steers were fed eragrostis hay with the addition of alfalfa hay, and their rumen fluid was collected using four folds of clean cheese cloth into a pre-heated thermos flask. The rumen fluid was quickly sent to the nutrition lab of the University of Pretoria's Department of Animal Science.

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In a water bath with a temperature of 39 °C, the rumen fluid and the buffer were combined in a 3:5 ratio and continuously flushed with CO_2. The mixture was used as inoculum and dispersed (40 mL) into the serum vials, containing two different substrates (400 mg each) as follows: *Eragrostis curvula* hay, ECH (ash, 45.9 g/kg; DM, 925.2 g/kg; CP, 79.9 g/kg; ADF, 397.2 g/kg; ADL, 77.7 g/kg; NDF, 725.6 g/kg) and total mixed ration, TMR (ash, 72.4 g/kg; DM, 942.9 g/kg; CP, 166.9 g/kg; ADF, 240.5 g/kg; ADL, 78.2 g/kg; NDF, 368.9 g/kg). For each substrate, treatments comprised of (1) feed alone (2) feed + unencapsulated mimosa tannin at 20 g/kg DM, UMT (3) feed + encapsulated mimosa tannin in sunflower oil at 10 g/kg DM, EMT^{S10} (4) feed + encapsulated mimosa tannin in sunflower oil at 20 g/kg DM, EMT^{S20} (5) feed + encapsulated mimosa tannin in sunflower oil at 30 g/kg DM, EMT^{S30}. Only one level of non-encapsulated tannin was tested based on the preliminary *in vitro* results that recorded a significant drop in CH_4 without decline in IVOMD at 20 g/kg DM dose. The vials were instantly locked with stoppers and revolved in a shaker at 120 rpm and at 39 °C. All the tannin treatments and substrate alone (control) were incubated in four replicates while blanks were added in triplicates for all the four incubation cycles carried out.

A pressure transducer (PX4200-015GI; Omega Engineering Inc., Laval, QC, Canada) connected to a data logger (digital tracker 220 series indicators; Omega Engineering Inc.) was used to record the amount of gas produced during *in vitro* incubation (Theodorou *et al.*, 1994). The volume of gas developed in the bottles at 3, 6, 12, 24 and 48 hours were monitored directly from the screen of data logger as soon as the transducer was attached to each bottle. The cumulative gas volume logged in per square inch (psi) for all the hours were changed to millilitres (mL) with the aid of an ANKOM technology formula (Pressure to Gas Production Conversion, 2014), shown in equation (5.1) below.

$$V_x = V_j P_{psi} X\ 0.068004084 \qquad (5.1)$$

Where, V_x = gas quantity (mL), V_j = vial headspace, and P_{psi} = data logger pressure reading in psi.

Gas production characteristics were estimated using non-linear model (Ørskov and McDonald, 1979) as presented in equation (5.2).

$$y = b\ (1 - e^{-ct}) \qquad (5.2)$$

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Where, y = amount of gas generated at t = time; b = quantity of gas from the insoluble but slowly fermentable fibre fraction; and c = rate of fermentation of 'b'.

With a few minor modifications, the method of Tavendale *et al.* (2005), for measuring CH_4 concentration was used. Following gas readings at 3, 6, 12, 24 and 48 hours, 10 mL luer-locked syringes were used to draw gas samples from each bottle and inject them into the flame ionization sensor-attached SRI GC (GC 8610C BTU Gas Analyzer System, Bad Honnef, Germany). The GC calibration was done using standard methane and carbon dioxide (Gemeda and Hassen, 2015). The GC calibration curves generated were used to determine the CH_4 concentrations of UMT, EMT^{S10}, EMT^{S20} and EMT^{S30}.

The incubation process was stopped after 48 hours of gas sampling by placing the container in a cold room to prevent ongoing microbial activity, and the contents of the bottle were separated using a centrifuge at 2500 rpm for 15 minutes. The supernatants were then filtered off, and 5 mL of each sample was pipetted into two different vials, one of which contained 1 mL of 25% orthophosphoric acid and used for VFAs determination according to the procedure of Webb (1994), and the other 1 mL of 0.5 M H_2SO_4 used for NH_3N determination following the instruction of Broderick and Kang (1980).

In vitro organic matter digestibility evaluation

The digestibility of the two substrates (TMR and ECH) was assessed using the two-step digestion method proposed by Tilley and Terry (1963) and Engels and Van der Merwe (1967), after the addition of UMT, EMT^{S10}, EMT^{S20} and EMT^{S30}. Artificial saliva was formulated, put in a water bath that was heated to 39 °C, and continuously flushed with saturated CO_2. Three Pinzyl steers with rumen cannula were used to get the rumen fluid samples, which were then compressed through clean cheese-cloth into a warm thermos flask and taken to the laboratory. The artificial saliva and rumen fluid were immediately mixed in the laboratory at the ratio of 3:1, while maintaining constant flushing with saturated CO_2 in a water bath set at 39 °C.

In the first step, 20 mL solution of the rumen fluid and saliva were poured into the tubes already added with the two different substrates (200 mg each) mixed with either UMT, EMT^{S10}, EMT^{S20} or EMT^{S30} following similar treatments connotation as stated above during *in vitro* gas trial. Thereafter, the tubes were covered with stoppers and digested in a shaker at 100 rpm set at a temperature of 39 °C for 48 h. Following the digestion, the supernatants were discarded

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after 15 min separation using centrifuge at 2500 rpm. In the second step, sample tubes were splashed with 20 mL acid-pepsin mixture and incubated at 100 rpm for another 48 h and heated at 39 °C. The digested samples were removed, centrifuged for 15 minutes at 2500 rpm, and the supernatants were then dried for 18 hours at 105 °C in an oven. After measuring the weight of the dried samples, the weight of the ash was determined after it had been heated for three hours at 550 °C. The original sample weights, dried sample weights, and weights of residual ash were used to calculate the digestibility of the ECH and TMR supplemented with UMT, EMT^{S10}, EMT^{S20}, and EMT^{S30}. In each digestion cycle, the substrates alone, all tannin treatments, and blanks were duplicated three times. There were four different cycles.

Statistical analysis

The SAS 9.4 (SAS Institute Inc., Cary, NC, USA) mixed model approach was used to statistically analyze the data from the current study. The model statement was stated in equation (5.3) below:

$$Y_{hijk} = \mu + C_h + S_i + T_j + R_k + (ST)_{ij} + \varepsilon_{hijk} \qquad (5.3)$$

Where, Y_{ijk} = mean of observation, μ = overall mean, C_h = effect of incubation cycle (run), S_i = effect of substrates (ECH and TMR), T_j = effect mimosa tannins (UMT, EMT^{S10}, EMT^{S20} and EMT^{S30}), R_k = effect of run within treatment, $(ST)_{ij}$ = effect of interaction between substrate and tannin, and ε_{hijk} = residual error. Substrate, tannin and substrate – tannin interactions were set at fixed effects, while run within treatment and incubation cycle were specified as random effects. Tukey was used to separate the means with significant differences at the 5% level of probability.

5.3 Result

The results of the *in vitro* tests on *Eragrostis curvula* hay (ECH) and total mixed rations (TMR) with encapsulated mimosa tannin in sunflower oil (EMT^{S}) at 10 (EMT^{S10}), 20 (EMT^{S20}), and 30 g/kg DM (EMT^{S30}) as well as unencapsulated mimosa tannin at 20 g/kg DM of feed (UMT) are shown in Table 5.1. In terms of the production of gas, methane (CH_4), total volatile fatty acids (TVFA), ammonia nitrogen (NH_3N), and *in vitro* organic matter digestibility (IVOMD), the results showed that the effect of interaction between substrate type and mimosa tannin extracts was not significant. Irrespective of the addition of mimosa tannin extracts, the proportion of total gas, CH_4, IVOMD, NH_3N and TVFA were higher ($p < 0.05$) in the TMR

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than the ECH substrate. The primary outcome of tannin extracts showed that addition of both EMT^{S} and UMT considerably reduced gas, CH_4, and IVOMD. However, NH_3N and TVFA were not impacted by EMT^{S} and UMT ($p > 0.05$).

In general, compared to substrates alone, UMT inclusion reduced total gas and CH_4 yields by 13.2% and 16%, respectively, for ECH, and a decrease of 14% and 13.2%, respectively for TMR diet. While, inclusion of EMT^{S20} resulted in 11% reduction in gas and 18.4% in CH_4 compared with mainly ECH diet, and lead to 15% and 13.9% decline in gas and CH_4, respectively for TMR substrate. Furthermore, addition of EMT^{S30} reduced total gas and CH_4 by 14.2% and 19.2%, respectively, for ECH substrate, while, gas and CH_4 dropped by 18% and 19.7%, respectively related to TMR diet alone. However, for both substrates, IVOMD decreased ($p = 0.01$) only at 30 g/kg inclusion level of encapsulated mimosa tannin. Addition of EMT^{S30} resulted in 3.6% and 4.9% reduction in IVOMD compared to ECH and TMR diets, respectively.

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Table 5.1 Effects of mimosa tannin encapsulated in sunflower oil (EMT^S) and unencapsulated mimosa tannin (UMT) and on *in vitro* organic matter digestibility, gas, methane and rumen fermentation characteristics of total mixed ration (TMR) and *Eragrostis curvula* hay (ECH) diets.

Treatment [a]		Gas (mL/g)	CH_4 [b] (mL/g)	IVOMD [c] (g/Kg)	NH_3N [d] (mg/100 mL)	TVFA [e] (mmol/L)
ECH						
Control		184.1^a	5.3^a	570.6^a	17.0	81.3
UMT		159.7^b	4.5^{bc}	563.5^a	13.8	84.7
EMT^{S10}		177.6^a	4.7^b	565.3^a	13.8	84.1
EMT^{S20}		163.8^b	4.3^c	563.1^{ab}	16.1	85.8
EMT^{S30}		157.9^b	4.3^c	549.9^b	15.2	95.9
TMR						
Control		199.1^a	5.5^a	727.6^a	25.0	98.6
UMT		171.2^b	4.8^b	714.7^a	21.4	89.0
EMT^{S10}		197.9^a	5.3^a	717.9^a	21.9	87.8
EMT^{S20}		169.6^{bc}	4.8^b	711.6^a	22.7	91.5
EMT^{S30}		162.6^c	4.5^c	691.7^b	23.7	89.9
SEM		5.69	0.20	0.75	2.21	6.37
p – values [f]	S	<0.01	<0.01	<0.01	<0.01	0.04
	T	<0.01	<0.01	0.01	0.10	0.41
	S*T	0.08	0.15	0.74	0.96	0.08

[a] UMT = unprotected mimosa tannin at 20 g/kg DM; EMT^{S10}, EMT^{S20} & EMT^{S30} = mimosa tannin encapsulated in sunflower oil at inclusion level of 10, 20 & 30 g/kg DM, respectively; SEM = standard error of mean.

[b] CH_4 = methane.

[c] IVOMD = *in vitro* organic matter digestibility.

[d] NH_3N = ammonia nitrogen.

[e] TVFA = total volatile fatty acids.

[f] *p* - values: S = substrate effect; T = tannin effect; S*T = effect of interaction of substrate and tannin. Mean values with uncommon superscripts across similar column differed significantly ($p < 0.05$).

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The ratios of gas, CH_4, IVOMD and TVFA from ECH and TMR substrates supplemented with UMT, EMT^{S10}, EMT^{S20} and EMT^{S30} is shown in Table 5.2. The effects of interaction between the substrates and mimosa tannin types were recorded on gas per unit IVOMD (p = 0.01) and CH_4 per unit TVFA (p = 0.04).

Table 5.2 Ratios of gas, methane (CH_4), *in vitro* organic matter digestibility (IVOMD) and total volatile fatty acids (TVFA) as influenced by the inclusion of encapsulated mimosa tannin in sunflower oil (EMT^S) and unencapsulated mimosa tannin (UMT).

Treatment [a]		**CH_4/Gas**	**CH_4/IVOMD (mL/g)**	**Gas/IVOMD (mL/Kg)**	**CH_4/TVFA**	**Gas/TVFA**
ECH						
Control		0.029^{a}	0.094^{a}	3.23^{a}	0.066^{a}	2.27^{a}
UMT		0.028^{ab}	0.079^{b}	2.83^{c}	0.053^{b}	1.89^{ab}
EMT^{S10}		0.026^{c}	0.082^{b}	3.14^{ab}	0.056^{b}	2.13^{ab}
EMT^{S20}		0.027^{bc}	0.077^{b}	2.91^{bc}	0.051^{b}	1.92^{ab}
EMT^{S30}		0.027^{bc}	0.078^{b}	2.87^{bc}	0.047^{b}	1.70^{b}
TMR						
Control		0.028^{a}	0.076^{a}	2.74^{a}	0.057^{ab}	2.03^{ab}
UMT		0.028^{a}	0.068^{b}	2.40^{b}	0.055^{ab}	1.94^{bc}
EMT^{S10}		0.027^{b}	0.074^{a}	2.76^{a}	0.062^{a}	2.31^{a}
EMT^{S20}		0.028^{a}	0.067^{b}	2.38^{b}	0.052^{ab}	1.87^{c}
EMT^{S30}		0.028^{a}	0.065^{b}	2.35^{b}	0.050^{b}	1.81^{c}
SEM		0.001	0.004	0.10	0.004	0.10
p – values[b]	S	0.36	<0.01	<0.01	0.61	0.85
	T	0.01	<0.01	<0.01	<0.01	<0.01
	S*T	0.09	0.14	0.01	0.04	0.16

[a] UMT = unprotected mimosa tannin at inclusion level of 20 g/kg DM; EMT^{S10}, EMT^{S20} & EMT^{S30} = mimosa tannin encapsulated in sunflower oil at inclusion level of 10, 20 & 30 g/kg DM, respectively; SEM = standard error of mean.

[b] p - values: S = substrate effect; T = tannin effect; S*T = effect of interaction of substrate and tannin. Mean values across similar column with uncommon superscripts differed significantly ($p < 0.05$).

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However, there were no significant interaction between substrate and tannin sources on gas/TVFA, CH_4/gas and CH_4/IVOMD. Regardless of the tannin inclusion, CH_4/IVOMD and gas/IVOMD were less ($p < 0.01$) in the TMR than ECH substrate. The main effect showed statistical influence of UMT and EMT^S on CH_4/gas, CH_4/IVOMD, gas/IVOMD, CH_4/TVFA and gas/TVFA. The addition of UMT had no impact on CH_4/gas in either substrate when compared to control ($p > 0.05$). Nevertheless, for both ECH and TMR feeds, addition of UMT decreased gas/IVOMD significantly. In addition, UMT reduced ($p < 0.01$) CH_4/IVOMD and CH_4/TVFA for ECH, while, for TMR substrate, only CH_4/IVOMD was affected by UMT.

Generally, inclusion of UMT reduced CH_4/IVOMD by 16% compared to ECH alone and 10.5% decline related to mainly TMR, while, CH_4/TVFA dropped by 19.7% for ECH and 3.5% for TMR, whereas, UMT decreased the concentrations of gas/IVOMD by 12.4% for both substrates. Equally, the proportion of CH_4/gas was lower ($p < 0.01$) at EMT^{S10} for TMR substrate while, for ECH diet CH_4/gas declined at all dosages of EMT^S. Compared with substrate alone, all inclusion levels of encapsulated mimosa reduced CH_4/gas, CH_4/IVOMD and CH_4/TVFA significantly for ECH diet. For TMR substrate, CH_4/gas decreased ($p < 0.01$) at EMT^{S10}, while, CH_4/IVOMD dropped at EMT^{S20} and EMT^{S30} doses, whereas, CH_4/TVFA decreased ($p < 0.01$) at EMT^{S30} only. For both ECH and TMR substrates, gas/IVOMD was significantly influenced by the addition of EMT^{S20} and EMT^{S30}. However, gas/TVFA dropped ($p < 0.01$) at EMT^{S30} for ECH diet and at EMT^{S20} and EMT^{S30} for TMR substrate. Compared to substrates alone, Addition of EMT^{S10} reduced CH_4/gas by 10.5%, CH_4/IVOMD by 12.8%, CH_4/TVFA by 15.2% and gas/TVFA by 6.2%, for ECH substrate, while, for TMR diet, EMT^{S10} decreased CH_4/gas and CH_4/IVOMD by 3.6% and 2.6%, respectively. Likewise, compared to ECH diet alone, inclusion of EMT^{S20} resulted in 6.9% reductions in CH_4/gas, 18.1% in CH_4/IVOMD, 9.9% in gas/IVOMD, 22.7% in CH_4/TVFA and 15.4% in gas/TVFA. However, for TMR substrates, EMT^{S20} led to decline in 11.8% CH_4/IVOMD, 13.4% gas/IVOMD, 8.8% CH_4/TVFA and 7.9% gas/TVFA. Furthermore, addition of EMT^{S30} reduced CH_4/gas by 6.9%, CH_4/IVOMD by 17%, gas/IVOMD by 11.2%, CH_4/TVFA by 28.8% and gas/TVFA by 21.1% when compared with mainly ECH substrate. Nevertheless, in relation to TMR diet alone, EMT^{S30} decreased CH_4/IVOMD by 14.5%, gas/IVOMD by 14.2%, CH_4/TVFA by 12.3% and gas/TVFA by 10.8%.

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The summary of gas production indices from ECH and TMR substrates incubated with unencapsulated and various levels of encapsulated mimosa tannin extracts (Table 5.3).

Table 5.3 Influence of encapsulated mimosa tannin in sunflower oil (EMT^{S}) and unencapsulated mimosa tannin (UMT) on gas production indices from total mixed ration (TMR) and *Eragrostis curvula* hay (ECH) substrates.

Treatment [a]		**b (mL/g)**[b]	**c (mL/h)**[c]	**EGP (mL/g)**[d]
ECH				
Control		241.8^{a}	0.032	90.0^{a}
UMT^{20}		236.8^{a}	0.025	77.4^{ab}
EMT^{S10}		227.0^{ab}	0.033	89.8^{a}
EMT^{S20}		230.4^{ab}	0.025	81.0^{ab}
EMT^{S30}		206.6^{b}	0.028	70.3^{b}
TMR				
Control		232.8^{a}	0.039	104.1^{a}
UMT^{20}		204.3^{bc}	0.038	87.6^{b}
EMT^{S10}		225.7^{a}	0.040	104.3^{a}
EMT^{S20}		208.1^{b}	0.036	84.7^{b}
EMT^{S30}		195.0^{c}	0.037	82.9^{b}
SEM		11.96	0.003	3.48
p – values[e]	S	0.01	<0.01	<0.01
	T	0.01	0.06	<0.01
	S*T	0.49	0.49	0.36

[a] UMT = unprotected mimosa tannin at inclusion level of 20 g/kg DM; EMT^{S10}, EMT^{S20} & EMT^{S30} = mimosa tannin encapsulated in sunflower oil at inclusion level of 10, 20 & 30 g/kg DM, respectively; SEM = standard error of mean.

[b] b = gas volume from slowly fermentable fraction.

[c] c = rate of fermentation of fraction b.

[d] EGP = effective gas production.

[e] *p* - values: S = substrate effect; T = tannin effect; S*T = effect of interaction of substrate and tannin. Mean values with uncommon superscripts across similar column differed significantly ($p < 0.05$).

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The results demonstrated that the interaction between substrate type and mimosa tannin extracts had no appreciable impact on effective gas production (EGP), gas volume from slowly fermentable fibre fractions (b), or fermentation rate (c). However, the main effect of substrate indicated that 'b' was higher ($p < 0.01$) in ECH diet, while EGP and 'c' were significantly higher in TMR substrate. The simple effect of encapsulated and unencapsulated tannin extracts, revealed that only EMT^{S30} affected 'b' and EGP ($p < 0.01$) for ECH, while for TMR diet, 'b' and EGP reduced significantly at UMT and EMT^{S20} – EMT^{S30} compared with controls. However, only a tendency for decline in 'c' was recorded ($p = 0.06$) after inclusion of UMT, EMT^{S20} and EMT^{S30} in both substrates.

In general, compared with control, UMT reduced b, c and EGP outputs by 2.1%, 21.9% and 14%, respectively, for ECH and 12%, 2.6% and 15.9%, respectively for TMR substrate. Whereas, addition of EMT^{S20} led to 4.7% reduction in b, 21.9% in c and 10% in EGP, related to ECH alone, and resulted in 10.6% 7.7% and 18.6% decline in b, c and EGP, respectively compared to TMR substrate. Also, incubation of EMT^{S30} decreased the concentrations of b, c and EGP by 14.6%, 12.5% and 21.9%, respectively, for ECH substrate, while, b, c and EGP declined by 16.2%, 5.1% and 20.4%, respectively for TMR diet.

Table 5.4 illustrates the impact of encapsulated and non-encapsulated tannin extracts on the molar proportion of volatile fatty acids (VFAs) in total mixed ration and *Eragrostis curvula* hay substrates. The findings showed that for all VFA concentrations and the acetate: propionate ratio, there were no impacts ($p > 0.05$) of the interaction between substrate type and tannin extract. According to the main effect of tannins, addition of both EMT^{S} and UMT had no effect on the molar concentrations of the VFAs, although there was a propensity for tannin influence on the iso-butyrate and acetate: propionate ratio ($p = 0.05$) regardless of the substrate type. The acetate to propionate ratio and all other molar proportions of VFAs, with the exception of propionic acid, were, nevertheless, significantly influenced ($p = 0.01$) by the substrate type.

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Table 5.4 Mean volatile fatty acids from total mixed ration and *Eragrostis curvula* hay as affected by the inclusion of encapsulated mimosa tannin in sunflower oil (EMT^{S}) and unencapsulated mimosa tannin (UMT).

Treatment [a]		**AA**[b]	**PA**[c]	**IBA**[d]	**BA**[e]	**IVA**[f]	**VA**[g]	**AA: PA**[h]
ECH								
Control		73.0	15.8	1.5	5.7	2.3	1.7	4.6
UMT^{20}		72.1	16.1	1.5	5.9	2.5	1.9	4.5
EMT^{S10}		71.4	16.2	1.6	5.4	2.6	1.9	4.4
EMT^{S20}		72.7	15.5	1.5	6.0	2.5	1.8	4.7
EMT^{S30}		71.5	15.9	1.8	6.3	2.6	1.9	4.5
TMR								
Control		70.1	15.3	1.7	7.8	3.0	2.2	4.7
UMT^{20}		68.3	16.7	1.8	8.0	3.0	2.1	4.1
EMT^{S10}		68.3	16.4	1.7	7.9	3.3	2.5	4.2
EMT^{S20}		69.8	16.3	1.6	7.6	2.8	1.9	4.3
EMT^{S30}		68.5	16.7	1.7	8.1	2.9	2.1	4.1
SEM		0.44	0.40	0.07	0.28	0.15	0.12	0.13
p-values[i]	S	<0.01	0.18	0.01	<0.01	<0.01	<0.01	0.01
	T	0.10	0.19	0.05	0.43	0.23	0.14	0.05
	S*T	0.85	0.38	0.40	0.74	0.40	0.24	0.40

[a] UMT = unprotected mimosa tannin at inclusion level of 20 g/kg DM; EMT^{S10}, EMT^{S20} & EMT^{S30} = mimosa tannin encapsulated in sunflower oil at inclusion level of 10, 20 & 30 g/kg DM, respectively; SEM = standard error of mean.

[b] AA = Acetic acid;

[c] PA = Propionic acid;

[d] IBA = Iso-butyric acid;

[e] BA = butyric acid;

[f] IVA = Isovaleric acid;

[g] VA = Valeric acid;

[h] AA: PA = Acetic acid: Propionic acid ratio.

[I] *p* - values: S = substrate effect; T = tannin effect; S*T = effect of interaction of substrate and tannin. Mean values with uncommon superscripts across similar column differed significantly ($p < 0.05$).

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5.4 Discussion

The amount of *in vitro* gas and CH_4 was significantly reduced in this study when both encapsulated and unencapsulated acacia tannins were added at a dosage of 20 g/kg without affecting the substrates' digestibility or rumen fermentation characteristics. This suggests that mimosa tannins' influence may be linked to their interference with methanogens activities. Tannins' ability to inhibit the production of methane was attributed to their ability to either directly poison the bacteria that produce it or indirectly reduce the degradation of fiber and the digestion of organic materials (Animut *et al.*, 2008; Patra, 2012; Hristov *et al.*, 2013). The present finding compares favourably with the report of Ibrahim and Hassen (2022), who obtained approximately 7.4%, 6.9% and 6.6% reduction in gas as well as 16%, 12.9% and 10.8% decrease in CH_4 after incubation of 2% DM level of pentanol purified, ethyl acetate purified and unpurified mimosa tannin extracts, respectively. Furthermore, Adejoro *et al.* (2018), observed that inclusion of mimosa tannins encapsulated with lard and palm oil reduced *in vitro* gas by 6.8% and 7.2%, respectively, while CH_4 dropped by 16.3% and 12.4%, respectively. However, some studies found that mimosa tannin included at lower levels (less than 50 g/kg of feed DM) did not have any effect on the concentrations of gas, methane, ammonia nitrogen, and total volatile fatty acids (Hassanat and Benchaar, 2013; Adejoro *et al.*, 2020). According to numerous reports, the dosage of extracts supplied affects how much CH_4 and rumen microbial fermentation occur as a result of tannin supplementation (Patra and Saxena, 2011; Jayanegara *et al.*, 2012; García *et al.*, 2017).

The TMR diet had higher levels of CH_4, IVOMD, NH_3N, and TVFA than the ECH substrate. This might be explained by an increase in the rumen's microbial population, which would speed up fermentation in a diet high in protein. In agreement with our finding, earlier investigations also reported higher CH_4 and rumen fermentation characteristics in high protein based substrate than fibre based diet (Hassanat and Benchaar, 2013; Adejoro *et al.*, 2018). Akanmu *et al.* (2020), also recorded lower concentrations of total gas, methane, digestibility, total volatile fatty acids and ammonia nitrogen in *Eragrostis curvula* hay supplemented with some medicinal plant extracts when compared with total mixed ration and Lucerne hay. However, higher methane output was observed in ruminants fed poor quality forages (Forabosco *et al.*, 2017; Haque, 2018). The discrepancy with regards to influence of different tannin sources on methane and rumen fermentation parameters could be linked to variations in their structure and biological activity.

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Moreover, addition of 20 g/kg level of encapsulated mimosa tannin in sunflower oil resulted in less CH_4 volume compared to equivalent dose of non-encapsulated mimosa tannin. This could be traced to the inherent bioactive properties of sunflower oil which might complement the tannin towards suppressing the methanogens. A substantial decrease in methane and ammonia nitrogen productions were reported when sunflower oil was supplemented at 50 g/kg of feed DM in ruminants (Eckard *et al*., 2010).

By and large, interaction of diet and mimosa tannin sources were recorded on CH_4/TVFA and gas/IVOMD. In addition, the concentrations of CH_4/IVOMD and gas/IVOMD were greater in high fibre diet (ECH) than in high protein substrate (TMR). This further confirmed that the methane mitigation effects of encapsulated and unencapsulated mimosa tannin were more pronounced in suppressing the proliferation and activities of *Methanogenic archaea* than their impact on fibre and dry matter degradation. Tannin additives are reported to reduce CH_4 production by deterring the multiplication of methanogens or via decrease in feed digestion and interference with the activities of protozoa (Goel *et al*., 2011; Jayanegara *et* al. 2012). The findings of the current study correspond with the earlier reports on acacia tannins (Adejoro *et al*., 2018), and some medicinal plant extracts (Akanmu *et al*., 2020).

In both total mixed ration and *Eragrostis curvula* hay diets, EMT^{S20} produced less CH_4 per unit IVOMD and CH_4 per unit TVFA compared to UMT. This might be associated to the excellent emulsification of sunflower oil which ensured slowed and sustained tannin dissolution during incubation process. Thus, encapsulated tannin offered greater efficiency in terms of rumen fermentation process than the unencapsulated mimosa. In line with this finding, Ibrahim and Hassen (2022), recorded lower percent reductions (–8%) in CH_4/IVOMD when unprotected mimosa tannin extract was incubated together with *Eragrostis curvula* hay at 20 g/kg DM.

The current findings showed that while the rate of fermentation and effective gas production were higher in the total mixed ration diet, the volume of gas from the slowly fermentable fraction was higher in the eragrostis hay substrate. This might be explained by an increase in rumen fermentation activity brought on by a high protein diet's increased microbial population. Moreover, addition of both EMT^{S20} and UMT resulted in lower proportion of ‘b’ and EGP. Tannins are well recognized to inhibit rumen microbial development and activity as well as potential gas production by decreasing feed digestibility (Beauchemin *et al.*, 2007; Patra and

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Saxena, 2010). In agreement with our findings, Ibrahim and Hassen (2022) found that adding pentanol-, ethyl-, and unpurified mimosa tannin extracts to an *Eragrostis curvula* hay substrate resulted in a decrease in b. On the other hand, when encapsulated mimosa tannins in palm oil and lard were added individually to either eragrostis or total mixed ration substrate, Adejoro *et al*. (2018) observed an increase in b. The lower tannin release rate seen for palm oil (20%) and lard microparticles (19%) compared to the tannin concentration released by sunflower oil microcapsule (22%) employed in the present investigation may be responsible for the increase in gas volume from the slowly fermentable fraction found by the author.

Acetic acid and acetate: propionate ratios were higher in ECH diet, while, iso-butyric, butyric, iso-valeric and valeric acids were greater in TMR substrate. It is widely known that the rumen microorganisms' ability to multiply and carry out their various functions is heavily influenced by the substrate (Hassanat and Benchaar, 2013; Adejoro *et al*., 2018). However, several studies (Min *et al*., 2003; Beauchemin *et al.*, 2008) found that lower inclusion levels of tannins (50 g/kg DM) had little to no effect on rumen fermentation parameters. Our findings from the current investigation support this claim. Similar to this, Hervás *et al.* (2003a) and Getachew *et al.* (2008), reported that adding tannin to ruminants' diets in amounts less than 50 g/kg DM had no impact on the concentration of volatile fatty acids. However, Hassanat and Benchaar (2013), found that incubating *Acacia mearnsii* tannin extracts at 5% of the substrate DM resulted in significant decreases in the molar concentrations of acetic acid, butyric acid, valeric acid, iso-butyric acid, and iso-valeric acid. Likewise, inclusion of higher dosages of condensed tannins in the feed was found to decrease the volume of branched-chain volatile fatty acids (Singh *et al.*, 2005). Furthermore, addition of appreciable amount of tannin extracts were reported to depress acetate by poisoning the acetic acid producing bacteria without affecting propionate formation leading to decrease in acetic: propionic acid ratio (Beauchemin *et al.*, 2007; Castro-Montoya *et al*., 2011).

5.5 Conclusion

Following the outcomes of this investigation, inclusion of both encapsulated and non-encapsulated mimosa tannin at a dosage of 20 g/kg diet DM decreased methane and gas production by a significant amount while having no effect on the production of ammonia nitrogen, total volatile fatty acids, or the digestibility of substrates. *In vitro* gas, CH_4, IVOMD, NH_3N, TVFA, b and EGP were higher in total mixed ration diet compared to *Eragrostis*

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curvula hay substrate. In comparison to unprotected mimosa tannin at inclusion level of 20 g/kg feed DM, encapsulated mimosa tannin produced less CH_4 volume, CH_4 per unit IVOMD, and CH_4 per unit TVFA.

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CHAPTER SIX

Effect of non-encapsulated and encapsulated mimosa (*Acacia mearnsii*) tannins on growth performance, nutrient digestibility, methane and rumen fermentation of South African mutton Merino ram lambs

Abstract

The use of mimosa tannins as feed additives in contemporary ruminant nutrition studies has gained wider acceptance, because of their potential to reduce enteric methane (CH_4) and enhance dietary protein utilization. However, tannin's astringency and quick dissolution decrease feed intake and digestibility. Microencapsulation technology could be adopted to neutralize the astringency and ensure controlled tannin solubility across ruminant digestive tract. The present study examined the influence of supplementing unencapsulated and encapsulated Mimosa tannins on performance, digestibility, CH_4 and rumen fermentation in South African mutton Merino sheep. A total of 40 weaned ram lambs of 96 days old (34 – 35 kg body weight, BW) were randomly allocated to one of the four dietary treatments in a RCBD as follows: total mixed ration, (T1, TMR alone); TMR + Monensin at 75 mg/kg DM (T2, Monensin); TMR + unencapsulated Mimosa tannin at 20 g/kg DM (T3, UMT) and TMR + encapsulated Mimosa tannin in sunflower oil at 20 g/kg DM equivalent (T4, EMT^S). The lambs' feed intake and weight changes were recorded followed by digestibility and methane measurement. Finally, rumen fluids were sampled and analysed for volatile fatty acids (VFAs) and ammonia nitrogen (NH_3N). Compared with the TMR alone, UMT and EMT^S increased intake of DM (T1, 1328 g/day vs T3, 1578 and T4, 1569 g/day; P = 0.05), NDF (T1, 344 g/day vs T3, 434 and T4, 416 g/day; P = 0.01) and ADF (T1, 174 g/day vs T3, 241 and T4, 218 g/day; P = 0.02). However, only UMT decreased (P = 0.01) digestibility of DM (T3, 640 g/kg vs T1, 746 g/kg) and CP (T3, 734 g/kg vs T1, 829 g/kg). Furthermore, UMT increased (P = 0.01) faecal N excretion in g/head/day (T3, 13.7 vs T1, 8.03). UMT and EMT^S reduced CH_4 in g/kg DM-intake (T3, 15.3 and T4, 14.8; vs T1, 18.9; P = 0.04). However, NH_3N and total VFAs were not influenced (P > 0.05) by the inclusion of UMT and EMT^S. It was concluded that UMT and EMT^S could be utilized in mitigating enteric CH_4 while enhancing nutrient intake when supplemented at 20 g/kg DM of feed. Further studies should be conducted on the total tannin concentration of the encapsulated mimosa tannin in sunflower oil.

Keywords: Encapsulation; Merino lambs; methane; mimosa tannin; nutrient digestibility; rumen fermentation.

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6.1 Introduction

Mitigation of enteric methane (CH_4) emission became a principal area of recent investigations in ruminant nutrition, because of the significant influence of CH_4 to global warming and loss of dietary energy. Methane is produced in the rumen by the bacteria known as *Methanogenic archaea*, using carbon dioxide, CO_2 and hydrogen gas, H_2 (Mcallister and Newbold, 2008). About 95% of enteric CH_4 is produced in the rumen which account for about 70 – 100 tera-gram of CH_4 yield annually (Gerber *et al.*, 2013), and around 12% of animals' energy loss (Piñeiro-Vázquez *et al*., 2015). This led to the adoption of numerous dietary modifications to reduce methane emission by the ruminants which include improved forage quality, plant breeding and dietary additives such tannins, saponins, ionophores among others (Eckard *et al*., 2010). Monensin, the most extensively used ionophores which improve feed efficiency and with a methane mitigation potential, is a polyether antibiotic, which is orally fed as a sodium salt (Yang *et al*., 2007). However, in 2006, the European Union (EU) banned the use of antibiotics as feed additive due to its residual effects on human health (Millet and Maertens, 2011). Thus, utilization of plant secondary substances especially tannins gained more acceptance, because of their efficiency, safety and availability (Soltan *et al.*, 2013).

Tannins are high molecular weight polyphenolic compounds classified into hydrolysable and condensed tannins based on their chemical structure and properties (Hassanpour *et al.*, 2011). Nevertheless, condensed tannins are more extensively studied because of their abundance, anti-methanogenic activities and non-toxic to animals compared to hydrolysable tannins (Martin *et al*., 2010). *Acacia mearnsii* popularly known as Mimosa or Black wattle tannin is reported to be a rich source of both condensed and hydrolysable tannins (Bhatta *et al.*, 2009), and is widely available in Southern Africa (Galatowitsch and Richardson, 2005). Numerous studies revealed that Mimosa tannin extracts were successfully utilized in manipulating rumen fermentation, methane emission and protein metabolism in ruminants, but with some adverse effects on intake and digestibility because of its astringency and instability across various pH of the gastrointestinal tract (Carulla *et al.*, 2005; Grainger *et al.*, 2009), especially when fed at higher dosage ($\geq$ 50 g/kg DM).

Hence, microencapsulation of Mimosa tannin using oily substances could mask the bitter taste thereby reducing CH_4 without adverse effects on intake and digestibility. Among the widely available lipids, sunflower oil is found in abundance and could serve as suitable encapsulant

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for tannin due to its low viscous nature and excellent emulsification (Flanagan *et al*., 2006) as well as its tasteless and desirable aroma (Bakry *et al.*, 2016), in addition to its CH_4 abatement potential (Eckard *et al*., 2010). Recent *in vitro* studies have shown that Mimosa tannins encapsulated in either palm oil or sunflower oil reduced CH_4 yields (Adejoro *et al*., 2018), and slowed down quick tannin solubility in various pH simulating rumen, abomasum and small intestine (Adejoro *et al*., 2018; Ibrahim and Hassen, 2021). In addition, the use of encapsulated mimosa tannin as additive has been found to mask the tannins' astringency (Adejoro *et al*., 2020). However, to the best of our knowledge there is little or no data available on the long term effects of lipid-based encapsulated mimosa tannin on animal performance. The present study, therefore, aimed at evaluating the influence of unencapsulated and encapsulated mimosa tannin in sunflower oil on feed intake, growth performance, nutrient digestibility, methane and rumen fermentation of South African mutton Merino ram lambs.

6.2 Materials and Method

Animal ethics

The animal management procedure followed during this investigation was reviewed and approved by the animal ethics committee of the University of Pretoria with approval number: EC075-17. The committee acted in accordance with the recommendations specified in the South African National Standard 10,386 on the handling of animals for research purposes. This study was carried out at the small ruminant section of the University of Pretoria experimental farm (Pretoria, South Africa).

Preparation of experimental feed additives

The mimosa tannin used as feed additive in this study was obtained from UCL Company (Pty) Ltd. Dalton, South Africa. Before the experiment, the tannin powder was analysed in the laboratory to ascertain the concentrations of its total tannin (TT) following the procedure of Makkar *et al*. (1993), condensed tannin (CT) as described by Porter *et al*. (1986) and hydrolysable tannin (HT) according to the method of Singh *et al.* (2005). The tannin powder constituted of 677.6 g/kg DM of TT, 221.7 g/kg DM of CT and 463.8 g/kg DM of HT (Ibrahim and Hassen, 2022). The mimosa tannin was then encapsulated with sunflower oil using solid-in-oil-water method according to Ibrahim and Hassen (2021). Briefly, solid-in-oil portion was reconstituted by weighing 8.5 g of the tannin into a beaker containing 30 mL sunflower oil solution in dichloromethane (50 mg/mL), added with 0.5% (w/v) of Span80 and agitated for

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two minutes using magnetic stirrer set at 400 revolutions per minute, rpm. While, the aqueous solution was formed by mixing 300 mL water and 1% (w/v) of Tween80 using an iron rod homogenizer (PRO400DS, Pro Scientific Inc., Oxford, CT 06478, USA) at 20,000 rpm for three minutes. The two solutions were added together and agitated for three hours using a magnetic stirrer set at 800 rpm to evaporate the dichloromethane. The encapsulated tannin was squeezed using cheese cloth, rinsed with water and freeze-dried for 5 days. The dried mimosa tannin encapsulated in sunflower oil was ground to powder using pestle and mortar, and refrigerated before use.

Animals, experimental design and treatments

A total of 40 South African mutton Merino ram lambs of 96 days old (34 – 35 kg body weight) were used for the experiment. The lambs were grouped based on age and allocated to four dietary treatments in five blocks each with two lambs per cage in a randomised complete block design. The treatments were T1: Total mixed ration only, TMR (negative control); T2: TMR + Monensin @ 75 mg/kg DM of feed (positive control) based on manufacturers' recommendation; T3: TMR + Unencapsulated Mimosa tannin @ 20 g/kg DM (UMT) and T4: TMR + Encapsulated Mimosa tannin in sunflower oil @ 20 g/kg DM equivalent (EMT^S).

Dietary composition and growth performance trial

The diet adopted in this study was a total mixed ration formulated by a commercial feed company (AFGRIFEEDS Ltd, South Africa) to meet the growth and maintenance of growing lambs using Agricultural Modelling and Training System (AMTS) program which uses NRC (2007) standard as shown in Table 6.1. The diet was formulated to meet an average daily gain (ADG) of around 250 g/head/day. To facilitate intake and limit selection, all diets were thoroughly blended after mixing all the ingredients using a vertical mixer.

The animal trial took place between 20th of August 2019 to 24th January of 2020. The lambs were first adapted to the experimental diets for 4 weeks, during which the diets were gradually introduced to the lambs at 25 %, 50 %, 75 % and 100 % in 4 successive weeks, to replace the commercial pellets earlier fed to the animals. Following the adaptation period, the lambs were given their individual diets for a continuous period of eleven (11) weeks. The experimental diets were administered in the morning (07h00) and afternoon (16h00) with a left-over allowance of 2 %, while clean water was given *ad libitum*. During the growth trial, the average

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feed intake of the two lambs in each pen for the five blocks of four treatments were recorded on daily basis by subtracting the orts from the feed offered, while the individual lamb body weight was recorded every week using electric weighing scale. Feed conversion ratio was obtained as the fraction of dry matter intake to average daily gain.

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Table 6.1 Ingredients and Chemical Composition of the Experimental Diets fed to South African mutton Merino Sheep

Ingredient (g/kg)	**Control**	**Monensin**	**UMT**	**EMT^{S}**
Soybean meal	170	170	167	165
Yellow maize	280	280	275	272
Medicago sativa hay	200	200	196	194
Eragrostis curvula hay	227	227	223	221
Molasses	60.0	60.0	58.8	58.3
Wheat offal	50.0	50.0	49.0	48.6
Urea	8.00	7.99	7.84	7.77
Vitamin premix[a]	5.00	5.00	4.90	4.86
Monensin[b]	0.00	0.75	0.00	0.00
UMT[c]	0.00	0.00	19.6	0.00
EMT^{Sd}	0.00	0.00	0.00	28.2
Total volume	1000	1000	1000	1000
Chemical composition (g/kg)				
Dry matter	935	935	934	938
Organic matter	877	878	881	887
Crude Protein	217	215	201	207
Neutral Detergent Fibre	267	274	283	277
Acid Detergent Fibre	135	152	156	146

[a] Vitamin premix provided the following per kilogram diet: vit A, 18,000 iu; vit D, 3920 iu; vit E, 2.45 iu; Zn, 5.0 mg; Mn, 4.1 mg; Cu, 0.5 mg; Se, 0.2 mg; Mg, 28 mg; and Co, 0.3 mg.

[b] Monensin: @ 75 mg/kg DM of feed.

[c] UMT: unencapsulated Mimosa tannin @ 20 g/kg DM of feed.

[d] EMT^{S}: encapsulated Mimosa tannin in sunflower oil @ 20 g/kg DM of feed equivalent.

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Nutrient digestibility and nitrogen balance trial

Immediately, after the growth performance experiment, a total of twenty (20) lambs (involving one representative animal from each pen for the 5 blocks of 4 dietary treatment group) were taken to separate metabolic cages and attached with faecal bags, while urine bottles were fitted to the mouth of urine pan underneath the cage. The lambs were adapted to the cages for a period of 7 days, thereafter total feed offered, orts, faeces and urine output were collected and recorded for 5 days. The feed, orts and faeces were sampled every day and kept at −20 °C in the laboratory. The urine collected inside the bottles were sampled in plastic container already added with Sulphuric acid (10 %, v/v) to prevent N-volatilization and then frozen. At the end of the 5-days sample collection, the individual ram diet, orts, faeces and urine were pooled across days and sub-sampled for chemical analysis. A representative sample of the feed, orts and faeces was measured and dried at 105 °C in the oven for 18 hours thereafter, analysed for initial dry matter, while the remaining samples were dried at 55 °C for 48 hours, milled to pass a 1mm sieve, and analysed for chemical composition.

Method of the Association of Official and Analytical Chemists, AOAC (2000) was adopted for the analyses of dry matter (934.01), ash (942.05) and crude protein (968.06). The feeds, orts and faeces samples were evaluated for dry matter and ash by oven-drying at 105 °C and ashed at 550 °C in a furnace. Leco instrument analyses the Nitrogen value in the sample, and a conversion factor is used to determine the CP. Neutral detergent fibre (NDF) and acid detergent fibre (ADF) were determined using ANKOM filter bag technique as described by Van Soest *et al*. (1991).

Methane quantification

At the end of the digestibility trial, lambs were moved to the small ruminant open-circuit respiratory chambers in 5 batches (4 animals per cycle) for methane evaluation following the procedure documented by Adejoro *et al*. (2020) with slight modification. Prior to the experiment, the whole room where methane chambers were installed was thoroughly cleaned and disinfected, the probes attached to the pipes were dusted to allow uninterrupted air flow, while each cage was cleaned and the floor was covered with mat to provide a conducive environment for the sheep. Methane recovery trials were carried out for each chamber by injecting a known concentration of standard methane (Methane Cylinder 8.1 kg N 3.5, AFROX LTD Germiston, GT, South Africa) to pre-calibrate the chambers a day before the

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commencement and the day after each cycle. Recovery percentages ranging from 76 % to 96 % were achieved, and used for correction.

During methane estimation, 4 lambs representing each dietary treatment (from the same block) were randomly assigned to an individual chamber and adapted for 24 hours followed by methane measurement in the 4 successive days, during which time the animals were moved to the next cage every 24 hours so that all the sheep had passed through the 4 chambers sequentially in order to reduce confounding effect associated with any variation in chambers and methane recovery. Experimental diets were offered to the lambs every morning and daily feed intake was recorded, while clean water was supplied *ad libitum*. Every morning, the chambers were cleaned, mat replaced and the animals locked for about 23 hours, during which hot wire anemometers (fitted with automatic data loggers) were used to monitor the speed of the airflow within each cage. The gas emitted by the sheep and the ambient air in front of each chamber over the 23 hours period were sucked using an 8-channel peristaltic pump (Masterflex 77292−50 L/S, Cole-Palmer Instr., IL, USA), set at time dispose mode with 2-minutes on and 2-minutes off and then collected in 10 litres SKC gas sampling bags (SKC SamplePro$^{(R)}$ FlexFilm Sample Bag, Inc. Johannesburg, South Africa). The PVC tubes (4 mm x 6 mm) firmly attached to luer-locked syringes were connected to each bag and sampled the gas manually in 6 replicates. The gas samples for each batch were immediately taken to the gas chromatography (GC) room and injected manually into the gas chromatography (8610C BTU Gas analyser GC System, SRI Instruments, Bad Honnef, Germany) fitted with a flame ionization detector. The methane peaks resulted from the gas sampled from 5 representative animals for each of the four dietary treatments were converted into methane concentration (mL) using standard curve generated with the Peak simple software.

Determination of rumen fermentation parameters

Following the methane estimation in the chamber, the lambs were taken to abattoir for slaughter. The sheep were rendered unconscious with the aid of an electrical stunner followed by immediate bleeding and evisceration using a sharp knife. The rumen was quickly removed and the content emptied and thoroughly mixed in a plastic bucket. Subsequently, 4 layers of cheesecloth was used to squeeze the rumen fluids into small plastic bucket. Syringes were used to sample 100 mL of rumen fluids into a bottle containing 20 mL of 25 % Orthophosphoric acid (for volatile fatty acid, VFA determination), and 90 mL fluid sampled in another bottle

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containing 15 mL of 0.5M Sulphuric acid (for ammonia nitrogen, NH_3N analysis) as described by Adejoro *et al*. (2020). All the rumen fluid samples were kept in cooler boxes containing ice blocks and immediately transported to the laboratory and stored in the freezer at –20 °C before analysis.

The rumen fluid samples for VFA analysis were defrosted and centrifuged at 4500 rpm for 15 minutes and, thereafter sieved into GC tubes using Micropore filter (0.45 µm). Samples were injected into GC (Shimadzu GC-2010 Tracera; Shimadzu corp., Kyoto, Japan) connected with a 30 m Inert Cap Pure Wax column with Barrier Ionization Discharge (BID) detector (Webb, 1994). While, rumen fluids for NH_3N determination were thawed, centrifuged and analysed using phenol-hypochlorite reagent spectrophotometry as described by Broderick and Kang (1980).

Statistical analysis

All statistical analyses were carried out using the general linear model procedure of SAS 9.4 (SAS Inst. Inc.; Cary, NC, USA). Average daily gain (ADG) of individual animal was obtained from the regression of the weekly body weight gains against time. The experimental design was randomized complete block design and the model used for data analysis was as follows:

$$y_{ij} = \mu + Block + A_i + \varepsilon_{ij}$$

where yij = observation j at different dietary additives (i; monensin, unencapsulated mimosa tannin or encapsulated mimosa tannin in sunflower oil); µ= overall mean; Block = effect of blocking (initial weight); Ai= effect of additives, and εij = random error with mean of 0 and variance σ2. Where significant differences existed, Tukey HSD was used for mean separation at $P \leq 0.05$.

6.3 Results

Growth performance

The influence of adding unencapsulated Mimosa tannins (UMT) and encapsulated Mimosa tannins in sunflower oil (EMT^S) on body weight gain and dry matter intake of South African mutton Merino ram lambs is summarised in Table 6.2. The results showed that inclusion of UMT and EMT^S did not affect ($P > 0.05$) individual lamb's final weight, total weight gain, dry

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matter intake (DMI), average daily gain (ADG) and feed conversion ratio (FCR) compared with total mixed ration alone and Monensin diet.

Nutrient intake and digestibility

Table 6.3 shows the effects of inclusion of UMT and EMT^S on nutrient intake and digestibility of South African mutton Merino ram lambs. The findings revealed that inclusion of UMT and EMT^S increased (P = 0.05) intake of feed dry matter, organic matter (P = 0.04), neutral detergent fibre (P = 0.01) and acid detergent fibre (P = 0.02) compared with control diet. However, intake of dietary protein was not influenced (P > 0.05) by the inclusion of both the non-encapsulated and encapsulated Mimosa tannin additives. Generally, addition of UMT and EMT^S at 20 g/kg raised the intake of dietary dry matter by 18.9% vs 18.2%, organic matter by 19.1% vs 18.8%, neutral detergent fibre by 26.3% vs 21.1%, and acid detergent fibre by 38.4% vs 25.1%, respectively.

With regards to nutrient digestibility, only UMT was found to reduce (P = 0.01) dry matter, organic matter and crude protein compared with the total mixed ration alone. In general, the digestibility of DM, OM and CP dropped by 14.2%, 14% and 11.4%, respectively, after inclusion of UMT at 20 g/kg of feed DM.

Nitrogen balance

The summary of nitrogen balance of South African mutton Merino sheep fed total mixed ration and added with either unencapsulated, encapsulated Mimosa tannins or Monensin is shown in Table 6.4. The results indicated that nitrogen intake, nitrogen excreted, urinary nitrogen and retained nitrogen were not affected (P > 0.05) by the inclusion of both the non-encapsulated and encapsulated Mimosa tannin additives. However, addition of UMT increased (P = 0.01) the excretions of faecal nitrogen (in g/head/day and g/kg N-intake) when compared with the control diet. Generally, addition of UMT raised nitrogen excretion in the faeces by 71.3% compared to lambs fed total mixed ration alone.

Methane quantification

Table 6.5 presents the effects of Monensin and Mimosa tannin additives on in vivo methane emission by South African mutton Merino sheep. The findings showed that both UMT and EMT^S reduced CH_4 in g/kg DM-intake (P = 0.04) compared to control diet. However, the effect of non-encapsulated and encapsulated Mimosa tannins additives were not significant with

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regards to CH_4 in g/kg NDF-intake, CH_4 in g/kg DM-digested and CH_4 in g/kg NDF-digested. Methane yield (in g/kg DM-intake) dropped by 19% and by 22% for UMT and EMT^S diets, respectively.

Rumen fermentation parameters

The influence of non-encapsulated and encapsulated Mimosa tannin on rumen fermentation parameters of South African mutton Merino ram lambs is presented in Table 6.6. The results revealed that all the additives had no effects ($P > 0.05$) on rumen ammonia nitrogen concentration. Similarly, total and individual volatile fatty acids were not affected ($P > 0.05$) by the inclusion of unencapsulated and encapsulated Mimosa tannins or Monensin.

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Table 6.2 Growth performance of South African mutton Merino ram lambs fed total mixed ration with Monensin and Mimosa tannin additives

	Dietary Treatments					
Parameters	**Control**	**Monensin[a]**	**UMT[b]**	**EMT[Sc]**	**SEM**	***p* – values**
Initial weight (kg)	34.6	34.8	34.4	35.4	1.88	0.56
Final weight (kg)	52.4	52.1	52.1	51.8	1.78	0.98
Total weight gain (kg)	17.7	17.3	17.5	16.5	0.67	0.67
Dry matter intake (g/day)	1400	1371	1256	1372	62.5	0.43
Average daily gain (g/day)	170	169	169	160	7.73	0.78
Feed conversion ratio	8.33	8.62	7.67	8.76	0.50	0.47

[a] Monensin: @ 75 mg/kg DM of feed.

[b] UMT: unencapsulated Mimosa tannin @ 20 g/kg DM of feed.

[c] EMT[S]: encapsulated Mimosa tannin in sunflower oil @ 20 g/kg DM of feed equivalent.

SEM = Standard error of mean. Mean values are based on 2 animals per block and 5 blocks per treatment.

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Table 6.3 Nutrient intake and apparent digestibility of South African mutton Merino ram lambs fed total mixed ration with Monensin and Mimosa tannin additives

	Dietary Treatments					
Parameters	**Control**	**Monensin[a]**	**UMT[b]**	**EMT[Sc]**	**SEM**	***p* – values**
Nutrient intake (g/day)						
DM	1328 [b]	1329 [b]	1578 [a]	1569 [a]	84.1	0.05
OM	1254[b]	1254 [b]	1494 [a]	1490 [a]	79.2	0.04
CP	291	294	320	330	21.3	0.48
NDF	344 [b]	327 [b]	434 [a]	416 [a]	26.3	0.01
ADF	174 [c]	181[bc]	241[a]	218[ab]	16.0	0.02
Apparent digestibility (g/kg)						
DM	746[a]	684[bc]	640 [c]	706 [ab]	16.1	0.01
OM	761 [a]	700[bc]	655[c]	722[ab]	15.4	0.01
CP	829 [a]	801[ab]	734[c]	793[ab]	13.0	0.01
NDF	511	303	378	462	53.8	0.10
ADF	429	314	333	407	65.0	0.58

[a] Monensin: @ 75 mg/kg DM of feed.

[b] UMT: unencapsulated Mimosa tannin @ 20 g/kg DM of feed.

[c] EMT[S]: encapsulated Mimosa tannin in sunflower oil @ 20 g/kg DM of feed equivalent.

Means with uncommon superscripts within a row differed significantly ($p \leq 0.05$). Mean values are based on one animal per block and 5 blocks per treatment. SEM = Standard error of mean; DM = dry matter; OM = organic matter; CP = crude protein; NDF = neutral detergent fibre; ADF = acid detergent fibre.

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Table 6.4 Nitrogen balance of South African mutton Merino ram lambs fed total mixed ration with Monensin and Mimosa tannin additives

Parameters	Dietary Treatments				SEM	*p* – values
	Control	Monensin[a]	UMT[b]	EMT[S c]		
Nitrogen intake (g/head/day)	46.5	47.1	51.3	52.8	3.41	0.48
Nitrogen-excreted (g/head/day)	27. 6	27.8	36.4	30.5	2.80	0.11
Faecal nitrogen (g/head/day)	8.03[b]	9.47[b]	13.7[a]	10.7[b]	0.96	0.01
Urinary nitrogen (g/head/day)	19.5	18.1	22.7	19.7	2.48	0.60
Retained nitrogen (g/head/day)	19.0	19.7	14.9	22.4	2.90	0.36
Faecal nitrogen (g/kg N-intake)	171 [b]	199 [b]	266 [a]	206 [b]	13.0	0.01
Urinary nitrogen (g/kg N-intake)	421	399	449	368	48.3	0.66
Retained nitrogen (g/kg N-intake)	409	403	285	426	48.2	0.19

[a] Monensin: @ 75 mg/kg DM of feed.

[b] UMT: unencapsulated Mimosa tannin @ 20 g/kg DM of feed.

[c] EMT[S]: encapsulated Mimosa tannin in sunflower oil @ 20 g/kg DM of feed equivalent.

Means with different superscripts within a row differed significantly ($p \leq 0.05$). Mean values are based on one animal per block and five blocks per treatment. SEM = Standard error of mean; N = Nitrogen.

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Table 6.5 Methane emissions of South African mutton Merino ram lambs fed total mixed ration with Monensin and Mimosa tannin additives

	Treatments					
Parameters	**Control**	**Monensin[a]**	**UMT[b]**	**EMT[Sc]**	**SEM**	***p* – values**
CH_4, g/kg DM-intake	18.9[a]	16.3[ab]	15.3[b]	14.8[b]	1.02	0.04
CH_4, g/kg NDF-intake	70.3	62.4	60.4	58.7	5.78	1.50
CH_4, g/kg DM-digested	34.0	31.4	37.8	32.4	1.96	0.12
CH_4, g/kg NDF-digested	139	155	169	135	27.6	0.60

[a] Monensin: @ 75 mg/kg DM of feed.

[b] UMT: unencapsulated Mimosa tannin @ 20 g/kg DM of feed.

[c] EMT^S: encapsulated Mimosa tannin in sunflower oil @ 20 g/kg DM of feed equivalent.

Means with different superscripts within a row differed significantly ($p \leq 0.05$). Mean values are based on one animal per block and five blocks per treatment. SEM = Standard error of mean; CH_4 = Methane.

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Table 6.6 Rumen characteristics of South African mutton Merino ram lambs fed total mixed ration with Monensin and Mimosa tannin additives

	Treatments					
Parameters	**Control**	**Monensin[a]**	**UMT[b]**	**EMT[S] [c]**	**SEM**	***p* – values**
Ammonia nitrogen (mg/dL)	28.6	32.4	27.6	26.4	2.46	0.31
TVFA (mmol/L)	120	116	116	122	13.0	0.98
Acetate (mol/100 mol)	60.7	59.2	58.9	59.8	1.13	0.59
Propionate (mol/100 mol)	20.4	23.0	23.4	21.7	1.45	0.23
Isobutyrate	2.04	1.77	1.60	1.86	0.24	0.64
Butyrate (mol/100 mol)	11. 7	11.0	11.2	11.5	0.55	0.84
Isovalerate	3.49	3.10	3.35	3.50	0.36	0.87
Valerate (mol/100 mol)	1.73	1.69	1.62	1.69	0.07	0.74
Acetate: propionate ratio	3.00	2.65	2.60	2.79	0.21	0.29

[a] Monensin: @ 75 mg/kg DM of feed.

[b] UMT: unencapsulated Mimosa tannin @ 20 g/kg DM of feed.

[c] EMT[S]: encapsulated Mimosa tannin in sunflower oil @ 20 g/kg DM of feed equivalent.

SEM = Standard error of mean. Mean values are based on one animal per block and five blocks per treatment.

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6.4 Discussion

Growth performance

Sheep are widely reported to secrete salivary muco-protein rich in proline, which have a high capacity to bind with tannins (especially when fed at low concentration) and formed tannin-protein complexes; that are stable across the various pH ranges of the gastrointestinal tract, and thus, cancel the tannins' adverse effect on palatability, feed intake and digestibility (Austin *et al*., 1989; Hagerman and Butler, 1991; Narjisse *et al*., 1995; Foley *et al*., 1999). In concurrence with our finding, numerous studies have shown that inclusion of tannins below 50 g/kg DM of feed did not reduce voluntary feed intake and weight gain in sheep (Waghorn *et al*., 1994; Barry and McNabb, 1999; Hervas *et al*., 2003a; Frutos *et al*., 2004a). However, reduction in dry matter intake and weight gains were reported in sheep (Priolo *et al*., 2000; Carulla *et al*., 2005) and cows (Eckard *et al*., 2010), when tannins were added at higher (≥50 g/kg DM of feed) amount. The influence tannins' bitter taste on intake and digestibility is well known (Eckard *et al*., 2010; Martin *et al*., 2010), and strongly linked to tannin concentration (Frutos *et al*., 2004b; Piñeiro-Vázquez *et al*., 2015). Nevertheless, the degree to which it affects dry matter intake and daily gains in ruminants continue to differ (Animut *et al*., 2008).

Nutrient intake and digestibility

The increase in dry matter, organic matter, neutral detergent fibre and acid detergent fibre intake recorded in the current study for unencapsulated and encapsulated treatments could be attributed to increase in dietary intake as a result of neutralization of tannin's astringency owing to the sheep ability to secrete salivary proteins rich in proline (Frutos *et al*., 2004b). This result compares favourably with the findings of Adejoro *et al*. (2020), where unencapsulated Mimosa tannin additive did not affect DM, OM, CP, NDF and ADF intake of Merino lambs when added at 42 g/kg DM of feed. Zhang *et al.* (2019), also reported that addition of 30 g/kg DM of condensed tannins sources from bayberry and *Acacia mangium* as well as Valonia hydrolysable tannin did not reduce nutrient intake of lactating dairy cows. Similar trend was also recorded by Barry and McNabb (1999), when tannin rich *Lotus pedunculatus* additives were included in the diet of grazing sheep at 34 – 44 g/kg DM. In contrast to our findings, others have reported reduction in nutrient intake when Mimosa tannin extract were added above 20 g/kg DM of feed (Carulla *et al*., 2005; Grainger *et al*., 2009). According to Frutos *et al*. (2004b) and Piñeiro-Vázquez *et al*. (2015), the anti-nutritional effect of tannins is strongly related to their level of inclusion.

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The reduction in digestibility revealed that unencapsulated Mimosa tannin acted more on the highly soluble protein and organic matter fraction than fibre portion of the diets due to its quick solubility in the rumen compared to the encapsulated tannin, of which the tannin dissolution was delayed by the lipid capsule (Adejoro *et al*., 2018; Ibrahim and Hassen, 2021). Numerous literature have indicated that tannins exhibit different affinity levels for dietary dry matter, fibre and protein (Makkar, 2003; Beauchemin *et al*., 2007; Piñeiro-Vázquez *et al*., 2015). In agreement with the present finding, Beauchemin *et al*. (2007), recorded a significant reduction in dietary protein degradation with no effect on NDF and ADF digestibility after inclusion of quebracho tannin in the diet of cattle at 20 g/kg DM. While, Carulla *et al*. (2005) reported substantial decrease in fibre and protein degradation on sheep added with mimosa tannin extract at 25 g/kg DM level. Similarly, Adejoro *et al*. (2020) also observed a significant reduction in DM, OM, CP, NDF and ADF digestibility in South African mutton Merino sheep after adding mimosa tannin at 42 g/kg DM of feed.

Nitrogen balance

The increased in faecal nitrogen excretion obtained for non-encapsulated diet could be traced to higher protein intake (320.4 g/day) of the UMT group compared to 290.8 g/day for control group as recorded in the current study. Adejoro *et al*. (2020), reported that mimosa tannin reduced soluble protein degradation in the rumen, facilitated nitrogen flow to the lower gut and increased faecal-N excretion as against urine-N. The results of this study are also in consistent with the earlier reports that tannins did not influence nitrogen retention in cattle and sheep (Tiemann *et al*., 2008a; Stewart *et al*., 2019; Adejoro *et al*. 2020). However, Grainger *et al*. (2009) observed an enhanced nitrogen utilization with Mimosa tannin addition in the diet of dairy cows, attributed to the improvement in protein flow to the duodenum and shift in nitrogen excretion from urine to faeces. Moreover, the review by Waghorn (2008), showed that absorption of bypassed protein depends on the degree of tannin-protein binding and amount of nutrients required by the animals. Aboagye *et al*. (2018), also noted that the nitrogen content of the faeces which is more stable is not easily lost to the environment and thus, more environmentally friendly. However, urinary-N is largely urea and therefore more rapidly released to the surface as source of atmospheric nitrous oxide and nitrate contamination in water bodies (Eckard *et al*., 2010).

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Methane

The observed decrease in enteric methane emission showed that the impact of Mimosa tannin is likely related to suppression of dry matter and fibre degradation than directly affecting methanogens (Lawal, 2022). The present result compares favourably with that of Carulla *et al*. (2005), who reported around 13% decrease in CH_4 at 25 g/kg DM inclusion of Mimosa tannin extract in sheep. Tan *et al*. (2011) also reported CH_4 reduction (–33%) at 20 g kg^{-1} DM of *L. leucocephala* extracts. It has been well documented that tannins modulate enteric CH_4 emission through reduction in feed intake and digestibility or toxicity to *Methanogenic archaea* (Animut *et al*., 2008; Jayanegara *et al*., 2012; Hristov *et al*., 2013). However, some studies revealed that inclusion of tannins at 20 g/kg DM did not reduce methane emission in sheep (Hervás *et al*., 2003b; Adejoro *et al*., 2020). These variations could be attributed to differences in the tannin sources, structure and their biological activity.

Rumen fermentation parameters

Several studies reported little or no impact of tannins on rumen fermentation parameters when added at lower (20 g/kg DM) concentration (Hervas *et al*., 2003a; Carulla *et al*., 2005; Getachew *et al*., 2008; Hassanat and Benchaar, 2013). In agreement with our finding, Carulla *et al.* (2005); Aboagye *et al*. (2018); Adejoro *et al*. (2020) did not observe a significant effect of tannin on TVFA. In contrast, when Mimosa tannins were added at ≥50 g/kg DM there were significant decreases in the proportions of total volatile fatty acids, acetate, butyrate, valerate and branched-chain VFA (Hassanat and Benchaar, 2013). Similarly, reduction in the concentrations of NH_3N and TVFAs have been found due to the establishment of tannin–protein and tannin–carbohydrate bonds that are not easily digestible (Makkar, 2005; Martınez *et al*., 2006) or tannin's toxicity to rumen microorganisms (Bento, 2005; Bhatta *et al*., 2009) as well as delay in cellulolytic activity and longer retention times (Priolo *et al*., 2000). Moreover, higher inclusions of tannin are found to reduced acetic acid production by inhibiting the activities of acetate forming bacteria (Castro-Montoya *et al*., 2011), while, enhancing the concentrations of propionic acid giving rise to reduction in acetate to propionate ratio (Beauchemin *et al*., 2007). Similarly, addition of adequate proportion of tannins in the diets of ruminants have been reported to reduce iso-butyric acid and iso-valeric acid productions (Singh *et al*., 2005; Getachew *et al*., 2008; Bhatta *et al*., 2009). According to Hassanat and Benchaar (2013), these branched-chain VFAs are produced from the breakdown of the carbon skeleton of amino acids by rumen microorganisms.

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6.5 Conclusion

Based on the results of this study it was concluded that both non-encapsulated Mimosa tannin and encapsulated Mimosa tannin in sunflower oil as additives could be used in modulating rumen fermentation, reduce enteric methane emission as well as increase faecal nitrogen excretion when added at 20 g/kg DM of feed. Among the two tannins studied, the encapsulated Mimosa tannin reduced more methane emitted by South African mutton Merino sheep without affecting nutrient digestibility compared to similar inclusion level of unencapsulated Mimosa tannin. Further studies should be conducted on the total tannin concentration of the encapsulated mimosa tannin in sunflower oil.

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CHAPTER SEVEN

General Conclusion, Recommendation and Critical Evaluation

7.1 Conclusion

The current research sought to use *A. mearnsii* tannin as natural alternative to reduce enteric methane emission while promoting dietary protein utilization by overcoming negative effects of tannin on normal rumen function, feed consumption and digestibility. Firstly, purification of *A. mearnsii* tannin extract was studied to increase the proportion of condensed tannin and later on encapsulation of tannin was studied to conceal the bitter taste and slow down tannin quick dissolution in the GIT. It was concluded that purifying of *A. mearnsii* tannin with organic solvents increased the proportion of condensed tannin component, while purifying with ethyl acetate resulted in a higher rise in condensed tannin fraction. Soxhlet extraction of acacia tannin with pentanol and ethyl acetate decreased gas and methane production with slight reduction on digestibility at 30 g/kg DM. Moreover, lesser dosage of purified tannin extracts showed similar impact on gas and methane comparable with higher inclusion level of non-purified tannin.

Sunflower oil and palm oil microparticles exhibited an excellent encapsulation efficiencies, their particles were smaller in size, lighter in density and delayed tannin release in the gastrointestinal tract simulated buffers compared to unprotected tannin. However, non-encapsulated tannin bound and released more protein in the buffers simulating rumen and abomasum. While, encapsulated tannin in sunflower and palm oils released more protein in small intestine simulated buffer. Among the two oil wall materials used for encapsulation, sunflower oil microparticles bound and released more protein than palm oil microcapsules. Addition of encapsulated and non-encapsulated acacia tannin at 20 g/kg DM level decreased methane and total gas yield without affecting ammonia nitrogen, volatile fatty acids and digestibility. Moreover, encapsulated tannin reduced more methane yield compared to equivalent dosage of free tannin. Nevertheless, 30 g/kg inclusion of encapsulated tannin reduced *in vitro* feed digestibility. *In vivo* tests revealed that compared to unencapsulated tannin and monensin, *A. mearnsii* tannin encapsulated in sunflower oil at a dosage of 20 g/kg feed DM reduced methane emitted by sheep while increasing intake of dry matter, organic matter, and fiber without lowering digestibility of dry matter, organic matter, and crude protein. Thus, supplementing encapsulated acacia tannin at 20 g/kg feed DM level could be used to replace the use of ionophore as safer natural alternative additive and eco-friendly approach of ruminant animal production under organic farming condition.

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7.2 Recommendations

- It is recommended that the ethyl acetate can be adopted in the tannin purification to increase condensed tannin concentration.

- Microencapsulation of tannin with sunflower oil and palm oil can be recommended for neutralizing tannin's astringency and promoting dietary protein binding and sustained release to the target site.

- It is recommended that inclusion of encapsulated and non-encapsulated *A. mearnsii* tannin in the diet of small ruminant should'nt go beyond 20 g/kg DM for the purpose of enteric methane mitigation without affecting nutrient intake and digestibility.

- Further research should be done to look at the lipid stability, fatty acid transfer rate, and antioxidant properties of the sunflower and palm oils used as wall materials in the encapsulated tannin microcapsules.

- The concentration of total tannin in the encapsulated material should be analysed in the future studies to know the exact amount given to the animals for the purpose of standardisation and easy comparison.

7.3 Critical evaluation of study

It is imperative to reflect on certain essential matters that were not incorporated at the start or during the implementation of the present study, nonetheless would have enhanced the findings and conclusion of this investigation.

- The hydrolysable tannin content was estimated (obtained) by subtracting the concentration of condensed tannins from the total tannin. Total tannin was first analysed using Folin – Ciocalteu method (expressed as tannic acid equivalent), whereas Butanol – HCl technique was used to determine the condensed tannin and expressed as leucocyanidin equivalent. Thus, there are some limitations of estimating the hydrolysable tannin content by difference, which suggest for the need to consider alternative methods that recommended for the individual tannin constituent to be analysed using an appropriate tannin standard with comparable molecular structure. If suitable standard and the apparatus for the determination of each tannin component are readily available, more precise concentrations of hydrolysable tannin, condensed

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tannin, total tannin, tannin phenol and non-tannin phenol will be obtained. Hence, such an approach should be adopted in the subsequent studies.

- The *in vitro* tannin release as well as protein binding and release trials were conducted using mainly the encapsulated and non-encapsulated tannin additives and the buffer media simulating gastrointestinal tract pH, devoid of rumen fluid and the substrate. Although the technique of using buffer media to stimulate what happens at different GIT segment proved to be effective, it also has some limitations because of the absence of rumen microorganisms and their feeds which are well documented to play a key role in rumen fermentation. Hence, further *in vitro* studies should include rumen fluid and the substrate commonly utilized by the animals for better results.

- During an *in vitro* gas trial, several inclusion levels of encapsulated mimosa tannin (10, 20 and 30 g/kg DM) were tested with a single inclusion level (20 g/kg DM) of unprotected or unencpasulated tannin. This is based on the preliminary experiment that showed 20 g/kg DM dose to reduce CH_4 with little impact on digestibility. However, using only one level of unencapsulated tannin may not provide sufficient comparison with the various levels of encapsulated tannin. Thus, the fact that unprotected tannin level was also tested for each encapsulation level would have better demonstrated the results.

- The SRI GC adopted for CH_4 quantification was calibrated using CH_4 standard and the calibration curves plotted were used to estimate the CH_4 concentration from the unknown gas samples. The CH_4 standards and the working conditions of the equipment could lead to some discrepancies in the results which might be different from one cycle of incubation to another. Nevertheless, in the present investigation, repeated batches were conducted within a short period of time while maintaining similar working conditions to avoid any significant influence on the treatments.

- The estimation of CH_4 emitted by the animals inside the methane chamber depend on the negative air generated from within the cages which guaranteed a single direction of the air coming out of the chambers. The open circuit respiratory cages were adequately airtight, to curtail loss of the air from the chamber. But, errors might occur due to air ducting and mixing during gas sampling into the collecting bags from the individual

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chambers. Thus, carrying out recovery tests before and after each cycle is important to get a result which served as correction factors. At the same time, representative animals from each dietary treatment were rotated after 24 hours across all the chambers in order to expose all the animals to the different chamber conditions and prevent confounding effects associated with chamber performance for better comparison of the results among the treatments.

- For the *in vivo* experiment, rumen fluids for ammonia nitrogen and volatile fatty acids analyses were collected at the abattoir after slaughtering the sheep. This is due to the fact that animal ethics committee didn't approved the use of stomach tubes for rumen fluid collection because of the perceived risk of animal welfare violation. The rumen fluid collection at the abattoir may affect the quality of the results although, the samplings were done immediately after slaughter and the preservatives were quickly added to the rumen fluid samples and kept in an ice block before transportation to the laboratory and analysis in the laboratory. Thus, it is important to include rumen cannulated animals in the trial in conformity with the ideal method of rumen fluid collection.

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REFERENCES

Abedi, A., Rismanchi, M., Shahdoostkhany, M., Mohammadi, A., 2016. Microencapsulation of Nigella sativa seeds oil containing thymoquinone by spray-drying for functional yogurt production. Intern. J. Food Sci. Technol. 2280–2289. https://doi.org/10.1111/ijfs.13208.

Aboagye, I.A., Oba, M., Castillo, A.R., Koenig, K.M., Iwaasa, A.D., Beauchemin, K.A., 2018. Effects of hydrolyzable tannin with or without condensed tannin on methane emissions, nitrogen use, and performance of beef cattle fed a high-forage diet. J. Anim. Sci. 96, 5276–5286.

Adejoro, F.A., Hassen, A., Thantsha, M.S., 2019a. Characterization of starch and gum Arabic-maltodextrin microparticles encapsulating Acacia tannin extract and evaluation of their potential use in ruminant nutrition. Asian-Australas J. Anim. Sci. 32(7), 977–987.

Adejoro, F.A., Hassen, A., Akanmu, A.M., 2019b. Effect of lipid-encapsulated acacia tannin extract on feed intake, nutrient digestibility and methane emission in sheep. Animals 9, 863. https://doi:10.3390/ani9110863.

Adejoro, F.A., Hassen, A., 2017. Effect of supplementing or treating *Eragrostis curvula* hay with urea or nitrate on its digestibility and in vitro fermentation. South African J. Anim. Sci. 47(2), 168. https://doi.org/10.4314/sajas.v47i2.8.

Adejoro, F.A., Hassen, A., Thantsha, M.S., 2018. Preparation of acacia tannin loaded lipid microparticles by solid-in-oil-in-water and melt dispersion methods, their characterization and evaluation of their effect on ruminal gas production in vitro. PLos One 13(10), 1–15. https://doi.org/10.1371/journal.pone.0206241.

Adejoro, F.A., Hassen, A., Akanmu, A.M., Morgavi, D.P., 2020. Replacing urea with nitrate as a non-protein nitrogen source increases lambs' growth and reduces methane production, whereas acacia tannin has no effect. Anim. Feed Sci. Technol. 259, 114360. https://doi.org/10.1016/j.anifeedsci.2019.114360.

Afsana, K., Shiga, K., Ishizuka, S., Hara, H., 2004. Reducing effect of ingesting tannic acid on the absorption of iron, but not of zinc, copper and manganese by rats. Biosci. Biotechnol. Biochem. 68(3), 584–92. https://doi.org/https://doi.org/10.1271/bbb.68.584.

Akanmu, M.A., Hassen, A., Adejoro, F.A., 2020. Gas Production, Digestibility and Efficacy of Stored or Fresh Plant Extracts to Reduce Methane Production on different Substrates. Animals 10, 146. https://doi:10.3390/ani10010146.

Akanmu, A.M., Hassen, A., 2017. The use of certain medicinal plant extracts reduced in vitro methane production while improving in vitro organic matter digestibility. Anim. Prod. Sci. 58(5), 900–908. https://doi.org/10.1071/AN16291.

Alexandratos, N., Bruinsma, J., 2012. World agriculture towards 2030/2050: the 2012 revision.

UNIVERSITEIT VAN PRETORIA
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YUNIBESITHI YA PRETORIA

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ESA Working paper No. 12–03. FAO, Rome. Accessed on 31/12/2022 from www.fao.org/economic/esa.

Alvarez-Hess, P.S., Moate, P.J., Williams , S.R.O., Jacobs, J.L., Beauchemin, K.A., Hannah, M.C., Durmic, Z., Eckard, R.J., 2019. Effect of combining wheat grain with nitrate, fat or 3-nitrooxypropanol on in vitro methane production. Anim. Feed Sci. Technol. 256, 114237. https://doi.org/DOI 10.1016/j.anifeedsci.2019.114237.

Anantasook, N., Wanapat, M., Cherdthong, A., Gunun, P., 2013. Effect of Plants Containing Secondary Compounds with Palm Oil on Feed Intake, Digestibility, Microbial Protein Synthesis and Microbial Population in Dairy Cows. Asian-Australas. J. Anim. Sci. 26(6), 820–826.

Anantasook, N., Wanapat, M., Cherdthong, A., Gunun, P., 2014. Effect of tannins and saponins in Samanea saman on rumen environment, milk yield and milk composition in lactating dairy cows. J. Anim. Physiol. Anim. Nutr. 99(2), 335–344. https://doi.org/10.1111/jpn.12198.

Animut, G., Puchala, R., Goetsch, A.L., Patra, A.K., Sahlu, T., Varel, V.H., Wells, J., 2008. Methane emission by goats consuming diets with different levels of condensed tannins from lespedeza. Anim. Feed Sci. Technol. 144(3–4), 212–227. https://doi.org/10.1016/j.anifeedsci.2007.10.014.

AOAC, 2002. Official Method of Analysis. Association of Official Analytical Chemists, Washington, DC, USA,

AOAC, 2000. Official Methods of Analysis, 17th ed. Association of Official Analytical Chemists, Inc, Arlington, VA, USA.

Arbenz, A., Avérous, L., 2015. Chemical modification of tannins to elaborate aromatic biobased macromolecular architectures. Green Chem. 2626–2646. https://doi.org/10.1039/c5gc00282f.

Arthington, J.D., Brown, W.F., 2005. Estimation of feeding value of four tropical forage species at two stages of maturity. J. Anim. Sci. 83, 1726–1731.

Attwood, G., McSweeney, C., 2008. Methanogen genomics to discover targets for methane mitigation technologies and options for alternative H_2 utilisation in the rumen. Austral. J. Experim. Agric. 48, 28–37.

Augustin, M.A., Sanguansri, L., Margetts, C., Young, B.J.F.A., 2001. Microencapsulating food ingredients. Food Aust. 53, 220–223.

Austin, P.J., Suchar, L.A., Robbins, C.T., Hagerman, A.E., 1989. Tannin-binding proteins in saliva of deer and their absence in saliva of sheep and cattle. J. Chem. Ecol. 15(4), 1335–1347. https://doi.org/10.1007/BF01014834.

UNIVERSITEIT VAN PRETORIA
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Avila, A.S., Zambom, M.A., Faccenda, A., Fischer, M.L., Anschau, F.A., Venturini, T. Tinini, R.C.R., Dessbesell, J.G., Faciola, A.P., 2020. Effects of Black Wattle (Acacia mearnsii) Condensed Tannins on Intake, Protozoa Population, Runinal Fermentation and Nutrient Digestibility in Jersey Steers. Animals 10, 1011. https://doi.org/doi:10.3390/ani10061011.

Bainbridge, M., Kraft, J., 2016. Total-Tract Digestibility to Achieve an Optimal Transfer Efficiency of Fatty Acids to Milk Fat. PLoS One 11(10), 1–18.

Bakry, A.M., Abbas, S., Ali, B., Majeed, H., Abouelwafa, M.Y., Mousa, A., Liang, L., 2016. Microencapsulation of Oils: A Comprehensive Review of Benefits, Techniques, and Applications. Compr. Rev. Food Sci. Food Safety 15(1), 143–182. https://doi.org/10.1111/1541-4337.12179.

Barry, T.N., McNabb, W.C., 1999. The implications of condensed tannins on the nutritive value of temperate forages fed to ruminants. Brit. J. Nutr. 81, 263-272.

Bayat, A., Shingfield, K.J., 2012. Overview of nutritional strategies to lower enteric methane emissions in ruminants. Maatal. Päivät. 1–7.

Beauchemin, K.A., McGinn, S.M., Martinez, T.F., McAllister, T.A., 2007. Use of condensed tannin extract from quebracho trees to reduce methane emissions from cattle. J. Anim. Sci. 85(8), 1990–1996. https://doi.org/10.2527/jas.2006-686.

Beauchemin, K.A., Kreuzer, M., O'Mara, F., McAllister, T.A., 2008. Nutritional management for enteric methane abatement: A Review. Austral. J. Experim. Agric. 48(2), 21-27. https://doi.org/10.1071/ea07199.

Belanche, A., Pinloche, E., Preskett, D., Newbold, C.J., 2016. Effects and mode of action of chitosan and ivy fruit saponins on the microbiome, fermentation and methanogenesis in the rumen simulation technique. FEMS Microbiol. Ecol. 92, 1-7.

Belanche, A., Patra, A.K., Morgavi, D.P., Suen, G., Newbold, C.J., Yáñez-Ruiz, D.R., 2021. Gut microbiome modulation in ruminants: Enhancing advantages and minimizing drawbacks. Frontiers Microbiol. 11, 3452.

Benchaar, C., Calsamiglia, S., Chaves, A.V., Fraser, G.R., Colombatto, D., McAllister, T.A., Beauchemin, K.A., 2008. A review of plant-derived essential oils in ruminant nutrition and production. Anim. Feed Sci. Technol. 145, 209–228.

Bento, M.H.L., Makkar, H.P.S., Acamovic, T., 2005. Effect of mimosa tannin and pectin on microbial protein synthesis and gas production during in vitro fermentation of 15N-labelled maize shoots. Anim. Feed Sci. Technol. 123(124), 365–377.

Besharati, M., Maggiolino, A., Palangi, V., Kaya, A., Jabbar, M., Eseceli, H., Palo, P.D., Lorenzo, J.M., 2022. Tannin in Ruminant Nutrition: Review. Molecules 27, 8237. https://doi.org/10.3390/molecules27238273.

UNIVERSITEIT VAN PRETORIA
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YUNIBESITHI YA PRETORIA

© University of Pretoria

Bhatta, R., Uyeno, Y., Tajima, K., Takenaka, A., Yabumoto, Y. Nonaka, I., Enishi, O., Kurihara, M., 2009. Difference in the nature of tannins on in vitro ruminal methane and volatile fatty acid production and on methanogenic archaea and protozoal populations. J. Dairy Sci. 92(11), 5512–5522. https://doi.org/10.3168/jds.2008-1441.

Bhatta, R., Saravanan, M., Baruah, L., Sampath, K.T., Prasad, C.S., 2013a. Effect of plant secondary compounds on in vitro methane, ammonia production and ruminal protozoa population. J. Appl. Microbiol. 115(2), 455–465. https://doi.org/10.1111/jam.12238.

Bhatta, R., Enishi, O., Yabumoto, Y., Nonaka, I., Takusari, N., Higuchi, K., Tajima, K., Takenaka, A., Kurihara, M., 2013b. Methane reduction and energy partitioning in goats fed two concentrations of tannin from Mimosa spp. J. Agric Sci. 151(1), 119–128. https://doi.org/10.1017/S0021859612000299.

Biondi, L., Randazzo, Cinzia L., Russo, N., Pino, A., Natalello, A., Hoorde, K.V., Caggia, C., 2019. Dietary Supplementation of Tannin-Extracts to Lambs: Effects on Meat Fatty Acids Composition and Stability and on Microbial Characteristics. Foods 8, 469. https://doi:10.3390/foods8100469.

Bodas, R., Prieto, N., García-gonzález, R., Andrés, S., Giráldez, F.J., López, S., 2012. Manipulation of rumen fermentation and methane production with plant secondary metabolites. Anim. Feed Sci. Technol. 176(1–4), 78–93. https://doi.org/10.1016/j.anifeedsci.2012.07.010.

Boussaada, A., Arhab, R., Ferrara, M., 2018. Effect of Eucalyptus globulus leaves extracts on in vitro rumen fermentation, methanogenesis, degradability and protozoa population. Ann. Anim. Sci. 18(3), 753–767. https://doi.org/10.2478/aoas-2018-0006.

Breslin, P.A.S., Gilmore, M.M., Beauchamp, G.K., Green, B.G., 1993. Psychophysical evidence that oral astringency is a tactile sensation. Chem. Senses 18, 405-417.

Broderick, G.A., Kang, J.H., 1980. Automated simultaneous determination of Ammonia and total amino acids in ruminal fluid and in vitro media 1. J. Dairy Sci. 63, pp. 64–75.

Burke, J.M., Miller, J.E., Terrill, T.H., Mosjidis, J.A., 2014. The effects of supplemental sericea lespedeza pellets in lambs and kids on growth rate. Livest. Sci. 159(1), 29–36. https://doi.org/10.1016/j.livsci.2013.10.030.

Busquet, M., Calsamiglia, S., Ferret, A., Carro, M.D., Kamel, C., 2005. Effect of garlic oil and four of its compounds on rumen microbial fermentation. J. Dairy Sci. 88, 4393–4404.

Cabral Filho, S., Abdalla, A., Bueno, I., Oliveira, A., 2013. Effect of sorghum tannins in sheep fed with high-concentrate diets. Arq. Bras. De Med. Veterin. E Zootec. 65, 1759–1766.

Callaway, T.R., Carneiro De Melo, A.M.S., Russell, J.B., 1997. The effect of nisin and monensin on ruminal fermentations in vitro. Curr. Microbiol. 35, 90–96. https://doi.org/doi:10.1007/s002849900218.

UNIVERSITEIT VAN PRETORIA
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YUNIBESITHI YA PRETORIA

© University of Pretoria

Calsamiglia, S., Busquet, M., Cardazo, P.W., Castillejos, L., Ferret, A., 2007. Invited review: essential oils as modifiers of rumen microbial fermentation. J. Dairy Sci. 90, 2580–2595.

Cann, A.F., Liao, J.C., 2010. Pentanol isomer synthesis in engineered microorganisms. Appl. Microbiol. Biotechnol. 85, 893–899. https://doi.org/10.1007/s00253-009-2262-7.

Carulla, J.E., Kreuzer, M., Machmüller, A., Hess, H.D., 2005. Supplementation of Acacia mearnsii tannins decreases methanogenesis and urinary nitrogen in forage-fed sheep. Austral. J. Agric. Res. 56, 961–970. https://doi.org/10.1071/ar05022.

Castellanos, I.J., Carrasquillo, K.G., López, J.D.J., Alvarez, M., Griebenow, K., 2001. Encapsulation of bovine serum albumin in poly(lactide-co-glycolide) microspheres by the solid-in-oil-in-water technique. J. Pharm. Pharmacol. 53(2), 167–178. https://doi.org/10.1211/0022357011775361.

Castillo, C., Benedito, J.L., Méndez, J., Pereira, V., López-Alonso, M., Miranda, M., Hernández, J., 2004. Organic acids as a substitute for monensin in diets for beef cattle. Anim. Feed Sci. Technol. 115, 101–116. https://doi.org/doi:10.1016/j.anifeedsci.2004.02.001.

Castro-Montoya, J.M., Makkar, H.P.S., Becker, K., 2011. Chemical composition of rumen microbial fraction and fermentation parameters as affected by tannins and saponins using an in vitro rumen fermentation system. Can. J. Anim. Sci. 91, 433–448.

ChaichiSemsari, M., MaheriSis, N., Sadaghian, M.., Eshratkhah, B., Hassanpour, S., 2011. Effects of administration of industrial tannins on nutrient excretion parameters during naturally acquired mixed nematode infections in Moghani sheep. J. Amer. Sci. 7(6), 245-248.

Choudhury, P.K., Salem, A.Z.M., Jena, R., Kumar, S., Singh, R., Puniya, A.K., 2015. Rumen Microbiology: An Overview. In: Puniya K.A., Singh, R., Kamra, N.D. (Eds.), Rumen Microbiology: From Evolution to Revolution. New Delhi: Springer. 3-16.

Cieslak, A., Zmora, P., Pers-Kamczyc, E., Szumacher-Strabel, M., 2012. Effects of tannins source (Vaccinium vitis idaea L.) on rumen microbial fermentation in vivo. Anim. Feed Sci. Technol. 176(1–4), 102–106. https://doi.org/10.1016/j.anifeedsci.2012.07.012.

Conklin, D.E., 1995. Digestive physiology and nutrition. Biol. Lobster Homarus Am. 441–463.

Cottle, D.J., Nolan, J.V., Wiedemann, S.G., 2011. Ruminant enteric methane mitigation: A review. Anim. Prod. Sci. 51, 491-514.

Davies, R., 2016. Effect of the temperature on dynamic viscosity, density and flow rate of some vegetable oils. J. Sci. Res. Engr. Technol. 1, 14–24.

Dawra, K., 1988. Protein-Binding Capacity of Microquantities of Tannins. Anal. Biochem. 170, 50–53.

UNIVERSITEIT VAN PRETORIA
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YUNIBESITHI YA PRETORIA

© University of Pretoria

Denninger, T.M., Schwarm, A., Birkinshaw, A., Terranova, M. Dohme-meier, F., 2020. Immediate effect of Acacia mearnsii tannins on methane emissions and milk fatty acid profiles of dairy cows. Anim. Feed Sci. Technol. 261, 114388. https://doi.org/10.1016/j.anifeedsci.2019.114388.

Desai, K.G.H., Park, H.J., 2005. Recent developments in microencapsulation of food ingredients. Drying Technol. 23, 7, 1361 - 1394. https://doi.org/10.1081/DRT-200063478.

de Wit, M.P., Crookes, D.J., Van Wilgen, B.W., 2001. Conflicts of interest in environmental management: estimating the costs and benefits of a tree invasion. Biol. Invas. 3, 167–178.

Dey, A., De, P.S., 2014. Influence of condensed tannins from Ficus bengalensis leaves on feed utilization, milk production and antioxidant status of crossbred cows. Asian-Australas. J. Anim. Sci. 27(3), 342–348. https://doi.org/10.5713/ajas.2013.13295.

Dickinson, E., 2011. Double emulsions stabilized by food biopolymers. Food Biophys. 6, 1–11.

Ding, X., Long, R., Zhang, Q., Huang, X., Guo, X., Mi, J., 2012. Reducing methane emissions and the methanogen population in the rumen of Tibetan sheep by dietary supplementation with coconut oil. Trop. Anim. Health Prod. 44, 1541–1545.

Doce, R.R., Belenguer, A., Toral, P.G., Hervás, G., Frutos, P., 2013. Effect of the administration of young leaves of Quercus pyrenaica on rumen fermentation in relation to oak tannin toxicosis in cattle. J. Anim. Physiol. Anim. Nutr. 97, 48-57.

Dohme, F., Machmüller, A., Wasserfallen, A., Kreuzer, M., 2001. Ruminal methanogenesis as influenced by individual fatty acids supplemented to complete ruminant diets. Lett. Appl. Microbiol. 32, 47–51.

Douglas, G.B., Stienezen, M., Waghorn, G.C., Foote, A.G., Purchas, R.W., 1999. Effect of condensed tannins in birdsfoot trefoil (Lotus corniculatus) and sulla (Hedysarum coronarium) on body weight, carcass fat depth, and wool growth of lambs in New Zealand. New Zeal. J. Agric. Res. 42, 55–64. https://doi.org/doi:10.1080/00288233.1999.9513353.

Dschaak, C.M., Williams, C.M., Holt, M.S., Eun, J.-S., Young, A.J., Min, B.R., 2011. Effects of supplementing condensed tannin extract on intake, digestion, ruminal fermentation, and milk production of lactating dairy cows. J. Dairy Sci. 94(5), 2508–2519. https://doi.org/10.3168/jds.2010-3818.

Eckard, R.J., Grainger, C., de Klein, C.A.M., 2010. Options for the abatement of methane and nitrous oxide from ruminant production: A review. Lives. Sci. 130(1–3), 47–56. https://doi.org/10.1016/j.livsci.2010.02.010.

Engels, E.A.N., Van der Merwe, F.J., 1967. Application of an in vitro technique to South African forages with special reference to the effect to certain factors on the results. South

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© University of Pretoria

African J. Agric. Sci. (10), 983–995.

Eugène, M., Masse, D., Chiquette, J., Benchaar, C., 2008. Meta-analysis on the effects of lipid supplementation on methane production in lacting dairy cows. Can. J. Anim. Sci. 88, 331–334.

Fang, Z., Bhandari, B., 2010. Encapsulation of polyphenols - A review. Trends Food Sci. Technol. 21(10), 510–523. https://doi.org/10.1016/j.tifs.2010.08.003.

Filippich, L. J., Zhu, J., Oelrichs, P., Alsalami, M.T., Doig, A.J., Cao, G.R., English, P.B., 1991. Hepatotoxic and nephrotoxic principles in Terminalia oblongata. Res. Veterin. Sci. 50, 170-177.

Flanagan, J., Flanagan, J., Singh, H., 2006. Microemulsions: A Potential Delivery System for Bioactives in Food. Crit. Rev. Food Sci. Nutr. 46, 221–237. https://doi.org/10.1080/10408690590956710.

Foley, W.J., Iason G.R., MacArthur, C., 1999. Role of secondary metabolites in the nutritional ecology of mammalian herbivores: how far have we come in 25 years? In: Jung, H.J.G., Fahey, G.C.Jr. (Eds.), Nutritional ecology of herbivores. American Society of Animal Science, Illinois (USA), 130-209.

Forabosco, F., Chitchyan, Z., Mantovani, R., 2017. Methane, nitrous oxide emissions and mitigation strategies for livestock in developing countries: A review. S. Afr. J. Anim. Sci. 47, 268–280.

Frutos, P., Hervás, G., Giráldez, F. J., Mantecón, A.R., 2004b. Review. Tannins and ruminant nutrition. Spanish J. Agric. Res. 2(2), 191–202. https://doi.org/10.5424/73.

Frutos, P., Raso, M., Hervas, G., Mantecón, A.R., Perez V., Giráldez, F.J., 2004a. Is there any detrimental effect when a chestnut hydrolyzable tannins extract is included in the diet of finishing lambs? Anim. Res. 56, 127-136.

Galatowitsch, S., Richardson, D.M., 2005. Riparian scrub recovery after clearing of invasive alien trees in headwater streams of the Western Cape, South Africa. Biolog. Conserv. 122(4), 509–521. https://doi.org/10.1016/j.biocon.2004.09.008.

Galindo, J., González, N., Delgado, D., Sosa, A., Marrero, Y., Gonzalez, R., Aldama, A.I., Moreira, O., 2008. Efecto modulador de Leucaena leucocephala sobre la microbiota ruminal. Zootecn. Trop. 26, 249-252.

García, E.M., Cherry, N., Lambert, B.D., Muir, J.P., Nazareno, M.A., Arroquy, J.I., 2017. Exploring the biological activity of condensed tannins and nutritional value of tree and shrub leaves from native species of the Argentinean Dry Chaco. J. Sci. Food Agric. 97, 5021–5027.

Gemeda, B.S., Hassen, A., 2015. Effect of tannin and species variation on in vitro digestibility,

UNIVERSITEIT VAN PRETORIA
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YUNIBESITHI YA PRETORIA

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gas, and methane production of tropical browse plants. Asian-Austral. J. Anim. Sci. 28(2), 188–199. https://doi.org/10.5713/ajas.14.0325.

Gerber, P.J., Hristov, A.N., Henderson, B., Makkar, H., Oh, J., Lee, C., Meinen, R., Montes, F., Ott, T., Firkins, J., Rotz, A., Dell, C., Adesogan, A.T., Yang, W. Z., Tricarico, J.M., Kebreab, E., Waghorn, G., Dijkstra, J., Oosting, S., 2013. Technical options for the mitigation of direct methane and nitrous oxide emissions from livestock: a review. Animal 7(2), 220–234. https://doi.org/10.1017/S1751731113000876.

Getachew, G., Pittroff, W., Putnam, D.H., Dandekar, A., Goyal, S., DePeters, E.J., 2008. The influence of addition of gallic acid, tannic acid, or quebracho tannins to alfalfa hay on in vitro rumen fermentation and microbial protein synthesis. Anim. Feed Sci. Technol. 140(3–4), 444–461. https://doi.org/10.1016/j.anifeedsci.2007.03.011.

Gharsallaoui, A., Roudaut, G., Chambin, O., Voilley, A., Saurel, R., 2007. Applications of spray-drying in microencapsulation of food ingredients: An overview. Food Res. Int. 40, 1107–1121. https://doi.org/10.1016/j.foodres.2007.07.004.

Goel, G., Makkar, H.P.S., 2012. Methane mitigation from ruminants using tannins and saponins. Trop. Anim. Health Prod. 44, 729–39.

Goel, G., Puniya, AK., Singh, K., 2007. Phenotypic characterization of tannin-protein complex degrading bacteria from faeces of goat. Small Rumin Res. 69, 217-220.

Goel, N., Sirohi, S.K., Dwivedi, J., 2011. Studies on the effects of methanolic extract of Cinnamomum zeylanicum on in vitro methane inhibition and rumen fermentation patterns. J. Chem. Pharm. Res. 3(6), 609 – 615.

Goel, G., Makkar, H.P.S., Becker, K., 2008. Effects of Sesbania sesban and Carduus pycnocephalus leaves and Fenugreek (Trigonella foenum-graecum L.) seeds and their extracts on partitioning of nutrients from roughage- and concentrate-based feeds to methane. Anim. Feed Sci. Technol. 147(1–3), 72–89. https://doi.org/10.1016/j.anifeedsci.2007.09.010.

Gomez, J.A., Tejido, M.L., Carro, M.D., 2005. Influence of disodium malate on microbial growth and fermentation in rumen-simulation technique fermenters receiving medium- and high-concentrate diets. Br. J. Nutr. 93, 479–484. https://doi.org/doi:10.1079/bjn20041367.

Grainger, C., Clarke, T., Auldist, M.J., Beauchemin, K.A., McGinn, S.M., Waghorn, G.C., Eckard, R.J., 2009. Potential use of Acacia mearnsii condensed tannins to reduce methane emissions and nitrogen excretion from grazing dairy cows. Can. J. Anim. Sci. 89(2), 241–251. https://doi.org/10.4141/cjas08110.

Greathead, H., 2003. Plants and plant extracts for improving animal productivity. Proc. Nutr. Soc. 62, 279–290.

Guitart, R., Croubels, S., Caloni, F., Sachana, M., Davanzo, F., Vandenbroucke, V., Berny, P.,

UNIVERSITEIT VAN PRETORIA
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YUNIBESITHI YA PRETORIA

© University of Pretoria

2010. Animal poisoning in Europe. Part 1: Farm livestock and poultry. Vet. J. 183(3), 249-254. https://doi.org/https://doi.org/10.1016/j.tvjl.2009.03.002.

Gujrathi, A.M., Babu, V.B., 2007. Environment friendly products from black wattle. Energy Edu. Sci. Technol. 19, 37-44.

Guo, Y.Q., Liu, J.X., Lu, Y., Zhu, W.Y., Denman, S.E., McSweeney, C., 2008. Effect of tea saponin on methanogenesis, microbial community structure and expression of mcrA gene, in cultures of rumen micro-organisms. Lett. Appl. Microbiol. 47, 421–426.

Hagerman, A.E., Butler, L.G., 1978. Protein precipitation method for the quantitative determination of tannins. J. Agric. Food Chem. 26, 809–812.

Hagerman, A.E., Charles, T. Robbins, Y.W., Thomas, C.W, Claire, M., 1992. Tannin chemistry in relation to digestion. J. Range Manage. 45, 57–62.

Hagerman, A.E., Butler, L.G., 1991. Tannins and lignins. In: Herbivores: their interactions with secondary plant metabolites, Vol I: The chemical participants, Rosenthal, G.A., Berenbaum M.R. (Eds.), Academic Press, NY (USA), 355-388.

Hagerman, A.E., Butler, L.G., 1980. Determination of Protein in Tannin-Protein Precipitates. J. Agric. Food Chem. 28(5), 944–947. https://doi.org/10.1021/jf60231a010.

Hagerman, A.E., Rice, M.E., Ritchard, N.T., 1998. Mechanisms of protein precipitation for two tannins, pentagalloyl glucose and epicatechin (16) (4 - 8) catechin (procyanidin). J Agric. Food Chem. 46, 2590-2595.

Haque, M.N., 2018. Dietary manipulation: A sustainable way to mitigate methane emissions from ruminants. J. Anim. Sci. Technol. 60, 15. https://doi.org/https://doi.org/10.1186/s40781-018-0175-7.

Häring, D.A., Scharenberg, A., Heckendorn, F., Dohme, F., Luscher, A., Maurer, V., Suter, D. Hertzberg, H., 2008. Tanniferous forage plants: Agronomic performance, palatability and efficacy against parasitic nematodes in sheep. Renew. Agric. Food Syst. 23, 19-29.

Harris, S., Clark, D., Laboyrie, P., 1998. Birdsfoot trefoil-an alternative legume for New Zealand dairy pastures. NZ Grassland Assoc. 60, 99–103.

Hassanat, F., Benchaar, C., 2013. Assessment of the effect of condensed (acacia and quebracho) and hydrolysable (chestnut and valonea) tannins on rumen fermentation and methane production in vitro. J. Sci. Food Agric. 93(2), 332–339. https://doi.org/10.1002/jsfa.5763.

Hassanpour, S., 2011. Effect of condensed tannin on controlling faecal protein excretion in nematode-infected sheep. J. Amer. Sci. 7(5), 896–900.

UNIVERSITEIT VAN PRETORIA
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Hassanpour, S., Maheri-Sis, N., Eshratkhah, B., Mehmander, F.B., 2011. Plants and secondary metabolites (Tannins): A Review. Int. J. Forest, Soil Eros. 1(1), 47–53.

Hassanpour, S., Mehmandar, F.B., 2012. Anthelmintic effects of Acacia mearnsii (Wattle tannin) in small ruminants; A Review. J. Comp. Clin. Pathol. Res. 1(1), 1–8.

Herrero, M., Thornton, P.K., Gerber, P., Reid, R.S., 2009. Livestock, livelihoods and the environment: understanding the trade-offs. Curr. Opin. Environ. Sustain. 1, 111–120.

Herrero, M., Gerber, P.J., Garnett, T., 2011. Livestock and greenhouse gas emissions: The importance of getting the numbers right. Anim. Feed Sci. Technol. 166–167, 779–782. https://doi.org/10.1016/j.anifeedsci.2011.04.083.

Hervas, G., Perez, V., Giraldez, F.J., Mantecon, A.R., Almar M.M., Frutos, P., 2003b. Intoxication of sheep with quebracho tannin extract. J. Comp. Pathol. 129, 44-54.

Hervás, G., Frutos, P., Giraldez, F.J., Mantecon, A.R., Maria, C., Alvarez, D.P., 2003a. Effect of different doses of quebracho tannins extract on rumen fermentation in ewes. Anim. Feed Sci. Technol. 109(1–4), 65–78. https://doi.org/10.1016/S0377-8401(03)00208-6.

Hindrichsen, I.K., Kreuzer, M., Machmuller, A., Bach Knudsen, K.E., Madsen, J., Wettstein, H.R., 2003. Methane release and energy expenditure of dairy cows fed concentrates characterized by different carbohydrates. In: Souffrant, W.M., Metges, C.C. (Eds.), Proceedings of the EAAP-Symposium on Energy and Protein Metabolism and Nutrition. 413–416.

Ho, K.K.Y., Lee, J.W., Durand, G., Majumder, S., Liu, A., 2017. Protein aggregation with poly (-vinyl) alcohol surfactant reduces double emulsion-encapsulated mammalian cell-free expression. PLoS One 12(3), e0174689.

Hook, S.E., Wright, A.D.G. McBride, B.W., 2010. Methanogens: Methane producers of the rumen and mitigation strategies. Archaea 2010, 50–60. https://doi.org/10.1155/2010/945785.

Hristov, A.N., Oh, J., Firkins, J.L., Dijkstra, J., Kebreab, E., Waghorn, G., Makkar, H.P.S., Adesogan, A.T., Yang, W., Lee, C., Gerber, P.J., Henderson, B., Tricarico, J.M., 2013. SPECIAL TOPICS-Mitigation of methane and nitrous oxide emissions from animal operations: I. A review of enteric methane mitigation options. J. Anim.Sci. 91(11), 5045–5069. https://doi.org/10.2527/jas.2013-6583.

Huang, X.D., Liang, J.B., Tan, H.Y., Yahya, R., Khamseekhiew, B., Ho, Y.W., 2010. Molecular weight and protein binding affinity of Leucaena condensed tannins and their effects on in vitro fermentation parameters. Anim. Feed Sci. Technol. 159(3–4), 81–87. https://doi.org/10.1016/j.anifeedsci.2010.05.008.

Huang, X.D., Liang, J.B., Tan, H.Y., Yahya, R., Ho, Y.W., 2011. Effects of Leucaena condensed tannins of differing molecular weights on in vitro CH_4 production. Anim. Feed Sci.

UNIVERSITEIT VAN PRETORIA
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Technol. 166–167, 373–376. https://doi.org/10.1016/j.anifeedsci.2011.04.026.

Hulshof, R.B.A., Berndt, A., Gerrits, W.J.J., Dijkstra, J., van Zijderveld, S.M., Newbold, J.R., Perdok, H.B., 2012. Dietary nitrate supplementation reduces methane emission in beef cattle fed sugarcane-based diets. J. Anim. Sci. 90, 2317–2323. https://doi.org/doi:10.2527/jas.2011-4209.

Ibrahim, S.L., Hassen, A., 2021. Characterization, density and in vitro controlled release properties of Mimosa (Acacia mearnsii) tannin encapsulated in palm and sunflower oils. Animals 11, 2919. https://doi.org/10.3390/ani11102919.

Ibrahim, S.L., Hassen, A., 2022. Effects of graded levels of mimosa (Acacia mearnsii) tannin purified with organic solvents on gas, methane and in vitro organic matter digestibility of Eragrostis curvula hay. Animals 12, 562. https://doi.org/10.3390/ani12050562.

IPCC, Intergovernmental Panel on Climate Change 2007. Climate Change 2007: The Physical Science Basis Contribution of Working Group I to the Fourth Assessment Report of the Intergovernmental Panel on Climate Change. In: Solomon, S., Qin, D., Manning, M., Chen, Z., Marquis, M., Averyt, K.B., Tignor, M., Miller, H.L. (Eds.), Cambridge University Press, Cambridge, United Kingdom and New York, NY, USA. pp. 996.

Islam, M., Lee, S., 2019. Advanced estimation and mitigation strategies: a cumulative approach to enteric methane abatement from ruminants. J. Anim. Sci. Technol. 61, 3, 122-137. https://doi.org/10.5187/jast.2019.61.3.122.

Jafari, S.M., Assadpoor, E., He, Y., Bhandari, B., 2008. Encapsulation Efficiency of Food Flavours and Oils during Spray Drying. Drying Technol. 26, 7, 816–835. https://doi.org/10.1080/07373930802135972.

Janssen, P.H., 2010. Influence of hydrogen on rumen methane formation and fermentation balances through microbial growth kinetics and fermentation thermodynamics. Anim. Feed Sci. Technol. 160(1–2), 1–22.

Jayanegara, A., Wina, E., Soliva, C.R., Marquardt, S., Kreuzer, M., Leiber, F., 2011. Dependence of forage quality and methanogenic potential of tropical plants on their phenolic fractions as determined by principal component analysis. Anim. Feed Sci. Technol. 163(2–4), 231–243. https://doi.org/10.1016/j.anifeedsci.2010.11.009.

Jayanegara, A., Goel, G., Makkar, H.P.S., Becker, K., 2015. Divergence between purified hydrolysable and condensed tannin effects on methane emission, rumen fermentation and microbial population in vitro. Anim. Feed Sci. Technol. 209, 60–68. https://doi.org/10.1016/j.anifeedsci.2015.08.002.

Jayanegara, A., Leiber, F., Kreuzer, M., 2012. Meta-analysis of the relationship between dietary tannin level and methane formation in ruminants from in vivo and in vitro experiments', J. Anim. Physiol. Anim. Nutr. 96(3), 365–375. https://doi.org/10.1111/j.1439-0396.2011.01172.x.

UNIVERSITEIT VAN PRETORIA
UNIVERSITY OF PRETORIA
YUNIBESITHI YA PRETORIA

© University of Pretoria

Jayanegara, A., Togtokhbayar, N., Makkar, H.P.S., Becker, K., 2009. Tannins determined by various methods as predictors of methane production reduction potential of plants by an in vitro rumen fermentation system. Anim. Feed Sci. Technol. 150, 230–237. https://doi.org/10.1016/j.anifeedsci.2008.10.011.

Jerónimo, E., Pinheiro, C., Lamy, E., Teresa, M., Sales-baptista, E., Lopes, O., Capela, F., 2015. Tannins in ruminant nutrition: impact on animal performance and quality of edible products. In: Combs, C.A. (Ed.), Chapter 5. Tannins: Biochemistry, Food Sources and Nutritional Properties. Nova Science Publishers Inc., New York, USA. pp. 43.

Jeyanathan, J., Martin, C., Morgavi, D.P., 2014. The use of direct-fed microbials for mitigation of ruminant methane emissions: a review. Animal 8, 250–261. https://doi.org/doi 10.1017/S1751731113002085.

Johnson, D.E., Ward, G.M., 1996. Estimates of animal methane emissions. Environ. Monit. Assess. 42, 133–141.

Johnson, K.A., Johnson, D.E., 1995. Methane emissions from cattle. J. Anim. Sci. 73, 2483–2492. https://doi.org/doi:/1995.7382483x.

Jones, G.A., McAllister, T.A., Muir, A.D., Cheng, K.-J., 1994. Effects of sainfoin (Onobrychis viciifolia Scop.) condensed tannins on growth and proteolysis by four strains of ruminal bacteria. Appl. Environ. Microbiol. 60(4), 1374–1378. https://doi.org/doi://10.1128/aem.60.4.1374-1378.1994.

Jordan, E., Lovett, D., Monahan, F., Callan, J., Flynn, B., O'Mara, F., 2006. Effect of refined coconut oil or copra meal onmethane output and on intake and performance of beef heifers. J. Anim. Sci. 84, 162–170.

Jouany, J.-P., Morgavi, D.P., 2007. Use of 'natural' products as alternatives to antibiotic feed additives in ruminant production. Animal 1(10), 1443–1466. https://doi.org/10.1017/S1751731107000742.

Kamra, D.N., Pawar, M., Singh, B., 2012. Effect of plant secondary metabolites on rumen methanogens and methane emissions by ruminants. In: Patra, A.K. (ed.), Chapter 12. Dietary Phytochemicals and Microbes. Springer Netherlands., pp. 351–370. https://doi.org/10.1007/978-94-007-3926-0_12.

Kamra, D.N., 2005. Rumen microbial ecosystem. Curr. Sci. 89(1), 124–135. https://www.jstor.org/stable/24110438.

Kar, S., Kundu, B., Reis, R.L., Sarkar, R., Nandy, P., Basu, R., Das, S., 2019. Curcumin ameliorates the targeted delivery of methotrexate intercalated montmorillonite clay to cancer cells. Eur. J. Pharm. Sci. 135, 91–102.

Karamać, M., 2009. Chelation of Cu(II), Zn(II), and Fe(II) by tannin constituents of selected

UNIVERSITEIT VAN PRETORIA
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YUNIBESITHI YA PRETORIA

© University of Pretoria

edible nuts. Intern. J. Mol. Sci. 10(12), 5485–5497. https://doi.org/https://doi.org/10.3390/ijms10125485.

Kardel, M., Taube, F., Schulz, H., Schutze, W., Gierus, M., 2013. Different approaches to evaluate tannin content and structure of selected plant extracts - review and new aspects. J. Appl. Bot. Food Qual. 166, 154–166. https://doi.org/10.5073/JABFQ.2013.086.021.

Kaske, M., Engelhardt, W.V., 1990. The effect of size and density on mean retention time of particles in the gastrointestinal tract of sheep. British J. Nutr. 63(3), 457–465. https://doi.org/10.1079/bjn19900133.

Knapp, J.R., Laur, G.L., Vadas, P.A., Weiss, W.P., Tricarico, J.M., 2014. Invited review: Enteric methane in dairy cattle production: Quantifying the opportunities and impact of reducing emissions. J. Dairy Sci. 97(6), 3231–3261. https://doi.org/10.3168/jds.2013-7234.

Kobayashi, Y., 2010. Abatement of methane production from ruminants: Trends in the manipulation of rumen fermentation. Asian-Aust. J. Anim. Sci., 410–416. https://doi.org/doi:10.5713/ajas.2010.r.0123.

Krishnan, S., Bhosale, R., Singhal, R.S., 2005. Microencapsulation of cardamom oleoresin: Evaluation of blends of gum arabic, maltodextrin and a modified starch as wall materials. 61, 95–102. https://doi.org/10.1016/j.carbpol.2005.02.020.

Krueger, W., Gutierrez-Banuelos, H., Carstens, G.E., Min, B.R., Pinchak, W.E., Gomez, R.R., Anderson, R.C., Krueger, N.A., Forbes, T.D.A., 2010. Effects of dietary tannin source on performance, feed efficiency, ruminal fermentation, and carcass and non-carcass traits in steers fed a high-grain diet. Anim. Feed Sci. Technol. 159, 1-9. https://doi.org/https://doi.org/10.1016/j.anifeedsci.2010.05.003.

Ku-vera, J.C., Jiménez-ocampo, R., Valencia-salazar, S.S., Montoya-flores, M.D., Molina-botero, I.C., Arango, J., Gómez-bravo, C.A., Aguilar-pérez, C.F., Solorio-sánchez, F.J., 2020. Role of Secondary Plant Metabolites on Enteric Methane Mitigation in Ruminants. Frontiers Vet. Sci. 7, 584. https://doi.org/10.3389/fvets.2020.00584.

Kumar, R., Horigome, T., 1986. Fractionation, characterization and protein precipitating capacity of the condensed tannins from Robinia pseudoacacia (L.) leaves. J. Agric. Food Chem., 34, 3, 487–489. https://doi.org/10.1021/jf00069a029.

Kumar, R., Singh, M., 1984. Tannins: their adverse role in ruminant nutrition. J. Agr. Food Chem. 32, 3, 447-453. https://doi.org/10.1021/jf00123a006.

Kumar, S., Choudhury, P.K., Carro, M.D., Griffith, G.W., Dagar, S.S., Puniya, M., Calabro, S., Ravella, S.R., Dhewa, T., Upadhyay, R.C., Sirohi, S.K., Kundu, S.S., Wanapat, M., Puniya, A.K., 2013. New aspects and strategies for methane mitigation from ruminants. Appl. Microbiol. Biotechnol. 1-15. https://doi.org/10.1007/s00253-013-5365-0.

Landau, S., Perevolotsky, A., Bonfil, D., Barkai, D., Silanikove, N., 2000. Utilization of low

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UNIVERSITY OF PRETORIA
YUNIBESITHI YA PRETORIA

© University of Pretoria

quality resources by small ruminants in Mediterranean agro-pastoral systems: The case of browse and aftermath cereal stubble. Livest. Prod. Sci., 64, 39–49.

Lascano, C., Avila, P., Stewart, J., 2003. Intake, digestibility and nitrogen utilization by sheep fed with provenances of Calliandra calothyrsus Meissner with different tannin structure. Arch. Latinoam. Prod. Anim. 11, 21–28.

Lawal, I.B., 2022. Unpublished data from M. Sc. Dissertation. Department of Animal Science, University of Pretoria, South Africa.

Leahy, S.C., Janssen, P.H., Attwood, G.T., Mackie, R.I., McAllister, T.A., Kelly, W.J., 2022. Electron flow: key to mitigating ruminant methanogenesis. Trends Microbiol. 30(3), 209–212.

Lee, S.H., Shinde, P.L., Choi, J.Y., Kwon, I.K., Lee, J.K., Pak, S.I., Choi, W.T., Chae, B.J., 2010. Effects of tannic acid supplementation on growth performance, blood hematology, iron status and faecal microflora in weanling pigs. Livest. Sci. 131((2-3), 281-286. https://doi.org/https://doi.org/10.1016/j.livsci.2010.04.013.

Lee, S.S., Hsu, J.T., Mantovani, H.C., Russell, J.B., 2002. The effect of bovicin HC5, a bacteriocin from Streptococcus bovis BC5, on ruminal methane production in vitro. *FEMS* Microbiol. Lett. 217, 51–55.

Leng, R.A., 2008. The potential of feeding nitrate to reduce enteric methane production in ruminants. A Report to the Department of Climate Change Commonwealth Government of Australia, Canberra, ACT, Australia. Retrieved on 31/12/2022 at www.penambulbooks.com.

Li, Z., Liu, N., Cao, Y., Jin, C., Li, F., Cai, C., Yao, J., 2018. Effects of fumaric acid supplementation on methane production and rumen fermentation in goats fed diets varying in forage and concentrate particle size. J. Anim. Sci. Biotechnol. 9(21), 1–9. https://doi.org/10.1186/s40104-018-0235-3.

Lin, B., Lu, Y., Salem, A.Z.M., Wang, J.H., Liang, Q., Liu, J.X., 2013. Effects of essential oil combinations on sheep ruminal fermentation and digestibility of a diet with fumarate included. Anim. Feed Sci. Technol. 184, 24–32. https://doi.org/doi:10.1016/j.anifeedsci.2013.05.011.

Liu, H.W., Dong, X.F., Tong, J.M., Zhang, Q., 2011. A comparative study of growth performance and antioxidant status of rabbits when fed with or without chestnut tannins under high ambient temperature. Anim. Feed Sci. Technol. 164(1-2), 89-95. https://doi.org/https://doi.org/10.1016/j.anifeedsci.2010.09.020.

Liu, H.W., Zhou, D.W., Li, K., 2013. Effects of chestnut tannins on performance and antioxidative status of transition dairy cows. J. Dairy Sci. 96(9), 5901-5907. https://doi.org/https://doi.org/10.3168/jds.2013-6904.

UNIVERSITEIT VAN PRETORIA
UNIVERSITY OF PRETORIA
YUNIBESITHI YA PRETORIA

© University of Pretoria

Llonch, P., Haskell, M.J., Dewhurst, R.J., Turner, S.P., 2017. Review: current available strategies to mitigate greenhouse gas emissions in livestock systems: an animal welfare perspective. Animal 11(2), 274–284. https://doi.org/10.1017/S1751731116001440.

Lochab, B., Shukla, S., Varma, I.K., 2014. Naturally occurring phenolic sources: monomers and polymers. RSC Adv. 4, 21712–21752.

Macheboeuf, D., Lassalas, B., Ranilla, M.J., Carro, M.D., Morgavi, D.P., 2006. Dose response effect of diallyl disulfide on ruminal fermentation and methane production in vitro. Reprod. Nutr. Dev. 46, S103.

Macheboeuf, D., Morgavi, D.P., Papon, Y., Mousset, J.L., Arturo-Schaan, M., 2008. Dose-response effects of essential oils on in vitro fermentation activity of the rumen microbial population. Anim. Feed Sci. Technol. 145, 335–350. https://doi.org/doi:10.1016/j.anifeedsci.2007.05.044.

Mahanani, M.M.P., Kurniawati, A., Hnim, C., Anas, M.A., Yusiati, L.M., 2020. Effect of (Leucaena leucocephala) Leaves as Tannin Source on Rumen Microbial Enzyme Activities and In Vitro Gas Production Kinetics. IOP Conf. Ser.: Earth Environ. Sci. 478 012088. https://doi.org/10.1088/1755-1315/478/1/012088.

Maheri-Sis, N., Chaichi Semsari, M., Eshratkhah, B., Sadaghian, M., Gorbani, A., Hassanpour, S., 2011. Evaluation of the effects of Quebracho condensed tannin on faecal egg counts during naturally acquired mixed nematode infections in Moghani sheep. Annals Biol. Res. 2(2), 170-174.

Makkar, H.P.S., Dawra, R.K., Singh, B., 1987. Protein Precipitation Assay for Quantitation of Tannins: Determination of Protein in Tannin-Protein Complex. Analyt. Chem. 166, 435–439.

Makkar, H.P.S., Bluemmel, M., Borowy, N.K., Becker, K., 1993. Gravimetric determination of tannins and their correlations with chemical and protein precipitation methods. J. Sci. Food Agric. 61, 161–165.

Makkar, H.P.S., 2000. Quantification of Tannins in Tree and Shrub Foliage. A laboratory manual for the FAO/IAEA Co-ordinated Research Project on Use of Nuclear and Related Techniques to Develop Simple Tannin Assays for Predicting and Improving the Safety and Efficiency of Feeding Ruminants on Tanniniferous Tree Foliage. https://doi.org/10.1007/978-94-017-0273-7.

Makkar, H.P.S., 2003. Effects and fate of tannins in ruminant animals, adaptation to tannins, and strategies to overcome detrimental effects of feeding tannin-rich feeds. Small Rum. Res. 49, 241–256. https://doi.org/10.1016/S0921-4488(03)00142-1.

Makkar, H.P.S., 2005. In vitro gas methods for evaluation of feeds containing phytochemicals. Anim. Feed Sci. Technol. 123(124), 291–302.

UNIVERSITEIT VAN PRETORIA
UNIVERSITY OF PRETORIA
YUNIBESITHI YA PRETORIA

© University of Pretoria

Mamvura, C.I., Cho, S., Mbiriri, D.T., Lee, H., Choi, N., 2014. Effect of Encapsulating Nitrate in Sesame Gum on In vitro Rumen Fermentation Parameters. Asian-Australas. J. Anim. Sci. 27(11), 1577–1583.

Marino, D.J., 2005. Ethyl Acetate. Encyclopedia of Toxicology (Second Edition), 277-279. https://doi.org/10.1016/B0-12-369400-0/00390-2.

Martin, C., Morgavi, D.P., Doreau, M., 2010. Methane mitigation in ruminants: from microbe to the farm scale. Animal, 4(03), 351–365. https://doi.org/10.1017/s175173110999620.

Martínez, J.P.Q., Ruiz, J.C.R., Campos, M.R.S., 2018. Release kinetic studies of Stevia rebaudiana extract capsules from sodium alginate and inulin by ionotropic gelation. Adv. Mater. Sci. Eng. 2018.

Martınez, T.F., McAllister, T.A., Wang, Y., Reuter, T., 2006. Effects of tannic acid and quebracho tannins on in vitro ruminal fermentation of wheat and corn grain. J. Sci. Food Agric. 86, 1244–1256.

McAllister, T.A., Bae, H.D., Yanke, L.J., Cheng, K.J., Muir, A., 1994. Effect of condensed tannins from birdsfoot trefoil on endoglucanase activity and the digestion of cellulose filter paper by ruminal fungi. Can. J. Microbiol. 40, 298–305. https://doi.org/doi:10.1139/m94-048.

Mcallister, T.A., Newbold, C.J., 2008. Redirecting rumen fermentation to reduce methanogenesis. Austral. J. Experim. Agric. 48, 7–13.

McAllister, T.A., Okine, E.K., Mathison, G.W., Cheng, K.J., 1996. Dietary, environmental and microbiological aspects of methane production in ruminants. Can. J. Anim. Sci. 76, 231–243.

McDonald, P., Edwards, R., Greenhalgh, J.F.D., Morgan, C.A., Sinclair, L.A., Wilkinson, R.G., 2011. Animal Nutrition. 7th Ed., Harlow, Pearson education, Prentice Hall, England. pp 712.

McIntosh, F.M., Williams, P., Losa, R., Wallace, R.J., Beever, D.A., Newbold, C.J., 2003. Effects of essential oils on ruminal microorganisms and their protein metabolism. Appl. Environ. Microbiol. 69(8), 5011–5014. https://doi.org/10.1128/AEM.69.8.5011-5014.2003.

McNabb, W.C., Peters, J.S., Foo, L.Y., Waghorn, G.C., Jackson, F.S., 1998. Effects of condensed tannins prepared from several forages on the in vitro precipitation of ribulose-1,5-biphosphate carboxylase (Rubisco) protein and its digestion by trypsin (EC2.4.21.1). J. Sci. Food Agric. 77, 201–212.

McNabb, W.C., Waghorn, G.C., Peters, J.S., Barry, T.N., 1996. The effect of condensed tannins in Lotus pedunculatus on the solubilization and degradation of ribulose-1,5-bisphosphate carboxylase (EC 4.1.1.39; Rubisco) protein in the rumen and the sites of Rubisco digestion. Br. J. Nutr. 76, 535–549. https://doi.org/doi:10.1079/BJN19960061.

UNIVERSITEIT VAN PRETORIA
UNIVERSITY OF PRETORIA
YUNIBESITHI YA PRETORIA

© University of Pretoria

McNabbl, W.C., Waghorn, G.C., Barry, T.N., Shelton, I.D., 1993. The effects of condensed tannins in Lotus pedunculutus on the digestion and metabolism of methionine, cystine and inorganic sulphur in sheep. Br. J. Nutri. 70, 641–661.

McNeill, D., Osborne, N., Komolong, M., Nankervis, D., 1998. Condensed tannins in the genus Leucaena and their nutritional significance for ruminants. In Proceedings of the ACIAR Workshop, Hanoi, Vietnam, 9–14 February 1998; ACIAR: Canberra, Australia., 205–214.

McSweeney, C.S., Palmer, B., McNeill, D.M., Krause, D.O., 2001. Microbial interactions with tannins: Nutritional consequences for ruminants. Anim. Feed Sci. Technol. 91(1–2), 83–93. https://doi.org/10.1016/S0377-8401(01)00232-2.

Mehran, M., Masoum, S., Memarzadeh, M., 2020. Microencapsulation of Mentha spicata essential oil by spray drying: Optimization, characterization, release kinetics of essential oil from microcapsules in food models. Ind. Crops Prod. 154, 112694.

Méndez-Ortiz, F.A., Sandoval-Castro, C.A., Ventura-Cordero, J., Sarmiento-Franco, L.A., Torres-Acosta, J.F.J., 2018. Condensed tannin intake and sheep performance: A meta-analysis on voluntary intake and live weight change. Anim. Feed Sci. Technol. 245, 67–76.

Menke, K.H., Steingass, H., 1988. Estimation of the energetic feed value obtained from chemical analysis and in vitro gas production using rumen fluid. Anim. Res. Dev. 28, 7–55.

Mergeduš, A., Janžekovi, M., Škorjanc, D., Šumenjak, T.K., 2022. Growth Performance, Meat Quality, and Fecal Microbial Population in Limousin Bulls Supplemented with Hydrolyzable Tannins. Agric. 12, 939. https://doi.org/10.3390/ agriculture12070939.

Mijena, D., Getiso, A., 2021. Feeding and Nutritional Strategies to Reduce Methane Emission from Large Ruminants: Review. J. Aquac. Livest. Prod. 2(1), 1–9.

Miller, T.L., 1995. Ecology of methane production and hydrogen sinks in the rumen. Rumin. Physiol. Dig. Metab. Growth, Reprod. 317–331.

Millet, S., Maertens, L., 2011. The European ban on antibiotic growth promoters in animal feed: From challenges to opportunities. Vet. J. 187(2), 143–144. https://doi.org/10.1016/j.tvjl.2010.05.001.

Min, B.R., Hart, S.P., 2003. Tannins for suppression of internal parasites. J. Anim. Sci. 81, 102-109.

Min, BR., Solaiman, S., Shange, R., Eun, J.S., 2014. Gastrointestinal bacterial and methanogenic archaea diversity dynamics associated with condensed tannin-containing pine bark diet in goats using 16S rDNA amplicon pyrosequencing. Int. J. Microbiol. 11.

Min, B.R., Barry, T.N., Attwood, G.T., McNabb, W.C., 2003. The effect of condensed tannins

UNIVERSITEIT VAN PRETORIA
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YUNIBESITHI YA PRETORIA

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on the nutrition and health of ruminants fed fresh temperate forages: A review. Anim. Feed Sci. Technol. 106(1–4), 3–19. https://doi.org/10.1016/S0377-8401(03)00041-5.

Min, B.R., Pinchak, W.E., Anderson, R.C., Fulford, J.D., Puchala, R., 2006. Effects of condensed tannins supplementation level on weight gain and in vitro and in vivo bloat precursors in steers grazing winter wheat. J. Anim. Sci. 84(9), 2546–2554. https://doi.org/10.2527/jas.2005-590.

Min, B.R., Pinchak, W.E., Anderson, R.C., Puchala, R., 2015. Bloat Mitigation Potential of Plant Tannins and Yucca Extracts based on in Vitro Ruminal Fermentation and Methane Gas Production from Wheat Forage. Int. J. Res. Studies Agric. Sci. 1(6), 1–13.

Min, B.R., Castleberry, L., Allen, H., Parker, D., Waldrip, H., Brauer, D., Willis, W., 2019. Associative effects of wet distiller's grains plus solubles and tannin-rich peanut skin supplementation on in vitro rumen fermentation, greenhouse gas emissions, and microbial changes. J. Anim. Sci. 97(11), 4668-4681. https://doi:10.1093/jas/skz317.

Minho, A.P., Bueno, I.C.S., Gennari, S.M., Jackson, F., Abdalla, A.L., 2008. In vitro effect of condensed tannin extract from acacia (Acacia mearnsii) on gastrointestinal nematodes of sheep. Brazilian J. Vet. Parasitol. 17(1), 144–148. http://www.ncbi.nlm.nih.gov/pubmed/20059834.

Mir, Z., Acharya, S.N., Mir, P.S., Taylor, W.G., Zaman, M.S., Mears, G.J., Goonewardene, L.A., 1997. Nutrient composition, in vitro gas production and digestibility of fenugreek (Trigonella foenum-graecum) and alfalfa forages. Can. J. Anim. Sci. 77, 119–124.

Missio, A.L., Tischer, B., dos Santos, P.S.B., Codevilla, C., de Menezes, C.R., Barin, J.S., Haselein, C.R., Labidi, J., Gatto, D.A., Petutschnigg, A., Tondi, G., 2017. Analytical characterization of purified mimosa (Acacia mearnsii) industrial tannin extract: Single and sequential fractionation. Separ. Purif. Technol. 186, 218–225. https://doi.org/10.1016/j.seppur.2017.06.010.

Moreira, G.D., Lima, P.d.M.T., Borges, B.O., Primavesi, O., Longo, C., McManus, C., Abdalla, A., Louvandini, H., 2013. Tropical tanniniferous legumes used as an option to mitigate sheep enteric methane emission. Trop. Anim. Health Prod. 45(3), 879–882. https://doi.org/10.1007/s11250-012-0284-0.

Morgavi, D.P., Forano, E., Martin, C., Newbold, C.J., 2010. Microbial ecosystem and methanogenesis in ruminants. Animal, 4:7, 1024-1036. https://doi.org/10.1017/S1751731110000546.

Moss, A.R., Jouany, J.-P. Newbold, J., 2000. Methane production by ruminants: its contribution to global warming. Annal. Zootech. 49(3), 231–253. https://doi.org/10.1051/animres:2000119.

Mould, F.L., Morgan, R., Kliem, K.E., Krystallidou, E., 2005. A review and simplification of the in vitro incubation medium. Anim. Feed Sci. Technol. 123-124, 155–172.

UNIVERSITEIT VAN PRETORIA
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https://doi.org/10.1016/j.anifeedsci.2005.05.002.

Mueller-Harvey, I., 2006. Unravelling the conundrum of tannins in animal nutrition and health. J. Sci. Food Agric. 86, 2010–2037. ttps://doi.org/10.1002/jsfa.2577.

Munin, A., Edwards-lévy, F., 2011. Encapsulation of Natural Polyphenolic Compounds; a Review. Pharm. 3, 793-829. https://doi.org/10.3390/pharmaceutics3040793.

Murdiati, T., McSweeney, C., Lowry, J., 1992. Metabolism in sheep of gallic acid, tannic acid and hydrolysable tannin from terminalia oblongata. Aust. J. Agric. Res. 43, 1307–1319.

Murray, R., Bryant, A., Leng, R., 1976. Rates of production of methane in the rumen and large intestine of sheep. Br. J. Nutr. 36, 1–14.

Nagpal, R., Puniya, A.K., Griffith, G.W., Goel, G., Puniya, M., Sehgal, J.P., Singh, K., 2009. Anaerobic rumen fungi: potential and applications. Agric. Important Micro-Organisms II, 375–393.

Narjisse, H., Elhonsali, M.A., Olsen, J.D., 1995. Effects of oak (Quercus ilex) tannins on digestion and nitrogen balance in sheep and goats. Small Rum. Res., 18, 201-206.

National Research Council, N.R.C., 2007. Nutrient requirements of small ruminants: Sheep, goats, cervids, and new world camelids. National Academy of Sciences, Washington DC, US. The national Academy Press. pp. 384.

Naumann, H.D., Hagerman, A.E., Lambert, B.D., Muir, J.P., Tedeschi, L.O., Kothmann, M.M. 2014. Molecular weight and protein-precipitating ability of condensed tannins from warm-season perennial legumes. J. Plant Interactions 9(1), 212–219. https://doi.org/10.1080/17429145.2013.811547.

Naumann, H.D., Tedeschi, L.O., Zeller, W.E., Huntley, N.F., 2017. The role of condensed tannins in ruminant animal production: Advances, limitations and future directions. Revista Brasileira de Zootecnia 46(12), 929–949. https://doi.org/10.1590/S1806-92902017001200009.

Nawab, A., Tang, S., Gao, W., Li, G., Xiao, M., An, L., Wu, J., Liu, W., 2020. Tannin Supplementation in Animal Feeding; Mitigation Strategies to Overcome the Toxic Effects of Tannins on Animal Health : A Review. J. Agric. Sci. 12(4), 217–230. https://doi.org/10.5539/jas.v12n4p217.

Newbold, C., Ramos-Morales, E., 2020. Ruminal microbiome and microbial metabolome: effects of diet and ruminant host. Animal 14(1), 78–86.

Newbold, C.J., Lopez, S., Nelson, N., Ouda, J.O., Wallace, R.J., Moss, A.R., 2005. Propionate precursors and other metabolic intermediates as possible alternative electron acceptors to methanogenesis in ruminal fermentation in vitro. Br. J. Nutr. 94, 27–35.

UNIVERSITEIT VAN PRETORIA
UNIVERSITY OF PRETORIA
YUNIBESITHI YA PRETORIA

© University of Pretoria

https://doi.org/doi:10.1079/BJN20051445.

Niwinska, B., 2013. Digestion in Ruminants. In: Chang, C. (Eds.), Chapter 11 - Carbohydrates - Comprehensive Studies on Glycobiology and Glycotechnology. INTECH, pp. 245–258.

Nolan, J.V., Hegarty, R.S., Hegarty, J., Godwin, I.R., Woodgate, R., 2010. Effects of dietary nitrate on fermentation, methane production and digesta kinetics in sheep. Anim. Prod. Sci. 50, 801–806.

Novilla, M.N., 2018. Ionophores. In: Gupta, R.C. (Eds.), Veterinary toxicology: basic and clinical principles. Third edition. Chapter 78. pp. 1073–1092.

Ogawa, S., Yazaki, Y., 2018. Tannins from Acacia mearnsii De Wild. Bark: Tannin Determination and Biological Activities. Molecules 23, 837. https://doi.org/10.3390/molecules23040837.

Ogino, A., Orito, H., Shimadad, K., Hirooka, H., 2007. Evaluating environmental impacts of the Japanese beef cow–calf system by the life cycle assessment method. Anim. Sci. J. 78, 424–432.

Ørskov, E.R., McDonald, I., 1979. The estimation of protein degradability in the rumen from incubation measurements weighted according to rate of passage. J. Agric. Sci. Cambr. 92:(1970), 499–503.

Papas, A.M., Sniffen, C.J., Muscato, T.V., 1984. Effectiveness of Rumen-Protected Methionine for Delivering Methionine Postruminally in Dairy Cows. J. Dairy Sci. 67(3), 545–552. https://doi.org/10.3168/jds.s0022-0302(84)81337-5.

Pathak, A.K., Dutta, N., Banerjee, P.S., Goswami, T.K., Sharma, K., 2016. Effect of condensed tannins supplementation through leaf meal mixture on voluntary feed intake, immune response and worm burden in Haemonchus contortus infected sheep. J. Parasit. Dis. 40, 100–105.

Patra, A., Park, T., Kim, M., Yu, Z., 2017. Rumen methanogens and mitigation of methane emission by anti-methanogenic compounds and substances. J. Anim. Sci. Biotechnol. 8(1), 1–18. https://doi.org/10.1186/s40104-017-0145-9.

Patra, A.K., Saxena, J., 2009. Dietary phytochemicals as rumen modifiers: a review of the effects on microbial populations. Antonie Van Leeuwenhoek. 96, 363–375.

Patra, A.K., Saxena, J., 2011. Exploitation of dietary tannins to improve rumen metabolism and ruminant nutrition. J. Sci. Food Agric. 91, 24–37.

Patra, A.K., Yu, Z., 2014. Effects of vanillin, Quillaja saponin, and essential oils on in vitro fermentation and protein-degrading microorganisms of the rumen. Appl. Microbiol. Biotechnol. 98, 897-905.

UNIVERSITEIT VAN PRETORIA
UNIVERSITY OF PRETORIA
YUNIBESITHI YA PRETORIA

© University of Pretoria

Patra, A.K., Yu, Z., 2015. Effects of adaptation of in vitro rumen culture to garlic oil, nitrate, and saponin and their combinations on methanogenesis, fermentation, and abundances and diversity of microbial populations. Front Microbiol. 6, 1434.

Patra, A.K., 2012. Enteric methane mitigation technologies for ruminant livestock: A synthesis of current research and future directions. Environ. Monitor. Assess. 184(4), 1929–1952. https://doi.org/10.1007/s10661-011-2090-y.

Patra, A.K., Kamra, D.N., Agarwal, N., 2006. Effect of plant extracts on in vitro methanogenesis, enzyme activities and fermentation of feed in rumen liquor of buffalo. 128, 276–291. https://doi.org/10.1016/j.anifeedsci.2005.11.001.

Patra, A.K., Saxena, J., 2010. Phytochemistry. A new perspective on the use of plant secondary metabolites to inhibit methanogenesis in the rumen. Phytochem. 71(11–12), 1198–1222. https://doi.org/10.1016/j.phytochem.2010.05.010.

Pattanaik, B.N., Mandalia, H.C., 2011. Ethyl Acetate: Properties, Production Processes and Applications - A Review. Int. J. Curr. Res. Rev. 03(12), 23–40.

Pereira, A.M., Lurdes, M.D., Enes, N., Borba, A.E.S., 2022. Alternative pathways for hydrogen sink originated from the ruminal fermentation of carbohydrates: Which microorganisms are involved in lowering methane emission ? Anim. Microb. 4(1), 1–12. https://doi.org/10.1186/s42523-021-00153-w.

Pérez, V., Doce, R.R., García-Pariente, C., Hervás, G., Carmen Ferreras, M., Mantecón, Á.R., Frutos, P., 2011. Oak leaf (Quercus pyrenaica) poisoning in cattle. Res. Vet. Sci. 91(2), 269-277. https://doi.org/10.1016/j.rvsc.2010.12.015.

Perry, B., Sones, K., 2007. Poverty reduction through animal health. Sci. 315, 5810, 333–334. https://doi.org/10.1126/science.1138614.

Piñeiro-Vázquez, A.T., Canul-Solís, J.R., Alayón-Gamboa, J.A., Chay-Canul, A.J., Ayala-Burgos, A.J., Aguilar-Pérez, C.F., Solorio-Sánchez, F.J., Ku-Vera, J.C., 2015. Potential of condensed tannins for the reduction of emissions of enteric methane and their effect on ruminant productivity. Arch. Med. Vet. 47, 263–272.

Pineiro-Vazquez, A.T., Jimenez-Ferrer, G., Alayon-Gamboa, J.A., Chay-Canul, A.J., Ayala-Burgos, A.J., Aguilar-Perez, C.F., Ku-Vera, J.C., 2018. Effects of quebracho tannin extract on intake, digestibility, rumen fermentation, and methane production in crossbred heifers fed low-quality tropical grass. Trop. Anim. Health Prod. 50, 29–36. https://doi.org/DOI 10.1007/s11250-017-1396-3.

Porter, L.J., Hrstich, L.N., Chans, B.G., 1986. The Conversion of Procyanidins and Prodelphinidins to Cyanidin and Delphinidin. Phytochem. 2, 223–230. https://doi.org/10.1016/S0031-9422(00)94533-3.

UNIVERSITEIT VAN PRETORIA
UNIVERSITY OF PRETORIA
YUNIBESITHI YA PRETORIA

© University of Pretoria

Pressure to Gas Production Conversion, 2014. Ankom Technology: Mecedon, NY, USA. https://www.ankom.com/sites/default/files/documentfiles/RFS005_Pressure_to_Gas_Production_Conversion.pdf. Accessed on 13/07/2018.

Prevolnik, M., Škrlep, M., Brus, M., Pugliese, C., Čandek-Potokar, M., Škorjanc, D., 2012. Supplementing pig diet with 0.2% sweet chestnut (Castanea sativa Mill.) wood extract had no effect on growth, carcass or meat quality. Acta Agricul. Slov. 100(3), 83-88.

Priolo, A., Waghorn, G.C., Lanza, M., Biondi, L., Pennisi, P., 2000. Polyethylene glycol as a means for reducing the impact of condensed tannins in carob pulp: effects on lamb growth performance and meat quality. J. Anim. Sci. 78(4), 810–816. https://doi.org/10.2527/2000.784810x.

Rahman, K., 2007. Studies on free radicals, antioxidants, and co-factors. Clin. Interv. Aging 2, 219–236.

Reed, J.D., 1995. Nutritional Toxicology Polyphenols in of Tannins and Related Forage Legumes. J. Anim. Sci. 73, 1516–1528. https://doi.org/doi:/1995.7351516x.

Ribeiro, L.G., Machado, F.S., Campos, M.M., Guimaraes, R., Tomich, T.R., Reis, L.G., Coombs, C., 2015. Enteric methane mitigation strategies in ruminants: a review. Rev. Columb. Cie. Pec. 28, 124–143. https://doi.org/10.17533/udea.rccp.v28n2a02.

Rira, M., Morgavi, D.P., Genestoux, L., Djibiri, S., Sekhri, I., Doreau, M., 2019. Methanogenic potential of tropical feeds rich in hydrolyzable tannins. J. Anim. Sci. 97, 2700–2710. https://doi.org/10.1093/jas/skz199.

Robertson, L.J., Waghorn, G.C., 2002. Dairy industry perspectives on methane emissions and production from cattle fed pasture or total mixed rations in New Zealand. Proc. New Zeal. Soc. Anim. Prod. 62, 213–218.

Rojas-downing, M.M., Nejadhashemi, A.P., Harrigan, T., Woznicki, S.A., 2017. Climate Risk Management Climate change and livestock: Impacts, adaptation, and mitigation. Clim. Risk Man. 16, 145–163. https://doi.org/10.1016/j.crm.2017.02.001.

Romero-Pérez, A., Okine, E.K., Guan, L.L., Duval, S.M., Kindermann, M., Beauchemin, K.A., 2016. Effects of 3-nitrooxypropanol and monensin on methane production using a forage-based diet in rusitec fermenters. Anim. Feed Sci. Technol. 220, 67–72. https://doi.org/DOI 10.1016/j.anifeedsci.2016.07.013.

Rossi, F., Maurizio, M., Francesco, M., Giovanna, C., Gianfranco, P., 2003. Rumen degradation and intestinal digestibility of rumen protected amino acids: Comparison between in situ and in vitro data. Anim. Feed Sci. Technol. 108(1–4), 223–229. https://doi.org/10.1016/S0377-8401(03)00131-7.

Russell, J.B., 2002. Rumen Microbiology and and Its Role in Ruminant Nutrition. Publisher:

UNIVERSITEIT VAN PRETORIA
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Department of Microbiology, Cornell University, Ithaca, NY, USA.

Salami, S.A., Valenti, B., Bella, M., O'Grady, M.N., Luciano, G., Kerry, J.P., Jones, E., Priolo, A., Newbold, C.J., 2018. Characterisation of the ruminal fermentation and microbiome in lambs supplemented with hydrolysable and condensed tannins. FEMS Micorbiol. Ecol. 94(5), 1–13.

Saminathan, M., Tan, H.Y., Sieo, C.C., Abdullah, N., Ling Wong, C.M.V., Abdulmalek, E., Ho, Y.W., 2014. Polymerization degrees, molecular weights and protein-binding affinities of condensed tannin fractions from a leucaena leucocephala hybrid. Molecules 19(6), 7990–8010. https://doi.org/10.3390/molecules19067990.

Sanchez, V., Baeza, R., Galmarini, M.V., Zamora, M.C., Chirife, J., 2013. Freeze-Drying Encapsulation of Red Wine Polyphenols in an Amorphous Matrix of Maltodextrin. Food Bioprocess Technol. 6(5), 1350–1354. https://doi.org/10.1007/s11947-011-0654-z.

Santoso, B., Kume, S., Nonaka, K., Kimura, K., Mizokoshi, H., Gamo, Y., Takahashi, J., 2003. Methane emission, nutrient digestibility, energy metabolism and blood metabolites in dairy cows fed silages with and without galacto-oligosaccharides supplementation. Asian-Austral. J. Anim. Sci. 16, 534–540.

Santoso, B., Mwenya, B., Sar, C., Gamo, Y., Kobayashi, T., Morikawa, R., Kimura, K., Mizokhoshi, H., Takahashi, J., 2004. Effects of supplementing galactooligosaccharides, Yucca schidigera or nisin on rumen methanogenesis, nitrogen and energy metabolism in sheep. Livest. Prod. Sci. 91, 209–217.

Sar, C., Mwenya, B., Pen, B., Morikawa, R., Takaura, K., Kobayashi, T., 2005. Effect of nisin on ruminal methane production and nitrate/nitrite reduction in vitro. Aust. J. Agric. Res. 56, 803–810.

Schofield. P., 2000. Gas Production Methods. In: D'Mello, J.P.F. (Eds.), Chapter 10 - Farm Animal Metabolism and Nutrition. CABI Publishing, New York, USA. pp. 443.

Schofield, P., Pell, A.N., 1995. Validity of Using Accumulated Gas Pressure Readings to Measure Forage Digestion In Vitro. J. Dairy Sci. 78(10), 2230–2238. https://doi.org/10.3168/jds.S0022-0302(95)76850-3.

Schofield, P., Pitt, R.E., Pell, A.N., 1994. Kinetics of fiber digestion from in vitro gas production J. Anim. Sci. 72, 2980-2991. https://doi.org/10.2527/1994.72112980x.

Seeram, N., Lee, R., Hardy, M., Heber, D., 2005. Rapid large scale purification of ellagitannins from pomegranate husk, a by-product of the commercial juice industry. Sep. Purif. Technol. 41(1), 49–55. https://doi.org/10.1016/j.seppur.2004.04.003.

Sejian, V., Bhatta, R., Malik, P.K., Madiajagan, B., Al-Hosni, Y.A.S.., Sullivan, M., Gaughan, J.B., 2016. Livestock as Sources of Greenhouse Gases and Its Significance to Climate Change.

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YUNIBESITHI YA PRETORIA

© University of Pretoria

In: Moya, B.L., Pous, J. (Eds.), Chapter 11 - Greenhouse Gases. pp. 1–19. Intech. https://dx.doi.org/10.5772/62135.

Sharp, R., Ziemer, C.J., Stern, M.D., Stahl, D.A., 1998. Taxon-specific associations between protozoal and methanogen populations in the rumen and a model rumen system. FEMS Microbiol. Ecol., 26, 71–78. doi:10.1016/S0168-6496(98)00024-5.

Shreck, A., 2013. Use of alkaline treated crop residues as partial grain replacements for finishing cattle. Unpublished Doctoral Dissertation; University of Nebraska-Lincoln. USA.

Silanikove, N., Perevolotsky, A., Provenza, F.D., 2001. Use of tannin-binding chemicals to assay for tannins and their negative postingestive effects in ruminants. Anim. Feed Sci. Technol. 91(1–2), 69–81. https://doi.org/10.1016/S0377-8401(01)00234-6.

Singh, B., Sahoo, A., Sharma, R., Bhat, T.K., 2005. Effect of polethylene glycol on gas production parameters and nitrogen disappearance of some tree forages. Anim. Feed Sci. Technol. 123-124, 351–364. https://doi.org/10.1016/j.anifeedsci.2005.04.033.

Sirohi, S.K., Goel, N., Pandey, P., 2012. Efficacy of different methanolic plant extracts on anti-methanogenesis, rumen fermentation and gas production kinetics in vitro. Open Vet. J. 2, 72–77.

Smith, P., Martino, D., Cai, Z., Gwary, D., Janzen, H., Kumar, P., McCarl, B., Ogle, S., O'Ma ra, F., Rice, C., Scholes, B., Sirotenko, O., 2007. Agriculture. In: Metz, O.R., Davidson, P.R., Bosch, R.D., Meyer, L.A. (Eds.), Climate Change: Mitigation. Contribution of Working Group III to the Fourth Assessment Report of the Intergovernmental Panel on Climate Change, Cambridge, United Kingdom, Cambridge University Press.

Soares, S., Mateus, N., De Freitas, V., 2007. Interaction of different polyphenols with bovine serum albumin (BSA) and human salivary alpha-amylase (HSA) by fluorescence quenching. J. Agric. Food Chem. 55, 6726-6735.

Soltan, Y.A., Patra, A.K., 2021. Ruminal Microbiome Manipulation to Improve Fermentation Efficiency in Ruminants. In: Kumar, D.A. (Ed.), Animal Feed Science and Nutrition - Production, Health and Environment; Intech: London, England. pp. 1-21.

Soltan, Y.A., Morsy, A.M., Sallam, S.M.A., Louvandini, H., Abdalla, A.L., 2012. Comparative *in vitro* evaluation of forage legumes (prosopis, acacia, atriplex, and leucaena) on ruminal fermentation and methanogenesis. J. Anim. Feed Sci. 21(4), 759–772. https://doi.org/10.22358/jafs/66148/2012.

Soltan, Y.A., Morsy, A.M., Sallam, S.M.A., Lucas, R.C., Louvandini, H., Kreuzer, M., Abdalla, A.L., 2013. Contribution of condensed tannins and mimosine to the methane mitigation caused by feeding Leucaena leucocephala. Arch. Anim. Nutr. 67(3), 169–184. https://doi.org/10.1080/1745039X.2013.801139.

UNIVERSITEIT VAN PRETORIA
UNIVERSITY OF PRETORIA
YUNIBESITHI YA PRETORIA

© University of Pretoria

Sophal, C., Khang, D.N., Preston, T.R., Leng, R.A., 2013. Nitrate replacing urea as a fermentable N source decreases enteric methane production and increases the efficiency of feed utilization in Yellow cattle. Livest. Res. Rural Dev. 25, 1–7.

Steinfeld, H., Gerber, P., Wassenaar, T., Castel, V., Rosales, M., de Haan, C., 2006. Livestock's long shadow environmental issues and options. Livestock Environment and Development (LEAD), and Food and Agricultural Organization (FAO). pp. 416. Retrieved on 29/7/2022 from http://www.virtualcentre.org.

Stewart, E.K., Beauchemin, K.A., Dai, X., MacAdam, J.W., Christensen, R.G., Villalba, J.J., 2019. Effect of tannin-containing hays on enteric methane emissions and nitrogen partitioning in beef cattle. J. Anim. Sci. 97, 3286–3299.

Stover, M.G., Watson, R.R., Collie, R.J., 2016. Chapter 2 - Pre- and Probiotic Supplementation in Ruminant Livestock Production. In: Probiotic, Prebiotic and Symbiotic. Biocactive Foods in Health Promotion. pp. 25–36.

Sun, K., Liu, H., Fan, H., Liu, T., Zheng, C., 2021. Research progress on the application of feed additives in ruminal methane emission reduction: a review. Peer J. 9, e11151. https://doi.org/10.7717/peerj.11151.

Takahashi, J. 2006. Emission of GHG from livestock production in Japan. In: Soliva, C.R., Takahashi, J., Kreuzer, M. (Eds.), Greenhouse gases and animal agriculture: An update. International congress series 1293. Elsevier, Amsterdam, Netherlands. pp. 1–20.

Tan, H.Y., Sieo, C.C., Abdullah, N., Liang, J.B., Huang, X.D., Ho, Y.W., 2011. Effects of condensed tannins from Leucaena on methane production, rumen fermentation and populations of methanogens and protozoa in vitro. Anim. Feed Sci. Technol. 169(3–4), 185–193. https://doi.org/10.1016/j.anifeedsci.2011.07.004.

Tavendale, M.H., Meagher, L.P., Pacheco, D., Walker, N., Attwood, G.T., Sivakumaran, S., 2005. Methane production from in vitro rumen incubations with Lotus pedunculatus and Medicago sativa, and effects of extractable condensed tannin fractions on methanogenesis. Anim. Feed Sci Technol. 123-124, 403–419. https://doi.org/10.1016/j.anifeedsci.2005.04.037.

Taylor, J., Taylor, J.N.R., Belton, P.S., Minnaar, A., 2009. Kafirin Microparticle Encapsulation of Catechin and Sorghum Condensed Tannins. J. Agric. Food Chem. 57, 7523–7528. https://doi.org/10.1021/jf901592q.

Teather, R., Forster, R., 1998. Manipulating the rumen microflora with bacteriocins to improve ruminant production. Can. J. Anim. Sci. 78, 57–69.

Tedeschi, L.O., Muir, J.P., Naumann, H.D., Norris, A.B., Ramírez-restrepo, C.A., Mertens-talcott, S.U., 2021. Nutritional Aspects of Ecologically Relevant Phytochemicals in Ruminant Production. Front. Vet. Sci. 8, 628445. https://doi.org/10.3389/fvets.2021.628445.

Terrill, T.H., Waghorn, G.C., Woolley, D.J., McNabb, W.C., Barry, T.N., 1994. Assay and

UNIVERSITEIT VAN PRETORIA
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YUNIBESITHI YA PRETORIA

© University of Pretoria

digestion of 14C-labelled condensed tannins in the gastrointestinal tract of sheep. Br. J. Nutr. 72, 467–477. https://doi.org/doi:10.1079/BJN19940048.

Theodorou, M.K., Williams, B.A., Dhanoa, M.S., McAllan, A.B., France, J., 1994. A simple gas production method using a pressure transducer to determine the fermentation kinetics of ruminant feeds. Anim. Feed Sci. Technol. 48, 185–197. https://doi.org/10.1016/0377-8401(94)90171-6.

Tiemann, T.T., Lascano, C.E., Wettstein, H.-R., Mayer, A.C., Kreuzer, M., Hess, H.D., 2008b. Effect of the tropical tannin-rich shrub legumes Calliandra calothyrsus and Flemingia macrophylla on methane emission and nitrogen and energy balance in growing lambs. Animal 2, 790–799.

Tiemann, T.T., Avila, P., Ramírez, G., Lascano, C.E., Kreuzer, M., Hess, H.D., 2008a. In vitro ruminal fermentation of tanniniferous tropical plants: Plant-specific tannin effects and counteracting efficiency of PEG. Anim. Feed Sci. Technol. 146, 222–241. https://doi.org/10.1016/j.anifeedsci.2007.12.009.

Tilley, J.M.A., Terry, R.A., 1963. A two-stage technique for the in vitro digestion of forage crops. J. Brit. Grassland Soc. 18, 104–111.

Tolve, R., Galgano, F., Condelli, N., Cela, N., Lucini, L., Caruso, M.C., 2021. Optimization model of phenolics encapsulation conditions for biofortification in fatty acids of animal food products. Foods 10, 881. https://doi.org/10.3390/foods10040881.

Tondi, G., Pizzi, A., 2009. Tannin-based rigid foams: Characterization and modification. Ind. Crops Prod. 29, 356–363. https://doi.org/10.1016/j.indcrop.2008.07.003.

Ungerfeld, E.M., Kohn, R.A., 2008. The role of thermodynamics in the control of ruminal fermentation. Rumin. Physiol. 90, 8686. https://doi.org/10.3920/978-90-8686-566-6.

United Nation, 2013. World population projected to reach 9.6 billion by 2050. United Nations Department of Economic and Social Affairs. Retrieved on 31/12/2022 from http://www.un.org/development/desa/en/news/population/un-report-world-population-project/htm.

Van Der Watt, E., Pretorius, J.C., 2001. Purification and identification of active antibacterial components in Carpobrotus edulis L. J. Ethnopharmacol. 76, 87–91. https://doi.org/10.1016/S0378-8741(01)00197-0.

van Niekerk, W.A., Hassen, A., Snyman, L.D., Rethman, N.F.G., Coertze, R.J., 2009. Influence of mineral composition and rumen degradability of Atriplex nummularia (Hatfield Select F1) plants on selection preference of sheep. Afr. J. Range Forage Sci. 26, 91–96.

Van Soest, P.J., Robertson, J.B., Lewis, B.A., 1991. Symposium: carbohydrate methodology, metabolism, and nutritional implications in dairy cattle. J. Dairy Sci. 74, 3583–3597.

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Van Soest, P.J., 1994. Nutritional Ecology of Ruminants. 2nd Edition, Cornell University Press, Ithaca, London. pp. 122.

van Zijderveld, S.M., Fonken, B., Dijkstra, J., Gerrits, W.J.J., Perdok, H.B., Fokkink, W., Newbold, J.R., 2011. Effects of a combination of feed additives on methane production, diet digestibility, and animal performance in lactating dairy cows. J. Dairy Sci. 94, 1445–1454. https://doi.org/doi:10.3168/jds.2010-3635.

van Zijderveld, S.M., Gerrits, W.J.J., Apajalahti, J.A., Newbold, J.R., Dijkstra, J., Leng, R.A., Perdok, H.B., 2010. Nitrate and sulfate: Effective alternative hydrogen sinks for mitigation of ruminal methane production in sheep. J. Dairy Sci. 93, 5856–5866. https://doi.org/doi:10.3168/jds.2010-3281.

Vargas-ortiz, L., Andrade-yucailla, V., Barros-rodr, M., Lima-orozco, R., Mac, E., Contreras-barros, K., Guishca-cunuhay, C., 2022a. Influence of Acacia Mearnsii Fodder on Rumen Digestion and Mitigation of Greenhouse Gas Production. Animals 12, 2250. https://doi.org/10.3390/ani12172250.

Vargas-ortiz, L., Chavez-garcia, D., Barros-rodr, M., Andrade-yucailla, V., Lima-orozco, R., Mac, E., Guishca-cunuhay, C., Zeidan, A., Salem, M., 2022b. Rumen Function and In Vitro Gas Production of Diets Influenced by Two Levels of Tannin-Rich Forage. Fermentation 8, 607. https://doi.org/10.3390/ fermentation8110607.

Vasta, V., Nudda, A., Cannas, A., Lanza, M., Priolo, A., 2008. Alternative feed resources and their effects on the quality of meat and milk from small ruminants. Anim. Feed Sci. Technol. 147, 223–246. https://doi.org/10.1016/j.anifeedsci.2007.09.020.

Veneman, J.B., Muetzel, S., Hart, K.J., Faulkner, C.L., Moorby, M., Perdok, H.B., 2015. Does Dietary Mitigation of Enteric Methane Production Affect Rumen Function and Animal Productivity in Dairy Cows ? PLoS One 10(10), 1–18.

Versfeld, D.B., Le Maitre, D.C., Chapman, R.A., 1998. Alien invading plants and water resources in South Africa. A Preliminary Assessment. A Report to the Water Research Commission. WRC Report No. 99/98. pp. 443.

Waghorn, G.C., Tavendale, M.H., Woodfield, D.R., 2002. Methanogenesis from forages fed to sheep. Proc. New Zeal. Grassl. Assoc. 64, 159–165.

Waghorn, G., 2008. Beneficial and detrimental effects of dietary condensed tannins for sustainable sheep and goat production — Progress and challenges. Anim. Feed Sci. Technol. 147, 116–139. https://doi.org/10.1016/j.anifeedsci.2007.09.013.

Waghorn, G.C., Shelton, I.D., McNabb, W.C., 1994. Effects of condensed tannins in Lotus pedunculatus on its nutritive value for sheep 1. Nitrogenous aspects. J. Agric. Sci. 123, 99-107.

Wallace, R.J., Wood, T.A., Rowe, A., Price, J., Yanez-Ruiz, D.R., Williams, S.P., Newbold,

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C.J., 2006. Encapsulated fumaric acid as a means of decreasing ruminant methane emission. International Congress Series. 1293, 148–151. https://doi.org/10.1016/j.ics.2006.02.018.

Wang, Y., Douglas, G.B., Waghorn, G.C., Barry, T.N., Foote, A.G., 1996. Effect of condensed tannins in Lotus corniculatus upon lactation performance in ewes. J. Agric. Sci. 126, 353–362.

Webb, E.C., 1994. Synthesis of long chain fatty acids in ruminants and their effects on meat quality. pp. 58-59.

Wischer, G., Greiling, A.M., Boguhn, J., Steingass, H., Schollenberger, M., Hartung, K., Rodehutscord, M., 2014. Effects of long-term supplementation of chestnut and valonea extracts on methane release, digestibility and nitrogen excretion in sheep. Animal 8(6), 938–948. https://doi.org/10.1017/s1751731114000639.

Wolin, M.J., Miller, T.L., Stewart, C.S., 1997. Microbe-Microbe Interactions. In: Hobson, P.J., Stewart, C.S. (Eds.), The Rumen Microbial Ecosystem. Blackie Academic & Professional, London, pp. 467–491.

Wood, T.A., Wallace, R.J., Rowe, A., Price, J., Yáñez-Ruiz, D.R., Murray, P., Newbold, C. J., 2009. Encapsulated fumaric acid as a feed ingredient to decrease ruminal methane emissions. Anim. Feed Sci. Technol. 152, 62–71. https://doi.org/10.1016/j.anifeedsci.2009.03.006.

Woodward, S.L., Waghorn, G.C., Ulyatt, M.J., Lassey, K.R., 2001. Early indications that feeding Lotus will reduce methane emissions from ruminants. Proc. New Zeal. Soc. Anim. Prod. 61, 23–26.

Wu, H., Meng, Q., Zhou, Z., Yu, Z., 2019. Ferric citrate, and nitrate, saponin and their combinations affect in vitro ruminal fermentation, production of sulphide and methane and abundance of select microbial populations. J. Appl. Microbiol. 127, 150–158. https://doi.org/10.1111/jam.14286.

Yang, W.Z., Benchaar, C., Ametaj, B.N., Chaves, A.V., He, M.L., McAllister, T.A., 2007. Effects of garlic and juniper berry essential oils on ruminal fermentation and on the site and extent of digestion in lactating cows. J. Dairy Sci. 90, 5671–5681.

Zhang, J., Xu, X., Cao, Z., Wang, Y., Yang, H., Azarfar, A., Shengli, L., 2019. Effect of Different Tannin Sources on Nutrient Intake, Digestibility, Performance, Nitrogen Utilization, and Blood Parameters in Dairy Cows. Animals 9, 507. https://doi.org/10.3390/ani9080507.

Zhang, Z., Wang, Y., Si, X., Cao, Z., Li, S., Yang, H., 2020. Rumen methanogenesis, and fermentation, rumen, rumen fermentation and microbial community response to nitroethane, 2-nitroethanol, and 2-nitro-1-propanol: an in vitro study. Animals 10, 479. https://doi.org/10.3390/ani10030479.

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Zhou, H.C., Lin, Y.M., Wei, S.D., Tam, N.F.Y., 2011. Structural diversity and antioxidant activity of condensed tannins fractionated from mangosteen pericarp. Food Chem. 129, 1710–1720. https://doi.org/10.1016/j.foodchem.2011.06.036.

Zhou, X., Meile, L., Kreuzer, M., Zeitz, J.O., 2013. The effect of lauric acid on methane production and cell viability of Methanobrevibacter ruminantium. Adv. Anim. Biosci. 4(2), 458.

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