The use of encapsulated condensed tannin extract and β-carotene supplementation to manipulate oxidative status, production and reproduction performance in sheep

By

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Submitted in partial fulfilment of the requirements for the degree of

DOCTOR OF PHILOSOPHY:

ANIMAL SCIENCE

In the Department of Animal Science
Faculty of Natural & Agricultural Sciences
University of Pretoria

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Pretoria
November 2022

(1) In the name of Allah the Entirely Merciful, the Especially Merciful. (2) [All] praise is [due] to Allah, Lord of the worlds – (3) The Entirely Merciful, the Especially Merciful, (4) Sovereign of the Day of Recompense. (5) It is You we worship and You we ask for help. (6) Guide us to the straight path – (7) The path of those upon whom You have bestowed favor, not of those who have evoked [Your] anger or of those who are astray.

Holy Quran (Surah al-Fatihah)
Saheeh International Quran English Meanings

Declaration

I, Osman Merghani Makki Ahmed declare that this thesis which I hereby submit for the degree Ph.D. (Animal Science) at the University of Pretoria is my own work and has not previously been submitted by me for a degree at this or any other tertiary institution.

Signed…….

Date……07/11/2022……..

Preface

This thesis has been prepared under the supervision of Prof. Abubeker Hassen in the Department of Animal and Wildlife Sciences, University of Pretoria and Prof. Khoboso Lehloenya, Department of Agriculture, University of Zululand, South Africa. It consists of six chapters that include a literature review and manuscripts published in or prepared for submission to peer-reviewed journals. Chapter One reviews the literature on oxidative stress, biomarkers and assays and also addresses the sources, bioavailability, antioxidant capacity, potential effects of supplementation on production and reproduction and possible mechanisms for these effects for both condensed tannin and β-carotene.

Chapters Two, Three, Four and Five are formatted according to the guidelines of the specific journals. Chapter Two is a scientific paper entitled "Effect of *Acacia mearnsii* Tannin Extract Supplementation on Reproductive Performance and Oxidative Status of South African Mutton Merino Rams", and was published in *Animals*. Chapter Three is a meta-analysis paper entitled "Threshold inclusion level of tannin extract in Sheep and Cattle Feed: A meta-analysis" was written to be submitted to the journal of *Animal Feed Science and Technology*. Chapter Four contains a research manuscript prepared for submission to *Antioxidants* journal entitled "Effects of ß-carotene Supplementation and Age on the Oxidative Status, Production and Reproductive Performance of Grazing Merino Ewes". In Chapter Five, a review manuscript entitled "The effect of β-carotene supplementation below or above requirement on the production and reproduction performance of the female sheep and goats" was written to be submitted to the *South African Journal of Animal Science*. The final chapter of the thesis contains the conclusion and recommendations of the research findings and areas for further research as well as a critical evaluation of the study.

Journal article

Ahmed, O., Lehloenya, K., Mphaphathi, M. and Hassen, A., 2021. Effect of *Acacia mearnsii* Tannin Extract Supplementation on Reproductive Performance and Oxidative Status of South African Mutton Merino Rams. Animals, 11(11), p.3266. DOI: https://www.mdpi.com/2076-2615/11/11/3266.

Conference abstract

Ahmed, O.M.M., Hassen, A. & Lehloenya, K.C., 2019. Effect of β-carotene supplementation on production and reproduction traits in Merino ewes (Ovis aries). 51rst South African Society for Animal Science Congress, Bloemfontein, Free State, South Africa. Abstract available at: https://www.sasas.co.za/wp-content/uploads/2019/09/51st-SASAS-Congress-Book-of-Abstracts-final-27_08_2019.pdf.

Dedication

To the soul of my beloved mother (Nawal)

To my beloved father

To my loving wife (Hana) and kids (Nawal and Mohammed)

To my brothers (Mohammed & Mosap), sister (Sara), friends and fellow members

I dedicate this work.

Acknowledgements

My first and foremost thanks to the Almighty God "Allah" who gave me the strength, patience and knowledge I needed to complete this project.

I am immensely grateful to my supervisors Prof. Abubeker Hassen and Prof. Khoboso Lehloenya for their support, encouragement, guidance and input during my studies.

I am indebted to the University of Pretoria (UP) and the Sudanese Ministry of Higher Education and Scientific Research for their financial support. I am also grateful to my employer, the University of Khartoum (UofK), for granting me study leave to pursue my PhD.

I am deeply grateful to my mother (رحمها الله) and father for their love, prayers, care and sacrifices in bringing me up and preparing me for my future. I am especially grateful to my wife Hana and our children Nawal and Mohammed for their love, prayers, understanding and constant support.

List of Abbreviations

ABTS	2,2'-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid)
ADF	Acid detergent fibre
ADFD	Acid detergent fibre digestibility
AEC	Animal ethics committee
ARC	Agricultural Research Council
BW	Bodyweight
CIDR	Controlled internal drug released device
CL	Corpus luteum
CP	Crude protein
CPD	Crude protein digestibility
CRBD	Completely randomized block design
CRD	Completely randomised design
CT	Condensed tannin
DCM	Dichloromethane
DHA	Docosahexaenoic acid
DM	Dry matter
DMI	Dry matter intake
DMI	Dry matter intake
DMPD	N, N-dimethyl-p-phenylenediamine dihydrochloride
E1	Estrone
E2	Estradiol
EIA	Enzyme immunoassay

ET	Electron transfer
ETE	Encapsulated tannin extract
FSHR	Follicle-stimulating hormone receptor
GLM	General linear model
GSH-Px	Glutathione peroxidase
HAT	Hydrogen atom transfer
HNE	4-hydroxy-2-nonenal
HT	Hydrolysable tannin
IGF-1	Insulin-like growth factors
LHR	luteinizing hormone receptor
LSD	Least Significant Differences
MBW	Metabolic bodyweight
MDA	Malondialdehyde
NDF	Neutral detergent fibre
NDFD	Neutral detergent digestibility
NRC	National Research Council
NRF	National Research Foundation
OM	Organic matter
OMD	Organic matter digestibility
P4	Progesterone
PBP	Phospholipid-binding protein
PCET	Proton-coupled electron transfer
R^2	Determination coefficient

RMSE	Root mean squared error
ROS	Reactive oxygen species
RS	Reactive species
RSR	RMSE-observations standard deviation ratio.
RV	Relative variance
SE	Standard error
SOD	Superoxide dismutase
StAR	Steroidogenic acute regulatory
T3	Triiodothyronine
T4	Thyroxine
TBARS	Thiobarbituric acid-reactive substances
TE	Tannin extract
TEAC	Trolox equivalent antioxidant capacity
TMR	Total mixed ration

Table of contents

Declaration i
Preface ii
Dedication iv
Acknowledgements v
List of Abbreviations vi
Table of contents ix
List of Tables xiv
List of Figures xvii
ABSTRACT 19
GENERAL INTRODUCTION 23
Introduction 23
Project theme 26
Aim of the study 26
Specific objectives 26
Hypotheses 27
CHAPTER ONE 28
LITERATURE REVIEW 28
1.1 Oxidative stress 28
1.2 Oxidative status biomarkers 29
1.3 Antioxidant activity/capacity assays 32
1.4 Condensed tannin sources and extraction 34
1.5 Bioavailability of condensed tannin in ruminants 37
1.6 Tannin extracts as antioxidant sources and potential effects on production and reproduction performance 37
1.7 Possible mechanisms of tannin supplementation benefits on oxidative status production and reproduction: 40
1.8 Which level of condensed tannin supplementation is recommended 41
1.9 ß-carotene sources and extraction 42
1.10 Bioavailability of ß-carotene in ruminant 43

1.11 ß-carotene as an antioxidant source and potential effects on production and reproduction 45
1.12 Possible mechanisms of supplemental ß-carotene on oxidative status, production and reproduction 48
1.13 Which level of ß-carotene supplementation is recommended: 48
1.14 Conclusions 49
CHAPTER TWO 51
Effect of Acacia mearnsii Tannin Extract Supplementation on Reproductive Performance and Oxidative Status of South African Mutton Merino Rams 51
Simple Summary 51
Abstract 51
2.1 Introduction 52
2.2 Materials and methods 54
2.2.1 Experimental location and ethical clearance 54
2.2.2 Materials 55
2.2.3 Encapsulation of the tannin extract 55
2.2.4 Animals and treatments 55
2.2.5 Bodyweight and testicular measurements 56
2.2.6 Ambient temperature 57
2.2.7 Semen collection 57
2.2.8 Semen evaluations 58
2.2.8.1 Subjective evaluation 58
2.2.8.2 Objective evaluation 58
2.2.9 Blood collection and analysis 58
2.2.9.1 Oxidative status 59
2.2.9.2 Hormonal status 59
2.2.10 Statistical analyses 60
2.3 Results and discussion 60
2.4 Conclusions 74
2.5 Funding 75

2.11 Appendix 2.A 76
CHAPTER THREE 77
Abstract: 78
3.1 Introduction: 79
3.2 Material and methods: 82
3.2.1. Data set creation: 82
3.2.2. Inclusion and exclusion criteria 83
3.2.3 Data extraction and description 84
3.2.4 Parameter's response calculation 84
3.2.5 Statistical analysis: 85
3.3 Results 87
3.3.1 Threshold inclusion levels of tannin extract for DMI 87
3.3.2 Threshold inclusion levels of tannin extract for feed digestibility 93
3.3.2.1 Threshold inclusion levels of tannin extract for organic matter digestibility (OMD) 93
3.3.2.2 Threshold inclusion levels of tannin extract for crude protein digestibility (CPD) 97
3.3.2.3 Threshold inclusion levels of tannin extract for neutral detergent fibre digestibility (NDFD) 101
3.3.2.4 Threshold of TE inclusion levels on acid detergent fibre digestibility (ADFD): 104
3.3.3 Effect of TE inclusion on nitrogen balance 107
3.3.3.1 Effect of TE inclusion on faecal nitrogen in sheep and cattle 107
3.3.3.2 Effect of TE inclusion on urinary nitrogen in sheep and cattle 110
3.3.3.3 Effect of TE inclusion on retained nitrogen in sheep and cattle 112
3.3.4 Effect of TE inclusion on weight gain (WG) in sheep and cattle: 115
3.4 Discussion 118
3.4.1 Threshold inclusion levels of tannin extract for DMI 118
3.4.2 Threshold inclusion levels of tannin extract for Feed digestibility 120
3.4.2.1 Threshold inclusion levels of tannin extract for organic matter digestibility (OMD) 120

3.4.2.2 Threshold inclusion levels of tannin extract for crude protein digestibility.....121
3.4.2.3 Threshold inclusion levels of TE for NDFD and ADFD.....122
3.4.3 Effect of TE inclusion on nitrogen balance.....123
3.4.3.1 Effect of TE inclusion on faecal nitrogen in sheep and cattle.....123
3.4.3.2 Effect of TE inclusion on urinary nitrogen in sheep and cattle.....123
3.4.4 Effect of TE inclusion on weight gain in sheep and cattle:.....125
3.4.5 General feed digestibility thresholds for TE inclusion in cattle and sheep:.....125
3.5 Application on the outcomes: Directing the research of TE inclusion co-benefit on the ruminant reproduction performance:.....126
3.6 Conclusions and recommendations:.....127
3.10 Appendix 3:.....129
3.10.1 Appendix 3A:.....129
3.10.2 Appendix 3B:.....130
3.10.3 Appendix 3C:.....135
CHAPTER FOUR.....139
Effects of ß-carotene Supplementation and Age on the Oxidative Status, Production and Reproductive Performance of Grazing South Africa Merino Ewes.....139
Abstract.....139
4.1 Introduction.....140
4.2 Material and methods.....142
4.2.1 Materials.....142
4.2.2 Animals and Treatments.....142
4.2.3 Blood Sample Collection.....143
4.2.4 Bodyweight of Ewes and Lambs.....143
4.2.5 Ultrasonographic examinations.....143
4.2.6 Oestrus synchronization and hand mating.....144
4.2.7 Oxidative status.....144
4.2.8 Estrogen and Progesterone Analysis.....144
4.2.9 Statistical analyses.....145
4.3 Results and discussions:.....146

4.3.1 Effect of ß-carotene supplementation, physiological condition and age on the bodyweight of ewes 146
4.3.2 Effect of ß- carotene supplementation, physiological condition and age on oxidative status of ewes 147
4.3.3 Effect of ß-carotene supplementation, time of sampling (physiological condition) and age of animals on progesterone concentration 151
4.3.4 Effect of ß-carotene supplementation, physiological condition and age of animals on estrogen concentration 152
4.3.5 Effect of ß-carotene supplementation on the birth weight of lambs 153
4.3.6 Effect of carotene supplementation on the survival rate of lambs 155
4.3.7 Effect of ß-carotene supplementation and age on the litter size of lambs 156
4.4 Conclusions 158
CHAPTER FIVE 160
The effect of β-carotene supplementation below or above requirement on the production and reproduction performance of the female sheep and goats - A review 160
Abstract 160
5.1 Introduction 161
5.2 Methods 161
5.3 Oxidative stress 162
5.4 β-carotene 163
5.4.1 β-carotene functions 163
5.4.2 β-carotene requirements and toxicity 164
5.5 The effect of β-carotene supplementation below or above the requirement 165
5.6 Conclusions and recommendations 169
CHAPTER SIX 170
6.1 Conclusions 170
6.2 Recommendations 171
6.3 Areas for further research 171
6.4 Critical Evaluation 172
REFERENCES 175

List of Tables

Chapter 1

Table 1.1 The advantages and disadvantages of various oxidative stress biomarkers. Adapted from (Celi 2011) 30
Table 1.2 Summary of studies that have assessed the effect of supplemental tannin extracts on oxidative status, production and reproduction performance of sheep 39
Table 1.3 Chosen natural carotene formulations obtained from plant material. Adapted from (Bogacz-Radomska and Harasym 2018) 43
Table 1.4 Carotenoid and retinol concentrations in sheep, goats, and cattle grazing the same pasture. Adapted from (Yang et al. 1992)..... 45
Table 1.5 Summary of studies that have assessed the effect of supplemental ß-carotene on the production and reproduction performance of sheep 47

Chapter 2

Table 2.1 Effects of season and tannin supplementation on bodyweight and testicular measurements (Mean ± SE) of South African Mutton Merino rams ……..…. 62
Table 2.2 The effect of supplementation treatments on the semen quality parameters (Mean ± SE) of South African Mutton Merino rams …..……. 64
Table 2.3 The effects of supplementation treatments on sperm viability and morphology (Mean ± SE) of South African Mutton Merino rams ……. 65
Table 2.4 Effects of tannin type and tannin supplementation level on the testicular length and some semen quality parameters of Merino rams …… 70
Table 2.5 Pearson's Correlation Coefficient between weight, some testicular measurements, semen quality, and hormonal status variables in the study ……. 71
Table 2.A.1 The initial data of the experimental groups in the study ……. 76

Chapter 3

Table 3.1 Descriptive statistics of DMI, OMD, CPD, NDFD, ADFD, faecal N, urinary N, retained N with TE level and body weight used in the studies included in this meta-analysis to determine the responses to TE inclusion in sheep and cattle …….. 89
Table 3.2 Description of animals and feeds used to determine DMI response to TE inclusion in the studies of this meta-analysis 92

Table 3.3 Results of regression and the goodness of fit for the best fit model used to predict DMI response (% of the control) for TE inclusion (g/100 g of DM) 92
Table 3.4 Description of animals, feeds and methods used to determine OMD digestibility in the studies of this meta-analysis. 94
Table 3.5 Results of regression, the goodness of fit and joint tests analysis for the best fit models for OM digestibility response (%) to TE inclusion in sheep and cattle. 95
Table 3.6 Description of animals, feeds and methods used to determine CPD in this meta-analysis..... 98
Table 3.7 Results of regression, the goodness of fit and joint tests analysis for the best fit model for crude protein (CP) digestibility response (%) to TE inclusion (g/100 g of DM) in sheep and cattle. 100
Table 3.8 Description of animals, feeds and methods used to determine NDFD in the studies of this meta-analysis 102
Table 3.9: Results of regression, the goodness of fit and joint tests analysis for the best fit model for NDF digestibility response (%) to TE inclusion (g/100 g of DM) in sheep and cattle.... 103
Table 3.10 Description of animals, feeds and methods used to determine ADFD in the studies of this meta-analysis 105
Table 3.11: Results of regression, the goodness of fit and joint tests analysis for the best fit model for ADF digestibility response (%) to TE inclusion (g/100 g of DM) in sheep and cattle. 106
Table 3.12 Description of animals, feeds and methods used to determine faecal N in the studies of this meta-analysis 108
Table 3.13: Regression, the goodness of fit and joint tests analysis of the best fit model for faecal N response (% control) to TE inclusion (g/ kg MBW) in sheep and cattle..... 109
Table 3.14 Description of animals, feeds and methods used to determine urinary N in the studies of this meta-analysis 110
Table 3.15: Regression, the goodness of fit and joint tests analysis of the best fit model for urinary nitrogen response (% control) to TE inclusion (g/kg MBW) in sheep and cattle 112
Table 3.16 Description of animals and feeds used to determine retained N response to TE inclusion in the studies of this meta-analysis..... 113

Table 3.17 Regression, the goodness of fit and joint tests analysis of the best fit model for retained nitrogen response (% control) to TE inclusion (g/kg MBW) in sheep and cattle ...114
Table 3.18 Description of animals and feeds used to determine WG response in the studies included in this meta-analysis.....116
Table 3.19: Results of regression, the goodness of fit for the best fit models of WG response (%) to TE inclusion (g/100 g of DM) in the sheep and cattle diets. 117
Table 3A1: Summary of the studies included in the meta-analysis…….. 129

Chapter 4

Table 4.1: Effect of β-carotene supplementation on oestrous response, oestrous duration, oestrous onset, conception rate and corpus luteum size at 12 d after hand mating …… 157
Table 4.2 Effect of age on oestrous response, oestrous duration, oestrous onset, conception rate and corpus luteum size at 12 d after hand mating ……. 158

Chapter 5

Table 5.1 A list of peer-reviewed published articles that show the level of β-carotene supplemented, the level of β-carotene offered already in the diet and the animal requirements of β-carotene used in each study …… 168

List of Figures

Chapter 1

Figure 1.1: “Schematic and simplified representation of molecular interactions among some of the most common biomarkers of oxidative damage and antioxidant defences. Taken from (Beaulieu and Costantini, 2014) 32
Figure 1.2: Molecular structure of tannic acid and condensed tannin (adapted from (Gnanamani et al. 2001)) 35
Figure 1.3 Steps for commercial production of black wattle TE. Adapted from the NTE Company (Pty) Ltd website https://www.nte.co.za/contact-us/.....36
Figure 1.4 Molecular structure of ß-carotene 42

Chapter 2

Figure 2.1 Effect of time and supplementation treatments on the hormonal and oxidative status (Mean ± SE) of South African Mutton Merino rams …….…… 66

Chapter 3

Figure 3.1 Actual (unweighted) observations with the predicted line of DMI response (%) to TE inclusion level as g/100 g of DM (Model DMI) ……..….. 93
Figure 3.2 Actual (unweighted) observations with the linear predicted lines for the Effect of TE source on the OMD response (%) to TE inclusion level as g/100 g of DM ……97
Figure 3.3 Actual (unweighted) observations with the linear predicted lines for the effect of animal species on CPD response to TE inclusion level as g/100 g of DM ……..……101
Figure 3.4 Actual (unweighted) observations with the predicted lines for the effect of TE source on NDFD response (%) to TE inclusion level as g/100 g of DM ……..… 104
Figure 3.5 Actual (unweighted) observations with the predicted lines for the effect of TE source on ADFD response (%) to TE inclusion level as g/100 g of DM …….. 107
Figure 3.6 Actual (unweighted) observations with the quadratic predicted lines for the effect of the supplementation period on the retained N response (%) to TE inclusion level …… 115
Figure 3.7 Actual (unweighted) observations with linear and quadratic prediction lines for weight gain response to TE inclusion level as g/100 g of DM ……. 118

Chapter 4

Figure 4.1 Effect of physiological conditions on body weight (kg) of ewes …….....147

Figure 4.2: Effect of interaction between physiological condition and ß-carotene supplementation on oxidative status of ewes……..149
Figure 4.3: Effect of oestrous synchronization on the oxidative status of ewes…….150
Figure 4.4 Effect of the interaction between age and sampling time on the progesterone concentration in ewes …….....152
Figure 4.5 Effect of litter size on body weight (kg) of lambs at birth and 15 days old …… 154
Figure 4.6: Effect of litter size on survival rate (kg) of lambs up to 15 days old …….....156
Figure 4.7: Effect of β-carotene supplementation on the frequencies of singles, twins, triplets and quadruplets in merino ewes …… 156

The use of β-carotene and encapsulated condensed tannin to manipulate oxidative status, production and reproduction performance in sheep

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ABSTRACT

The study aimed to investigate the supplementation effect of *Acacia mearnsii* tannin extract and ß-carotene on the production and reproduction performance of sheep. To achieve this aim four studies (two experiments, one meta-analysis and one literature review) were conducted with the following specific objectives: The objective of study 1 was to investigate the supplementation effects of *Acacia mearnsii* tannin extract (TE) and encapsulated tannin extract (ETE) on reproduction performance and oxidative status of South African Mutton Merino rams. The objective of study 2 was to perform a meta-analysis to identify the threshold level of tannin extracts (black wattle, quebracho, and chestnut) on feed intake and digestibility in sheep and cattle. Study 3 was conducted to evaluate the effects of ß-carotene supplementation and age on the oxidative status of grazing merino ewes during breeding, pregnancy, parturition and early lactation as well as on their production and reproduction performance. The objective of study 4 was to review the literature to assess the effects of below and above requirement ß-carotene supplementation on the production and reproduction performance of female goats and sheep.

In the first trial, thirty merino rams were divided into five groups: 0.0 g TE (control), 1.5 g TE, 3 g TE, 1.5 g ETE, and 3 g ETE supplemented daily for 16 weeks transiting from autumn to winter. Bodyweight and testicular measurements were recorded biweekly. Semen and blood samples were collected weekly during the last five weeks of supplementation. In the second trial, 104 ewes were divided into four groups drenched daily with 100 mg, 75 mg, 50 mg or 0.0 mg (control) of ß-carotene in a completely randomized block design. Supplementation lasted 28 days before oestrous synchronization; 14 days during oestrous synchronization (CIDR) and hand-mating; 18 days post-hand-mating; 30 days before lambing and 15 days after lambing. All animals were grazed on Kikuyu grass (*Pennisetum clandestinum*). Six ewes from each group (total = 24) were plasma sampled to measure oxidative and hormonal status during the experiment. The data for the meta-analysis were extracted from forty-three peer-reviewed articles and one PhD thesis that met the selection criteria. We proposed and used the change percentage to calculate the responses of the dependent variables to the inclusion of TE. The change percentage compares the tannin-treated group with the control group in each study. Therefore, feed intake, digestibility parameters, N balance parameters, and weight gain were calculated in response to the addition of TE in each applicable study. Statistical analyses of the data were performed using the RStudio integrated tool. Study names were treated as random effects, whereas TE level, TE type or source, animal species and supplementation period were treated as fixed effects. Data used to review the supplementation effect of β-carotene on the production and reproduction performance of female sheep and goats were extracted from thirteen peer-reviewed published articles that met the selection criteria. We estimated the animal requirement for β-carotene, the amount of β-carotene provided to the animal in the diet and the amount of β-carotene supplementation used

in each study. Those estimations were done to indicate whether the diet fulfils the animal requirement of β-carotene or not.

Results showed that the increase in the ETE from 1.5 to 3 g/animal/day increased the testicular length and sperm concentration, as well as decreased the percentages of low and non-progressive spermatozoa ($p < 0.05$). Simultaneously, the increase in the TE from 1.5 to 3 g/animal/ day decreased semen volume and elevated the percentage of abnormal sperm ($p < 0.05$). The results suggest that the encapsulation of TE affords the maximum benefit of the TE on sperm quality. Treatments with TE and ETE did not affect the hormonal and oxidative status. Testicular measurements were significantly higher in autumn compared to winter. The plasma level of cortisol significantly correlates negatively with sperm motility, viability, and acrosome integrity.

The results of the meta-analysis showed that tannin sources and tannin type had no significant effect on feed intake, and the level of 3.5 g/100 DM is suggested as the TE threshold for feed intake in sheep and cattle. The levels of 5.7, 2.5, and 2.7 g/100 g DM are proposed as organic matter digestibility (OMD) thresholds for chestnut, quebracho and black wattle TE in sheep and cattle, respectively. The levels of 1 g and 2.3 g/100 g DM are proposed as the crude protein digestibility (CPD) thresholds for TE in sheep and cattle, respectively. The levels of 2.4, 2.5, and 0.5 g/100 g DM are proposed as neutral detergent fibre digestibility (NDFD) thresholds for chestnut, quebracho, and black wattle TE, respectively. For acid detergent fibre digestibility (ADFD), the thresholds were 3.2 and 1.8 g/100 g DM for chestnut and quebracho, respectively, while only 1 g/100 g of black wattle TE decreased ADFD by 15%. Our meta-analysis revealed that the CP digestibility model has the strongest and most accurate prediction of the response to TE inclusion compared to the other digestibility parameters studied (OM,

NDF and ADF). Therefore, based on the CP digestibility response, we propose to consider 1 and 2.3 g/100 g DM TE as thresholds for digestibility in cattle and sheep, respectively. The proposed thresholds showed a negligible effect on weight gain in cattle and sheep after ingestion of TE.

This study is the first to report that supplemental β-carotene above 75 mg/animal/day may act as a pro-oxidant in ruminants under oxidatively stressed conditions such as parturition. Age did not affect the oxidative status of the ewes. The supplemental ß-carotene did not affect bodyweight, oestrous parameters, corpus luteum size, conception rate and litter size. The supplemental β-carotene or age did not affect the lamb's survival rate or weight at birth or 15 days old. The survival rate of singles and twins is higher than that of triplets and quadruplets. Older ewes (4-6 y) showed earlier and longer duration of oestrus than the younger ones (1-3 y).

The finding of the review work showed that β-carotene supplementation enhances the production and reproduction performance of the sheep when fed on a carotene-deficient diet. β-carotene supplementation may increase the levels of some hormones such as progesterone in the blood of sheep and goats when fed on a diet that fulfils their requirements of β-carotene. However, this supplementation of β-carotene above the requirements seems to not affect the production and reproduction parameters such as body weight, milk production, oestrous parameters, conception rate, pregnancy rate, and litter size.

The results of this study provide more insight into the optimum application of supplemental tannin extract and ß-carotene as a source of antioxidants in ruminants in general and in sheep in particular.

GENERAL INTRODUCTION

Introduction

In mammalian cells, enzymatic and non-enzymatic processes can generate a group of oxidants called reactive oxygen species (ROS) (Bayr 2005). ROS typically act as secondary messengers, controlling normal physiological functions (Valko et al. 2007). However, ROS can add oxygen or remove hydrogen from the target molecule, leaving its outer orbit with an unpaired electron, thus rendering it unstable (Lykkesfeldt and Svendsen 2007). Antioxidant systems, which involve both enzymatic and non-enzymatic antioxidants, protect the body naturally (Sies 1997, Kabel 2014). Nevertheless, ROS levels can exceed the organism's antioxidant capacity, resulting in oxidative stress (Miller et al. 1993, Weiss 1998, Agarwal et al. 2020).

In males, testicular cells divide rapidly and consume a large amount of mitochondrial oxygen (Asadi et al. 2017). In addition, testicular tissues are rich in unsaturated fatty acids, making the male reproductive system suspectable to oxidative stress (Asadi et al. 2017). In the scenario of oxidative stress, ROS may decrease sperm motility and harm the DNA and plasma membrane (Saleh and Agarwal 2002, Baker and Aitken 2004). ROS have been reported to cause 30% to 80% of male subfertility cases (Showell et al. 2014). Among females, oxidative stress may induce granulosa cell apoptosis (Tilly and Tilly 1995), endometriosis, polycystic ovarian syndrome, spontaneous abortion, preeclampsia, recurrent pregnancy loss and unexplained infertility (Agarwal et al. 2012).

Thus, supplemental antioxidants are proposed as a tool for breaking down the oxidative chain reaction and improving animal reproduction and production performance (Celi 2011, Showell et al. 2014, Asadi et al. 2017). Synthetic antioxidants are broadly used in animal

nutrition (Salami et al. 2016), but consumer safety concerns have recently sparked interest in natural antioxidants (Soldado et al. 2021). Several plant-derived antioxidants such as flavonoids, polyphenols, and carotenoids are proposed to fulfil this role (Scalbert and Williamson 2000, Kadifkova Panovska et al. 2005).

Indeed, condensed tannin is a promising antioxidant source for animal nutrition (Huang et al. 1992, Deng et al. 2016). In South Africa, tannin is mainly extracted from the bark of black wattle (*Acacia mearnsii*), one of the richest sources of tannin (Roux et al. 1995). Given the vulnerability of the male reproductive system to oxidative stress, tannin extract could be used to improve rams' oxidative statuses and, as a result, their reproduction performance. However, tannin's antioxidant ability must be balanced against its potential to diminish nutrient digestibility and feed intake (Dschaak et al. 2011). Researchers believe that tannin's negative impacts on feed intake and digestibility are due to its bitterness and remarkable ability to bind with protein and other feed components at high concentrations (Dschaak et al. 2011, Patra and Saxena 2011, Adejoro et al. 2018). Thus, tannin-related adverse effects on feed intake and digestibility can be reduced by lowering tannin levels in the diet or encapsulating tannins to slow down their release in the digestive tract (Adejoro et al. 2018). Indeed, utilising tannin extracts within the safe limit necessitates determining the tannin extracts' threshold levels on ruminant feed intake and digestibility and understanding how source of tannin and species of animals affect the threshold levels.

There are pieces of evidence that normal physiological conditions such as breeding, pregnancy, parturition and lactation may trigger oxidative stress in ewes (Kamiloğlu et al. 2006, Piccione et al. 2008, Mohebbi-Fani et al. 2012, Ognik et al. 2015, Lotfollahzadeh et al. 2016, Santarosa et al. 2021), goats (Di Trana et al. 2006, Celi et al. 2008, Jozwik et al. 2010,

Zobel et al. 2015) and cows (Drackley 1999, Castillo et al. 2005). It also has been observed in several species, including sheep, that age affects their vulnerability to oxidative stress (Ashok and Ali 1999, Andriollo-Sanchez et al. 2005, Salar-Amoli and Baghbanzadeh 2010). In animals, β-carotene functions primarily as pro-vitamin A (Smith 1998, Nagarajan et al. 2017) and as a scavenger of free radicals, especially singlet state oxygen (Stahl and Sies 2005). The supplementation of ewes with ß-carotene may improve oxidative status during the so-called oxidatively stressed conditions and thereby improve their production and production outcomes. Nonetheless, the use of ß-carotene supplementation as therapy for oxidative stress is controversial (Lykkesfeldt and Svendsen 2007). For example, Some authors reported a positive impact for β-carotene supplementations (Peirce 1954, Salem et al. 2015), while others reported no effect on the production and reproduction performance (Brozos et al. 2007, Meza-Herrera et al. 2011, Köse et al. 2013, Meza-Herrera et al. 2014, Gore 2016, Meza-Herrera et al. 2017) indicating the need to systematically review the available literature on ß-carotene supplementation effect. Besides others, two of the reasons for the controversial result might be the physiological condition at which the ß-carotene was supplemented and the age of the animals used. The NRC has recommended a minimum requirement for ß-carotene in ruminant diets based on the animal's physiological stage (NRC 2007). Therefore, the disparity in results might also be a result of the level of ß-carotene used in the diets being under or above those requirements.

This study investigated the effects of supplemental condensed tannin extract in rams and ß-carotene in ewes on their oxidative status, production and reproduction performance. The study also analyzed the available literature in the form of meta-analysis to identify the threshold level of tannin extracts (black wattle, quebracho, and chestnut) on feed intake,

digestibility, and N balance in sheep and cattle, and a systemic review of the literature to assess the effects of ß-carotene supplementation below and above the requirement on female goat and sheep production and reproduction performance.

The results of this study may give more insight into the optimum application of supplemental tannin extract and ß-carotene as a source of antioxidants in ruminants in general and in sheep in particular.

Project theme

Enhancing animal production and reproduction, focusing on the use of antioxidants supplementation in animals (sheep) as a tool to improve their oxidative status and thus their production and reproduction performance.

Aim of the study

The overall objective of this thesis is to determine the effects of black wattle tannin extract and ß-carotene supplementation on the oxidative status, production and reproduction performance of sheep.

Specific objectives

1. To investigate the supplementation effects of *Acacia mearnsii* tannin extract (TE) and encapsulated tannin extract (ETE) on testicular measurements, semen quality, hormonal status, and oxidative status of South African Mutton Merino rams.
2. To evaluate seasonal variations in the testicular measurements of South African Mutton Merino rams during the transition from autumn to winter.

3. To perform a meta-analysis to determine TE threshold inclusion levels (Blackwattle, quebracho and chestnut) on feed intake, digestibility, N balance and weight gain in sheep and cattle.
4. To evaluate the effect of ß-carotene supplementation and age on the oxidative status of grazing merino ewes during breeding, pregnancy, parturition and early lactation as well as on their production and reproduction performance.
5. To conduct a review of the literature to assess the effects of ß-carotene supplementation below and above the requirement on female goat and sheep production and reproduction performance.

Hypotheses

The tested null hypotheses were:

H0: Supplemental tannin extract has no significant effects on the oxidative status, production and reproduction performance of merino rams.

H0: Supplemental ß-carotene has no significant effects on the oxidative status, production and reproduction performance of merino ewes.

CHAPTER ONE

LITERATURE REVIEW

1.1 Oxidative stress

"Oxidative stress is an imbalance between oxidants and antioxidants in favour of the oxidants, leading to a disruption of redox signalling and control and/or molecular damage" (Sies and Jones 2007). Under pathophysiological conditions, oxidants are produced at increased rates, but they are also formed as a normal byproduct of aerobic metabolism under normal conditions (Sies 1997). Furthermore, oxidants such as reactive oxygen species (ROS) are engaged in a variety of physiological processes, so a certain level is preferable (Sugino 2006, Celi 2011). Unfortunately, this level is unknown for ruminants at various physiological stages (Celi 2011). The term reactive oxygen species (ROS) refers to both free radicals like superoxide anion radical (O2_) and hydroxyl radical (OH) and their non-radical intermediates like hydrogen peroxide (Burton and Jauniaux 2011, Demir et al. 2020). Free radicals are molecules or molecular fragments that have unpaired electrons, making them very reactive chemically, such as the free hydroxyl radical (·OH) (Slater 1988). Free radicals highly react with any surrounding molecules such as proteins, lipids, nucleic acids and sugars (Tirzitis and Bartosz 2010). So, when ROS generation exceeds the antioxidants' neutralizing capacity, the radical and non-radical ROS can initiate a chain of reactions that cause extensive damage to DNA, proteins and lipids (Ames et al. 1993, Al-Sowayan 2015). Oxidative stress has direct effects on the animal, including peroxidative alteration in membranes and other cellular structures, and an indirect effect by interfering with metabolic functions, such as causing a decrease in the NADPH available for physiological processes (Miller et al. 1993).

1.2 Oxidative status biomarkers

The study of oxidative stress in animals, particularly ruminants, is still in its early stages (Celi 2011). Although researchers may have access to a variety of rapidly evolving methodologies for assessing oxidative status, choosing the best method can be challenging (Celi 2011). Table 1.1 summarizes the advantages and drawbacks of various biomarkers commonly used to assess oxidative stress.

Table 1.1 The advantages and disadvantages of various oxidative stress biomarkers. Adapted from (Celi 2011)

Biomarker	Assay procedure	Advantages	Disadvantages
MDA	Colirometry, luminometry, chemiluminescence, HPLC, GC/MS	Sensitive and reproducible	Non-specific product of lipid peroxidation; interferes with TBARS assay
TBARS	Spectrometry, luminometry chemiluminescence	Rapid, popular, easy, and economical	Non-specific, non-reproducible, no quantitative relationship with lipid peroxidation
F2-Isoprostane	EIA, ELISA, HPLC, GC/MS	Specific, reproducible, sensitive	Expensive, auto-oxidation of samples, sample derivitisation required
ORAC	Fluorescence	Sensitive and covers a wide variety of antioxidants	Requires spectro-fluorimeter; AAPH used a free radical source undergoes spontaneous decay and it is sensitive to temperature
FRAP	Spectrometry	Serum dilution effect not seen	Fe may generate free radicals; not every free radical reduces Fe; GSH is not measured
TEAC	Spectrometry	Extremely fast and simple	Results vary with sample dilution; antioxidant used may interact with solvent molecules; specificity varies
TRAP	Chemiluminescence	Gives an idea of the rate of free radical formation	Antioxidant employed may not trap all types of free radicals
ROMs	Spectrometry	Extremely fast, simple; only 5–20 μL of plasma/serum required; can be performed directly in whole blood, inflammatory fluids, cell extracts and respiratory condensate	Inhibited by sodium azide
BAP	Spectrometry	Extremely fast, simple and covers a wide variety of antioxidants; only 5–20 μL of plasma/serum required	Can be performed only in plasma and serum samples; hyperlipemic samples can underestimate results

2 AAPH, 2,2′-azobis(2-amidinopropane) dihydrochloride; BAP, biological antioxidant potential; EIA, enzyme immunoassay; ELISA, enzyme-linked immunosorbent assay; FRAP, ferric reducing ability of plasma; GC, gas chromatography; HPLC, high-pressure liquid chromatography; MDA, malonildialdehyde; MS, mass spectrometry; ORAC, oxygen radical absorbance capacity; ROMs, reactive oxygen metabolites; TBARS, thiobarbituric acid reactive substances; TEAC, trolox equivalent antioxidant capacity; TRAP, total radical antioxidant potential.

Biomarkers of oxidative damage present the oxidative stress end-points (Sohal and Orr 2012), whereas biomarkers of antioxidant defences provide information about how the organism responds to the oxidative stressor (Beaulieu and Costantini 2014). Indeed understanding those interactions, helps select the appropriate biomarkers required to obtain as much detail about the animal's oxidative status as possible. (Beaulieu and Costantini 2014) illustrated those interactions between oxidative damage and antioxidant defences biomarkers in animals as seen in Figure 1. For example, the fatty acids oxidation increases the presence of oxidative damage derivatives known as hydroperoxides. Those hydroperoxides are converted later to lipid peroxidation end-products such as malondialdehyde (MDA), isoprostanes and 4-hydroxy-2-nonenal (HNE). The MDA and HNE may form adducts with proteins, forming protein carbonyls. Nonetheless, reactive species (RS) may oxidise proteins directly to protein carbonyls. Indeed, many peroxidation pathways may result in form of MDA, HNE and isoprostanes. Enzyme glutathione peroxidase reduces hydroperoxides to alcohols. The process includes the oxidation of glutathione (the cofactor) and the reduction back by the glutathione reductase enzyme. Enzymatic and non-enzymatic antioxidants can neutralize the effect of ROS and other RS. The action of non-enzymatic antioxidants such as vitamins E, vitamin C, polyphenols and carotenoids can be quantified using a variety of in vitro antioxidant capacity assays. Assays are also available to quantify the activity of enzymes the organism uses to repair damage to DNA, RNA or telomeres.

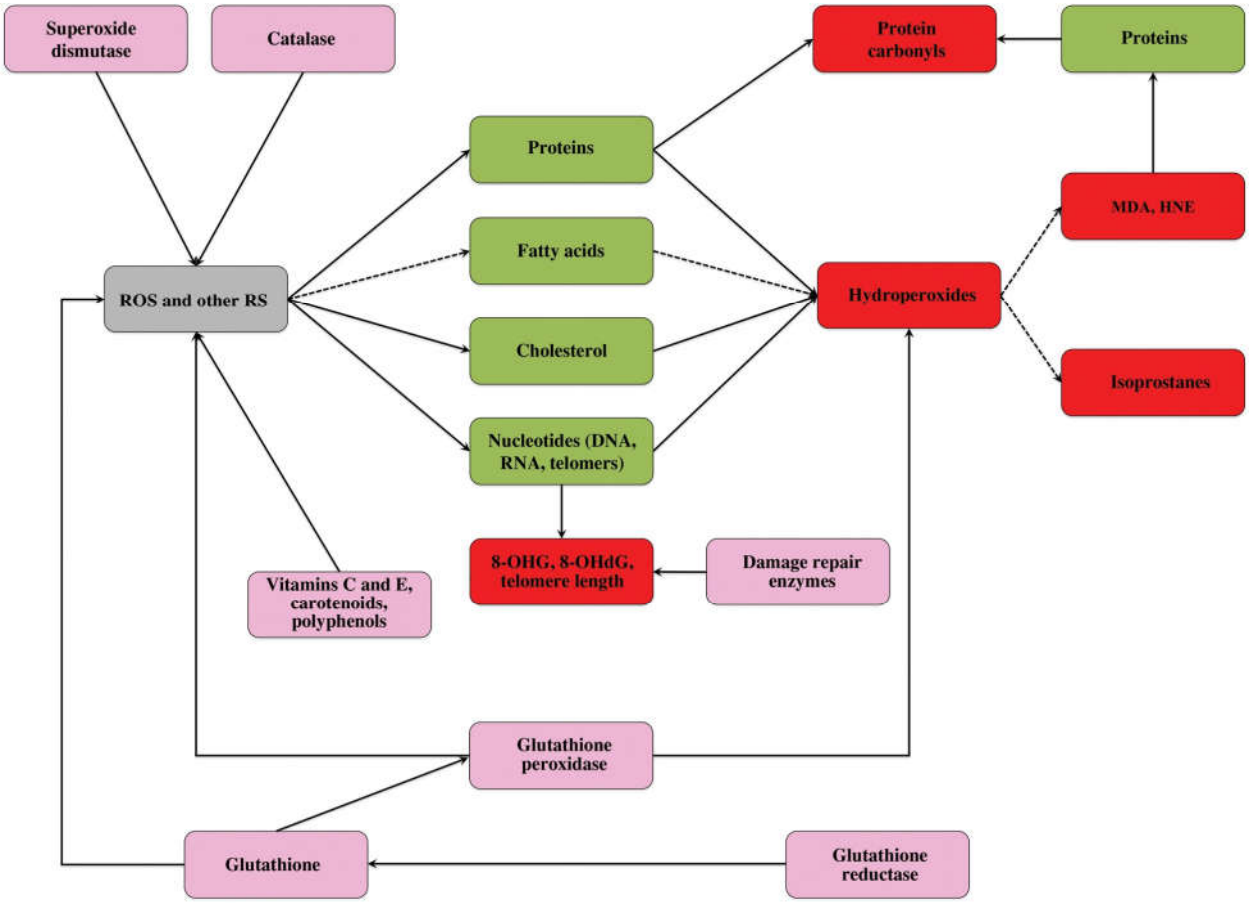

Figure 1.1: "Schematic and simplified representation of molecular interactions among some of the most common biomarkers of oxidative damage and antioxidant defences. Dashed lines indicate the fatty acid peroxidation chain. 8-OHG, 8-hydroxyguanosine; 8-OHdG, 8-hydroxy-2′-deoxyguanosine. Key: green, substrates that can be oxidized; grey, radical and non-radical reactive species; pink, antioxidant molecules (including damage repair enzymes); and red, oxidative damage compounds"(Beaulieu and Costantini, 2014)." Taken from (Beaulieu and Costantini, 2014)

1.3 Antioxidant activity/capacity assays

Assays for monitoring antioxidant activity/capacity utilize a variety of mechanisms, which include hydrogen atom transfer (HAT), electron transfer (ET), and mixed-mode

(ET/HAT) assays (Apak et al. 2016). HAT-based methods assess an antioxidant compound's ability to scavenge ROS such as peroxyl radical (ROO•) via hydrogen donation (Apak et al. 2016). The radicals of the antioxidant (A•) and the aryloxyl (ArO•) are usually stabilized by resonance. The ORAC assay, TRAP assay and TOSC assay are examples of HAT-based assays (Apak et al. 2016).

On the other hand, the ET-based method measures the antioxidant capacity via the reducing action of an oxidant that changes colour when reduced (ET) (Kimondo 2020). In these assays, reactions are slower than those of HAT-based and pH and solvent-dependent (Apak et al. 2016). Therefore, most ET reactions happen at a faster rate at high pH (Apak et al. 2016). Instead of biologically relevant reactive species, ET-based (or reduction-based) assays may employ artificial probes that change colour or fluorescence when reduced by antioxidants (such as peroxyl radicals) (Apak et al. 2016). The change in probe absorbance represents the sample's total concentration of antioxidants, also known as total antioxidant capacity (Apak et al. 2016).

The mixed-mode assays (ET- and HAT-based) typically depend on antioxidants that scavenge a stable radical chromophore (DPPH• and ABTS•+) or fluorophore (Apak et al. 2016). HAT, ET, and proton-coupled electron transfer (PCET) mechanisms may perform varying roles in those mixed-mode assays, depending on pH, solvent, and other parameters (Apak et al. 2016). The ABTS/Trolox equivalent antioxidant capacity (TEAC) assay, the DPPH radical scavenging assay, and the N, N-dimethyl-p-phenylenediamine dihydrochloride (DMPD) radical scavenging assay are three of the most common mixed-mode assays (Apak et al. 2016, Munteanu and Apetrei 2021). The DMPD assay is based on the fact that in the presence of a suitable oxidant, DMPD converts to coloured DMPD•+ at weakly acidic pH. DMPD•+ exhibits the highest absorbance at 505 nm (Apak et al. 2016). The decolourization

reaction proceeds quickly, and the stable endpoint is used to calculate antioxidant potency (Apak et al. 2016). DMPD is commonly used to assess the antioxidant capacity of natural products such as fruit juices (Apak et al. 2016). Mehdi and Rizvi successfully modified the DMPD-based method to measure the human plasma oxidative status as they age (Mehdi and Rizvi 2013). In the existence of oxidants such as ROS or ferric iron, the DMPD is converted to the quinonic DMPD•+, which is scavenged by antioxidants found in the studied matrices (Apak et al. 2016).

As a result, the DMPD method can assess both the antioxidant capacity and the oxidative status of unknown samples (Apak et al. 2016). The assay measures the hydroperoxides produced from the oxidation of lipids, amino acids and peptides to estimate the oxidative status/capacity in the plasma (Verde et al. 2002).

1.4 Condensed tannin sources and extraction

Phenolics are aromatic benzene ring compounds with single or more hydroxyl groups, including derivatives such as esters, glycosides and methyl ethers (Harborne 1989, Tyagi et al. 2020). Researchers have discovered more than 8,000 phenolic structures, including simple molecules such as phenolic acids and complex polymers like tannins (Dai and Mumper 2010). Hence, phenolics are the most abundant and widely distributed secondary metabolites in plants (Lattanzio 2013). Originally, tannins were defined as substances found in vegetable extracts that turned animal skins into durable leather (Jansman 1993). Tannins can be grouped based on their structure into hydrolysable and condensed tannins. Condensed tannins are composed of flavan-3-ol monomer subunits, whereas hydrolyzable tannins contain glucosides from gallic acid or ellagic acid (Haslam 1998, Laghi et al. 2010).

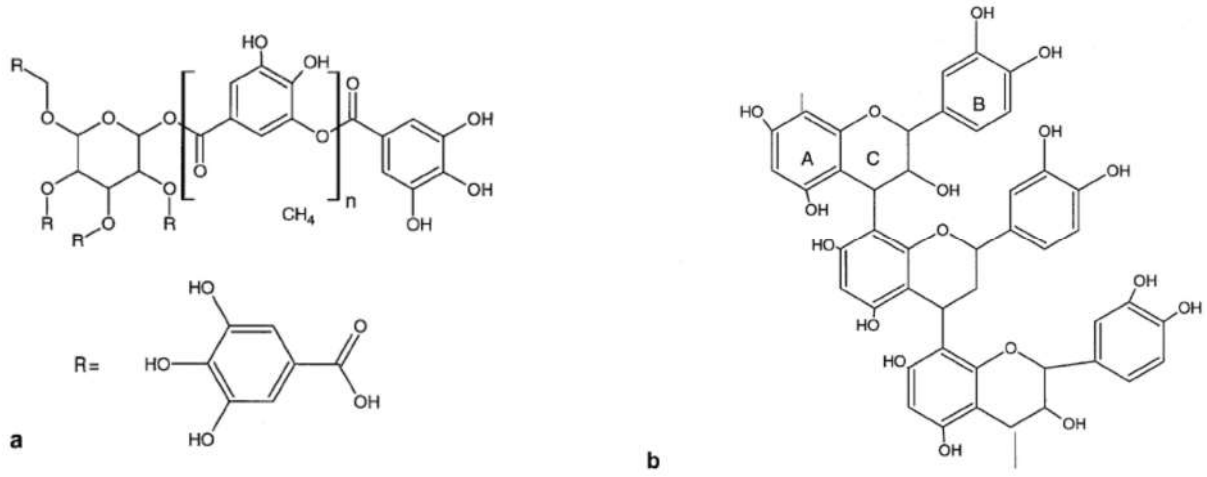

Figure 1.2: Molecular structure of tannic acid (a) and condensed tannin (b) (adapted from (Gnanamani et al. 2001))

Blackwattle (*Acacia mearnsii*) and quebracho tannins are one of the most common sources of natural condensed tannins extract used in animal feed (Frutos et al. 2004, Castillo et al. 2020, Soldado et al. 2021). Other sources such as grape pomace and grape seed extract are also used (Jerónimo 2011, Soldado et al. 2021). Currently, black wattle, also known as mimosa, is the main source of condensed tannin extract worldwide, with an actual production of 112,000 tons per year (Arenillas et al. 2019). Blackwattle bark is one of the highest-yielding sources of condensed tannin extract (Searle 1997). Plants are extracted to concentrate desirable compounds (Shi et al. 2005). Polyphenol extraction involves first dissolving the polyphenols in the plant matrix and then diffusing them in a solvent medium (Kallithraka et al. 1995, Shi et al. 2005). The extraction can be done using one of the three main methods:

1- Organic solvents: the extraction of polyphenols in this method is dependent on the use of organic solvents. However, with organic solvents being harmful if ingested, membrane separation was suggested as an alternative (Shi et al. 2005).

2- Membrane separation: The solvents are still used in this method, but in smaller amounts, and membranes are used after the polyphenols have been separated and concentrated from the solvent-solute complex (Shi et al. 2005).

3- Supercritical fluid extraction SFE: is currently being used for large-scale production. In this method, polyphenols will be extracted using high pressure and considered the safest and quickest extraction method. Although the initial cost of such a system is high, long-term savings may be obtained compared to other methods (Shi et al. 2005). This method was reported to produce extracts with stronger antioxidant power than those produced by other methods (Tipsrisukond et al. 1998). The reason could be the absence of light and air, which reduces the degradation of the highly sensitive polyphenols (Tipsrisukond et al. 1998, Shi et al. 2005). Figure shows the commercial manufacturing steps of black wattle TE.

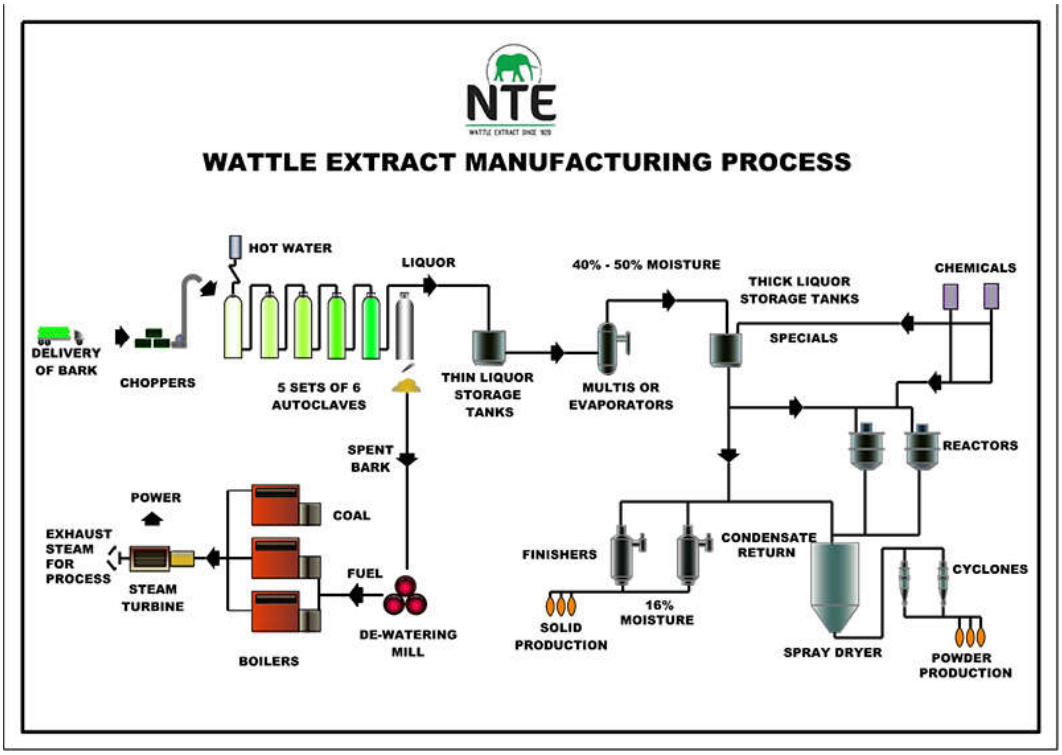

Figure 1.3 Steps for commercial production of black wattle TE. Adapted from the NTE Company (Pty) Ltd website https://www.nte.co.za/contact-us/

1.5 Bioavailability of condensed tannin in ruminants

Few studies have been conducted on polyphenol bioavailability in ruminants compared to monogastric animals (Gessner et al. 2017). Those studies suggest that ruminants absorb at least a small portion of polyphenols (Gessner et al. 2017). Despite that, it is difficult to obtain definitive conclusions about polyphenol bioavailability and further research is needed (D'Archivio et al. 2010). In particular, as far as we know, no study has been done on the bioavailability of condensed tannins in ruminants. Nevertheless, tannins' bioavailability is directly related to their polymerization degree and molecular weight as well as the animal's digestibility capacity (Fraga-Corral et al. 2021, Soldado et al. 2021). Condensed tannins are polymeric and have a high molecular weight, which may limit their bioavailability (Soldado et al. 2021). Therefore, the contribution of dietary condensed tannins to animal antioxidant status through absorption is probably limited. However, condensed tannins are thought to exert the majority of their biological activities in the gastrointestinal tract (Soldado et al. 2021). Condensed tannins can scavenge free radicals in the intestine, chelate metals, and prevent the production of toxic compounds resulting from lipid oxidation and peroxidation (Kerem et al. 2006). Moreover, many studies have shown that condensed tannins can protect other antioxidant molecules from oxidation, such as vitamin E (Lobón et al. 2017, Jerónimo et al. 2020). This would increase the amounts of antioxidant compounds along the gastrointestinal tract, contributing to enhancing the animal's antioxidant status (Soldado et al. 2021).

1.6 Tannin extracts as antioxidant sources and potential effects on production and reproduction performance:

Numerous tannin sources have been extensively studied for their effects on ruminal biohydrogenation, methane emissions, protein degradation, bloating, and parasite control

(Soldado et al. 2021). The tannin sources applied in these strategies included plants, part of plants, by-products and condensed tannin extracts (Soldado et al. 2021). However, few studies have assessed tannin's ability to improve animal antioxidant status and even fewer when limited to tannin extracts. Tannin extracts may represent antioxidants of great interest for animal nutrition.

For instance, condensed tannin extracted from *F. altissima* leaves protected plasmid DNA against hydroxyl radicals and prevented cellular damage caused by organic hydroperoxidet-BHP (Deng et al. 2016). It has been reported that among phenolics, condensed tannins quench peroxyl radicals 15–30 times better than simple phenolics (Hagerman et al. 1998). Table 1.2 summarises the studies that have assessed the outcoming effects of TE supplementation in sheep. The summary (Table 1.2) indicates that TE has the potential to serve as an antioxidant source and improve the antioxidant status of sheep. The improvement in antioxidant status was manifested by an increase (↑) in total antioxidant capacity, glutathione peroxidase, and superoxide dismutase, and a decrease (↓) in malondialdehyde (Table 1.2). However, some reports failed to observe a significant effect (=) on the oxidative or antioxidant status of the sheep (Table 1.2). Various factors may have contributed to the differences in outcomes, including the source of TE (e.g., chestnut and black wattle), type of tannin (hydrolysable *vs* condensed) and the level of TE used in each study (Table 1.2). The summary also indicates the potential for TE to improve spermatogenesis and general testicular health. Increased testicular antioxidant status, seminiferous tubule development, Sertoli cell number, testis weight, testis volume, and epididymis weight were evidence of this potential (Table 1.2).

Table 1.2 Summary of studies that have assessed the effect of supplemental tannin extracts on oxidative status, production and reproduction performance of sheep

Animal species	TE Source	CT level g/kg DM	Diet	Sample type	Outcomes	References
Lambs	Blackwattle TE	40	Concentrate based Diet	Meat	Fatty acids oxidation (=), vitamin E (=)	(Valenti et al. 2019)
Lambs	Chestnut TE	40	Concentrate based Diet	Meat	Fatty acids oxidation (=), vitamin E (=)	(Valenti et al. 2019)
Lambs	Chestnut TE	5 &10	Basal diet	Meat, serum and liver	SOD (↑), total antioxidant capacity (↑), GSH-Px (↑), MDA (↓), cortisol (↓), T3 (↓), T4 (↓), creatine kinase activity (↓), white blood cell count (↓), neutrophil count (↓), neutrophil: lymphocyte ratio (↓) & lymphocyte count (↑).	(Liu et al. 2016)
Lambs	Grape seed TE	0.05	Barley straw + concentrate ad libitum; grape pomace included in the concentrate	Meat	MDA (=), TBARS (=)	(Guerra-Rivas et al. 2016)
Lambs	Grape seed TE	3.6 & 7.2	Basic diet (Barley straw 26%, Corn 45.25 %, Malt root 8% and Concentrates 20.75 %)	Serum & testicular tissue	Testis weight (↑), testis volume (↑), epididymis weight (↑), antioxidant status of the testis (↑), SOD (↑), total antioxidant capacity (↑), testis seminiferous tubule development (↑), number of Sertoli cells (↑)	(Li et al. 2021)

(=) = Non significant, (↑) = statistically significant increase, (↓) = statistically significant decrease, TE = tannin extract, MDA = malondialdehyde, TBARS = thiobarbituric acid-reactive substances, SOD = superoxide dismutase, T3 = triiodothyronine, T4= thyroxine, GSH-Px = glutathione peroxidase, (*) In liver was not significant (=).

1.7 Possible mechanisms of tannin supplementation benefits on oxidative status production and reproduction:

Four possible potential mechanisms can be described as follows:

1- TE supplementation may selectively induce gene expression: According to Liu et al., grape pomace TE stimulates the transcription of the luteinizing hormone receptor (LHR) gene, thereby increasing steroidogenic acute regulatory (StAR) protein synthesis (Li et al. 2021). Steroid hormone biosynthesis is regulated by the (StAR) gene (Clark and Stocco 1996). In that context, TE supplementation may increase sensitivity to LH and FSH by stimulating the expression of follicle-stimulating hormone receptor (FSHR) and luteinizing hormone receptor (LHR) (Li et al. 2021). As well, low concentrations of tea tannins were reported to increase mRNA and protein expression of catalase and superoxide dismutase in goat skeletal muscle cells in vitro (Zhong et al. 2011).

2- TE may stimulate docosahexaenoic acid (DHA) synthesis (Li et al. 2021): DHA in the testis is believed to contribute to spermatogenesis and sperm quality as the decrease in its level in the sperm membrane is correlated with reduced fertility (Kelso et al. 1997, Zalata et al. 1998). In this regard, (Li et al. 2021) recently reported that grape pomace extract consumption increased the concentration of DHA in the testes.

3- Improving oxidative status in testes and preventing oxidative stress: (Li et al. 2021) reported that GPE increased total antioxidants capacity TAOC and SOD concentration in testis and up-regulated Cu-ZnSOD and GPX3 expression.

4- Indirect antioxidant effects: A sparing effect of polyphenols on other antioxidants like ascorbic acid and tocopherols is also suggested (Halliwell et al. 2005, Iglesias et al. 2012). For example,

researchers reported that supplemental quebracho condensed tannins may increase the level of vitamin E in sheep muscles (Lobón et al. 2017, Valenti et al. 2019).

1.8 Which level of condensed tannin supplementation is recommended

Tannins are well-known in plants for their anti-nutritional properties, which act as a natural defence mechanism against predators (Huang et al. 2018, Adejoro 2019). Therefore, the tannins' potential to serve as antioxidants in animals must be balanced against their ability to reduce feed intake and digestibility (Dschaak et al. 2011). These negative responses are related to the bitterness of tannins and their remarkable tendency to bind with dietary ruminal protein, starch, cellulose, and hemicellulose, as well as digestive enzymes and endogenous proteins when consumed in high concentrations (Butler 1992, Degen et al. 1995, Dschaak et al. 2011, Patra and Saxena 2011, Adejoro et al. 2018). Bitterness and the tendency to lower digestibility can be overcome by lowering the tannin amount in the diet and/or slowing its release rate in the digestive tract through encapsulation. The encapsulation of tannin extract slows the release rate of tannin extract in ruminants (Adejoro et al. 2018), hence extending its antioxidant activity. Animal poisoning linked to high tannin consumption has been well documented (Hervás et al. 2003). The presence of hydrolysable tannins has been largely blamed for the poisoning effect (McSweeney et al. 2001). The high level of phenols in the blood is the typical sign of tannin toxicity (Bausch and Carson 1981). Hydrolysable tannins are easily hydrolyzed by rumen microbes (Degen et al. 1995, Makkar and Singh 1995, McSweeney et al. 2001). On the other hand, condensed tannins are not directly harmful to animal organs due to their limited digestibility and absorption into the bloodstream (Terrill et al. 1994). A very high condensed tannin concentration, however, induces intestinal damage and hence increases the permeability of condensed tannin into the bloodstream,

potentially leading to toxicity symptoms (Reed 1995). Surprisingly, despite the accumulation of data from numerous in vivo experiments involving TE inclusion, no meta-analysis was performed to determine the threshold levels of TE on feed intake and digestibility in ruminant feed.

1.9 ß-carotene sources and extraction

Carotenoids are the most widespread group of naturally occurring pigments that range in hue from red to yellow and can be found in many fruits, vegetables, algae and mushrooms (Desmarchelier and Borel 2017). So far more than 600 carotenoids have been characterised (Namitha and Negi 2010). Chemically, carotenoids are divided into two classes: carotenes, which contain only hydrocarbons (e.g., α-Carotene, β-carotene and β-cryptoxanthin) and xanthophylls, which contain hydrocarbons and oxygen (e.g., Lutein, zeaxanthin, violaxanthin and fucoxanthin) (Namitha and Negi 2010). The best known of carotenoids is β-carotene which is a strongly coloured red-orange pigment and functions mainly as provitamin (A) in animals (Smith 1998).

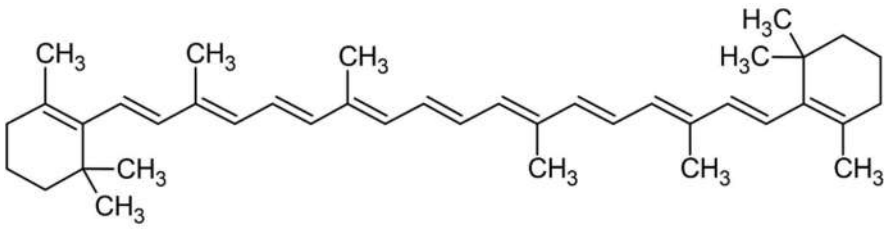

Figure 1.4 Molecular structure of ß-carotene

ß-carotene is widely used as a colouring, anti-inflammatory and antioxidant agent in food, pharmaceutical industries and cosmetics (Kyriakopoulou et al. 2015). Natural ß-carotene is primarily obtained through extraction from vegetable resources (e.g., carrots and palm oil) and microbial fermentation (e.g., microalga Dunaliella salina) (Ribeiro et al. 2011, Kulthe et al. 2016). Table 1.3 shows examples of natural carotenes extracted from plant materials.

Table 1.3 Chosen natural carotene formulations obtained from plant material. Adapted from (Bogacz-Radomska and Harasym 2018)

Product name	Plant material	Extraction method	Carotenes	Colour
Carotene mix	• Unrefined palm oil • Carrot • Alfalfa • Grass • Urtica	Solvent extraction: acetone, methyl ethyl ketone, methanol, ethanol, propane-2-ol, hexane, dichloromethane carbon dioxide.	• β-Carotene (85%) • α-Carotene (15%) • γ-Carotene trace amounts • $C_{40}H_{56}$	Orange
Lycopene oleoresin	Tomatos *Lycopersicon esculentum*	Solvent extraction: dichloromethane, carbon dioxide, ethyl acetat, acetone, propane-2-ol, methanol, ethanol, hexan	• Lycopene • $C_{40}H_{56}$	Orange-red

1.10 Bioavailability of ß-carotene in ruminant

Most studies on the bioavailability of carotenoids have been carried out on humans and rodents (Green and Fascetti 2016). Therefore, data on the carotenoids' passage through the gut is based on monogastric animals (Noziere et al. 2006). In steers and goats fed the same diet, lutein degradation differed slightly, but not ß-carotene degradation, suggesting insignificant species and molecular differences in ruminal ß-carotene digestion (Mora et al. 1999). (Mora et al. 1999) suggested that the disappearance of carotenoids from the rumen was probably due to their joining with the cellular content rather than their direct destruction or ruminal microorganism attack. This theory is supported by their finding that carotenoids disappear at high rates using the in-situ method in contrast to the virtually non-existent disappearance observed using the in vitro method in goats

and cattle. (Cardinault et al. 2006) suggested that rumen microorganisms may have the ability to synthesise ß-carotene and release conjugated xanthophylls in sheep. The colourless fat in sheep and goats indicates that ß-carotene does not accumulate in their adipose tissue (Fernandez et al. 1976). (Yang et al. 1992) determined carotenoids in various tissues of goats, sheep and cattle grazing the same pasture (Table 1.4). ß-carotene was not detectable in sheep or goat serum, but it was the dominant carotenoid in cattle. On the other hand, lutein was the dominant pigment in the serum and fat of goats and sheep. In the same context, in plasma, lutein has been identified as the main plasma carotenoid in sheep and goats, but zeaxanthin, and more rarely ß-carotene, have also been reported in sheep plasma (Prache et al. 2003, Cardinault et al. 2006). (Yang et al. 1992) found that retinol concentration in sheep livers was three times higher than in the livers of goats and cattle. However, the retinol concentration in serum and fat was similar for each species. It is thought that carotenoids are preferentially absorbed by lymphatic vessels in sheep, and the undetectable ß-carotene in plasma suggests a high conversion activity (Cardinault et al. 2006). Despite the absence of ß-carotene in other locations, it is proposed that goats and sheep have a mechanism for selectively depositing ß-carotene in the liver, which can be converted to retinol when needed (Yang et al. 1992).

Table 1.4 Carotenoid and retinol concentrations in sheep, goats, and cattle grazing the same pasture. Adapted from (Yang et al. 1992)

	Sheep	Goats	Cattle	s.e.d.
	Serum (μg/ml)			
Lutein	0.006[b]	0.004[b]	0.057[a]	0.0042
β-carotene	n.d.[A]	n.d.	2.19	0.006
Total C[B]	0.006[b]	0.004[b]	2.25[a]	0.116
Retinol	0.35	0.35	0.28	0.033
	Liver (μg/g)			
Lutein	n.d.	n.d.	0.30	0.010
β-carotene	0.87[b]	0.69[b]	7.01[a]	0.562
Total C	0.87[b]	0.69[b]	7.32[a]	0.564
Retinol	484.00[a]	136.00[b]	143.00[b]	39.40
	Subcutaneous fat (μg/g)			
Lutein	0.016[b]	0.010[b]	0.17[a]	0.040
β-carotene	n.d.	n.d.	0.81	0.169
Total C	0.016[b]	0.010[b]	0.97[a]	0.206
Retinol	0.92	1.18	0.99	0.212
Minolta b* value	6.4[b]	8.3[b]	20.8[a]	1.49

[A] Not detectable. [B] Lutein+ β-carotene.

1.11 ß-carotene as an antioxidant source and potential effects on production and reproduction

In animals, β-carotene functions mainly as pro-vitamin A (Smith 1998, Nagarajan et al. 2017) and as a free radical scavenger, particularly against the singlet state oxygen (Stahl and Sies 2005). The significance of β-carotene as a source of vitamin A in animal species is reviewed by (Green and Fascetti 2016). β-carotene scavenges peroxyl radicals and further degrades them to produce non-radical products; it may also bind to attacking free radicals to stop their reactions (Paiva and Russell 1999). β-carotene is lipophilic; scavenges radicals within the lipophilic compartment such as the cell membrane even more effectively than Vitamin E (Niki et al. 1995). However, some authors consider ß-carotene's effects to come from its impact on biochemical

systems and not from its antioxidant properties (Pryor et al. 2000). They argue that ß-carotene does not have a system that can donate a hydrogen atom or an electron to stabilise the oxidants. For example, both vitamin E and C have a hydroxyl group that is attached to an aromatic system, while ß-carotene has only a hydrocarbon compound (Pryor et al. 2000).

Regarding ß-carotene's effect on oxidative status in ruminants, studies were mostly on cows, while rarely conducted on goats and sheep. As far as we know, only two studies were conducted on goats (Gore and Lehloenya 2020) and sheep (Kamİloğlu et al. 2005). Both studies reported increases in glutathione peroxidase in response to supplemental ß-carotene. Table 1.5 summarizes studies that have examined the effect of supplemental ß-carotene on the production and reproduction of sheep and goats. Some authors reported a positive effect on ovulation rate (Meza-Herrera et al. 2013), oestrous response (Salem et al. 2015), oestrogen concentrations (Salem et al. 2015), progesterone concentration (Arellano-Rodriguez et al. 2009, Gore 2016), insulin concentration (Meza-Herrera et al. 2011), while others reported no significant effect on ovarian activity (Arellano-Rodriguez et al. 2007, Arellano-Rodriguez et al. 2009, Meza-Herrera et al. 2013, López-Flores et al. 2018), oestrus parameters (Köse et al. 2013, Gore 2016), litter size (Köse et al. 2013, Gore 2016), hormonal status (Oestrogen, P4 and insulin) (Brozos et al. 2007, Meza-Herrera et al. 2013, Gore 2016) .. etc. Such inconsistency, when usually reported is attributed to the variation in the amount of β-carotene supplemented, the amount of β-carotene in the animal diet and to the difference in the animal requirement for β-carotene. Assuming these variables can be extracted from those studies, a review of the supplemental β-carotene effects on the production and reproduction performance of sheep and goats is warranted.

Table 1.5 Summary of studies that have assessed the effect of supplemental ß-carotene on the production and reproduction performance of sheep

NO	Species	Type of the diet#	β-carotene supplementation (µg/kg BW/day)	Outcomes	References
1	Sheep	Chaffed wheaten straw, linseed meal, wheat and barley	25, 50, 75 and 100	Lambs survival rates: 48.3, 65, 66.7 and 62.2 %, respectively.	(Peirce 1954)
2	Sheep	Concentrate	100 (twice/ week)	Oestrous cases (↑) & oestrogen concentrations (↑)	(Salem et al. 2015)
3	Sheep	Concentrate, alfalfa hay and wheat straw	1644.74	BW (=), LH concentration (=), milk yield (=) & litter size (=).	(Brozos et al. 2007)
4	Sheep	Not mentioned	1000	Oestrus response (=), pregnancy rate (=), lambing rate (=) & litter size (=).	(Köse et al. 2013)
5	Goats	Total mixed ration (TMR) Total mixed ration (TMR)	2000 1000	Plasma P4 concentration (↑), GSH-Px activity (↑) milk yield (=), BW (=), oestrus parameters (=), oestrogen concentration (=), follicles number (=), largest follicle and CL size (=), gestation length (=), litter size (=) kids' birthweight (=).	(Gore 2016)
6	Goats	Alfalfa hay, corn silage and corn grain	1116	BW (=), total follicles (=), corpus luteum number (=), total ovarian activity (=).	(Arellano-Rodriguez et al. 2007)
7	Goats	Alfalfa hay, corn silage and corn grain	1116	Serum P4 concentration (↑), BW (=), total follicles number (=), corpus luteum number (=), total ovarian activity (=).	(Arellano-Rodriguez et al. 2009)
8	Goats	Alfalfa hay, corn silage and corn grain	1089	Total follicles number (=), corpus luteum number (=), total ovarian activity (=), insulin hormone (=).	(Meza-Herrera et al. 2013)
9	Goats	Alfalfa hay, corn silage and corn grain	1089	Total follicles number (=), corpus luteum number (=), total ovarian activity (=), growth hormone (↓) and IGF-1 (=).	(López-Flores et al. 2018)
10	Goats	Alfalfa hay, corn silage and corn grain	1089	Ovulation rate (↑) LH concentrations (↓).	(Meza-Herrera et al. 2013)
11	Goats	Alfalfa hay, corn silage and corn grain	2890	Goats reaching puberty % (=), BW (=), serum insulin concentration (↑)	(Meza-Herrera et al. 2011)
12	Goats	Alfalfa hay, corn silage and corn grain	2890	BW (=), goats reaching puberty % (=).	(Meza-Herrera et al. 2014)
13	Goats	Alfalfa hay, corn silage and corn grain	2890	BW (=).	(Meza-Herrera et al. 2017)

(=) = Non significant ($p > 0.05$), (↑) = statistically significant increase, (↓) = statistically significant decrease, BW = bodyweight, P4 = progesterone concentration, GSH-Px = glutathione peroxidase, IGF-1 = insulin-like growth factors. #The level of β-carotene in alfalfa hay (1 kg = 20.3 mg) where estimated according to Charkey et al (Charkey et al. 1961)

1.12 Possible mechanisms of supplemental ß-carotene on oxidative status, production and reproduction

As previously discussed, sheep and goats may have a different bioavailability for ß-carotene than cattle. Therefore, future studies are recommended to pay particular attention to how ß-carotene behaves in sheep and goats compared to cattle regarding its antioxidant activity. Despite being of direct effect or sparing effect, supplementation with ß-carotene has been reported to increase glutathione peroxidase in sheep (Kamİloğlu et al. 2005) and goats (Gore and Lehloenya 2020), suggesting a beneficial effect on oxidative status. Potential strategies by which supplemental ß-carotene may enhance the productive and reproductive status of sheep and goats may include:

- Oxidative damage to the reproduction performance is well documented in farm animals (Tilly and Tilly 1995, Saleh and Agarwal 2002, Baker and Aitken 2004, Agarwal et al. 2012) and supplemental ß-carotene working as an antioxidant may prevent such damage during oxidatively stressed conditions compared to non-supplemented animals.
- There is some evidence that β-carotene may stimulate steroid production (Arikan and Rodway 2000). In that regard, some authors reported that supplemental ß-carotene may enhance ovarian follicular development and ovulation rate in adult female goats (Arellano-Rodriguez et al. 2007, Meza-Herrera et al. 2013).

1.13 Which level of ß-carotene supplementation is recommended:

Animals are unable to synthesise carotenoids and obtain them from their diet (Britton and Khachik 2009, Kopsell and Kopsell 2010, Desmarchelier and Borel 2017). According to the National Research Council (NRC 2007), sheep and goats' requirements for vitamin A

during breeding and early gestation equal 157 µg of β-carotene/kg BW/day and 267 µg of β-carotene/kg BW/day during late pregnancy and lactation.

However, systems used to calculate animal nutrient requirements have traditionally estimated vitamin and trace element requirements for maintenance, growth, pregnancy, and lactation, taking into account the prevention of deficiency pathologies (Kamiloğlu et al. 2006). The extra supplementation of the animal with β-carotene may allow the animal to convert some of the β-carotene to vitamin A as usual and use the rest as antioxidants against oxidative stress. To our knowledge, there is no study on the toxicity of β-carotene in ruminants. Nevertheless, the treatment of high doses (approximately 180 mg of β-carotene/day) had no side effects in humans (Bendich 2001), except for hypercarotenemia in some subjects (Diplock 1995). Supplementation of experimental animals with about 17 times their requirements of β-carotene (Fiorelli et al. 2014) and up to 1000 mg/day of β-carotene per kg body weight showed no signs of toxicity (Woutersen et al. 1999). However, at the cellular level, β-carotene at high concentrations may act as a prooxidant and may lead healthy cells exposed to oxidative stress to neoplastic transformation (Palozza et al. 2001). Because of the oxidative attack, carotenoids degrade highly reactive aldehydes and epoxides that inhibit mitochondrial respiration, reduce protein sulfhydryl content, decrease redox state and GSH levels, and increase the accumulation of malondialdehyde (Siems et al. 2009).

1.14 Conclusions

- Tannin extracts have the potential to serve as antioxidants, however, that potential must be balanced against its tendency to reduce feed intake and nutrient digestibility of the animal.

- Despite the accumulation of data from numerous in vivo experiments involving TE inclusion, no meta-analysis was performed to determine the threshold levels of TE on feed intake and digestibility in ruminant feed.
- The majority of studies on the effects of ß-carotene on oxidative status in ruminants were conducted in cows, while goats and sheep were rarely studied.
- Results of the studies estimating the effect of supplemental ß-carotene on the production and reproduction of sheep and goats are controversial.

CHAPTER TWO

Effect of *Acacia mearnsii* Tannin Extract Supplementation on Reproductive Performance and Oxidative Status of South African Mutton Merino Rams

Published in Animals, 2021, 11, 3266. DOI:
https://doi.org/10.3390/ani11113266

Simple Summary

Nutrition and the seasons are two of the better-known variables that affect the reproduction performance of farm animals. In recent years, many antioxidants have been proposed as a tool to improve male reproduction performance. Although such antioxidants, in most cases, are expensive and artificial, tannin extract presents a cheap and natural source of antioxidants. This research evaluated the supplementation effects of tannin extract (TE) and encapsulated tannin extract (ETE) on testicular measurements, semen quality, hormonal status, and oxidative status, as well as the seasonal effect on the testicular measurements of South African Mutton Merino rams. The results suggest that encapsulation may afford the maximum benefit of TE on sperm concentration and motility. Treatments did not affect the hormonal and oxidative status. The testicular measurements were significantly higher in autumn compared to winter. The plasma level of cortisol correlates negatively with sperm mass motility, progressive motility, viability, and acrosome integrity.

Abstract

We investigated the supplementation effects of *Acacia mearnsii* tannin extract (TE) and encapsulated tannin extract (ETE) on the reproduction performance and oxidative status of South African Mutton Merino rams. We also studied the seasonal effects on testicular measurements. Thirty rams were divided into five groups: 0.0 g TE (control), 1.5 g TE, 3 g TE,

1.5 g ETE, and 3 g ETE supplemented daily for 16 weeks transiting from autumn to winter. Bodyweight and testicular measurements were recorded biweekly. Semen and blood samples were collected over five weeks. Results showed that the increase in the ETE from 1.5 to 3 g increased the testicular length and sperm concentration, as well as decreased the percentages of low and non-progressive spermatozoa ($p < 0.05$). Simultaneously, the increase in the TE from 1.5 to 3 g decreased semen volume and elevated the percentage of abnormal sperm ($p < 0.05$). The results suggest that the encapsulation of TE affords the maximum benefit of the TE on sperm quality. Treatments did not affect the hormonal and oxidative status. Testicular measurements were significantly ($p < 0.001$) higher in autumn compared to winter. The plasma level of cortisol significantly correlates negatively with sperm motility, viability, and acrosome integrity.

Keywords: encapsulated tannin; sheep; testicular measurements; semen quality; testosterone; cortisol; seasonality

2.1 Introduction

Reactive oxygen species (ROS) are critical for some sperm functions, such as sperm capacitation (Agarwal et al. 2012). However, ROS causes 30% to 80% of male subfertility cases when they exceed the body's antioxidant capacity (Agarwal et al. 2012). Testicular cells divide at a high rate, consume a high amount of mitochondrial oxygen, and testicular tissues also have a high level of unsaturated fatty acids which leave the male reproductive system susceptible to oxidative stress (Asadi et al. 2017). As a consequence of oxidative stress, ROS may reduce sperm motility and damage the DNA and plasma membrane (Saleh and Agarwal 2002, Baker and Aitken 2004). Antioxidant supplementation is suggested as a tool to break down the oxidative chain reaction and improve the process of spermatogenesis and thereby the sperm quality and general testicular health (Showell et al. 2014, Asadi et al. 2017). Numerous

antioxidants are proposed to achieve that goal, such as vitamin E, vitamin C, selenium, glutathione, and coenzyme Q10 (Sheweita et al. 2005, Lukusa and Lehloenya 2017). However, such antioxidants in most cases are expensive and artificial. Alternatively, cheap natural plant antioxidant sources are abundant and therefore, can be utilized. A polyflavonoid derived from chains of flavan-3-ol molecules, condensed tannin, represents an inexpensive and promising antioxidant source (Huang et al. 1992). For instance, the tannin-rich grape pomace concentrate has shown antioxidant activity equal to vitamin E when it was supplemented into the chicken diet (Brenes et al. 2008). Condensed tannins, extracted from *Ficus altissima* leaves, protected plasmid DNA and cells against oxidative damage (Deng et al. 2016). The addition of commercial tannins extracted from chestnut to the diet of heat-stressed lambs had improved the meat quality and oxidative status of those lambs (Liu et al. 2016). The supplementation of tannin-rich *Ficus infectoria* leaf meal improved the antioxidant status, daily gain and immunity in lambs (Dey et al. 2015) [11]. However, the potential of tannins to serve as antioxidants must be balanced against their potential to diminish nutrient digestibility and feed intake of the animal (Dschaak et al. 2011). Those negative responses relate to the bitterness and the high tendency of tannins to bind with dietary ruminal protein when a high concentration is consumed (Dschaak et al. 2011, Patra and Saxena 2011, Adejoro et al. 2018). These limitations can be controlled by reducing the amounts of tannin in the diet or/and slowing their release rate in the digestive tract by means of encapsulation (Adejoro et al. 2018). The encapsulation technique may slow the rate of tannin extract release in the ruminant, prolonging its antioxidant activity. The supplementation of tannin extract should not exceed 0.3 g/kg bodyweight/day (g/kg BW/d) since higher levels may decrease the feed intake and digestibility (Deaville et al. 2010, Kozloski et al. 2012). In South Africa, tannins are mainly extracted from the bark of *Acacia mearnsii*, which is one of the richest sources of tannin (Roux et al. 1995). We hypothesised

that supplementation of tannin extract would enhance the oxidative status and, thereby, the reproduction performance of the rams. The main objective of this study was to investigate the supplementation effects of *Acacia mearnsii* tannin extract (TE) and encapsulated tannin extract (ETE) on testicular measurements, semen quality, hormonal status, and oxidative status of South African Mutton Merino rams.

Seasonality of reproduction affects the productivity of small ruminants (El Kadili et al. 2019). Photoperiod is the key factor that influences the seasonal effects on the reproduction performance of the rams at high latitudes (Ghorbankhani et al. 2015). However, at lower latitudes, the effect of season on reproduction becomes less and a greater effect for nutrition arises (Ghorbankhani et al. 2015). In addition, besides the individual differences, there are major differences between the breeds in the length of the breeding season and sexual activity (Zamiri and Khodaei 2005). Therefore, the effect of season on testicular measurements and semen quality has been investigated in numerous sheep breeds (Cevik et al. 2017). As a secondary objective, we aimed to evaluate seasonal variations in the testicular measurements of South African Mutton Merino rams during the transition from autumn to winter. Correlation coefficients between bodyweight, some testicular measurements (testicular volume and scrotal circumference), semen quality parameter (semen volume, sperm concentration, pH, mass motility, progressive motility, viability, acrosome integrity), and hormonal status variables (testosterone and cortisol) were also reported in this study.

2.2 Materials and methods

2.2.1 Experimental location and ethical clearance

The study was approved by the Animal Ethics Committee (AEC) (Project number: EC056-17) and conducted at the experimental farm of the University of Pretoria in Hatfield,

Pretoria, South Africa. The geographical location of the farm was 25°44′30″ S, 28°15′30″ E and 1360 m above mean sea level (Van Niekerk et al. 2009).

2.2.2 Materials

Acacia mearnsii tannin extract was obtained from UCL Company (Pty) Ltd. Dalton, South Africa. Tween80, Span80, Dichloromethane (99.9%), N,N-Dimethyl-p-phenylenediamine dihydrochloride (DMPD) and Iron (III) chloride hexahydrate were purchased from (Sigma-Aldrich Ltd., St. Louis, MO, USA).

2.2.3 Encapsulation of the tannin extract

The *Acacia mearnsii* tannin extract used in this study consists of 65.8% total phenol, 58.5% total tannin, and 30.5% condensed tannin (Adejoro et al. 2019). The encapsulation of the *Acacia mearnsii* tannin extract was done as previously described by Adejoro et al. (Adejoro et al. 2018). In this process, the particles of tannin extract were micro-encapsulated with palm oil, while Span80 and Tween80 served as wall materials. Briefly, on a small scale, 8.5 g of the tannin extract was added and then mixed using magnetic stirrer to a mixture of 1.5 g palm oil, 0.15 g Span80, and 30 mL Dichloromethane (DCM). The resulting mixture was then gently added to 300 mL of 1.0% (w/v) aqueous solution of Tween 80 and homogenised for 3 min at 20,000 rpm using a bench-top homogeniser (PRO400DS, Pro Scientific Inc., Oxford, CT, USA). To evaporate the DCM; the resultant emulsion was rotated at 800 rpm for 3 h on a magnetic stirrer. Then the mixture was filtered and washed on a cheesecloth with 100 mL distilled water. The encapsulated tannin microparticles were collected, freeze-dried and stored at 4° C, until use.

2.2.4 Animals and treatments

The period of the trial was 16 weeks from autumn to winter (April–August). Thirty Merino rams, aged 12 months old, with an average bodyweight (BW) of 52.78 ± 0.79 kg, were

divided randomly into five groups (6 rams/group). The initial bodyweight, testicular parameters, semen volume, pH, colour, as well as sperm concentration, mass motility, and progressive motility were measured for the different groups. All rams grazed Kikuyu grass (*Pennisetum clandestinum*) during the day (08h00 to 16h00) while kept in sheds during the night. After eight weeks of the trial, the quality of Kikuyu grass dropped drastically; therefore, the rams were offered Eragrostis hay ad libitum in the afternoon. The rams were daily supplemented with either 0.0 g tannin extract (control), 1.5 g tannin extract (1.5 g TE), 3 g tannin extract (3 g TE), 1.5 g encapsulated tannin extract (1.5 g ETE), or 3 g encapsulated tannin extract (3 g ETE) for 16 weeks. The daily supplementation of 1.5 g and 3 g of TE or ETE approximately represent 0.03 and 0.06 g/kg BW/day, respectively. The specified amount of supplementation with TE and ETE for each ram was added to 15 g of crushed cornflakes, then mixed with 12 g of 50% water diluted molasses. Then a ball shape was formed from this mixture and provided to the animal fresh every morning. Tannin-free balls were provided to the rams in the control group. For ten days before the supplementation period, the rams were trained to consume the balls voluntarily while passing on standardised crash before going to the grazing field.

2.2.5 Bodyweight and testicular measurements

The bodyweight and testicular parameters of each ram were taken every two weeks. The bodyweight was measured using an electronic weighing balance (TAL-TEC, South Africa). The testicular measurements were taken after the rams were physically restrained in order to sit their rump on the floor (Williams 1990). The scrotal circumference (SC) was measured using a flexible measuring tape placed at the maximum diameter of the scrotal sac, as previously described by Martinez et al. (Martinez et al. 2012). The width and length of each testicle were measured using a digital vernier graduated in millimetres. When measured, both

testicles were force descended into the scrotum, and the epididymis head and tail were not included. The testicular volume (VT) was determined from the means of testicular width (a) and length (b) using the formula proposed by Steger and Wrobel (Steger and Wrobel 1994):

$$VT = (1/6) \times \pi \times a^2 \times b \times 0.945 \ [cm^3]. \quad (1)$$

2.2.6 Ambient temperature

Data for the ambient temperature were obtained from the Phytotron Department at the experimental farm of the University of Pretoria, Hatfield, Pretoria, South Africa. The average ambient temperatures represented the mean of the temperature—including the maximum and the minimum—throughout the day.

2.2.7 Semen collection

Semen was collected from rams weekly, starting twelve weeks after the first supplementation of tannin (total of five collections/ram). The 30 rams were divided into six groups of 5 rams in each. Each group (representing all the treatments) was designated to be collected on a certain day of the week. The semen was collected early morning (between 08h00 and 09h00) using an electro-ejaculator (Ramsem, South Africa) with a standardised rectal probe for a small ruminant as previously described by Lukusa and Lehloenya (Lukusa and Lehloenya 2017). However, the internal male accessory glands were stimulated through the rectum. The glands were gently massaged using a middle finger of a gloved hand for a few seconds until multiple flehmen responses were obtained. The stimulation was done directly before inserting the rectal probe to perform the electroejaculation process.

2.2.8 Semen evaluations

2.2.8.1 Subjective evaluation

The sperm concentration was estimated using haemocytometer (Hausser, Horsham, PA, USA) (Lukusa and Lehloenya 2017). The semen samples were diluted with distilled water 1:400 and the concentration of spermatozoa was calculated as follows:

Concentration (sperm/mL) = (*DilutionFactor*) (*Count in 5 squares*) (0.05×10^6) (2)

Sperm smears were prepared using nigrosin-eosin stain and the morphological abnormality, viability, and acrosome integrity of the spermatozoa were assessed (Barth and Oko 1989).

2.2.8.2 Objective evaluation

Semen samples were transferred in a water bath at 18° C to the laboratory of Germplasm Conservation and Reproductive Biotechnologies at the Agricultural Research Council (ARC) in Irene. The time taken from the collection site to the laboratory at ARC was 30 min. The sperm total motility (%), speed and progression of the sperm were determined using the Sperm Class Analyzer® system (Microptic, Spain). Semen samples were diluted with Tris-hydroxymethyl aminomethane (1:100) and 5 µL were placed on a pre-warmed glass slide and then mounted with a glass cover-slip before evaluation at a magnification of 10× (Nikon®, Shanghai, China).

2.2.9 Blood collection and analysis

Five rams from each experimental group were randomly chosen for blood sampling. Blood was sampled from the jugular vein using 18 G BD vacutainer® needle and heparinised BD vacutainer® tubes at day zero, and at the end of the eighth and sixteenth week of the supplementation treatments. The blood samples were centrifuged at 3000 rpm for 10 min and plasma aliquots were harvested and stored at −20 ∘C until subsequent analysis.

2.2.9.1 Oxidative status

Oxidative status was estimated by measuring intermediate products called hydroperoxides which originate from the oxidation of different types of molecules, such as lipids, peptides, and amino acids (Verde et al. 2002). The hydroperoxides or the plasma oxidant potential were measured as previously described by Mehdi and Rizvi (Mehdi and Rizvi 2013) with minor modifications. Briefly, 100 µL of plasma was added to 1.9 mL N,N-Dimethyl-p-phenylenediamine dihydrochloride (DMPD) (1 mM in acetate buffer). The solution was well mixed, incubated for 10 min and then centrifuged for 5 min at 3000× g. Absorbance was recorded at 505 nm against the blank solution and compared with the standard curve of ferric iron for final concentrations. The standard curve of DMPD–ferric iron (0.02–0.20 mM Fe(III)) was generated with highly significant correlation ($r = 0.998$, $p < 0.001$). The concentration of the ferric iron was obtained in accordance with an optical density (absorbance) of 1.00 = 0.137 mM ferric equivalents.

2.2.9.2 Hormonal status

Plasma samples were analysed for testosterone and cortisol concentrations using an enzyme immunoassay (EIA) performed on microtiter plates at the endocrine laboratory of the University of Pretoria. A detailed description of the assay component and cross-reactivity has been provided by Palme and Möstl for the cortisol EIA (Palme 1997) and the testosterone EIA (Palme and Möstl 1993). The sensitivity, intra, and inter-assay variation coefficients of the testosterone EIA used were 0.08 ng/mL, 5.75% to 7.53% and 11.04% to 13.94% respectively. The sensitivity, intra, and inter-assay variation coefficients of the cortisol EIA used were 0.02 ng/mL plasma, 6.21% to 6.98% and 7.01% to 10.35% respectively. Both tests were carried out in duplicates for each sample.

2.2.10 Statistical analyses

A 2 × 5 factorial model was used to analyse the data on the effects of the season (autumn and winter) and treatments (control, 1.5 g TE, 3 g TE, 1.5 g ETE, and 3 g ETE) on bodyweight and testicular measurements of the rams. Appropriate interactions were estimated, however, these were not significant in most cases; therefore, the main effect means were thus presented. Significant interactions were provided where appropriate. A completely randomised design (CRD) was also used to analyse the effect of treatments on semen quality parameters, hormonal and oxidative status, and the effect of time (day zero, 8 weeks, 16 weeks) on the oxidative and hormonal status. Correlations were estimated using Pearson's Correlation Coefficient. Means of significant differences were compared using Least Significant Differences (LSD) at a 5% significance level. All the data were conducted using SPSS (IBM SPSS Statistics software 20). Data were expressed as means ± standard error (SE).

2.3 Results and discussion

Experimental groups were not significantly different in their initial bodyweight, testicular parameters, semen volume, pH, colour, sperm concentration, mass motility, and progressive motility (Table 2.A1). Data on the effect of season and treatments on bodyweight and testicular measurements are presented in Table 2.1 No significant interactions between treatments and seasons on testicular measurements were found. We observed a significant seasonal effect on the testicular measurements of the rams (Table 2.1). Mean values of scrotal circumference, testicular volume, testicular width, and testicular length were significantly higher in autumn than those in winter. Authors in previous studies found a similar significant effect for the season on the testicular measurements (Kafi et al. 2004, Zamiri et al. 2010, Belkhiri et al. 2019). Level of nutrition, daily body growth, and the response of the pineal gland to the day length are the main factors that affect testicular measurements (Belkhiri et al. 2019).

In this study, the level of nutrition and body growth seems not to be the cause of the observed effect of season on testicular measurements, since the means of bodyweight remained almost the same between the two seasons (Table 2.1). The longest day in Pretoria was during December and the length of the day starts to decrease during January and February until it gradually becomes the shortest days during June and July (World.info). It is, therefore, concluded that the decreasing day length resulted in the secretion of melatonin from the pineal gland (Batailler et al. 2018) which led to increased testicular measurements via the control of the LH and FSH secretion from the pituitary gland (Avdi et al. 2004). Moreover, we found a significant positive correlation between average ambient temperature and testicular volume (r = 0.797, p = 0.010) together with scrotal circumference (r = 0.813, p = 0.008), which support the assumption of the seasonal effect on the testicular measurements. Bodyweight and testicular measurements were not affected by the treatments, except for the testicular length (p = 0.04). The group supplemented with 3 g ETE had a higher testicular length as compared to animals supplemented with 3 g TE and 1.5 ETE (Table 2.1). Some studies found a positive effect of antioxidant supplementation on the testicular length on rams (Zhao et al. 2017) and goat bucks (Zubair et al. 2017), while others reported no effect on rams (Elbaz and Abdel Razek 2019) and goat bucks (Lukusa and Lehloenya 2017). The positive effect of 3 g ETE supplementation on testicular length might be due to its antioxidants properties since the encapsulation process slows and maintains constant release of the tannin microparticles (Adejoro et al. 2018). The variations in reports could be attributed to the differences in the level and duration of the supplementation, and the type of antioxidants used.

Table 2.1 Effects of season and tannin supplementation on bodyweight and testicular measurements (Mean ± SE) of South African Mutton Merino rams

Factors	Parameters				
	Bodyweight (kg)	Scrotal Circumference (cm)	Testicular Volume (cm^3)	Testicular Width (mm)	Testicular Length (mm)
Season					
Autumn	52.9 ± 0.35	**29.8 ± 0.21** ^{a}	**150.0 ± 3.44** ^{a}	**56.5 ± 0.49** ^{a}	**92.8 ± 0.80** ^{a}
Winter	52.3 ± 0.41	**28.2 ± 0.20** ^{b}	**101.2 ± 3.08** ^{b}	**47.5 ± 0.52** ^{b}	**87.8 ± 0.88** ^{b}
***p*-value**	0.320	<0.001	<0.001	<0.001	< 0.001
Treatments					
Control	52.1 ± 0.69	28.8 ± 0.36	124.2 ± 6.51	51.7 ± 1.07	**90.0 ± 1.42** ^{ab}
1.5 g TE	52.6 ± 0.77	29.6 ± 0.38	134.3 ± 6.72	53.3 ± 1.03	**91.7 ± 1.53** ^{ab}
3.0 g TE	51.8 ± 0.38	28.7 ± 0.36	121.6 ± 5.81	51.9 ± 1.00	**88.3 ± 1.23** ^{b}
1.5 g ETE	52.9 ± 0.42	28.9 ± 0.35	118.5 ± 5.81	51.1 ± 0.97	**88.3 ± 1.31** ^{b}
3.0 g ETE	53.6 ± 0.60	29.0 ± 0.25	129.4 ± 6.11	52.1 ± 0.94	**93.1 ± 1.23** ^{a}
***p*-value**	0.220	0.299	0.233	0.409	0.040

^{a,b} Column means with different superscripts within season and treatment indicate significant differences ($p < 0.05$). TE = tannin extract; ETE = encapsulated tannin extract.

Results for the effects of supplementation treatments on semen quality are presented in Table 2.2. The treatments have a significant effect on semen volume (p = 0.042) and sperm concentration (p = 0.012). The groups supplemented with 1.5 g TE and 3 g ETE had a higher semen volume as compared to the group supplemented with 3 g TE and a higher sperm concentration as compared to the group supplemented with 1.5 ETE (Table 2.2). Similar improvements in semen volume and sperm concentration have been reported when antioxidants are supplemented in rams (Yue et al. 2010, Ozer Kaya et al. 2020), goat bucks (Arivazhagan et al. 2020), and rabbits (El-Ratel et al. 2021). However, some authors reported no effect of antioxidant supplementation on the semen volume and sperm concentration in rams (Cofré-Narbona et al. 2016, Piagentini et al. 2017) and bulls (Butt et al. 2019). Different results following the supplementation with antioxidants on semen volume and sperm concentration could be referred to the variations in the level or the duration of supplementation and the antioxidant type used. Since antioxidants presence increase spermatogenesis (El-Ratel et al. 2021), the positive effects of 1.5 g TE and 3 g ETE on semen volume and sperm concentration in the present study can be attributed to the potential antioxidant activity in tannin extract. The

supplementation with TE and ETE had no significant effect on semen pH and colour (Table 2.2). In both subjective and objective evaluations, the sperm motility parameters were not influenced by the supplementation treatments, except for the percentages of slow motility (p = 0.005) and non-progressive motility (p = 0.007). The group supplemented with 3 g ETE had lower percentages of the slow and non-progressive motility spermatozoa as compared to the control, 1.5 ETE and 3 g TE groups. The positive effects of supplementation with antioxidants on sperm motility have been reported in several studies (Stefanov et al. 2018, Steiner et al. 2018, Ozer Kaya et al. 2020). The mode of action for the improvement of sperm motility after antioxidant supplementations is still under investigation (Barbagallo et al. 2020). However, according to Zhu et al. (Zhu et al. 2019) and the recent review of Barbagallo et al. (Barbagallo et al. 2020), the antioxidants protect the gene expression system of the spermatozoa from ROS and, thus, maintain ATP generation in the mitochondria, which is important to fuel sperm linear motility (Zhu et al. 2019). Therefore, the encapsulation of a higher level of tannin extract (3 g ETE) could maintain the antioxidants release, which may protect the ATP generation system in the spermatozoa and thus results in better sperm motility.

Table 2.2 The effect of supplementation treatments on the semen quality parameters (Mean ± SE) of South African Mutton Merino rams

Variable	Treatments					*p*-Value
	Control	1.5 g TE	3 g TE	1.5 g ETE	3 g ETE	
Semen volume (mL)	**2.0 ± 0.14** ^{abc}	**2.4 ± 0.20** ^{a}	**1.6 ± 0.13** ^{c}	**1.9 ± 0.17** ^{bc}	**2.2 ± 0.28** ^{ab}	**0.042**
Semen pH	7.3 ± 0.04	7.3 ± 0.04	7.3 ± 0.07	7.3 ± 0.04	7.3 ± 0.05	0.610
Subjective evaluation						
Sperm concentration (10^9/mL)	**2.9 ± 0.26** ^{ab}	**3.6 ± 0.31** ^{a}	**3.0 ± 0.29** ^{ab}	**2.3 ± 0.16** ^{b}	**3.1 ± 0.20** ^{a}	**0.012**
Semen colour (1–4)	3.8 ± 0.08	3.6 ± 0.15	3.8 ± 0.13	3.7 ± 0.09	3.9 ± 0.06	0.320
Sperm mass motility (1–5)	4.3 ± 0.11	4.3 ± 0.12	4.2 ± 0.13	4.3 ± 0.14	4.2 ± 0.12	0.822
Sperm PM (%)	75.0 ± 1.02	75.0 ± 1.75	70.8 ± 1.96	71.1 ± 2.58	73.2 ± 1.90	0.343
Objective evaluation (SCA®)						
Sperm total motility (%)	77.7 ± 1.79	71.7 ± 3.36	74.7 ± 2.82	76.3 ± 2.79	78.6 ± 2.95	0.415
- Rapid motility (%)	47.1 ± 2.88	45.1 ± 3.60	44.1 ± 3.73	46.8 ± 3.32	55.9 ± 3.18	0.099
- Medium motility (%)	7.1 ± 0.57	6.6 ± 0.54	7.6 ± 0.68	7.5 ± 0.59	5.4 ± 0.65	0.077
- Slow motility (%)	**23.6 ± 1.47** ^{a}	**20.1 ± 1.25** ^{ab}	**23.0 ± 1.28** ^{a}	**22.1 ± 1.17** ^{a}	**17.3 ± 1.31** ^{b}	**0.005**
Sperm PM (%)	50.1 ± 2.80	48.0 ± 3.52	48.0 ± 3.60	50.2 ± 3.30	58.3 ± 3.12	0.143
- Rapid PM (%)	29.9 ± 2.36	29.2 ± 2.47	27.8 ± 2.45	28.9 ± 2.02	35.7 ± 2.58	0.154
- Medium PM (%)	20.2 ± 1.49	18.8 ± 1.61	20.3 ± 2.13	21.3 ± 1.92	22.7 ± 2.25	0.658
Sperm non-PM (%)	**27.7 ± 1.77** ^{a}	**23.7 ± 1.49** ^{ab}	**26.7 ± 1.54** ^{a}	**26.1 ± 1.34** ^{a}	**20.2 ± 1.67** ^{b}	**0.007**
Immotiltiy (%)	22.3 ± 1.79	28.4 ± 3.36	25.4 ± 2.82	23.7 ± 2.79	21.4 ± 2.95	0.415

$^{a–c}$ Row means with different superscripts within treatments indicate significant differences ($p < 0.05$). TE = tannin extract; ETE = encapsulated tannin extract; PM = progressive motility; SCA® = Sperm Class Analyser.

The effects of supplementation treatments on sperm viability, acrosome integrity, and abnormality are presented in Table 2.3. The supplementation treatments did not affect sperm viability (p = 0.791) and acrosome integrity (p = 0.366). However, the supplementation treatments had a significant influence on the sperm total abnormality percentage (Table 2.3). Rams supplemented with 1.5 g ETE, 3 g ETE, and 1.5 g TE had lower abnormal sperm percentages as compared to those supplemented with 3 g TE. The percentages of the sperm head, midpiece, and tail abnormality were not affected by the supplementation treatment (p = 0.777, p = 0.124 and p=0.351, respectively), but the sperm cytoplasmic droplet percentage did (p = 0.002). The group supplemented with 3 g TE had the highest sperm cytoplasmic droplet percentage. The sperm cytoplasmic droplet is the remnant formed after the phagocytosis of germ cells cytoplasm by the Sertoli cells during spermatogenesis (Cooper 2005). Incidence of sperm cytoplasmic droplets and disrupted sperm morphology are considered prime features of anomalous spermatozoa leading to oxidative stress in the spermatozoa (Dutta et al. 2019). In

rams, phospholipid-binding protein (PBP) is believed to induce the release of cytoplasmic droplets from epididymal sperm cells during spermatogenesis (Kuster and Althouse 2003). The synthesis of PBP was positively associated with the increased level of testosterone in the blood plasma (Matoušek and Staněk 1993). However, the plasma testosterone level in this study was not affected by the supplementation treatments (Figure 2.1), it is, therefore, difficult to explain the higher sperm cytoplasmic droplet present in the rams supplemented with 3 g TE.

Table 2.3 The effects of supplementation treatments on sperm viability and morphology (Mean ± SE) of South African Mutton Merino rams

Variable	Treatments					*p*-Value
	Control	1.5 g TE	3 g TE	1.5 g ETE	3 g ETE	
Viability (%)	72.3 ± 2.77	72.6 ± 2.48	75.5 ± 2.23	72.1 ± 2.81	70.8 ± 3.00	0.791
Acrosome integrity (%)	90.6 ± 0.84	87.9 ± 1.36	86.4 ± 1.49	87.5 ± 2.04	87.0 ± 1.96	0.366
Total abnormalities (%)	**9.2 ± 1.37** ^{ab}	**7.3 ± 1.54** ^{b}	**13.1 ± 2.74** ^{a}	**6.7 ± 0.99** ^{b}	**6.9 ± 1.36** ^{b}	**0.048**
Head abnormalities (%)	0.2 ± 0.09	0.3 ± 0.12	0.5 ± 0.31	0.3 ± 0.13	0.2 ± 0.10	0.777
Midpiece abnormalities (%)	5.1 ± 0.70	5.2 ± 1.12	7.3 ± 1.86	4.2 ± 0.63	3.4 ± 0.60	0.124
Tail abnormalities (%)	2.7 ± 0.81	1.4 ± 0.60	2.6 ± 0.76	1.3 ± 0.33	3.0 ± 1.02	0.351
Cytoplasmic droplets (%)	**1.3 ± 0.34** ^{b}	**0.4 ± 0.18** ^{b}	**2.7 ± 0.83** ^{a}	**0.9 ± 0.37** ^{b}	**0.4 ± 0.17** ^{b}	**0.002**

^{a,b} Row means with different superscripts within treatments indicate significant differences ($p < 0.05$). TE = tannin extract; ETE = encapsulated tannin extract.

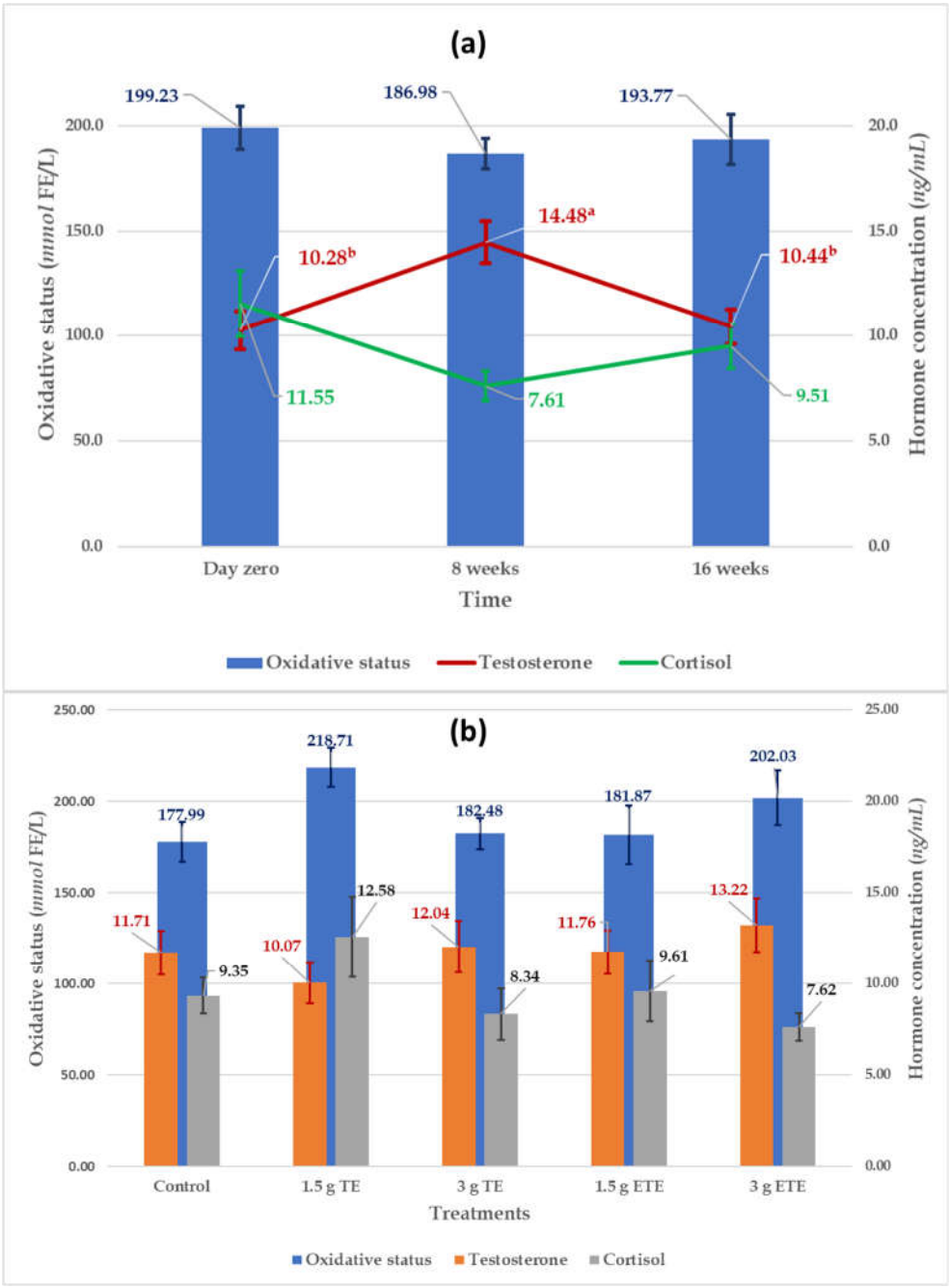

Figure 2.1 Effect of time (a) and supplementation treatments (b) on the hormonal and oxidative status (Mean ± SE) of South African Mutton Merino rams. [a,b] Means with different superscripts for the same parameter indicate significant differences ($p < 0.05$). TE = tannin extract; ETE = encapsulated tannin extract

The effects of supplementation treatments and time (day zero, 8 weeks, and 16 weeks) on the level of male hormone (testosterone), the stress hormone (cortisol) and oxidative status are presented in Figure 2.1. The supplementation treatments did not affect the oxidative status, testosterone and cortisol. However, despite the research done so far, the mechanism of action that tannins exert on animal tissues is still unknown (Fraga-Corral et al. 2020). The potential antioxidant effect might be a process that took place at the cellular level of the testicles and, therefore, was not detectable in the blood. Future works may consider measuring the oxidative status of the semen or/and testicular tissue. It is worth mentioning here that the 3 g ETE treatment appeared to have the highest level of testosterone and the lowest level of cortisol, but, statistically, was not significant (Figure 2.1). The plasma testosterone concentration was influenced by time, while the cortisol and the oxidative status were not. At week eight of the study period (Figure 2.1), the testosterone concentration showed a significant arising trend (14.48 ± 1.00 ng/ml) as compared to day zero (10.28 ± 0.93 ng/ml) and week sixteen (10.44 ± 0.82 ng/ml). Meaning that the testosterone concentration was higher in the second half of autumn (week eight = May) as compared to the first half of autumn (day zero = April) and the end of winter (week sixteen = August). These findings are comparable to the data reported by Sarlos et al. where the plasma concentration of testosterone continued to increase during the first half of autumn until it reached the maximum in the second half and then dropped in winter (Sarlós et al. 2013). The seasonal fluctuations in the plasma testosterone concentration can be attributed to the changes in melatonin secretion due to variation in the daylight length (Casao et al. 2010, Flores-Gil et al. 2020). Due to the regulating effect on the hypothalamus–pituitary–testicular axis, melatonin modulates the GnRH pulse activity, and gonadotropin and testosterone production (Casao et al. 2010).

There were significant interactions between tannin type and tannin supplementation level for testicular length, semen volume, sperm concentration, and percentages of slow motility sperm, non-progressive motility sperm, and sperm with cytoplasmic droplet (Table 2.4). In most cases, the group of TE at supplementation level of 1.5 g/d and the group of ETE at 3 g/d performed better in comparison with their counterparts (Table 2.4).

The results in this study revealed an interesting dose-dependent effect of supplementation with TE and ETE on the reproduction performance of the rams. The increase in TE from 1.5 g to 3 g resulted in negative effects in semen volume and sperm morphology. However, on the opposite, the increase in ETE from 1.5 g to 3 g had positive effects on testicular length, sperm concentration and motility. The most likely reason for this disparity is the encapsulation process. As mentioned above, the encapsulation process slows and maintains the constant release of the tannin during the day (Adejoro et al. 2018). Thus, sufficient time may be provided for the environment inside the digestive tract to absorb and utilise the available tannin which could explain the beneficial effects of the supplementation with 3 g ETE. Whereas, in the case of the un-encapsulated tannin extract (TE), the tannin dissolves faster and becomes immediately available (Adejoro et al. 2018). Therefore, the high quantity of the dissolved un-encapsulated tannin (3 g TE) might have worked as an anti-nutritional factor that led to the observed negative effects, since the tannin may combine with the dietary proteins, carbohydrate and minerals and complex with the secreted enzymes and endogenous enzymes, thus diminishing the digestion process (McSweeney et al. 2001, Gerlach et al. 2018). However, the low quantity of the dissolved un-encapsulated tannin (1.5 g TE) might be utilised as antioxidants and hence added its beneficial effects. It is crucial to note that the improvements due to supplementation with 1.5 g TE and 3 g ETE did not statistically differ from the control in most cases. This study is the first report on the effect of *Acacia mearnsii* supplementation

on the reproduction performance of rams. The daily supplements levels used in our study from TE or ETE were 1.5 g and 3 g per animal, which approximately represent 0.03 and 0.06 g/kg BW/day. These levels are low compared to the dietary tannin inclusion levels used to reduce enteric methane emissions in sheep (Adejoro et al. 2019), since we used the amount that is far from the threshold level to identify the least amount of the tannin extract, which can enhance the oxidative status of the rams. As a result, levels higher than 0.06 g/kg BW/day and lower or equal to the threshold level (0.30–0.70 g/kg BW) [15,16,63] were not covered in this study. Those high levels should be considered in future studies in an encapsulated form when provided to animals.

Table 2.4 Effects of tannin type and tannin supplementation level on the testicular length and some semen quality parameters of Merino rams (Mean ± SE)

Treatments		Parameters					
Tannin Type	**Tannin Level (g/day)**	**Testicular Length (mm)**	**Semen Volume (mL)**	**Sperm Concentration (10^9/mL)**	**Slow Motility Sperm (%)**	**Non-Progressive Motility Sperm (%)**	**Sperm with Cytoplasmic Droplet (%)**
TE	1.5	91.9 ± 1.17 ^{ab}	2.5± 0.61 ^{a}	3.6 ± 0.03 ^{a}	20.1 ± 1.53 ^{ab}	23.7 ± 1.68 ^{ab}	0.4 ± 1.44 ^{b}
	3	88.8 ± 0.95 ^{b}	1.7 ± 0.72 ^{b}	3.1 ± 0.03 ^{ab}	23.0 ± 1.41 ^{a}	26.7 ± 1.58 ^{a}	2.7 ± 2.64 ^{a}
ETE	1.5	88.6 ± 1.02 ^{b}	1.9 ± 0.74 ^{b}	2.4 ± 0.02 ^{b}	22.1 ± 1.32 ^{a}	26.1 ± 1.39 ^{a}	0.9 ± 1.97 ^{b}
	3	93.3 ± 0.93 ^{a}	2.1 ± 0.95 ^{ab}	3.2 ± 0.02 ^{a}	17.3 ± 1.72 ^{b}	20.2 ± 2.03 ^{b}	0.4 ± 1.47 ^{b}
ANOVA		***p*-value**	***p*-value**	***p*-value**	***p*-value**	***p*-value**	***p*-value**
Tannin Type		0.663	0.830	**0.032**	0.151	0.191	0.051
Tannin level		0.535	0.113	0.585	0.459	0.347	0.068
Tannin type × Tannin level		**0.003**	**0.010**	**0.006**	**0.003**	**0.004**	**0.003**

^{a,b} Column means with different superscripts within time and treatments indicate significant differences ($p < 0.05$). TE = tannin extract; ETE = encapsulated tannin extract.

Table 2.5 Pearson's Correlation Coefficient between weight, some testicular measurements, semen quality, and hormonal status variables in the study

Variable	Bodyweight	Testicular Volume	Scrotal Circum-ference	Semen Volume	Sperm Concen-tration	pH	Mass Motility	Progressive Motility	Viability	Acrosome Integrity	Testosterone
Testicular volume	0.320 ***	1									
Scrotal circumference	0.388 ***	0.728 ***	1								
Semen volume	0.266 *	0.319 **	0.409 ***	1							
Sperm concentration	0.046	0.257 *	0.231 *	0.205 ***	1						
pH	−0.188	−0.175	−0.112	−0.139	−0.056	1					
Mass motility	0.030	−0.077	0.001	−0.047	0.017	−0.243 *	1				
Progressive motility	−0.052	0.152	0.081	0.104	0.212 *	−0.152	0.421 ***	1			
Viability	0.164	−0.061	−0.092	−0.243 **	−0.008	−0.143	0.282 **	0.178 *	1		
Acrosome integrity	−0.005	−0.117	−0.078	−0.273 **	−0.006	−0.216 *	0.327 ***	0.196 *	0.608 ***	1	
Testosterone	0.205	0.033	0.019	0.202	−0.164	−0.312	0.068	−0.140	0.162	0.165	1
Cortisol	−0.028	0.196	0.295 *	0.191	−0.107	0.085	−0.530 **	−0.437 *	−0.537 **	−0.594 **	−0.123

* $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$.

Table 2.5 shows correlation coefficients between bodyweight, testicular measurements (testicular volume and scrotal circumference), semen quality (semen volume, sperm concentration, pH, mass motility, progressive motility, viability, acrosome integrity), and hormonal status variables (testosterone and cortisol) in Merino rams. Only significant correlations ($p < 0.05$) are discussed. Correlations above r=0.3 are acceptable and considered to have moderate relationship and correlations above r=0.7 are considered to have strong relationship. Bodyweight had a moderate significant correlation with testicular volume ($r = 0.320$, $p < 0.001$), scrotal circumference ($r = 0.388$, $p < 0.001$), and low but significant relationship with semen volume ($r = 0.266$, $p = 0.012$). These results of the positive correlation between bodyweight and testicular measurements agree with the reports from previous studies (Salhab et al. 2001, Elmaz et al. 2007, Hassan et al. 2009, Al-Kawmani et al. 2014). The correlation between testicular measurements and bodyweight could be because testicular measurements were relative to bodyweight when age was kept constant (Salhab et al. 2001, Al-Kawmani et al. 2014). Testicular volume had a strong and significant correlation with scrotal circumference (0.728, $p < 0.001$), moderate significant relationship with semen volume ($r = 0.319$, $p = 0.003$) and low but significant correlation with sperm concentration ($r = 0.257$, $p = 0.016$). In addition, the scrotal circumference was found to have a moderate and significant correlation with semen volume ($r = 0.409$, $p < 0.001$) besides low and significant association with sperm concentration ($r = 0.231$, $p = 0.031$) and cortisol concentration (0.295, $p = 0.010$). The positive correlation between testicular measurements (testicular volume plus scrotal circumference) and semen parameters agree with previous studies (Elmaz et al. 2007, Hassan et al. 2009, Allaoui et al. 2018). The positive correlation between testicular measurements and semen volume plus sperm concentration could be because

seminiferous tubules and germinal cells approximately make-up over 90% of the testicles (Yamamoto et al. 1995). The bigger testicles result in more seminiferous tubules and germinal cells which may increase semen production (volume) and sperm concentration (Nugraha et al. 2019). Semen volume showed a low and significant relationship with sperm concentration (r = 0.205, $p < 0.001$) and negative, low, and significant correlations with sperm viability ($r = -0.243$, p =0.004) and sperm acrosome integrity ($r = -0.273$, $p = 0.001$). A similar positive correlation between semen volume and sperm concentration were reported in previous studies (Webb et al. 2004, Hassan et al. 2009). As the spermatogenesis increases, the sperm concentration and semen volume increase and vice versa (Anawalt 2013), which may explain the observed positive correlation between semen volume and sperm concentration. Sperm concentration had very low but significant relation with progressive motility ($r = 0.212$, $p = 0.010$). The positive relationship between sperm concentration and progressive motility agrees with the report of Darbandi et al. (Darbandi et al. 2019). Semen pH showed a low, negative but significant association with sperm mass motility and acrosome integrity. A significant negative correlation between sperm motility and semen pH had been reported in different species including sheep (Moghaddam et al. 2012), buffaloes (Sajjad et al. 2007), and humans (Samavat et al. 2019). The negative correlation between sperm mass motility and semen pH could probably be due to the observation that spermatozoa with high mass motility consume more energy (fructose) and produce more by-products, such as lactic acid (Hafez and Hafez 2013), which may drop the semen pH faster than those with low mass motility. Sperm mass motility had moderate significant correlations with progressive sperm motility ($r = 0.421$, $p < 0.001$) and sperm acrosome integrity ($r = 0.327$, $p < 0.001$) along with low but significant relationship with sperm viability ($r = 0.282$, $p = 0.001$). The moderate

association between sperm mass motility and progressive motility agrees with the finding of Aller et al. (Aller et al. 2012). Very low but significant association were found between the progressive sperm motility and viability ($r = 0.178$, $p = 0.034$) plus sperm acrosome integrity ($r = 0.196$, $p = 0.019$). Sperm viability had a moderate positive correlation with sperm acrosome integrity ($r = 0.608$, $p < 0.001$). This could be because spermatozoa gradually lose their acrosome integrity when dies (Nagy et al. 2004). The cortisol showed a moderate negative correlation with sperm mass motility ($r = -0.530$, $p = 0.006$), progressive motility ($r = -0.437$, p=0.029), viability ($r = -0.537$, $p = 0.006$), and acrosome integrity ($r = -0.594$, $p = 0.002$). These negative correlations indicate an adverse effect of cortisol on semen quality parameters. This might be because the elevated level of cortisol reduces testosterone secretion (Palacios et al. 2016), which may negatively affect spermatogenesis and semen parameters, such as sperm motility and morphology (Sengupta and Dutta 2018)[81]. In addition, the cortisol leads to an increase in the reactive oxygen species (ROS) (Singh and Singh 2017) that may reduce sperm motility and damage the DNA and plasma membrane (Saleh and Agarwal 2002, Baker and Aitken 2004). However, the negative correlation between cortisol and semen quality parameters seems to differ between the species, as a similar negative correlation was reported in humans (Wdowiak et al. 2017), while the stallion's semen seems to be well protected against high levels of cortisol (Deichsel et al. 2015).

2.4 Conclusions

To our knowledge, this was the first experimental approach to study the effects of supplementation with encapsulated and non-encapsulated *Acacia mearnsii* tannin extract on the oxidative status and reproduction performance of rams. The supplementation with 1.5 g tannin extract (0.03 g/kg BW/d) and 3 g encapsulated tannin extract (0.06 g/kg BW/d) improved the

testicular length, semen volume, and sperm concentration, as well as reduced the percentages of spermatozoa with abnormal morphology and low or non-progressive motility. However, the increase in tannin extract supplementation from 0.03 to 0.06 g/kg BW/d reduced semen volume and increased the percentage of abnormal spermatozoa. Thus, to obtain maximum benefits from tannin extract supplementation, the encapsulated tannin extract should be at higher than 0.03 g/kg BW/d. Further studies to elucidate the mechanisms of action that tannin extract has on semen quality and quantity are warranted. Research into the supplementation effect of levels higher than 0.06 g/kg BW/d of encapsulated TE on the oxidative status and reproduction performance of the rams is also needed. Testicular measurements of the South African Mutton Merino rams are higher in autumn than in winter. The plasma level of cortisol may play a significant role in determining the sperm quality of the sheep since it correlates negatively with sperm mass motility, progressive motility, viability, and acrosome integrity.

2.5 Funding

The running cost of this research was funded by the National Research Foundation (NRF), South Africa through Grant No: 118518 and 116228.

2.11 Appendix 2.A

Table 2.B.1 The initial data of the experimental groups (Mean ± SE) in the study

Variable	Experimental Groups					*p*-Value
	Control	1.5 g TE	3 g TE	1.5 g ETE	3 g ETE	
Initial Bodyweight (kg)	52.7 ± 1.85	52.3 ± 2.42	52.4 ± 1.59	53.5 ± 1.37	53.0 ± 2.05	0.991
Initial scrotal circumference (cm)	31.6 ± 1.52	31.9 ± 1.39	30.5 ± 0.94	30.7 ± 1.35	30.4 ± 0.52	0.860
Initial testicular volume (cm^3)	179.5 ± 22.61	186.3 ± 21.80	182.8 ± 17.93	184.5 ± 15.42	192.8 ± 13.64	0.991
Initial testicular width (mm)	59.7 ± 3.14	61.7 ± 2.47	61.4 ± 2.25	62.4 ± 2.40	61.7 ± 1.49	0.951
Initial testicular length (mm)	98.8 ± 4.40	96.9 ± 4.57	96.8 ± 4.17	97.5 ± 2.52	99.0 ± 4.26	0.992
Initial semen volume (mL)	0.7 ± 0.11	0.7 ± 0.18	1.0 ± 0.14	0.9 ± 0.23	0.8 ± 0.19	0.728
Intial semen pH	5.8 ± 0.17	5.9 ± 0.37	5.7 ± 0.33	6.5 ± 0.62	6.5 ± 0.22	0.355
Initial sperm concentration (10^9/mL)	3.8 ± 0.90	3.9 ± 0.75	3.9 ± 0.69	3.9 ± 0.91	3.5 ± 0.80	0.997
Initial Semen colour (1–4)	3.2 ± 0.31	3.2 ± 0.48	3.7 ± 0.21	2.8 ± 0.40	3.5 ± 0.22	0.471
Initial Sperm mass motility (1–5)	3.3 ± 0.21	3.3 ± 0.56	3.5 ± 0.43	3.3 ± 0.61	3.8 ± 0.17	0.907
Initial progressive motility (%)	56.2 ± 4.86	54.5 ± 10.80	52.2 ± 9.31	51.1 ± 12.71	56.4 ± 6.71	0.991

TE = tannin extract; ETE = encapsulated tannin extract.

CHAPTER THREE

Threshold inclusion levels of tannin extracts in sheep and cattle feed: A meta-analysis

Scientific paper prepared to be submitted for publication

Abstract:

Tannin extracts (TE) supplements can be used as antioxidants, anti-methanogenic, anthelmintics, and many other applications. However, these applications are challenged by the reports of tannin's negative effects on feed intake and digestibility and thus animal performance. The objective of this meta-analysis is to identify thresholds for the three most used sources of TE in ruminants (black wattle, quebracho, and chestnut) that allow various applications and co-benefits without compromising animal performance. Data were extracted from forty-four scientific publications that met the selection criteria. We proposed the use of the change percentage to calculate the responses of the dependent variables to TE inclusion. The percentage change approach compares the tannin-treated group with the control group in each study. Hence, feed intake, digestibility parameters (organic matter (OM), crude protein (CP), nutrient detergent fibre (NDF) and acid detergent fibre (ADF), N balance parameters (faecal N, urinary N and retained N) and weight gain were calculated in response to TE inclusion in each applicable study. Statistical analyses were performed using R (version 4.2.1) and RStudio (version 2022.07.1+554). Different studies were treated as random effects, while TE level, TE source, animal species and supplementation period were treated as fixed effects. The results showed that tannin sources had no significant effect on feed intake and the level of 3.5 g/100 g of DM is suggested as the TE threshold for feed intake in sheep and cattle. The levels of 5.7, 2.5 and 2.7 g/100 g of DM are proposed as OM digestibility (OMD) thresholds for chestnut, quebracho and black wattle TE, respectively in sheep and cattle. The levels of 1 g and 2.3 g/100 g DM are proposed as CP digestibility (CPD) thresholds for TE in sheep and cattle, respectively. The levels of 2.4, 2.5 and 0.5 g/100 g DM are proposed as NDF digestibility (NDFD) thresholds for chestnut, quebracho and

black wattle TE, respectively. For ADF digestibility (ADFD), the thresholds were 3.2 and 1.8 g/100 g DM for chestnut and quebracho, respectively, while only 1 g/100 g of black wattle TE lowered ADFD by 15%. Our meta-analysis showed that the CPD model provides the strongest response to TE inclusion compared to the other digestibility parameters (OMD, NDFD and ADFD). Therefore, based on the CPD response, we propose to consider the TE inclusion level of 1 and 2.3 g/100 g of DM as thresholds for digestibility in cattle and sheep, respectively. The proposed thresholds showed a negligible effect on weight gain in cattle (+ 1.6%) and sheep (− 3.1%) after TE inclusion.

Keywords: chestnut; black wattle; quebracho: mixed model; percentage change

Abbreviations: ADF, acid detergent fibre; ADFD, acid detergent fibre digestibility; MBW, metabolic bodyweight; CP, crude protein; CPD, crude protein digestibility; CT, condensed tannins; DM, dry matter; DMI, dry matter intake; HT, hydrolysable tannins; NDF, neutral detergent fibre; NDFD, neutral detergent digestibility; OM, organic matter; OMD, organic matter digestibility; R2, determination coefficient; RMSE, root mean squared error; CCC, concordance correlation coefficient; RSR, RMSE-observations standard deviation ratio.

3.1 Introduction:

As a promising natural feed additive, tannin extracts (TE) have received significant attention recently. Tannins are water-soluble phenolic compounds of high molecular weight, found abundantly in plants (Westendarp 2006, Hassanpour et al. 2011). Based on their molecular structure, tannins are divided into hydrolyzable (molecular weight (MW) 500-3000 g/mol) and condensed tannins (MW up to 20,000 g/mol) (Denninger et al. 2020, Manzoor et al. 2021). Black wattle *(Acacia mearnsii*), quebracho (*Schinopsis quebracho-colorado*) and chestnut (*Castanea*

sativa) are the three most widely used TE in animal feed (Duke 2012). Chestnut TE is considered a source of hydrolysable tannins (HT), while black wattle and quebracho are regarded as condensed tannins (CT) sources (Missio et al. 2017). Tannin additives can be used as a tool to break down the oxidative chain reaction (antioxidant) (Brenes et al. 2008, Deng et al. 2016, Liu et al. 2016), reduce ruminant methane emissions (anti-methanogenic) (Beauchemin et al. 2007, Adejoro et al. 2019), control gastrointestinal parasites (anti-helminthics) (Athanasiadou et al. 2001, Minho et al. 2008) and many other applications. However, many of these opportunities and applications are challenged by the reports of the negative impact of TE on feed intake and digestibility and thus animal performance (Dschaak et al. 2011, Patra and Saxena 2011, Adejoro et al. 2018). A solution to these challenges can be found within the note derived from these reports that chances of negative consequences may increase as the TE inclusion level increases (Dschaak et al. 2011, Patra and Saxena 2011, Adejoro et al. 2018). Therefore, the solution is to identify the threshold levels of TE inclusion in animal feed that allow various applications and co-benefits without compromising the animal performance. This comes in handy for research stakeholders, who want quantitative knowledge on the animal response to TE dietary additives.

Surprisingly, considering the accumulation of data from many in vivo experiments done on TE inclusion in animal feed (especially for chestnut, black wattle and quebracho TE), no meta-analysis was done to identify those levels. Nontheless, a number of meta-analyses have been attempted to study the relationship between tannin supplementation and the animal performance. A recently published meta-analysis (Yanza et al. 2021) examined the effects of TE supplementation on ruminant (cattle, sheep and goats) performance and digestibility at various levels. Besides the TE level, the study examined the tannin-type effect (HT vs CT extract).

However, many factors such as TE source, animal species, supplementation period, diet's fibre level, diet's protein level, and animal growth stage may also play a role. Above that, a suitable approach to identify those levels is needed. Different approaches have been used to study the relationship between TE inclusion and animal performance (sheep and cattle) in several meta-analyses (Jayanegara and Palupi 2010, Jayanegara et al. 2012, Méndez-Ortiz et al. 2018, Jayanegara et al. 2019, Herremans et al. 2020, Purba et al. 2020, Orzuna-Orzuna et al. 2021, Orzuna-Orzuna et al. 2021, Yanza et al. 2021). One approach was to link the dependent variable, such as DMI (kg/day), to the tannin level (e.g., g/kg of DM). So, the influence of TE will be seen as a decrease or increase in the DMI (kg/day) regression line depending on the TE level used (g/kg of DM). However, the DMI of an individual animal depends on its weight, as heavy animals have a higher DMI than lighter ones, and cattle eat more than sheep. That will produce a source of variability in the data that has nothing to do with TE inclusion. Such variation in the data can be reduced by dividing the DMI by the body weight or the metabolic bodyweight of the animals treated with tannin (Jayanegara et al. 2012, Méndez-Ortiz et al. 2018). Nevertheless, we suggest another approach that might better suit the purpose of this meta-analysis: the percentage change, by comparing the tannin-treated group with the control in each study. That is to subtract the DMI (dependent variable) of the control from the DMI of the treated group and divide the result by the control's DMI, then multiply by 100. The result will be a percentage of the change due to TE inclusion with a positive (+) or negative (-) direction. With the use of appropriate statistical software, regression line/s can be drawn based on the generated observations, taking into account the factors that may affect the relationship between TE inclusion and DMI response (TE source,

animal species, etc.). The same principle can be applied to any dependent variables, such as animal digestibility and weight gain.

Nonetheless, observations within one study are more correlated than observations across studies, and there are unignorable differences in measurement accuracy within and across studies (St-Pierre 2001). The consequence of discounting these two facts is that the regression equation under consideration may create biased estimated parameters (St-Pierre 2001). Mixed models allow account for the effect of the study as a random effect and account for different weights for observations depending on the accuracy of their measurements (variation) (St-Pierre 2001, St-Pierre 2007). We hypothesis that the animal response to TE inclusion is affected by TE level, TE source, animal species, supplementation period, diet's fibre level, diet's protein level and animal growth stage. The objective of the present meta-analysis is to determine the TE threshold inclusion levels (black wattle, quebracho and chestnut) on feed intake and digestibility, as well as the effects of TE inclusion on N balance and weight gain in sheep and cattle.

3.2 Material and methods:

3.2.1. Data set creation:

Peer-reviewed articles, theses and dissertations for the subject under investigation were searched in "Web of Science" and "Google Scholar" databases. The following in-title keywords were used: "condensed tannin OR condensed tannins OR hydrolyzable tannin OR hydrolysable tannins OR mimosa tannin OR mimosa tannins OR quebracho tannin OR quebracho tannins OR chestnut tannin OR chestnut tannins OR chestnuts tannin OR chestnuts tannins OR *Castanea sativa* tannin OR *Castanea sativa* tannins *OR Acacia mearnsii* tannin OR *Acacia mearnsii* tannins OR black wattle tannin OR black wattle tannins AND ram OR rams OR ewe OR ewes OR lamb

OR lambs OR cattle OR cattles OR heifer OR heifers OR cow OR cows bull OR bulls OR calf OR calves OR Steer OR Steers OR Whether OR Whethers"

3.2.2. Inclusion and exclusion criteria

The online research resulted in 153 published documents. The following criteria were adapted to include or exclude the studies in our meta-analysis:

1. The study should be an in vivo experiment done on sheep or cattle.
2. The source of tannin used in sheep or cattle studies should be tannin extract not the plant or part of the plant.
3. We focused on the most used sources of tannin extract: black wattle, quebracho and chestnut TE. Studies on other sources of TE were excluded.
4. The TE used should be from one source. Studies, where the animal was provided with a mixture of TE, were excluded.
5. The animal should be healthy (e.g., studies in which TE is used as an anthelmintic agent were excluded).
6. The concentration of TE in the feed is reported or possible to be calculated.
7. The TE-containing feed should be consumed by the animal voluntarily. Studies, where the TE is infused in the animal ruminant, were excluded.
8. The TE-containing feed provided to the animals should be ad libitum or at least a refusal rate equal to or higher than 10% is reported.

However, the last two conditions were not taken into account for the data sets of digestibility and nitrogen balance parameters, while applied to DMI and weight gain data sets.

3.2.3 Data extraction and description

Out of the 153 published documents, forty-three peer-reviewed articles and one PhD thesis fit the selection criteria (Table 3A2). Our data set included six categorical predictors: (1) TE source consists of three categories: chestnut TE, black wattle TE, and quebracho TE; (2) Animal species included sheep and cattle. Data from sheep and cattle were included in the analysis to assess the significance and extent of differences between sheep and cattle in their responses to TE inclusion; (3) Supplementation periods were divided into two categories: short-term (≤ 1 month) and long-term (> 1 month); (4) Diet's protein levels were classified into low (< 18% CP) and high level (≥ 18% CP); (5) Diet fibre levels were categorized into a low level (≤ 38% NDF) and high level (> 38% NDF); (6) Animal growth stages were grouped into growing and mature. Young animals such as lambs and heifers were classified as growing, while mature animals such as rams, steers, bulls, cows, and ewes were classified as mature. It is crucial to note that lactating cows and ewes were only included in feed intake and digestibility data and were excluded from the N balance and weight gain data.

3.2.4 Parameter's response calculation

To make the comparison between different studies more applicable we proposed the use of the change percentage to calculate the dependent variables' responses to TE inclusion. For example, the following equation was used to calculate the DMI response to TE inclusion:

DMI response = ((DMI *t* – DMI *c*) ÷ DMI *c*) × 100

Where:

DMI *t* = DMI for the treated group, DMI *c* = DMI for the control group.

The same method was used to calculate the response of weight gain, digestibility parameters (OM, CP, NDF and ADF) and N balance parameters (Faecal N, urinary N and retained N). However, for nitrogen balance, the nitrogen (Faecal N, urinary N and retained N) in grams per 100 g intake was calculated for both the control and the treated group first before the calculation of the responses. TE inclusion level can be reported as a gram per kilogram of dry matter (g/kg of DM) of the feed or as a gram per kilogram of the animal's metabolic bodyweight (g/kg MBW). However, the scale of TE level as g/kg DM (e.g in DMI data, Min: 2.0, Max: 89.3) is different from that of TE level as g/kg MBW (e.g in DMI data, Min: 0.2, Max: 6.35). Therefore, the TE level as g/100 g of DM (e.g in DMI data, Min: 0.2, Max: 8.9) was used instead of g/kg DM. In this study, both expressions (g/100 g of DM and g/kg of MBW) were calculated and their relationship with the dependent variables was tested.

3.2.5 Statistical analysis:

Statistical analyses were carried out on data using R (version 4.2.1, 2022-06-23) and RStudio (version 2022.07.1+554 for Windows). Studies were treated as random effects whereas TE level, TE source, animal species, supplementation period, diet's fibre level, diet's protein level and animal growth stage were treated as fixed effects using the following mixed models:

Model 1: Linear model:

$$Y_{irsfvnak} = \mu + S_i + B_1X_{irsfvnak} + C_r + A_s + P_f + F_v + T_n + G_a + e_{irsfvnak}$$

Model 2: Quadratic model:

$$Y_{ijtfvnak} = \mu + S_i + B_1X_{ijtfvnak} + B_2X^2_{ijtfvnak} + C_r + A_s + P_f + F_v + T_n + G_a + e_{ijtfvnak}$$

Where: $Y_{irsfvnak}$, $Y_{ijtfvnak}$ = response variable; μ = overall mean; S_i = random effect of the *i*th study; B_1= linear regression coefficient; B_2= quadratic regression coefficient; X = value of the continues predictor variable (TE level); C_r = TE source (r = 1-3); A_s = animal species (s = 1-2); P_f = supplementation period (f= 1-2); F_v= fibre level; T_n = protein level (1-2); G_a = animal growth stage; $e_{irsfvnak}$: $e_{ijtfvnak}$ = residual errors.

The package lme4 (version 1.1-27.1) (Bates et al. 2015) was used to create those mixed models followed by lmertest using the function "step" to create a backward reduced fixed-effect table. Accordingly, best-fit models for each response variable were selected, in which only significant terms ($P < 0.05$) were retained. Regression coefficients, p-values, conditional ($R^2_{(c)}$) and marginal ($R^2_{(m)}$) determination coefficients (R^2) reported for the different models were estimated using sjPlot's tab_model function (version 2.8.10). Marginal $R^2_{(m)}$ is the variance explained by the fixed effects of the model, while conditional $R^2_{(c)}$ is the variance explained by both fixed and random effects of the model (Nakagawa and Schielzeth 2013). In addition, goodness-of-fit statistics: Akaki information criteria (AIC) and weighted root mean squared error (RMSE) were also reported for each model. The RMSE is a measurement of prediction accuracy, the smaller RMSE, the greater the accuracy of the model. As a rule of thumb, the RMSE should be less than half of the standard deviation (SD) (Singh et al. 2005, Moriasi et al. 2007). The ratio of RMSE to the standard deviation of the observations is called the RMSE-observations standard deviation ratio (RSR) (Singh et al. 2005, Moriasi et al. 2007). The lower RSR, the lower the RMSE, and the better the model performance (Moriasi et al. 2007). Lin's concordance correlation coefficient (CCC) was calculated for all models to measure the agreement between the observed and the model-predicted values. Following Altman's recommendation (Altman 1990) to interpret the CCC values greater than 0.80 as excellent agreement. Furthermore, the models were internally

validated with 1000 resampling using parametric bootstrapping with the bootMer function of the Package lme4 (version 1.1-30). The bootstrapping parameters' bias and SE were reported in the supplementary data of this article. For best-fit models with more than one term, joint tests were performed using the joint test function in the 'emmeans' R package (version 1.8.1-1). The joint test generates an F ratio and a p-value for each term in the model. In general, the higher the value of the statistic, the higher the ranking of the term. When found significant in a model, TE sources were pairwise compared using Tukey contrast (function emmeans) using also "emmeans" R package (version 1.8.1-1). Data observations were weighted by the inverse of their relative variance (1/RV). The relative variance (RV) was calculated by dividing the squared standard error mean (SEM^2) by the mean of each study. Then each weight was divided by the mean of all weights, and the resulting values were used as weighting factors in the analysis to maintain the expressions of dispersion in the original scale of the measurements (St-Pierre 2001). The presence of outliers was identified by examining studentized residuals with the Influence plot, provided within the car package (version 3.0-12); values beyond ± 3 SD were considered as outliers and, therefore, were removed from the dataset.

3.3 Results

Table 3.1 shows descriptive statistics for TE treatments (TE level), DMI, OMD, CPD, NDFD, ADFD, faecal N, urinary N, and retained N with body weight by TE source and animal species.

3.3.1 Threshold inclusion levels of tannin extract for DMI

A description of animals and feeds used to determine the DMI response to TE inclusion in the studies of this meta-analysis is presented in Table 3.2. The result showed that when used to

predict DMI response, the TE inclusion level as g/100 g of DM was a better presenter ($P < 0.01$) for TE level than as g/kg MBW ($P = 0.64$). The result also revealed a nonsignificant second-degree coefficient ($P = 0.46$) for the TE level (g/100 g of DM), indicating that DMI data support the linear model rather than the quadratic one. Table 3A1 shows the full model's estimates for potential predictors of DMI response. Backward stepwise selection revealed that except for the TE level, all potential predictors, namely TE source, animal species, diet's fibre level, supplementation period, protein level, and animal stage, were insignificant. This finding suggests that the DMI response may be best represented as a linear response to the TE inclusion level (g/100 g of DM). Table 3.3 displays this best-fit model for DMI response to TE inclusion and the goodness of fit (Model DMI). The fixed-effects of the model (TE inclusion level) explained 45% (R^2_m) of the DMI variance and 86% (R^2_c) of the variance when combined with the random-effect (different studies). The model RMSE was found to be 1.76 (Table 3.3). The smaller the RMSE, the better the model's accuracy, and it should be less than 0.5 of the SD of the observations (Moriasi et al. 2007). In other words, the RSR should be less than 0.5 (Moriasi et al. 2007). In this case, the RMSE of 1.76 corresponds to an RSR of 0.35. This indicates that the model has excellent prediction accuracy. Furthermore, the result revealed that Lin's concordance correlation coefficient (CCC) value was 0.93 (Table 3.3). Following Altman's recommendation (values greater than 0.80 are excellent) for interpreting the CCC, the result indicates excellent agreement between the predicted and actual responses. Table 3C1 shows the results of the model validation using bootstrapping. The bootstrapping result revealed almost no bias in the predictor estimate, so our final best-fit model was equivalent to the bootstrap model. This result strengthens the model's predictions as being reliable.

Table 3.1 Descriptive statistics of DMI, OMD, CPD, NDFD, ADFD, faecal N, urinary N, retained N with TE level and body weight used in the studies included in this meta-analysis to determine the responses to TE inclusion in sheep and cattle.

			TE level g/100 g of DM				DMI kg/d				Bodyweight (kg)			
Parameter	Categories	Obs./ Stud.	Mean	SD	Min	Max	Mean	SD	Min	Max	Mean	SD	Min	Max
TE source	Chestnut	13/ 7	1.4	1.3	0.3	5.0	6.3	4.6	0.8	13.0	294	244	25	717
	Quebracho	24/ 11	1.9	1.9	0.2	8.9	10.5	8.3	0.8	26.3	381	228	24	730
	Blackwattle	24/ 10	2.7	1.7	0.6	8.0	6.6	4.6	0.8	17.0	398	288	26	870
Animal species	Sheep	18/ 11	3.5	2.3	0.5	8.9	1.4	0.6	0.8	2.9	45	22	24	82
	Cattle	43/ 16	1.6	1.0	0.2	4.0	10.9	5.8	3.5	26.3	505	170	254	870
			TE level g/100 g of DM				OMD (%)				Bodyweight (kg)			
TE source	Chestnut	10/5	5.2	3.6	0.1	10	65.8	4.7	58.8	73.2	104	132	51	480
	Quebracho	26/10	2.2	1.6	0.4	6.0	65.8	9.8	44.2	79.4	372	201	50	730
	Blackwattle	27/13	2.7	1.9	0.6	7.6	69.6	7.2	51.2	81.0	280	292	25	752
Animal species	Sheep	27/14	4.2	2.8	0.1	10	66.3	6.9	51.2	79.4	52	17	25	76
	Cattle	36/14	2.0	1.3	0.4	6.0	68.2	9.1	44.2	81.0	456	195	156	752
			TE level g/100 g of DM				CPD (%)				Bodyweight (kg)			
TE source	Chestnut	9/4	3.2	3.3	0.1	8.3	63.4	10.7	50.7	81.2	121	159	51	480
	Quebracho	17/8	2.3	1.6	0.4	6.0	64.1	8.6	48.1	76.9	332	240	50	730
	Blackwattle	17/9	2.3	1.3	0.6	4.2	65.1	12.9	18.1	75.0	336	338	25	752
Animal species	Sheep	20/11	3.3	2.3	0.1	8.3	63.3	13.3	18.1	76.9	48	18	25	76
	Cattle	23/10	1.8	1.3	0.4	6.0	65.3	8.0	48.1	81.2	567	155	254	752

Table 3.1 continued

			TE level g/100 g of DM				NDFD (%)				Bodyweight (kg)			
Parameter	Categories	Obs./ Stud.	Mean	SD	Min	Max	Mean	SD	Min	Max	Mean	SD	Min	Max
TE source	Chestnut	9/4	3.2	3.3	0.1	8.3	57.0	15.8	44.2	83.9	121	159	51	480
	Quebracho	25/11	2.5	1.6	0.4	6.0	54.9	6.9	40.6	70.7	353	219	27	730
	Blackwattle	28/13	2.7	1.8	0.6	8.0	51.0	14.8	24.0	72.7	251	279	25	752
Animal species	Sheep	24/13	3.8	2.4	0.1	8.3	49.4	13.3	24.0	72.7	46	17	25	76
	Cattle	38/15	2.1	1.3	0.4	6.0	56.0	11.2	27.9	83.9	422	216	107	480
			TE level g/100 g of DM				ADFD (%)				Bodyweight (kg)			
TE source	Chestnut	9/4	3.2	3.3	0.1	8.3	49.4	14.1	37.0	73.4	121	159	51	480
	Quebracho	18/8	2.6	1.5	0.6	6.0	49.5	7.8	35.8	63.0	377	244	50	730
	Blackwattle	8/5	2.9	1.4	0.8	4.2	38.4	16.2	21.8	68.5	222	279	25	582
Animal species	Sheep	16/8	3.6	2.4	0.1	8.3	41.1	12.1	21.8	68.5	54	15	25	76
	Cattle	19/9	2.2	1.5	0.5	6.0	51.9	10.7	35.8	73.4	483	177	236	730
			TE level g/kg MBW				Faecal N (g/100 g N intake)				Bodyweight (kg)			
TE source	Chestnut	8/3	4.7	3.6	0.1	8.3	8.5	2.3	5.7	13.4	115	148	51	480
	Quebracho	12/4	2.2	1.6	0.5	5.0	23.4	11.9	9.6	42.1	276	91	27	350
	Blackwattle	24/12	3.3	2.3	0.8	8.0	23.0	23.4	0.9	72.2	142	187	25	582
Animal species	Sheep	24/12	4.5	2.7	0.1	8.3	9.5	6.7	0.9	37.2	45	20	25	76
	Cattle	20/7	1.8	1.1	0.5	4.5	33.6	20.9	10.2	72.2	329	134	156	582
			TE level g/kg MBW				Urinary N (g/100 g N intake)				Bodyweight (kg)			
TE source	Chestnut	6/2	5.7	2.7	1.5	8.3	9.7	3.4	7.4	16.6	136	169	65	480
	Quebracho	13/5	2.0	1.5	0.5	5.0	23.5	26.1	0.0	86.0	221	130	27	350
	Blackwattle	21/12	2.9	2.0	0.8	7.6	28.2	21.9	1.4	67.0	159	195	25	582
Animal species	Sheep	22/12	4.1	2.4	0.8	8.3	13.1	9.4	1.4	46.2	47	19	25	76
	Cattle	18/7	1.6	1.0	0.5	4.5	37.1	26.4	0.0	86.0	333	141	156	582

Table 3.1 continued

			TE level g/kg MBW				Retained N (g/100 g N intake)				Bodyweight (kg)			
Parameter	Categories	Obs./ Stud.	Mean	SD	Min	Max	Mean	SD	Min	Max	Mean	SD	Min	Max
TE source	Chestnut	8/3	4.3	3.5	0.1	8.3	9.8	17.5	1.2	52.7	114	148	51	480
	Quebracho	14/4	2.0	1.3	0.5	4.5	17.5	19.2	2.1	64.0	246	114	50	350
	Blackwattle	24/12	3.3	2.3	0.8	8.0	24.1	26.7	0.3	81.9	142	187	25	582
Animal species	Sheep	20/7	4.1	2.7	0.1	8.3	8.1	10.4	0.3	53.6	46	18	25	76
	Cattle	26/12	1.8	1.1	0.5	4.5	34.5	27.1	2.1	81.9	329	134	156	582
			TE level g/100 g of DM				WG (kg)				Bodyweight (kg)			
TE source	Chestnut	7/4	1.1	0.7	0.2	2.1	48.5	46.1	7.3	135.0	233	199	25	461
	Quebracho	13/6	1.9	2.4	0.2	8.9	87.1	95.0	6.0	270.0	283	227	24	542
	Blackwattle	12/5	2.9	1.0	1.2	4.2	126.1	137.5	9.3	399.6	308	210	27	540
Animal species	Sheep	12/8	3.1	2.3	0.5	8.9	10.6	3.0	6.0	16.6	32	5	24	40
	Cattle	20/7	1.5	1.1	0.2	3.5	142.9	108.8	21.3	399.6	432	94	254	542

Obs., number of observations used in the present work; Stud., number of studies used in the present work; DMI, dry matter intake; OMD, organic matter digestibility; CPD, crude protein digestibility; NDFD, neutral detergent fiber digestibility; WG, weight gain; SD, standard deviation; Min, minimum; Max, maximum

Table 3.2 Description of animals and feeds used to determine DMI response to TE inclusion in the studies of this meta-analysis

Item		Ob./Stu.	Description (observation number/studies number)
Animals	Sheep	18/11	Lambs (9/6), rams (3/1), wethers (1/1) non-lactating ewes (2/1), lactating ewes (3/2)
	Cattle	43/16	Steers (21/6), bulls (5/2), heifers (6/2), non-lactating cows (6/3), lactating cows (5/3)
Feed	Low fibre	31/14	TMR (17/8), finishing diet (7/2), concentrates (6/3), concentrates + forage (1/1),
	High fibre	30/13	TMR (17/8), forage (9/3), forage-based diet (2/1), silage (2/1)

Ob. = observation number, Stu. = study number

Figure 3. illustrates the predicted DMI response to the TE inclusion. The result illustrates that the inclusion of the TE in the feed caused a change in the animal's DMI depending on the levels of TE (g/100 g of DM) used (Figure 3.). When inclusion levels were less than 1 g/100 g of DM, TE caused a slight increase in DMI (Figure 3.1). Then, the animal's DMI decreases by 2.14% for each gram of TE inclusion, reaching 5% at around 3.5 g/100 g of DM. The inclusion level of 3.5 g /100 g DM can be identified as the threshold level for the TE in sheep and cattle when dietary TE is used for various applications and co-benefits.

Table 3.3 Results of regression and the goodness of fit for the best fit model used to predict DMI response (% of the control) for TE inclusion (g/100 g of DM) in the sheep and cattle diets.

Model DMI

Predictors	Estimates	SE	*p- value*
(Intercept)	2.32	1.20	0.058
TE level g/100 g	-2.1429	0.31	<0.001
Goodness of fit of model DMI			
R^2 marginal/ conditional	0.45/ 0.86	CCC	92.8
RMSE	1.76	AIC	372.91
Observations/ Studies number	61/27	Model type	Linear

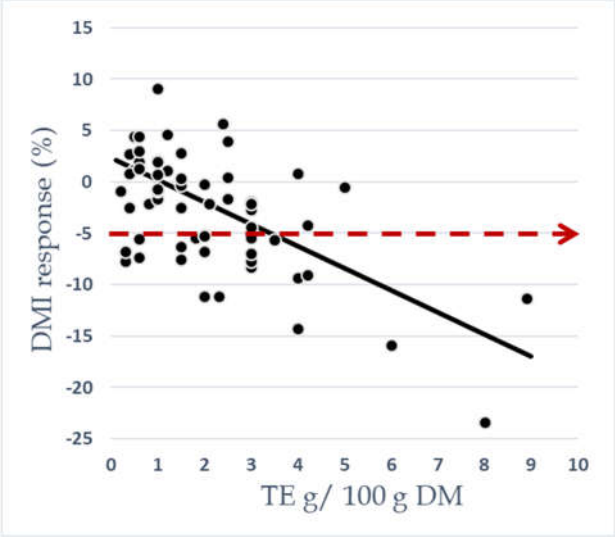

Figure 3.1 Actual (unweighted) observations with the predicted line of DMI response (%) to TE inclusion level as g/100 g of DM (Model DMI). The cross-point of the red arrow with the predicted lines indicates the proposed inclusion levels.

3.3.2 Threshold inclusion levels of tannin extract for feed digestibility

3.3.2.1 Threshold inclusion levels of tannin extract for organic matter digestibility (OMD)

A description of animals, feeds and methods used to determine OMD in the studies of this meta-analysis is presented in Table 3.4. The result showed that when used to predict OMD response, the TE inclusion level as g/100 g of DM ($P < 0.05$) performed better than the TE inclusion level as g/kg MBW ($P = 0.86$). Furthermore, the result revealed a significant second-degree coefficient ($P < 0.01$) for TE level, implying that DMI data may also support the quadratic term of TE level. Table 3B2 shows estimates for potential predictors of OMD response in the full model. Except for the TE level and TE source, the results of backward stepwise selection indicated that all potential predictors, namely animal species, supplementation period, diet's protein level, animal growth stage, and diet's fibre level, were not significant. This result implies that the OMD response to TE inclusion may be best represented as a linear or quadratic response to TE inclusion level (g/100 g DM) and TE sources.

Table 3.4 Description of animals, feeds and methods used to determine OMD digestibility in the studies of this meta-analysis.

Item		Ob./Stu.	Description (observation number/studies number)
Animal	Sheep	27/14	Lambs (7/4), rams (5/3), wethers (13/6) lactating ewes (2/1)
	Cattle	36/14	Steers (17/6), bulls (2/1), heifers (8/2), cows (3/1), lactating cows (6/4)
Feed	Low fibre	22/10	TMR (9/4), concentrates (4/2), fini.d. (4/1), conc.+ forage (4/2), concentrates + silage (1/1)
	High fibre	41/18	TMR (9/4), forage (13/6), forge-based diet (4/2), silage (4/2), concentrates + forage (11/4)
Digestibility type		63/28	Total tract digestibility (63/28)
Digestibility method		63/28	Total faces collection (46/21), external marker (10/4), internal marker (7/3)

Ob. = observation number, Stu. = studies number

The linear and quadratic best-fit regression models for OMD response to TE inclusion, as well as their goodness of fit, are shown in Table 3.5. TE inclusion level had the highest rank among the fixed effects of OMD models in both linear ($F = 29.01$, $P < 0.001$) and quadratic models ($F = 34.32$, $P < 0.0001$), followed by TE source in linear ($F = 3.44$ and, $P = 0.05$) and quadratic ($F = 2.62$ and, $P = 0.09$) models. In the linear and quadratic models, the fixed effects (TE inclusion level and TE source) explained 33 and 36% (R^2_m) of the OM digestibility variance, respectively (Table 3.5). When combined with the random effects, these effects explained 81 and 85% (R^2_c) of the variance in the linear and quadratic models, respectively. The RMSE for the linear and quadratic models were found to be 2.01 and 1.80, respectively (Table 3.5). The RMSE of 2.01 and 1.8 in this context correspond to RSR of 0.37 and 0.33, respectively. This indicates that both the linear and quadratic models have excellent prediction accuracy. Additionally, Lin's concordance correlation coefficient (CCC) value was found to be 0.91 for the linear model and 0.94 for the quadratic model (Table 3.5). The results show that both models have an excellent agreement between predicted and actual responses (Altman 1990). Tables 3C2 and 3C2 show the results of both models' bootstrapping validation.

The bootstrapping results revealed a negligible bias in the predictor estimates for both models, and thus our final best-fit models were nearly identical to the bootstrap models. This result adds to the reliability of the two models' predictions. However, the quadratic model appears to produce unrealistic predictions. For example, if the reader used the quadratic regression equation (Table 3.5) to predict the OMD response to the addition of 5 g/100 g DM of chestnut TE, the OMD would be predicted to decrease by 5.76%. Then, if the inclusion level is increased to 15 g/100 g of DM, an increase of 5.15% in the OMD is predicted, which is unrealistic because lower levels already caused a decrease in the OMD. As a result, the linear model may be preferred over the quadratic model for predicting the OMD response to TE inclusion.

Figure 3. depicts the effect of the TE source on the linear OMD response to the TE inclusion level. When the TE inclusion level was less than 2.5 g/100 g of DM, chestnut TE showed a slight increase in OMD. Then, as with quebracho and black wattle, increasing the TE inclusion (g/100 g of DM) caused a gradual decrease in the animal's OMD (Figure 3.). When chestnut, quebracho, and black wattle TE inclusion levels were 5.7, 2.5, and 2.7 g/100 g of DM, respectively, the OMD decreased by 5% (Figure 3.). Those levels which cause a decrease of 5% can be identified as the threshold level for chestnut, quebracho and black wattle TE on OMD when dietary TE is used for various applications and co-benefits. Nevertheless, the pairwise test showed no significant differences between the effect of the TE sources ($P > 0.05$) on OMD except between chestnut and black wattle TE ($P = 0.04$) in the linear model and as a trend ($P = 0.08$) in the quadratic one.

Table 3.5 Results of regression, the goodness of fit and joint tests analysis for the best fit models for OM digestibility response (%) to TE inclusion in sheep and cattle.

OMD Models					
Predictors	Model type	Estimates	SE		*p- value*
(Intercept)	L	-0.90	1.41		0.53
	Q	1.34	1.69		0.43
TE level g/100 g of DM	L	-1.55	0.29		<0.001
	Q	-3.369/0.223	0.78/0.09		<0.001/0.02
TE source [Chestnut]	L	4.81	1.85		0.01
	Q	4.17	1.82		0.03
TE source [Quebracho]	L	-0.20	1.93		0.92
	Q	-0.06	1.97		0.98
Goodness of fit of OMD models					
R^2 marginal/ conditional	L	0.33 /0.81	CCC	L	0.91
	Q	0.36 /0.85		Q	0.94
RMSE	L	2.01	AIC	L	368.37
	Q	1.80		Q	367.80
Observations number	63	Studies number		28	
Joint tests of OMD models					
Model term		Df	F ratio		*p-value*
TE level g/100 g of DM	L	1	29.01		<0.001
	Q	1	34.32		<0.0001
TE source	L	2	3.44		0.05
	Q	2	2.62		0.09

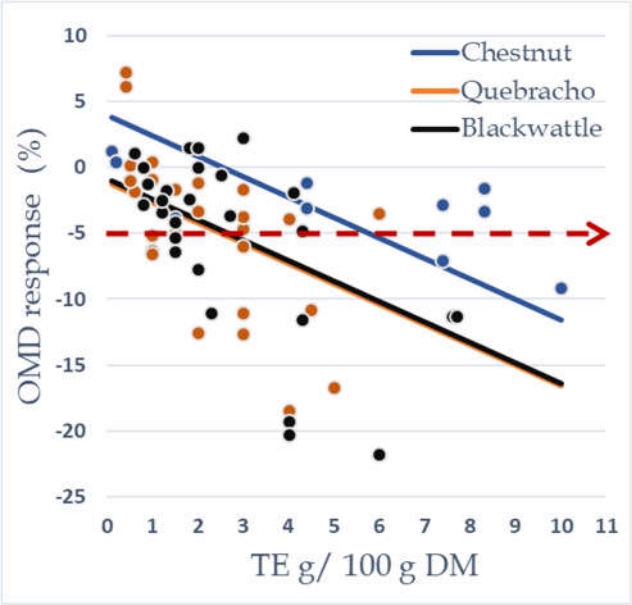

Figure 3.2 Actual (unweighted) observations with the linear predicted lines for the Effect of TE source on the OMD response (%) to TE inclusion level as g/100 g of DM. The cross-points of the red arrow with the predicted lines indicate the proposed threshold inclusion levels

3.3.2.2 Threshold inclusion levels of tannin extract for crude protein digestibility (CPD)

Table 3.6 summarizes the animals, feeds, and methods used to determine CPD in the studies of this meta-analysis. The result showed that the TE inclusion level as g/100 g of DM ($P < 0.01$) was a better presenter for TE inclusion level than as g/kg MBW ($P = 0.47$) when used to predict CPD response. Furthermore, the result showed a significant second-degree coefficient ($P < 0.05$) for TE level, suggesting that CPD data may also support the quadratic term of TE level. The full model estimates for all potential predictors are presented in Table 3B3. The backward stepwise selection revealed that except for TE level and animal species, potential predictors were insignificant, namely the diet's fibre content, supplementation period, protein level and animal growth stage.

Table 3.6 Description of animals, feeds and methods used to determine CPD in this meta-analysis

Item		Ob./Stu.	Description (observation number/studies number)
Animal	Sheep	20/11	Lambs (9/6), rams (4/2), wethers (5/2) lactating ewes (2/1)
	Cattle	23/10	Steers (5/2), bulls (2/1), heifers (6/2), non-lactating cows (3/1), lactating cows (7/4)
Feed	Low fibre	14/8	TMR (8/4), concentrates (4/2), concentrates + forage (1/1), concentrates + silage (1/1)
		29/13	TMR (11/5), forage (7/4), forage-based diet (1/1), forage + concentrates (10/3)
	High fibre		
Digestibility type		43/21	Total tract digestibility (43/21)
Digestibility method		43/21	Total faces collection (27/14), external marker (8/4), internal marker (8/3)

Ob. = observation number, Stu. = study number

The linear and quadratic best-fit regression models for CPD response to TE inclusion, as well as the goodness of fit, are shown in Table 3.7. The fixed effects of the model (TE inclusion level and animal species) explained 68% and 70% (R^2_m) of the CPD variance in the linear and quadratic models, respectively. When combined with the random effect (different studies) the fixed effects explained 91% (R^2_c) of the variance in both linear and quadratic models. Among the fixed effects of CPD model, TE inclusion level had the highest rank in the linear ($F = 83.53$, $P < 0.0001$) and quadratic ($F = 96.73$, $P < 0.0001$) model, respectively, followed by the animal species in the linear ($F = 5.99$, $P < 0.028$) and quadratic ($F = 7.14$, $P = 0.02$) model, respectively. The RMSE was found to be 1.82 and 1.65 for the linear and quadratic CPD models, respectively (Table 3.7). In this context, RMSEs of 1.82 and 1.65 correspond to RSR of 0.29 and 0.27, respectively, indicating that both models have excellent prediction accuracy. Moreover, the result showed that Lin's concordance correlation coefficient (CCC) value was found to be 0.96 for both models (Table 3.7). This result indicates excellent

agreement between the predicted and the actual responses for both models. The result of bootstrapping validation for both models is presented in Table 3C4 and Table 3C5. The bootstrapping result showed negligible bias in the predictors' estimates and hence, our final best-fit models were almost the same as the bootstrap models. This result may add further strength to the predictions of both models as reliable. However, the CPD quadratic model seems to produce unrealistic predictions. For example, if the quadratic equation (Table 3.7) is used to predict the CPD response in sheep to the addition of 5 g/100 g DM of TE, the CPD will be predicted to decrease by 14.78%. Then, if the inclusion level is raised to 16 g/100 g of DM, a 4.8% increase in CPD is predicted, which is unrealistic given that lower levels caused a decrease in CPD. As a result, we recommended using the linear model rather than the quadratic model to predict the CPD response to TE inclusion. Figure 3. illustrates the effect of the animal species on the linear CPD response to the TE inclusion level (Table 3.7). The inclusion of TE in the feed caused a gradual decrease in CPD depending on the levels of TE used (Table 3.7). The CPD decreased by 5% when TE inclusion levels of 1 g and 2.3 g /100 g DM is used in cattle and sheep, respectively (Table 3.7). We suggest those levels be identified as the TE threshold level in cattle and sheep on CPD when dietary TE is used for various applications and co-benefits.

Table 3.7 Results of regression, the goodness of fit and joint tests analysis for the best fit model for crude protein (CP) digestibility response (%) to TE inclusion (g/100 g of DM) in sheep and cattle.

CPD Models					
Predictors	Model type	Estimates	SE	*p- value*	
(Intercept)	L	-1.48	1.39	0.29	
	Q	- 0.47	1.55	0.76	
TE level g/100 g of DM	L	- 3.426	0.36	<0.001	
	Q	- 5.528/0.348	0.93/0.14	<0.001/0.02	
Animal species [sheep]	L	4.40	1.82	0.02	
	Q	4.63	1.76	0.02	
Goodness of fit of CPD models					
R^2 marginal/ conditional	L	0.70 / 0.91	CCC	L	0.96
	Q	0.68 / 0.91		Q	0.96
RMSE	L	1.82	AIC	L	244.20
	Q	1.64		Q	245.10
Observations number	43	Studies number		21	
Joint tests of CPD models					
Model term		Df	F ratio	*p-value*	
TE level g/100 g of DM	L	1	83.53	<0.0001	
	Q	1	96.73	<0.0001	
Animal species [Sheep]	L	1	5.99	0.028	
	Q	1	7.14	0.018	

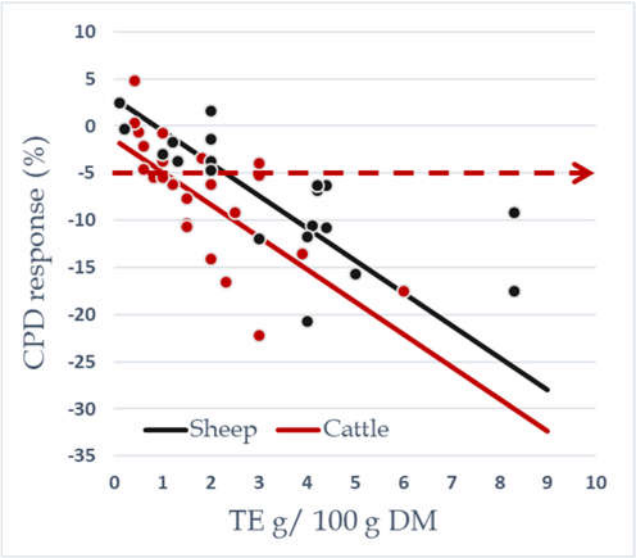

Figure 3.3 Actual (unweighted) observations with the linear predicted lines for the effect of animal species on CPD response (% control) to TE inclusion level as g/100 g of DM (Model CPD). The cross-points of the red arrow with the predicted lines indicate the proposed inclusion levels.

3.3.2.3 Threshold inclusion levels of tannin extract for neutral detergent fibre digestibility (NDFD)

Table 3.8 describes the animals, feeds, and methods used to determine NDFD in the studies of this meta-analysis. The result showed that the TE inclusion level as g/100 g of DM ($P < 0.01$) was a better presenter for TE inclusion level than as g/kg MBW ($P = 0.15$) when used to predict NDFD response. The result also revealed a non-significant second-degree coefficient ($P = 0.47$) for the TE level, suggesting that data for the NDFD support the linear form for the TE level more than the quadratic one. The full model estimates of all potential predictors for NDFD response are presented in Table 3B4. The result of the Backward stepwise showed that TE level and TE source were significant as fixed effects. Animal species and supplementation period showed a trend but were not significant as fixed effects. The rest of the potential predictors namely diet's protein level, animal stage and diet's fibre level were also

not significant. This finding suggests that the NDFD response to TE inclusion may be best represented as a linear response to TE inclusion level (g/100 g of DM) and TE sources.

Table 3.8 Description of animals, feeds and methods used to determine NDFD in the studies of this meta-analysis

Item		Ob./Stu.	Description (observations number/studies number)
Animal	Sheep	24/13	Lambs (9/6), rams (5/3), wethers (8/3) lactating ewes (2/1)
	Cattle	38/15	Steers (13/5), bulls (5/2), heifers (10/3), cows (3/1), lactating cows (7/4)
Feed	Low fibre	21/10	TMR (8/4), concentrates (6/3), concentrates + silage (3/1), concentrates + forage (4/2)
	High fibre	41/18	TMR (11/5), forage (15/7), forge-based diet (4/2), concentrates + forage (11/4)
Digestibility type		62/28	Total tract digestibility (62/28)
Digestibility method		62/28	Total faces collection (46/21) internal marker (4/3) external marker (8/4)
NDF type		62/28	NDF (50/23), NDFom (2/1), NDFomp (4/2), aNDF (3/1), asNDF (3/1)

Ob. = Observations number, Stud. = Studies numbers, NDF = neutral detergent fibre, NDFom = NDF corrected for ash, NDFomp = NDF corrected for ash and protein, aNDF = NDF with the addition of amylase, asNDF = NDF with the addition of amylase and sodium sulfite

Table 3.9 shows the best-fit regression model for NDF digestibility response to TE inclusion and the goodness of fit. Among the fixed effects of the NDFD response model, the TE inclusion level had the highest rank ($F = 73.27$, $P < 0.0001$), followed by the TE source ($F = 3.93$, $P = 0.04$). The fixed-effects of the model (TE inclusion level and TE source) explained 60% (R^2_m) of the variance in the NDFD data and 90% (R^2_c) of the variance when combined with the random-effect (different studies). The RMSE of the NDFD model was 2.82 (Table 3.9). The RMSE of 2.82 in this context correspond to an RSR of 0.26, thus indicating excellent prediction accuracy. The result also showed that Lin's concordance correlation coefficient

(CCC) value was found to be 0.96 (Table 3.9). This result indicates excellent agreement between the predicted and the actual responses. The outcome of the bootstrapping validation of the model validation is presented in Table 3C6. The bootstrapping result showed negligible bias in the estimates and hence, our final best-fit model was almost the same as the bootstrap model. This result further adds strength to the predictions of the model as reliable. Figure 3. illustrates the effect of the TE source on the predicted NDF digestibility to the TE inclusion level. Chestnut and quebracho TE caused a slight increase in NDFD when the inclusion levels were less than 1.2 g/100 g of DM. Then as with the black wattle, the inclusion of TE (g/100 g of DM) caused a gradual decrease in the animal's NDFD (Figure 3.). The NDFD decreased by 5% when chestnut, quebracho and black wattle inclusion levels were 2.4, 2.5 and 0.5 g/100 g of DM, respectively (Figure 3.). We suggest those levels which cause a decrease by 5% be identified as the threshold level for chestnut, quebracho and black wattle TE on NDFD for various applications and co-benefits. Nevertheless, the pairwise test showed no significant differences between chestnut and quebracho (P = 0.99), chestnut and Black wattle (P = 0.17) on NDFD, but a significant difference between quebracho and black wattle TE (P = 0.04).

Table 3.9: Results of regression, the goodness of fit and joint tests analysis for the best fit model for NDF digestibility response (%) to TE inclusion (g/100 g of DM) in sheep and cattle.

Model NDFD			
Predictors	Estimates	SE	*p- value*
(Intercept)	-3.12	2.42	0.206
TE level g/100 g of DM	-3.9474	0.45	<0.001
TE source [Chestnut]	7.42	3.96	0.066
TE source [Quebracho]	7.76	3.06	0.014
Goodness of fit of model NDFD			
R^2 marginal/ conditional	0.60 / 0.90	CCC	0.96
RMSE	2.82	AIC	417.97
Observations/ Studies number	62/ 28	Model	Linear
Joint tests of model NDFD			
Model term	Df	F ratio	*p-value*
TE level g/100 g of DM	1	73.25	<0.0001
TE source	2	3.93	0.0419

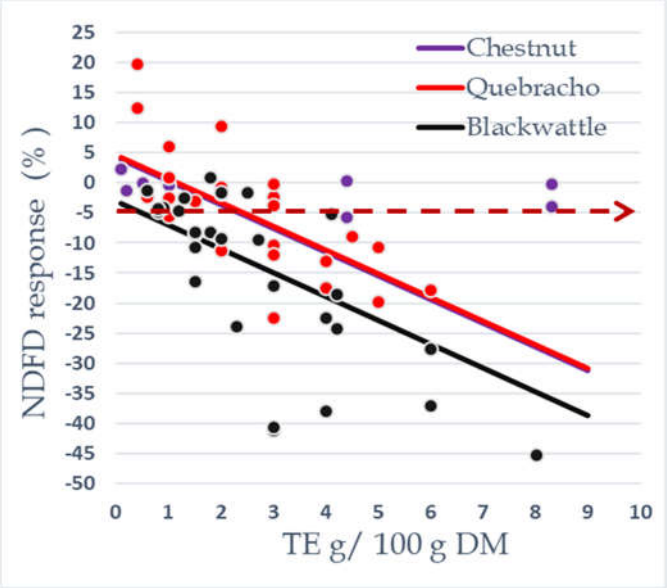

Figure 3.4 Actual (unweighted) observations with the predicted lines for the effect of TE source on NDFD response (%) to TE inclusion level as g/100 g of DM. The cross-points of the red arrow with the predicted lines indicate the proposed inclusion levels.

3.3.2.4 Threshold of TE inclusion levels on acid detergent fibre digestibility (ADFD):

Table 3.10 summarizes the animals, feeds, and methods used to determine ADFD in the studies of this meta-analysis. The result showed that the TE inclusion level as g/100 g of DM ($P < 0.05$) was a better presenter for TE inclusion level than as g/kg MBW ($P = 0.50$) when used to predict ADFD response. The second-degree coefficient for TE level (g/100 g of DM) also showed a trend but was not significant ($P = 0.09$), indicating that data for the ADFD support the linear term for TE level rather than the quadratic one. The full model estimates of all potential predictors for ADFD response are presented in Table 3B5. The backward stepwise selection showed that except for the TE level and TE source potential predictors namely animal species, diet's fibre level, supplementation period, protein level and animal stage were not

significant. This result indicates that ADFD response to TE inclusion may be best-represented as a linear response to TE inclusion level (g/100 g of DM) and TE sources.

Table 3.10 Description of animals, feeds and methods used to determine ADFD in the studies of this meta-analysis

Category		Ob./Stu.	Description (observation number/studies number)
Animal	Sheep	16/8	Lambs (6/3), rams (4/2), wethers (5/2) lactating ewes (2/1)
	Cattle	19/9	Steers (4/2), heifers (6/2), cows (2/1), lactating cows (7/4)
Feed	Low fibre	9/5	TMR (7/4), concentrates (2/1)
	High fibre	26/12	TMR (5/3), forage (7/4), forge-based diet (4/2), concentrates + forage (10/3)
Digestibility type		35/17	Total tract digestibility (35/17)
Digestibility method		35/17	Total faces collection (24/11) internal marker (4/2) external marker (7/4)

Ob. = Observations number, Stud. = Studies numbers

Table 3.11 shows the linear best-fit regression model for ADF digestibility response to TE inclusion level and the goodness of fit. Among the fixed effects of the model (ADFD), TE inclusion level had the highest rank (F = 15.62, P = 0.001), followed by the TE source (*F* = 5.407, P = 0.04). The fixed-effects of the ADFD model (TE inclusion level and TE source) explained 53% (R^2_m) of the variance in the ADFD data and 85% (R^2_c) of the variance when combined with the random effect (different studies). The model's RMSE was 3.67 (Table 3.11), corresponding to an RSR of 0.33 in this context. This indicates that the model's prediction accuracy is excellent. The concordance correlation coefficient (CCC) value was found to be 0.94 (Table 3.11). The result indicates excellent agreement between the predicted and the actual responses. The result of the model validation using bootstrapping is presented in Table 3C7. The bootstrapping result showed negligible bias in the estimates and hence, our final best-fit model was almost the same as the bootstrap model. This result strengthens the model's predictions as being reliable. Figure 3. illustrates the effect of the TE source on the predicted ADF digestibility response to the TE inclusion level. Chestnut TE showed a slight increase in

ADFD when the TE inclusion level was less than 1.6 g/100 g of DM. Then as with quebracho, the inclusion of TE (g/100 g of DM) caused a gradual decrease in the animal's ADFD (Figure 3.). The inclusion of black wattle TE (g/100 g of DM) caused a drastic decrease in the animal's ADFD (Figure 3.) down by 15% at 1 g/100 g of DM. The ADFD decreased by 5% when chestnut and quebracho TE inclusion levels were 3.2 and 1.8 g/100 g of DM, respectively (Figure 3.). Those levels which cause a decrease of 5% can be identified as the threshold level for chestnut and quebracho TE on ADFD when dietary TE is used for various applications and co-benefits. Nevertheless, the pairwise test showed no significant differences between chestnut and quebracho (0.64), a trend between quebracho and black wattle (0.07) and a significant difference between chestnut and black wattle TE (P = 0.04) on ADF digestibility.

Table 3.11: Results of regression, the goodness of fit and joint tests analysis for the best fit model for ADF digestibility response (%) to TE inclusion (g/100 g of DM) in sheep and cattle.

Model ADFD			
Predictors	Estimates	SE	*p- value*
(Intercept)	-12.33	5.05	0.02
TE level g/100 g of DM	-3.0270	0.73	<0.001
TE source [Chestnut]	16.96	5.98	0.008
TE source [Quebracho]	12.58	5.27	0.02
Goodness of fit of model ADFD			
R^2 marginal/ conditional	0.53/ 0.85	CCC	0.94
RMSE	3.67	AIC	246.23
Observations\ Studies number	35/17	Model	Linear
Joint tests of model ADFD			
Model term	Df	F ratio	*p- value*
TE level g/100 g of DM	1	15.62	0.001
TE source	2	5.41	0.042

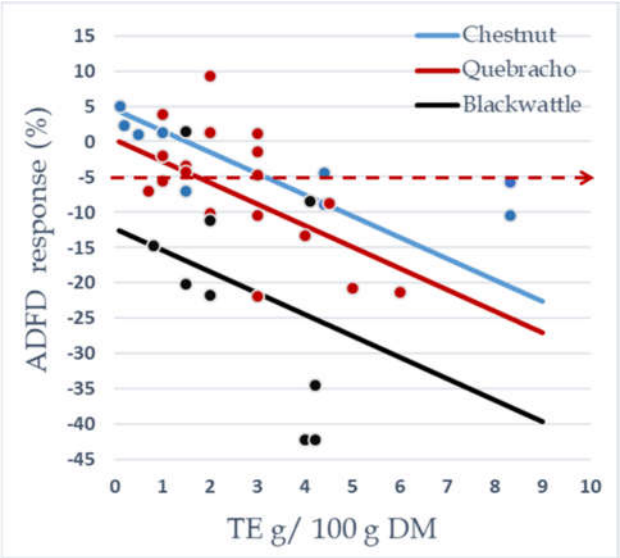

Figure 3.5 Actual (unweighted) observations with the predicted lines for the effect of TE source on ADFD response (%) to TE inclusion level as g/100 g of DM. The cross-points of the red arrow with the predicted lines indicate the proposed inclusion levels.

3.3.3 Effect of TE inclusion on nitrogen balance

3.3.3.1 Effect of TE inclusion on faecal nitrogen in sheep and cattle

Table 3.12 summarizes the animals, feeds, and methods used to determine faecal N in the studies of this meta-analysis. The result showed that the TE inclusion level as g/kg MBW ($P < 0.001$) was a better presenter for TE inclusion level than as g/100 g of DM ($P = 0.10$) when used to predict faecal N response. The result also revealed a non-significant second-degree coefficient ($P = 0.74$) for TE level (g/kg MBW), implying that data for faecal N may only support the linear term and not the quadratic. The full model estimates of all potential predictors for faecal N response are presented in Table 3B6. The backward stepwise selection revealed that, of the potential predictors investigated for faecal N response, TE level, TE source, diet's protein level, and supplementation period were significant. The rest of the potential predictors including animal species, diet fibre level and animal stage were not significant. This result indicates that faecal N response to TE inclusion may be best-represented

as a linear response to TE inclusion level (g/kg MBW), TE sources, diet protein level and supplementation period.

Table 3.12 Description of animals, feeds and methods used to determine faecal N in the studies of this meta-analysis

Category		Ob./Stu.	Description (observation number/studies number)
Animal	Sheep	24/12	Lambs (12/7), rams (1/1), wethers (11/4)
	Cattle	20/7	Steers (13/5), heifers (4/1), non-lactating cows (3/1)
Feed	Low fibre	17/8	TMR (6/3), concentrates (2/1), concentrates + silage (1/1), concentrates + forage (4/2), finishing diet (4/1)
	High fibre	27/11	TMR (4/1), forage (9/4), forge-based diet (4/2), forgae + concentrates (6/3), silage (4/1)
Output determination		44/19	Total faces collection (37/17), external marker (7/2)

Ob. = observations number, Stud. = studies numbers

Table 3.13 shows the best-fit regression model for faecal N response to TE inclusion and the goodness of fit. Among the fixed effects of the model, the TE inclusion level had the highest rank ($F = 151.25$, $P < 0.001$), followed by the TE source ($F = 20.34$, $P < 0.01$), protein level ($F = 11.71$, $P = 0.02$) and supplementation period ($F= 6.96$, $P = 0.02$). The fixed-effects of the Faecal -N model (TE inclusion level, TE source, protein level and supplementation period) explained 84% (R^2_m) of the variance in the Faecal N data and 95% (R^2_c) of the variance when combined with the random-effect (different studies). The faecal N model's RMSE was 4.12 (Table 3.13), equivalent to an RSR of 0.21, indicating excellent prediction accuracy. Furthermore, the model concordance correlation coefficient (CCC) value was 0.98 (Table 3.13), indicating excellent agreement between predicted and actual faecal N responses to TE inclusion. The bootstrapping validation result showed negligible bias in the estimates and hence, our final best-fit model was almost the same as the bootstrap model (Table 3C8). This result strengthens the model's predictions as being reliable. The pairwise test showed no significant differences between chestnut and quebracho ($P = 0.58$) and a significant difference between quebracho and black wattle (0.015) as well as between chestnut and black wattle TE

(P < 0.001) on faecal N response. The result showed that the black wattle increased the faecal N by 17.30 ± 3.17% higher than the chestnut TE when the same inclusion levels were used (Table 3.13). Similarly, black wattle increased the faecal N by 22.50 ± 6.10% higher than the quebracho TE when the same inclusion levels were used. These results indicate that black wattle had the strongest effect on the faecal N as compared to chestnut and quebracho (Table 3.13). The result also showed that diets with low protein (< 18%) have a higher level of faecal N by 13.5 ± 4.90 % as compared to diets with high levels of protein (> 18.0 %) when the same level of TE was used. The result also showed that TE supplementation for a longer period (> 1 month) may reduce the faecal nitrogen by 11.07 ± 4.06 % as compared to the short supplementation period when the same level of TE was used.

Table 3.13: Regression, the goodness of fit and joint tests analysis of the best fit model for faecal N response (% control) to TE inclusion (g/ kg MBW) in sheep and cattle.

Model Faecal -N			
Predictors	Estimates	SE	*p-value*
(Intercept)	2.10	4.26	0.63
TE level g/kg MBW (1rst degree)	14.348	1.15	<0.001
TE source [Chestnut]	-17.30	3.17	<0.001
TE source [Quebracho]	-22.50	6.10	<0.01
Diet's protein level [Low]	13.50	4.90	0.03
Supplementation period [Long]	-11.08	4.06	<0.01
Goodness of fit of model Faecal -N			
R^2 marginal/ conditional	0.84/ 0.95	CCC	0.98
RMSE	4.12	AIC	336.28
Observations/ Studies number	44/19	Model	Linear
Joint tests of model Faecal -N			
Model term	Df	F ratio	*p-value*
TE level g/kg MBW	1	151.25	<0.0001
TE source	2	20.34	< 0.01
Diet's protein level [low]	1	11.71	0.02
Supplementation period	1	6.96	0.02

3.3.3.2 Effect of TE inclusion on urinary nitrogen in sheep and cattle

Table 3.14 describes the animals, feeds, and methods used to determine urinary N in the studies of this meta-analysis. The result showed that the TE inclusion level as g/kg MBW ($P < 0.001$) was a better presenter for TE inclusion level than as g/100 g of DM ($P = 0.53$) when used to predict urinary N response. The result also revealed a non-significant second-degree coefficient ($P = 0.12$) for TE level (g/kg MBW), suggesting that data for urinary N may only support the linear form and not the quadratic. The full model estimates of all potential predictors for urinary N response are presented in Table 3B7. The result of backward stepwise selection showed that out of the potential predictors investigated for urinary N response TE level, diet protein level and supplementation period were significant. The rest of the potential predictors including animal species, TE source, diet's fibre level, and animal stage were not significant. This result indicates that urinary N response to TE inclusion may be best-represented as a linear response to TE inclusion level (g/kg MBW), the protein level in the diet and the supplementation period.

Table 3.14 Description of animals, feeds and methods used to determine urinary N in the studies of this meta-analysis

Category		Ob./Stu.	Description (observation number/studies number)
Animal	Sheep	22/12	Lambs (8/6), rams (4/2), wethers (10/4)
	Cattle	18/7	Steers (13/5), heifers (2/1), non-lactating cows (3/1)
Feed	Low fibre	17/8	TMR (6/3), concentrates (2/1), concentrates + silage (1/1), concentrates +fora. (4/2), finishing diet (4/1)
	High fibre	23/11	TMR (2/1), forage (9/5), forage-based diet (4/2), forage + concentrates (5/2), silage (3/1)
Urine determination		40/19	Total urine collection (33/17), internal marker (7/2)

Ob. = observations number, Stud. = studies numbers

Table 3.15 shows the best-fit regression model for urinary nitrogen response to TE inclusion and the goodness of fit. Among the fixed effects of the urinary N model (Table 3.15), the TE inclusion level had the highest rank ($F = 32.02$, $P = < 0.001$), followed by the diet

protein level (F = 7.63, P = 0.04) and supplementation period (*F* = 4.86, P = 0.04). The fixed-effects of the urinary-N model (TE inclusion level and supplementation period) explained 46% (R^2_m) of the variance in the Faecal N data and 89% (R^2_c) of the variance when combined with the random effect (different studies). The model's RMSE was 2.08 (Table 3.15), which is equivalent to 0.30 RSR and indicates excellent prediction accuracy. The model concordance correlation coefficient (CCC) value was 0.95 (Table 3.15), indicating excellent agreement between predicted and observed urinary N responses to TE inclusion. Furthermore, the bootstrapping result showed negligible bias in the estimates and hence, the final best-fit model was almost the same as the bootstrap model (Table 3C9). This result strengthens the model's predictions as being reliable. The result showed that diets with low protein (< 18%) had a lower level of urinary N by 6.81 ± 3.0% as compared to diets with a high level of protein (> 18.0 %) when the same level of TE was used (Table 3.15). The result also showed that TE supplementation for a longer period (> 1 month) increases the urinary N by 5.12 ± 2.23% as compared to the short supplementation period when the same level of TE was used (Table 3.15).

Table 3.15: Regression, the goodness of fit and joint tests analysis of the best fit model for urinary nitrogen response (% control) to TE inclusion (g/kg MBW) in sheep and cattle diets.

Model Urinary-N			
Predictors	Estimates	SE	*p- value*
(Intercept)	-3.33	2.67	0.23
TE level g/kg MBW	-4.69	0.82	< 0.001
Diet's protein level [Low]	-6.81	3.00	0.03
Supplementation period [> 1 month]	5.12	2.23	0.03
Goodness of fit of model Urinary-N			
R^2 marginal/ conditional	0.46/ 0.89	CCC	0.95
RMSE	2.08	AIC	266.25
Observations\ Studies number	40\19	Model	Linear
Joint tests of model Urinary-N			
Model term	Df	F ratio	*p-value*
TE level g/kg MBW	1	32.02	< 0.001
Diet's protein level	1	6.19	0.04
Supplementation period	1	4.86	0.04

3.3.3.3 Effect of TE inclusion on retained nitrogen in sheep and cattle

Table 3.16 shows the animals and feeds used to determine retained N response to TE inclusion in the studies of this meta-analysis. The result showed that the TE inclusion level as g/kg MBW ($P < 0.001$) was a better presenter for TE inclusion level than as g/100 g of DM ($P = 0.48$) when used to predict retained N response. Furthermore, the TE level (g/kg MBW) showed a significant second-degree coefficient ($P < 0.001$), suggesting that data for the retained response may support the quadratic term of the TE level. This result indicates that retained N response to TE inclusion may be best represented as a linear or quadratic response to TE inclusion level (g/100 g of DM). The Backward stepwise when TE fitted in the linear case showed that except for TE level all potential predictors for retained N response, namely TE source, animal species, supplementation period, diet's protein level, animal stage and diet's fibre level, were not significant. However, in addition to TE level (g/kg MBW), the result of

Backward stepwise, when TE fitted in the quadratic case, showed TE supplementation to be significant as a fixed effect.

Table 3.16 Description of animals and feeds used to determine retained N response to TE inclusion in the studies of this meta-analysis

Item		Ob./Stu.	Description (observation number/studies number)
Animal	Sheep	26/12	Lambs (11/6), rams (4/2), wethers (11/4)
	Cattle	20/7	Steers (13/5), heifers (4/1), non-lactating cows (3/1)
Feed	Low fibre	17/8	TMR (6/3), concentrates (6/2), concentrates + silage (1/1), concentrates + forage (4/2)
	High fibre	29/11	TMR (4/1), forage (11/4), forage-based diet (4/2), silage (3/1), forage + concentrates (7/3)

Ob. = observations number, Stud. = studies numbers

Table 3.17 shows the linear and quadratic best-fit regression models for retained nitrogen response to TE inclusion and the goodness of fit. Among the fixed effects of the quadratic retained N model (Table 3.17), the TE inclusion level had the highest rank ($F = 20.89$, $P < 0.001$), followed by the supplementation period ($F = 5.32$, $P = 0.03$). The fixed effects explained 0.27 and 0.34% (R^2_m) of the retained N response variance of data in the linear and quadratic models, respectively (Table 3.17). When combined with the random effect (different studies), the fixed effects explained 72 and 94% (R^2_c) of the variance in the linear and quadratic models, respectively. The RMSE were found to be 10.57 and 5.85 for the linear and quadratic retained N models, respectively (Table 3.17). In this context, RMSE of 10.57 and 5.85 correspond to RSRs of 0.50 and 0.22, respectively. This suggests that quadratic models have excellent prediction accuracy, whereas the linear model appears to have poor accuracy (RSR = 0.5). For the linear and quadratic models, the concordance correlation coefficient (CCC) values were 0.83 and 0.97, respectively (Table 3.17). Both CCC values are greater than 0.80 (Altman 1990), however, the quadratic model has better agreement than the linear model. Furthermore, the bootstrapping validation of both linear and quadratic models revealed negligible bias in the

predictor estimates, implying that our final best-fit models were nearly identical to the bootstrap models (Table 3C10 and Table 3C11). However, the bootstrapping SE for TE level in both models was nearly double that of the original, indicating that the accuracy of both models will be lower, in practice, than the rest of the models in this study.

Figure 3. shows the effect of supplementation period on the quadratic retained N response to TE inclusion level. When the inclusion levels were less than 2.8 g/kg MBW, supplementation of TE in animal feed caused an arc-like increase in the animal's retained N (Figure 3.). Above this level, the TE caused a gradual decrease in retained N. Long-term supplementation (> 1 month) of TE decreased retained N by 17.06±7.181% compared to short-term supplementation, when the same inclusions levels were used (Table 3.17). The results indicate that TE supplementation in sheep and cattle may reduce retained N compared to short supplementation.

Table 3.17 Regression, the goodness of fit and joint tests analysis of the best fit model for retained nitrogen response (% control) to TE inclusion (g/kg MBW) in sheep and cattle diets.

Retained N Models					
Predictors	Model type	Estimates	SE	*p- value*	
(Intercept)	L	13.438	6.26	< 0.05	
	Q	-9.06	8.32	0.28	
TE level g/kg MBW	L	-9.906	2.09	< 0.001	
	Q	18.927/-5.561	5.28/0.95	0.001/<0.001	
Supplementation period [Long]	Q	-17.06	7.18	0.02	
Goodness of fit of retained N models					
R^2 marginal/ conditional	L	0.27 /0.72	CCC	L	0.83
	Q	0.34/ 0.94		Q	0.97
RMSE	L	10.57	AIC	L	438.79
	Q	5.85		Q	412.46
Observations number	46	Studies number		19	
Joint tests of retained N models					
Model term		Df	F ratio	*p-value*	
TE level g/100 g of DM	Q	1	20.89	<0.001	
Supplementation period	Q	1	5.32	0.03	

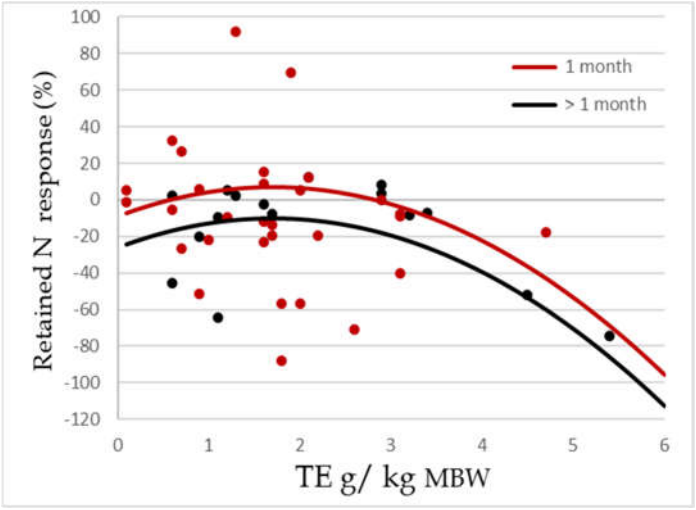

Figure 3.6 Actual (unweighted) observations with the quadratic predicted lines for the effect of the supplementation period on the retained N response (%) to TE inclusion level (g/kg MBW).

3.3.4 Effect of TE inclusion on weight gain (WG) in sheep and cattle:

Table 3.18 describes the animals and feeds used to determine WG response in the studies included in this meta-analysis. The result showed that the TE inclusion level as g/100 g of DM ($P < 0.01$) was a better presenter for TE inclusion level than as g/kg MBW ($P = 0.99$) when used to predict WG response. In addition, the result revealed a significant second-degree coefficient ($P < 0.01$) for the TE level, indicating that WG data may also support the quadratic term of the relationship. The full model estimates of all potential predictors for WG response are presented in Table 3B9. Except for the TE level, the backward stepwise selection indicated that all potential predictors, namely TE source animal species, supplementation period, diet's protein level, animal stage, and diet's fibre level, were not significant. This result indicates that the WG response to TE inclusion may be best-represented as a linear or quadratic response to TE inclusion level (g/100 g of DM).

Table 3.18 Description of animals and feeds used to determine WG response in the studies included in this meta-analysis

Category		Ob./Stu.	Category
Animal	Sheep	12/8	Lambs (9/7), rams (3/1)
	Cattle	20/7	Steers (15/5), heifers (2/1), bulls (3/1)
Feed	Low fibre	22/10	TMR (9/4), concentrates (1/1), concentrates + silage (4/2), finishing diet (7/2), concentrates + forage (1/1)
	High fibre	10/5	TMR (5/3), forage (3/1), forage-based diet (2/1)

Ob. = observations number, Stud. = studies numbers

Table 3.19 shows the linear and quadratic regression models for WG response to TE inclusion, as well as the goodness of fit. In the linear and quadratic models, the fixed effects of the WG response model (TE level g/100 g of DM) explained 38 and 34% (R^2_m) of the WG response variance, respectively. When combined with random effects (different studies), fixed effects explained 97% (R^2c) of the variance in both linear and quadratic models. In the linear and quadratic models, RMSE was found to be 1.48 and 1.03, respectively (Table 3.19). The RMSE of 1.48 and 1.03 in this context correspond to RSR of 0.13 and 0.09, respectively. This indicates that both the linear and quadratic models have excellent prediction accuracy. Moreover, the concordance correlation coefficient (CCC) values for the linear model were 0.98 and 0.99 for the quadratic model (Table 3.19), indicating excellent agreement between the predicted and actual WG responses, for both models. Additionally, bootstrapping validation revealed a negligible bias in predictor estimates for both models, indicating that the final best-fit models were nearly identical to the bootstrapped ones (Table 3C12 and Table 3C13). These findings support the reliability of the two models' predictions.

Table 3.19: Results of regression, the goodness of fit for the best fit models of WG response (%) to TE inclusion (g/100 g of DM) in the sheep and cattle diets.

WG Models					
Predictors	Model type	Estimates	SE	*p- value*	
(Intercept)	L	5.27	3.31	0.123	
	Q	-2.77	3.89	0.48	
TE level g/100 g of DM	L	-3.63	0.93	0.001	
	Q	3.08/-0.818	1.91/0.25	0.12/0.003	
Goodness of fit of model WG					
R^2 marginal/ conditional	L	0.38/ 0.97	CCC	L	0.98
	Q	0.34 / 0.97		Q	0.99
RMSE	L	1.48	AIC	L	226.22
	Q	1.03		Q	221.55
Observations number	32	Studies number		15	

Figure 3. displays the unweighted observations as well as the linear and quadratic prediction lines for the WG response to TE inclusion. In the linear model, the TE inclusion caused a slight increase in animal WG when the inclusion levels were less than 1.5 g/100 g of DM (Figure 3.). Above these levels, the TE caused a gradual decrease in the animals' WG. For example, animals lost 5% of their weight gain when the inclusion level was 2.9 g/100 g of DM. The quadratic model, on the other hand, showed a slightly negative effect on WG when the inclusion levels were less than 1.4 g/100 g of DM (Figure 3.). The model then showed a slight increase at inclusion levels greater than 1.4 but less than 2.3 g/100 g of DM. After this level (2.3 g/100 g of DM), the model showed a gradual decrease in animal WG and a 5% loss was reached at the inclusion level of 4.5 g/100 g of DM. The reduction in WG at very low levels of TE inclusion (0.1 - 1.4 g/100 g of DM) appears to be unrealistic, as most of the observations at those levels were positive, as shown in Figure 3.. The goodness of fit results, previously presented (Table 3.19), indicate that both the linear and quadratic models for WG response provided a good fit to the data. However, due to the presence of unrealistic predictions in the

quadratic model, the linear model might be recommended to calculate the subsequent response in weight gain to TE inclusion.

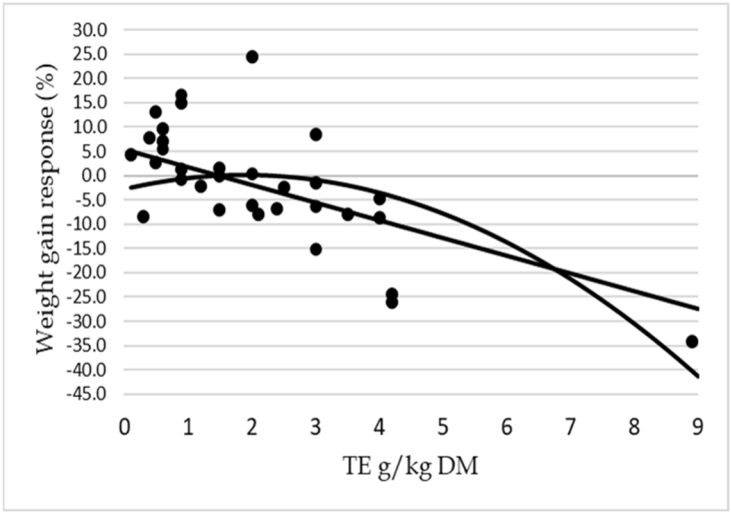

Figure 3.7 Actual (unweighted) observations with linear and quadratic prediction lines for weight gain response to TE inclusion level as g/100 g of DM.

3.4 Discussion

3.4.1 Threshold inclusion levels of tannin extract for DMI

The result showed that the TE inclusion level as g/100 g of DM was a better presenter for the DMI response than the TE as g/kg MBW. A possible reason is that the TE inclusion level as a g/kg MBW does not necessarily reflect the TE concentration in the feed but rather the daily amount consumed regardless of concentration, while the TE inclusion level as a g/100 g of DM does. The result indicates that the concentration of TE in the feed is the main factor that determines the response of the animal to increase or decrease his DMI, most properly through the effect of TE on the palatability of the feed. It has been documented that ruminants (goats, sheep and cattle) may show a preference for bitter taste at low concentrations (Lamy et al. 2011). The decrease in the DMI related to a high level of TE is probably due to the bitterness TE possesses on the animal mouth and the adverse post-ingestive influences on the oral cavity and foregut epithelium (Silanikove et al. 2001). Our result of the significant relationship

between TE inclusion level (g/100 g of DM) and feed intake agrees with the report of (Yanza et al. 2021) in their meta-analysis, where the ruminant's DMI (kg/d) decreased due to TE inclusion. However, they reported the ruminant's DMI (kg/d) decreased by a quadratic response in their study, while ours reported a linear response. In (Yanza et al. 2021) study more than 10 sources of TE were included, while this study included only the top three used (chestnut, quebracho and black wattle). The difference in the response type (linear vs quadratic) could be due to the differences in the data set between the two studies. Another reason could be the difference in the approach used to calculate the DMI response, as the percentage change approach, used in this study, might have reduced the variation in the data compared to reporting it as kg/day. The latter seems to be the case as (Yanza et al. 2021) study reported the linear model to be more significant when the DMI were calculated as g/ kg MBW. However, some recent meta-analyses reported that dietary tannins supplementation has no effect on the DMI in beef cattle (Orzuna-Orzuna et al. 2021) and sheep (Orzuna-Orzuna et al. 2021). The apparent contradiction of these reports with our result might be partially due to our selection criteria which allowed the TE inclusion to manifest its effect on the DMI (e.g., the animals should be fed at ad libitum the TE-containing feed). The other part could be the difference in the approach used to calculate the TE inclusion effect on the DMI. The suggested DMI threshold for TE inclusion in this meta-analysis (3.5 g/100 g of DM) was identified at a 5% reduction in DMI (Table 3.13). A 5% reduction in feed intake may be acceptable given that it was found to be statistically insignificant in many studies (Frutos et al. 2004, Piñeiro-Vázquez et al. 2018, Avila et al. 2020). Despite the statistical significance of the difference, the full model estimates (Table 3B1) indicated that the DMI of the sheep were higher by only 1.22 ± 2.44% than cattle when the same level of TE was used. This can be an indication that sheep and cattle seem to be alike in their DMI response to TE inclusion. However, sheep may select the feeds more than cattle

and thus their perception of feed palatability may differ. Nevertheless, there is a distinction to be made between providing ample choices and providing a selected diet; the bottom line is the latter (Forbes and Mayes 2002). The animals in the studies of this meta-analysis were provided with a selected diet (Table 3.2). These diets (dominated by TMRs and concentrates) have been structured to be balanced, which may also limit the animal's tendency for selection. Previous research on the response of ruminants to bitterness identified a substantial difference between goats on one side and sheep and cattle on the other (Goatcher and Church 1970, Lamy et al. 2011). In general, it was hard to distinguish between sheep and cattle in their responses to bitterness (Goatcher and Church 1970, Lamy et al. 2011). This meta-analysis implies that there is no considerable difference between sheep and cattle in their DMI response to TE inclusion when fed a selected diet.

3.4.2 Threshold inclusion levels of tannin extract for Feed digestibility

3.4.2.1 Threshold inclusion levels of tannin extract for organic matter digestibility (OMD)

The result suggests that chestnut TE had a significantly less negative effect on the OMD than the black wattle TE when the same inclusion levels were used. This finding agrees with the study of (Deaville et al. 2010) in sheep, who found that chestnut TE inclusion in silage produced higher OM digestibility values than those for black wattle TE when the same inclusion levels were used (Table 3.5). Researchers reported that condensed TE decreases in vitro OMD more than hydrolysable TE (Makkar et al. 1995, Jayanegara et al. 2015). The observed difference between the effects of black wattle and chestnut on OMD can be attributed to the higher condensed tannin content in black wattle TE. The observed negative effect of black wattle, quebracho and chestnut TE inclusion on OMD is probably a result of the complex-forming nature of TE with proteins and carbohydrates (Labieniec et al. 2003, Jayanegara and Palupi 2010). Our result of the significant relationship between TE inclusion level (g/100 g of

DM) and OMD agrees with the report of (Jayanegara and Palupi 2010) in their meta-analysis, where the ruminant's OMD (mg/g DM) decreased by the linear response to the condensed tannin inclusion level. The presence of unrealistic predictions in the quadratic model of OMD (Table 3.5), might be because quadratic fit tends to curve significantly upwards or downwards (Roussel et al. 2021) in order to fit the highest values or the lowest values of the data. It seems in this case the curve underestimated the initial OMD response to TE inclusion (at low levels) and may over estimated later the OMD response.

3.4.2.2 Threshold inclusion levels of tannin extract for crude protein digestibility (CPD)

The observed negative effect of TE inclusion on CPD is probably a result of the complex-forming nature of tannins with proteins (Labieniec et al., 2003, Jayanergara et al., 2010). The result suggests that sheep are slightly better than cattle (4.4±1.82%, P = 0.02) at tolerating the negative effect of the TE on CPD when the same levels of TE inclusion were used (Table 3.7, Figure 3.3). Surprisingly, this was not the case only when tannins were included in the diet, as previous reports indicated sheep were better at digesting CP than cattle (Südekum et al. 1995, Woods et al. 1999). Wood et al. reported a difference of 4.62% between sheep and cattle when fed on soya hulls and up to 16.73% when fed on palm kernel meal (Woods et al. 1999). A possible explanation for the difference in digestibility is that sheep may experience lower faecal metabolic losses (faecal matter of non-dietary origin) than cattle (Van Soest 1988, Van Soest 1994, Woods et al. 1999).

The presence of unrealistic predictions in the CPD quadratic model (Table 3.7) could be attributed to the fact that quadratic fit tends to curve significantly upwards or downwards (Roussel et al. 2021) to fit to the highest or lowest values of the data. In this case, it appears that the curve underestimated the initial CPD response to TE inclusion and may have overestimated the CPD response later. The recommended CPD threshold of sheep and cattle

for TE inclusion in this meta-analysis (1 g and 2.3 g /100 g DM, respectively) were identified at a 5% decrease in CPD (Table 3.7). A 5% reduction in CPD may be acceptable since it was statistically non-significant in many studies (Benchaar et al. 2008, AL-Dobaib 2009).

3.4.2.3 Threshold inclusion levels of TE for NDFD and ADFD

NDF is a measure of fibre content that includes cellulose, hemicellulose, and lignin (Cui et al. 2016). However, NDF also contains fibre-bound proteins and ashes (Galeano et al. 2021). The latter, like the first, may vary from sample to sample. As a result, a variety of methods for reporting NDF (Udén et al. 2005) are available such as traditional NDF, NDF corrected for ash (NDFom), and NDF corrected for ash and protein. This fact of multiple choices to report NDF is regarded as one of the limitations of using NDF in meta-analysis. That is, the method used to calculate NDF may introduce a source of variation into data that has nothing to do with the factor under investigation. In the studies of this meta-analysis, the dominant method used to measure NDF (Table 3.8) was the traditional NDF method (82.1%). However, other methods were also used including NDF corrected for ash (NDFom, 3.6%), NDF corrected for ash and protein (7.1%), NDF with the addition of amylase (3.6%) and NDF with the addition of amylase and sodium sulfite (3.6%). However, the source of variation due to different methods may be minimized using the approach of percentage change because the actual value of NDF is not used in the analysis but rather the NDF change due to the factor under investigation.

The result indicates that quebracho TE had a less negative effect (7.76 ± 2.99%) on the NDF than the black wattle TE when the same inclusion levels were used (Table 3.9). Similarly, the result suggests that chestnut TE has a less negative effect (16.96 ± 5.48%) on the ADFD than the black wattle TE when the same inclusion levels were used (Table 3.11). Those differences between the effect of TE sources on NDFD or ADFD could be due to their chemical

structures (Kardel et al. 2013, Dos Santos Grasel et al. 2016) that allow different levels of complex formation with the feed NDF or ADF content. This meta-analysis indicates that TE from black wattle has the highest negative effects on fibre digestibility in comparison to TE from chestnut and quebracho.

3.4.3 Effect of TE inclusion on nitrogen balance

3.4.3.1 Effect of TE inclusion on faecal nitrogen in sheep and cattle

The result indicates that TE from black wattle increase faecal nitrogen to a larger extent than quebracho TE (22±6.1%) or chestnut TE (17±3.17%) when the same inclusion levels were used (Table 3.13). In other words, the black wattle increases faecal N/N intake by 22.6% over quebracho TE, and by 17.17% over chest TE. The differences between TE sources in their effects on faecal N response could be due to their chemical structures (Kardel et al. 2013, Dos Santos Grasel et al. 2016) that allow different levels of complex formation with feed protein. The observed increase in the faecal N could be due to the complex-forming nature of TE (Labieniec et al. 2003, Jayanegara and Palupi 2010) with protein, which reduces protein digestibility. The result also showed that diets with low protein have a higher level of faecal N by 13.5 ± 4.90 % as compared to diets with high levels of protein when the same level of TE was used. Meaning due to TE inclusion, in a low protein diet, the proportion of faecal N/N intake will be higher compared to diets with a high level of N. This difference could be explained by the fact that when the same level of TE is used in a low-protein diet, TE binds to a greater proportion of the N/intake than in a high-protein diet.

3.4.3.2 Effect of TE inclusion on urinary nitrogen in sheep and cattle

The observed decrease in the urinary N due to TE inclusion is probably a result of the complex-forming nature of TE with proteins (Labieniec et al. 2003, Jayanegara and Palupi 2010) which reduced the N excretion via urine. The result showed that diets with low protein

had a lower level of urinary N (6.81 ± 3.0%) than diets with high protein when the same level of TE was used (Table 3.15). Meaning, due to the inclusion of TE in low-protein diets, urinary N/N intake will be lower than in diets with a high level of N. This difference could be explained by the fact that when the same level of TE is used, in a low-protein diet TE binds to a higher proportion of the N intake than it does in a high-protein diet, so faecal N/N intake increases, while urinary N/N intake decreases. In regards to the reduction effect of TE on urinary N, the result indicates also that ruminants may produce an acclimatization response with the long-term supplementation of TE. The acclimatization response may occur due to the adaptation of the ruminal microorganisms, or\and through changes in the amount of digestive enzymes, such as proline and trypsin which may help reduce the negative effect of TE on CP digestibility, and thus the urinary nitrogen response (Ahmed et al. 1991, Glendinning 1994, Bennick 2002, Ruiz González 2018, Orzuna-Orzuna et al. 2021).

4.3.3 Effect of TE inclusion on retained nitrogen in sheep and cattle

Although the retained-N model showed a high conditional R^2 value (94%), the model RMSE (5.85%) is the largest compared to all models in our study. The high RMSE may indicate that the Retained-N had the lowest prediction accuracy as compared to all studied predicted responses to TE inclusion. The previous meta-analysis reported different results regarding the retained N response to tannin inclusion. Some authors (Jayanegara and Palupi 2010, Jayanegara et al. 2012) found no significant relationship between tannin level and retained N (g/d), while (Yanza et al. 2021) reported a quadratic increase in the retained N as a response to TE inclusion (g/100 g N). The high RMSE in our study and the reported differences in response to tannin inclusion on retained N may be due to many factors affecting the utilization of dietary protein in animals, including physiological requirements, protein deposition genetic potential, metabolic and partitioning of absorbed amino acids (Kim and Pluske 2016).

3.4.4 Effect of TE inclusion on weight gain in sheep and cattle:

According to the linear model, the slight increase in WG when low inclusion levels of TE were used (< 1.5 g/100 g of DM) could be due to a slight increase in the feed intake, digestibility and N retention when low levels of TE were used as well. The later decrease in the animal weight response was probably correlated to the decrease in DMI and digestibility as well as N retention when high inclusion levels were used.

3.4.5 General feed digestibility thresholds for TE inclusion in cattle and sheep:

From the above-presented results, the model for CPD response had the highest conditional R^2 (0.91) compared to those of OMD, NDFD and ADFD responses (0.81,0.90 and 0.85, respectively). Moreover, the CPD response model had the lowest RMSE (1.82) compared to OMD, NDFD and ADFD responses (2.01, 2.82 and 3.67, respectively). In regards to CCC, the model of CPD shared the highest value (0.96) with the NDF model, followed by the model of ADFD (0.94) and OMD (0.91). Those statistics may indicate that CPD has the strongest response (relationship) to TE inclusion when compared to other digestibility parameters (OMD, NDFD and ADFD). Therefore, the TE threshold presented by the CPD model, 1 and 2.3 g/100 g of DM, might be identified as general digestibility thresholds for cattle and sheep, respectively. However, this difference between sheep and cattle in CPD was not detectable in weight gain response. The lack of difference in weight gain might reflect the complexity of the factors related to the retention of N in the animal body (Kim and Pluske 2016). The suggested cattle-threshold level (1 g/100 g of DM) increased the weight gain by 1.6%, while the sheep-threshold level (2.3 g/100 g of DM) decreased by 3.1%. The suggested thresholds for TE in sheep and cattle seem to be within the grey area, which slightly may increase or slightly decrease their weight gain. Thus, the inclusion of higher levels of TE may drastically reduce weight gain and animal performance.

3.5 Application on the outcomes: Directing the research of TE inclusion co-benefit on the ruminant reproduction performance:

The outcomes of this meta-analysis might guide research efforts on topics related to the inclusion of TE in ruminant feed, such as reducing methane emission, improving antioxidant status, enhancing immunity, and improving meat quality. Improving reproduction performance is another potential co-benefit of the TE in the ruminant feed (Ahmed et al. 2021). In terms of peer-reviewed articles, this topic has received limited attention. In the next few paragraphs, we discussed how our meta-analysis recommendations can be used to strategically direct future research in this area.

Banchero et al. (Banchero et al. 2012) supplemented grazing ewes with 6.5 and 9.3 g/100 g DM quebracho TE inclusion levels. Based on our recommendations, both levels exceeded the threshold level for TE inclusion in sheep feed (2.3 g/100 g DM). Both levels used reduced the pasture intake drastically by 23.8 and 66 %, respectively, compared to the control. However, the TE level of 6.5 g/100 g DM showed an increase in the ovulation rate of the ewes, while the higher level (93 g/kg DM) did not. Would the ewes ovulation rate increase if only supplemented by up to 2.3g/100g DM, which would grant no such drastic reduction in their feed intake?

In another study, grape seed TE was added to the diet of growing lambs at the level of 0.36 and 0.72 g/100 g DM. (Li et al. 2021). Both inclusion levels of grape seed TE increased testicular weight and volume as well as epididymal weight, with a slight increase in feed intake (3 and 6.6 %, respectively). Being used at levels lower than the thresh-hold levels, could other TE sources (e.g., black wattle, quebracho TE and chestnut) be used to achieve a similar improvement in the ruminant males?

Pregnant goats that graze natural range with bio-active condensed tannins were found to have higher birth weights and lower kids mortalities compared to goats that graze in the same range but with deactivated condensed tannins (Li et al. 2021). Could the inclusion of TE in the ruminant feed below the threshold level (2.3 g/100 g DM) lead to an increase in such parameters? With thresh-hold levels being known, the above three questions suggest a potential application of the TE as a tool to improve reproduction performance in ruminants without reducing feed intake or production performance (e.g., weight gain).

3.6 Conclusions and recommendations:

We conclude that the percentage change approach used in this meta-analysis could be a suitable tool for identifying the threshold levels of feed additives in animal studies. Our study revealed that several factors might affect the parameters' responses to TE inclusion (tannin source, animal species, diet's protein level and supplementation period), however, the most significant is the level of TE inclusion. The results disclosed that the TE inclusion level as g/100 g of DM had a significantly better relationship with DMI, digestibility parameters and weight gain responses than the inclusion level as g/kg MBW, while the opposite was true for all N balance parameters responses. The study identified the threshold levels of TE inclusion on DMI (bitterness threshold) at 3.5 g /100 g of DM for both sheep and cattle. Comparing black wattle with chestnut and quebracho TE, the results of this meta-analysis showed that black wattle TE had the strongest reduction effect on fibre digestibility. Furthermore, black wattle TE showed a stronger effect on increasing faecal N/N intake than chestnut TE or quebracho TE. Compared to all digestibility parameters studied in this meta-analysis (OMD, NDFD, and ADFD), CPD showed the strongest response to TE inclusion. Therefore, based on the CPD response, we suggested the inclusion levels at 1 and 2.3 g /100 g DM as digestibility threshold levels in cattle and sheep, respectively. The suggested levels showed a negligible effect on

weight gain response to TE inclusion in cattle and sheep. Accordingly, the inclusion of higher levels of TE may drastically reduce weight gain. As a result of knowing the threshold levels, TE inclusion in ruminant feed (sheep and cattle) can be used for various applications and co-benefits without compromising the performance of the animal. In light of this meta-analysis, it may be recommended to use the results regarding the extent and significance of each potential predictor on the animal's response to TE inclusion as a guide for future research on the inclusion of TE in animal feed.

3.10 Appendix 3:

3.10.1 Appendix 3A:

Table 3A2: Summary of the studies included in the meta-analysis

No	Study name	Diet used	Animal species	TE source	TE type	Inclusion method
1	(Aboagye et al. 2018)	TMR	Sheep	Black wattle	CT	In feed
2	(Adejoro et al. 2019)	TMR	Sheep	Black wattle	CT	In feed
3	(Adejoro et al. 2020)	Con	Sheep	Black wattle	CT	In feed
4	(Ahnert et al. 2015)	Forage + Conc	Cattle	Quebracho	CT	Intra-ruminally infused
5	(AL-Dobaib 2009)	TMR	Cattle	Black wattle	CT	In feed
6	(Avila et al. 2020)	TMR	Cattle	Black wattle	CT	In feed
7	(Beauchemin et al. 2007)	TMR	Sheep	Black wattle	CT	In feed
8	(Benchaar et al. 2008)	TMR	Cattle	Black wattle	CT	In feed
9	(Carulla et al. 2005)	Forage	Sheep	Black wattle	CT	In feed
10	(Costa et al. 2021)	Con	Cattle	Black wattle	CT	In feed
11	(Dallastra et al. 2018)	TMR	Cattle	Black wattle	CT	In feed
12	(Dawson et al. 1999)	Forage (PDG)	Sheep	Quebracho	CT	In feed
13	(Deaville et al. 2010)	Silage	Sheep	Black wattle	CT	In feed
14	(Dschaak et al. 2011)	TMR	Cattle	Black wattle	CT	In feed
15	(Ebert et al. 2017)	TMR	Sheep	Black wattle	CT	In feed
16	(Frutos et al. 2004)	Forage	Sheep	Chestnut	HT	In feed
17	(Gerlach et al. 2018)	TMR	Cattle	Black wattle	CT	In feed
18	(Henke et al. 2017)	Fin	Cattle	Chestnut	HT	In feed
19	(Junior et al. 2017)	TMR	Cattle	Chestnut	HT	In feed
20	(Kamel et al. 2018)	Conc	Sheep	Quebracho	CT	In feed
21	(Kapp-Bitter et al. 2020)	Conc	Cattle	Chestnut	HT	In feed
22	(Koenig et al. 2018)	TMR	Cattle	Chestnut	HT	In feed
23	(Kozloski et al. 2012)	Forage	Sheep	Black wattle	CT	Intra-ruminally infused
24	(Krueger et al. 2010)	Silage	Sheep	Chestnut	HT	In feed
25	(Liu et al. 2013)	Conc	Sheep	Chestnut	HT	In feed
26	(Liu et al. 2016)	TMR	Cattle	Quebracho	CT	In feed
27	(Mezzomo et al. 2011)	Forg-bas	Cattle	Quebracho	CT	In feed
28	(Norris et al. 2020)	Forg-bas	Cattle	Quebracho	CT	In feed
29	(Orlandi et al. 2015)	Forage + Conc	Cattle	Black wattle	CT	In feed
30	(Piñeiro-Vázquez et al. 2017)	Forage	Cattle	Quebracho	CT	In feed
31	(Piñeiro-Vázquez et al. 2018)	Forage	Cattle	Quebracho	CT	In feed

32	(Rivera-Méndez et al. 2017)	Forage	Cattle	Quebracho	CT	In feed
33	(Salawu et al. 1997)	Forage	Sheep	Quebracho	CT	In feed
34	(Sinz et al. 2019)	Forage + Conc	Sheep	Black wattle	CT	In feed
35	(Śliwiński et al. 2002)	Forage + Conc	Sheep	Chestnut	HT	In feed
36	(de Souza et al. 2021)	Silage + Conc	Sheep	Black wattle	CT	In feed
37	(Staerfl et al. 2012)	Fin	Cattle	Quebracho	CT	In feed
38	(Taha 2015)	Silage	Sheep	Chestnut	HT	In feed
39	(Toral et al. 2013)	TMR	Sheep	Quebracho	CT	In feed
40	(Tseu et al. 2020)	TMR	Cattle	Black wattle	CT	In feed
41	(Valenti et al. 2019)	Con	lambs	Blackwattle & chestnut	CT	In feed
42	(Vasta et al. 2009)	Forage + Conc	Sheep	Quebracho	CT	In feed
43	(Wischer et al. 2014)	Forage + Conc	Sheep	Chestnut	HT	In feed
44	(Zimmer and Cordesse 1996)	Forage	Sheep	Chestnut	HT	In feed

Con: concentrate, Fin: finishing diet, Forg-bas: forage-based diet, CT: condensed tannin, HT: hydrolysable tannin, PDG: Pelleted dried grass

3.10.2 Appendix 3B:

Table 3B1 Estimates of potential predictors for DMI response to TE inclusion in sheep and cattle

Predictors	Estimates	SE	P-value
(Intercept)	-1.23	3.38	0.72
TE inclusion level (g/100 g DM)	-2.24	0.36	<0.001
Diet’s fiber level [Low]	2.82	2.06	0.18
TE source [Chestnut]	2.64	2.34	0.27
TE source [Quebracho]	2.18	1.97	0.27
Animal species [Sheep]	1.22	2.44	0.62
Supplementation period [long]	1.23	2.03	0.55
Diet’s protein level [Low]	0.01	2.09	0.78
Animal stage [Mature]	-0.59	2.23	0.99

Table 3B2 Estimates of potential predictors for OMD response to TE inclusion in sheep and cattle

Predictors	Estimates	SE	P-value
(Intercept)	-0.96	3.57	0.79
TE inclusion level (g/100 g DM)	-1.51	0.31	<0.001
Diet's fiber level [Low]	0.19	2.44	0.94
TE source [Chestnut]	4.98	2.06	0.02
TE source [Quebracho]	-0.89	2.55	0.73
Animal species [Sheep]	-1.58	2.46	0.53
Supplementation period [long]	0.67	2.09	0.75
Diet's protein level [Low]	0.61	1.92	0.75
Animal stage [Mature]	0.51	2.40	0.83

Table 3B3 Estimates of potential predictors for CPD response to TE inclusion in sheep and cattle

Predictors	Estimates	SE	P-value
(Intercept)	-1.83	3.48	0.61
TE inclusion level (g/100 g DM)	-3.43	0.38	< 0.001
TE source [Chestnut]	1.79	2.95	0.56
TE source [Quebracho]	1.00	2.85	0.73
Animal species [Sheep]	4.36	2.82	0.14
Supplementation period [long]	-0.03	2.70	0.99
Diet's fiber level [Low]	1.30	2.34	0.59
Diet's protein level [Low]	-0.38	2.12	0.86
Animal stage [Mature]	-1.07	2.33	0.65

Table 3B4 Estimates of potential predictors for NDFD response to TE inclusion in sheep and cattle

Predictors	Estimates	SE	P-value
(Intercept)	-5.17	4.97	0.30
TE inclusion level (g/100 g DM)	-3.99	0.46	<0.001
TE source [Chestnut]	7.22	4.19	0.09
TE source [Quebracho]	8.9	3.91	0.03
Animal species [Sheep]	5.54	3.84	0.16
Supplementation period [long]	-6.01	2.09	0.04
Diet's fiber level [Low]	0.16	3.84	0.97
Diet's protein level [Low]	1.33	2.85	0.64
Animal stage [Mature]	-0.07	3.27	0.98

Table 3B5 Estimates of potential predictors for ADFD response to TE inclusion

Predictors	Estimates	SE	P-value
(Intercept)	-4.23	8.86	0.65
TE inclusion level (g/100 g DM)	-3.07	0.79	0.00
TE source [Chestnut]	14.79	7.01	0.08
TE source [Quebracho]	8.54	8.28	0.33
Animal species [Sheep]	0.33	6.25	0.96
Supplementation period [long]	-4.64	5.26	0.39
Diet's fiber level [Low]	-8.94	6.75	0.22
Diet's protein level [Low]	-4.30	4.58	0.38
Animal stage [Mature]	0.07	5.06	0.99

Table 3B6 Estimates of potential predictors for faecal N response to TE inclusion in sheep and cattle

Predictors	Estimates	SE	P-value
(Intercept)	-0.90	11.84	0.94
TE inclusion level (g/kg MBW)	14.52	1.17	<0.001
TE source [Chestnut]	-16.76	3.24	<0.001
TE source [Quebracho]	-21.04	10.00	0.06
Animal species [Sheep]	-1.69	9.17	0.86
Supplementation period [long]	-10.24	4.45	0.03
Diet's fiber level [Low]	5.00	6.74	0.48
Diet's protein level [Low]	13.24	6.72	0.09
Animal stage [Mature]	3.56	7.14	0.64

Table 3B7 Estimates of potential predictors for urinary N response to TE inclusion in sheep and cattle

Predictors	Estimates	SE	P-value
(Intercept)	-0.86	6.65	0.90
TE inclusion level (g/kg MBW)	-4.59	0.86	< 0.001
TE source [Chestnut]	-1.12	1.70	0.52
TE source [Quebracho]	-2.87	4.88	0.57
Animal species [Sheep]	-0.53	5.03	0.92
Supplementation period [long]	5.28	2.51	0.04
Diet's fiber level [Low]	-0.44	4.19	0.92
Diet's protein level [Low]	-4.31	4.14	0.33
Animal stage [Mature]	-4.22	4.35	0.36

Table 3B8 Estimates of potential predictors for retained N response to TE inclusion in sheep and cattle

Predictors	Estimates	SE	P-value
(Intercept)	-9.51	25.94	0.72
TE inclusion level (g/kg MBW)	-10.38	2.14	< 0.001
TE source [Chestnut]	2.73	21.34	0.90
TE source [Quebracho]	0.79	21.38	0.97
Animal species [Sheep]	18.58	18.36	0.34
Supplementation period [long]	-12.15	10.51	0.26
Diet's fiber level [Low]	14.63	17.87	0.43
Diet's protein level [Low]	-18.77	16.26	0.27
Animal stage [Mature]	24.74	15.68	0.16

Table 3B9 Estimates of potential predictors for weight gain response to TE inclusion in sheep and cattle

Predictors	Estimates	SE	P-value
(Intercept)	4.17	6.61	0.74
TE inclusion level (g/100 g)	-2.02	14.55	0.15
TE source [Chestnut]	1.19	17.62	0.86
TE source [Quebracho]	2.50	17.06	0.71
Animal species [Sheep]	4.58	9.39	0.76
Supplementation period [long]	-3.85	6.28	0.85
Diet's fiber level [Low]	-7.49	6.04	0.36
Diet's protein level [Low]	-6.11	20.71	0.47
Animal stage [Mature]	12.61	6.65	0.34

3.10.3 Appendix 3C:

Table 3C1 Bootstrapping statistics of DMI response model to TE inclusion in sheep and cattle

Predictors	Original estimates	Original SE	*Estimates bias*	*Boot SE*
(Intercept)	2.32	1.20	0.00	1.48
TE level g/ 100 g DM	-2.14	0.31	0.01	0.50

Table 3C2 Bootstrapping statistics of OMD response linear model to TE inclusion in sheep and cattle

Predictors	Original estimates	Original SE	*Estimates bias*	*Boot SE*
(Intercept)	-0.90	1.41	0.10	1.35
TE level g/ 100 g DM	-1.55	0.29	0.02	0.28
TE source [Chestnut]	4.81	1.85	0.09	1.89
TE source [Quebracho]	-0.20	1.93	-0.10	1.81

Table 3C3 Bootstrapping statistics of OMD response quadratic model to TE inclusion in sheep and cattle

Predictors	Original estimates	Original SE	*Estimates bias*	*Boot SE*
(Intercept)	1.34	1.69	0.13	1.65
TE level g/ 100 g DM [1st degree]	-3.37	0.78	0.04	0.75
TE level g/ 100 g DM [2nd degree]	0.22	0.09	0.00	0.08
TE source [Chestnut]	4.17	1.82	0.07	1.84
TE source [Quebracho]	-0.06	1.98	-0.11	1.86

Table 3C4 Bootstrapping statistics of CPD response linear model to TE inclusion in sheep and cattle

Predictors	Original estimates	Original SE	*Estimates bias*	*Boot SE*
(Intercept)	-1.48	1.39	0.03	1.40
TE level g/ 100 g DM	- 3.426	0.36	-0.01	0.45
Animal species [Sheep]	4.40	1.82	0.07	1.81

Table 3C5 Bootstrapping of CPD response quadratic model to TE inclusion in sheep and cattle

Predictors	Original estimates	Original SE	*Estimates bias*	*Boot SE*
(Intercept)	- 0.47	1.55	0.02	1.74
TE level g/ 100 g DM [1st degree]	- 5.528	0.93	0.00	1.15
TE level g/ 100 g DM [2nd degree]	0.348	0.14	0.00	0.15
Animal species [Sheep]	4.63	1.76	0.07	1.76

Table 3C6 Bootstrapping statistics of NDFD response linear model to TE inclusion in sheep and cattle

Predictors	Original estimates	Original SE	*Estimates bias*	*Boot SE*
(Intercept)	-3.12	2.42	0.03	2.63
TE level g/ 100 g DM	-3.95	0.45	0.02	0.61
TE source [Chestnut]	7.42	3.96	0.04	4.05
TE source [Quebracho]	7.76	3.06	-0.11	2.85

Table 3C7 Bootstrapping statistics of ADFD response linear model to TE inclusion in sheep and cattle

Predictors	Original estimates	Original SE	*Estimates bias*	*Boot SE*
(Intercept)	-12.33	5.05	0.07	4.72
TE level g/ 100 g DM	-3.0270	0.73	-0.02	0.77
TE source [Chestnut]	16.96	5.98	-0.03	5.70
TE source [Quebracho]	12.58	5.27	0.10	4.82

Table 3C8 Bootstrapping statistics of fecal N linear model to TE inclusion in sheep and cattle

Predictors	Original estimates	Original SE	*Estimates bias*	*Boot SE*
(Intercept)	2.10	4.26	0.07	4.52
TE level g/ 100 g DM	14.348	1.15	-0.01	1.63
TE source [Chestnut]	-17.30	3.17	-0.01	4.41
TE source [Quebracho]	-22.50	6.10	0.00	5.26
Diet's protein level [Low]	13.50	4.90	-0.09	4.44
Supplementation period [Long]	-11.08	4.06	0.07	4.38

Table 3C9 Bootstrapping statistics of urinary N linear model to TE inclusion in sheep and cattle

Predictors	Original estimates	Original SE	*Estimates bias*	*Boot SE*
(Intercept)	-3.33	2.67	-0.03	2.81
TE level g/ 100 g DM	-4.69	0.82	0.05	1.05
Diet's protein level [Low]	-6.81	3.00	-0.02	2.72
Supplementation period [Long]	5.12	2.23	-0.04	2.39

Table 3C10 Bootstrapping statistics of retained N response linear model to TE inclusion in sheep and cattle

Predictors	Original estimates	Original SE	*Estimates bias*	*Boot SE*
(Intercept)	13.438	6.26	0.13	8.42
TE level g/ 100 g DM	-9.906	2.09	0.03	4.04

Table 3C11 Bootstrapping statistics of retained N response quadratic model to TE inclusion in sheep and cattle

Predictors	Original estimates	Original SE	*Estimates bias*	*Boot SE*
(Intercept)	-9.06	8.32	-0.08	12.35
TE level g/ 100 g DM [1st degree]	18.93	5.28	0.35	9.96
TE level g/ 100 g DM [2nd degree]	-5.56	0.95	-0.06	1.52
Supplementation period [Long]	-17.06	7.18	-0.19	8.42

Table 3C12 Bootstrapping statistics for WG response linear model to TE inclusion in sheep and cattle

Predictors	Original estimates	Original SE	*Estimates bias*	*Boot SE*
(Intercept)	5.27	3.31	0.07	3.02
TE level g/ 100 g DM	-3.63	0.93	0.00	0.93

Table 3C13 Bootstrapping statistics for WG response quadratic model to TE inclusion in sheep and cattle

Predictors	Original estimates	Original SE	*Estimates bias*	*Boot SE*
(Intercept)	-2.77	3.89	0.12	3.37
TE level g/ 100 g DM [1st degree]	3.08	1.91	-0.02	1.76
TE level g/ 100 g DM [2nd degree]	-0.818	0.25	0.00	0.25

CHAPTER FOUR

Effects of ß-carotene Supplementation and Age on the Oxidative Status, Production and Reproductive Performance of Grazing South Africa Merino Ewes

Scientific paper prepared to be submitted for publication

Abstract

The effect of supplemental ß-carotene and age on the oxidative status of grazing ewes were investigated during breeding, pregnancy, parturition and early lactation as well as on their production and reproduction performance. Hundred and four ewes were stratified according to their age (young= 1-3 years and old= 4-6 years) and thereafter randomly allocated to four groups, in a completely randomized block design. Each group were drenched daily with 100 mg, 75 mg, 50 mg or 0.0 mg (control) of ß-carotene. Supplementation lasted 28 days before oestrous synchronization; 14 days during oestrous synchronization (CIDR) and hand-mating; 18 days post-hand-mating; 30 days before lambing and 15 days after lambing. All animals were grazed on Kikuyu grass (*Pennisetum clandestinum*). Six ewes from each group (total = 24) were sampled for blood to measure oxidative and hormonal status during the experiment. In this study, it was found that supplemental B-carotene above 75 mg/day may act as a pro-oxidant in ruminants under oxidatively stressed conditions such as parturition. Age did not affect the oxidative status of the ewes. The supplemental ß-carotene did not affect bodyweight, oestrous parameters, corpus luteum size, conception rate and litter size. The supplemental β-carotene or age did not affect the lamb survival rate or weight at birth or 15 days old. The survival rate of singles and twins was higher than that of triplets and quadruplets. Older ewes (4-6 y) showed earlier and longer duration of oestrus than the younger ones (1-3 y).

Keywords: Sheep, Hydroperoxides, CIDR, DMPD, progesterone, estrogen, oestrus parameters, litter size

4.1 Introduction

Oxidants such as the reactive oxygen species (ROS) help the cell to produce energy, transduce signals, expresses genes and maintain immunity (Bayr 2005, Valko et al. 2007). Conversely, when antioxidants in the body are overwhelmed by oxidants, ROS may alter the reduction-oxidation state, causing damage to DNA, protein, lipid and cell components (Ames et al. 1993, Miller et al. 1993, Bayr 2005). The damage occurs when oxidants remove hydrogen atoms or electrons from the target molecule or add oxygen, leaving it unstable and ready to attack nearby molecules (Lykkesfeldt and Svendsen 2007). The state in which oxidants exceed antioxidants is called oxidative stress, which is often the case during pathophysiological conditions (Sies 1997). However, in ewes (Kamiloğlu et al. 2006, Piccione et al. 2008, Mohebbi-Fani et al. 2012, Ognik et al. 2015, Lotfollahzadeh et al. 2016, Santarosa et al. 2021) as well as in goats (Di Trana et al. 2006, Celi et al. 2008, Jozwik et al. 2010, Zobel et al. 2015) and cows (Drackley 1999, Castillo et al. 2005) there are some evidences that normal physiological conditions such as breeding, pregnancy, parturition and lactation may trigger oxidative stress. It also has been observed in several species, including sheep, that age affects their vulnerability to oxidative stress (Ashok and Ali 1999, Andriollo-Sanchez et al. 2005, Salar-Amoli and Baghbanzadeh 2010). Oxidative stress affects animal production and reproduction performance. In females, oxidative stress may reduce litter size (Stier et al. 2012) and leads to granulosa cell apoptosis (Tilly and Tilly 1995), polycystic ovary syndrome, endometriosis, unexplained infertility, recurrent pregnancy loss, spontaneous abortion and preeclampsia (Agarwal et al. 2012). Thus, antioxidant supplementation during oxidatively stressed conditions is suggested as a tool to improve production and reproduction performance.

Recently, there has been a growing interest in using natural antioxidants to meet such a mission. One of the most studied and utilized natural sources of antioxidants is β-carotene (Choe and Min 2006, Lucas et al. 2020).

β-carotene is a red-orange pigment and one of a large group called carotenoids (Smith 1998). In animals, β-carotene functions primarily as pro-vitamin A (Smith 1998, Nagarajan et al. 2017) and as a scavenger of free radicals, especially singlet state oxygen (Stahl and Sies 2005). However, some authors consider the ß-carotene effects to come from its impact on biochemical systems and not from its antioxidant properties (Pryor et al. 2000). They argue that ß-carotene does not have a system that can donate a hydrogen atom or an electron to stabilise the oxidants. For example, both vitamin E and C have a hydroxyl group that is attached to an aromatic system, while ß-carotene has only a hydrocarbon compound (Pryor et al. 2000). Nonetheless, the use of ß-carotene supplementation as therapy for oxidative stress is controversial (Lykkesfeldt and Svendsen 2007). For example, Some authors reported a positive impact for β-carotene supplementations (Peirce 1954, Salem et al. 2015), while others reported no effect on the production and reproduction performance (Brozos et al. 2007, Meza-Herrera et al. 2011, Köse et al. 2013, Meza-Herrera et al. 2014, Gore 2016, Meza-Herrera et al. 2017). Besides others, two of the reasons for the inconsistent results might be the physiological condition at which the ß-carotene was supplemented and the age of the animals used. It is hypothesized that supplementation of ewes with ß-carotene may improve oxidative status during the so-called oxidatively stressed conditions and thereby improve their reproduction and production outcomes. The objective of the present study was to evaluate the effect of ß-carotene supplementation and age on the oxidative status of grazing South Africa Mutton merino ewes during breeding, pregnancy, parturition and early lactation as well as on their production and reproduction performance.

4.2 Material and methods

4.2.1 Materials

The ß-carotene (10%) was obtained from Pennville (Pty) Ltd, Pretoria, South Africa. The controlled internal drug releasing device (CIDR) and eCG hormone were obtained from RAMSEM (PTY) LTD, Bloemfontein, South Africa. N, N-Dimethyl-p-phenylenediamine dihydrochloride (DMPD) and Iron (III) chloride hexahydrate were purchased from Sigma-Aldrich (Ltd) (USA).

4.2.2 Animals and Treatments

The trial was conducted at the experimental farm of the University of Pretoria, South Africa from September 2017 to May 2018. The farm is located at latitude 25°44'30" south and longitude 28°15'30" east at an altitude of 1360 m (Van Niekerk et al. 2009). Hundred and four South African Mutton Merino ewes were stratified according to their age (young=1-3 years and old =4-6 years) and thereafter randomly allocated to four groups (A1, A2, A3 and C) in a completely randomized block design (CRBD). All animals were grazed on Kikuyu grass (*Pennisetum clandestinum*) during the day (8-9 h) and were penned at night. The ß-carotene product was dissolved in water to give a concentration of 25 mg/ml. Group A1, A2, A3 and C were daily drenched with 100 mg, 75 mg, 50 mg and 0.0 mg (Control) of ß-carotene, respectively. The treatments were administered to the ewes by using a drenching gun with the animals standing in a sheep chute right before grazing. The supplementation period was 105 days, which was divided into two periods: 60 days and 45 days. The first period lasted 28 days before oestrous synchronization; 14 days during oestrous synchronization and hand-mating; and 18 days post-hand-mating. The second period covered 30 days before lambing and 15 days after lambing. Ewes aged 1-3 years old were considered young and ewes aged 4-6 years were

considered old. The study ethical approval was obtained from the University of Pretoria Animal Ethics Committee (Reference (EC056-17)).

4.2.3 Blood Sample Collection

Blood samples of six ewes randomly chosen from each group (a total of 24 ewes) for blood sampling were collected. Blood was sampled using 18 G BD vacutainer® needle from the jugular venipuncture into heparinised BD vacutainer® tubes. The samples were collected on day zero (onset of supplementation), day 14, day 28 (day of CIDR insertion), day 40 (day of CIDR removal), day 41, day 42 (day of hand-mating), day 43, day 54 (day of CL measuring) and day 60 from the first supplementation period. During the second supplementation period, the sampling was done on day zero of supplementation, day 10, day 20, day 30 (day 1 after lambing) and day 45 (15 days after lambing). Blood samples collected were centrifuged for 10 minutes at 3000 rpm to recover plasma aliquots which were stored at - 20 °C for subsequent analysis.

4.2.4 Bodyweight of Ewes and Lambs

Bodyweights of the ewes were recorded on day zero, day 28 (day of CIDR insertion), day 40 (day of CIDR removal) and day 60 from the first supplementation period. During the second supplementation period, weighing was done on day zero (30 days before lambing), day 20 (10 days before lambing), day 30 (day 1 after lambing) and day 45 (15 days after lambing). The bodyweights of the lambs were measured on the day of birth and at 15 days old. The animals were weighed using an electronic weighing balance (TAL-TEC, South Africa).

4.2.5 Ultrasonographic examinations

Ultrasonographic examinations were performed using B-mode real-time ultrasound scanner (Aloka 500 SSD, Japan), connected to a transrectal probe with a 7.5 MHz linear array transducer (model UST-660-7.5). The examinations were done to measure the size of the

corpus luteum on day 12 post-hand mating and to confirm pregnancy on day 35 post-hand mating (Mogase et al. 2016, Gore and Lehloenya 2020).

4.2.6 Oestrus synchronization and hand mating

After the first 28 days of supplementation with ß-carotene, all ewes, oestrous cycles were synchronized with progesterone using controlled internal drug release devices (CIDR) which were inserted and remained intravaginally for 11 days. At CIDR withdrawal, the ewes were intramuscularly injected with 350 IU eCG hormone. Ewes were teased for three days (two times per day) with vasectomised ram after the withdrawal of CIDR. Oestrous response (%), the onset of oestrus (h) and Oestrous duration (h) were measured. Ewes were hand-mated twice at 48 and 60 h after CIDR withdrawal with a ram of proven fertility. After the second-hand mating, the rams were left with ewes for a joining period of approximately two oestrous cycles (34 days).

4.2.7 Oxidative status

The plasma oxidative status/capacity was assessed as mmol ferric equivalent (mM FE/L) using the DMPD-based method modified by Mehdi and Rizvi (Mehdi and Rizvi 2013). The assessment of the oxidative status was done by measuring intermediate products called hydroperoxides formed by the oxidation of lipids, peptides, and amino acids (Verde et al. 2002). A detailed description of the method used in this study is given elsewhere (Ahmed et al. 2021).

4.2.8 Estrogen and Progesterone Analysis

Plasma samples were analysed for Estrogen and Progesterone concentrations using an enzyme immunoassay (EIA) performed on microtiter plates at the endocrine laboratory of the University of Pretoria. Details of the two EIA's, are described by Schwarzenberger et al. (Schwarzenberger et al. 1996) and Palme and Möstl (Palme and Möstl 1993). The coefficient

of variance for the intra-assay variance ranged from 6.66% to 7.59% (progesterone) and 5.42 to 7.75% (estrogen), respectively. The coefficient of variance for the inter-assay variance ranged from 7.49% to 7.56% (progesterone) and 5.42% to 7.75% (estrogen), respectively. The sensitivity of the Progesterone (VUW) EIA used was 320 pg/mL plasma and the Estrogen (Estrone (E1) and Estradiol (E2) (VUW)) EIA used was 8 pg/mL plasma. Both tests were carried out in duplicates for each sample.

4.2.9 Statistical analyses

Statistical analyses were carried out using the integrated RStudio tool (RStudio 2021.09.1+372 for Windows). The procedure of general linear mixed model analysis of variance was conducted for bodyweight, antioxidant status and hormonal status, with treatment, sampling time and age as fixed effects and animal ID as random effects. The statistical model used was:

Y *ijkl* = μ + Animal *i* + Treat *j* + Sample *k* + Age *l* + (Treat *j* * Sample *k* * Age *l*)+ E *ijkl*

Where: Y ijk = response variable. μ = general mean. Animal *i* = random effect of the *i*th animal ID. Treat j = effect of the ß-carotene treatment. Sample *k* = effect of sampling time. Age *l* = effect of the age. Treat *j* * Sample *k* * Age *l* = interactions. E *ijkl* = experimental error.

Similarly, an analysis of variance was conducted for lambs, bodyweight at birth and 15 days old, with treatment, sex and litter size as fixed effects, and animal ID as random effects. The statistical model used was:

Y *ijkl* = μ + Animal *i* + Treat *j* + Sex *k* + Lsize (*l*) + E *ijkl*

Where:

Y ijk = response variable. μ = general mean. Animal *i* = random effect of the *i*th animal ID. Treat j = effect of the ß-carotene treatment (1-4). Sex *k* = effect of the sex of the lamb (1-2). Lsize *l* = effect of the litter size (1-4) of the lambs. E *ijkl* = experimental error.

The package lme4 (Package lme4 version 1.1-27.1) was used to create those mixed models (Bates et al. 2014). The general linear model (GLM) (Package stats version 4.1.3) procedure was used to analyze the effect of treatment and age on the oestorus duration, oestrous onset and corpus luteum size using the following model:

$Y_{ij} = \mu + Treat_i + Age_j + E_{ij}$

Where: Y ij = response variable, μ = general mean, Treat j = effect of the ß-carotene treatment (1-4), Age *j* = effect of the age of the ewe (1-2) and E *ij*= experimental error. Chi-square (Package stats version 4.1.3) was used for the analysis of oestrous response, conception rate, litter size and survival rate of the lambs. When found significant, variables and interactions were pairwise compared using Tukey contrast (Package emmeans version 1.7.1-1). Data were presented as means ± standard error (SE).

4.3 Results and discussions:

4.3.1 Effect of ß-carotene supplementation, physiological condition and age on the bodyweight of ewes

Our results show that ß- carotene supplementation had no significant effect on the bodyweight of ewes ($p = 0.978$). The average bodyweight were 69.5 ± 1.59, 68.8 ± 1.51, 69.7 ± 1.54 and 69.4 ± 1.57 for 100 mg, 75 mg, 50 mg and control group, respectively. This agrees with the lack of significant effect of ß-carotene supplementation on bodyweight previously reported in sheep (Brozos et al. 2007) and goats (Arellano-Rodriguez et al. 2009). A significant difference was recorded in bodyweight between old and young ewes ($p < 0.001$). Older ewes were heavier (74.0 ± 1.05) than their younger counterparts (64.7 ± 1.15). As shown in Figure 4.1, the different physiological conditions significantly affected the bodyweight of ewes during the experimentation period ($p < 0.001$). Ewes gained weight gradually and reached their peak during the last month of pregnancy (Figure 4.1). After lambing, the bodyweight of ewes

significantly decreased for two weeks. The ewes' weights may have decreased further during the early days of lactation because of the high demand for energy and protein as milk production is given priority over maintaining weight.

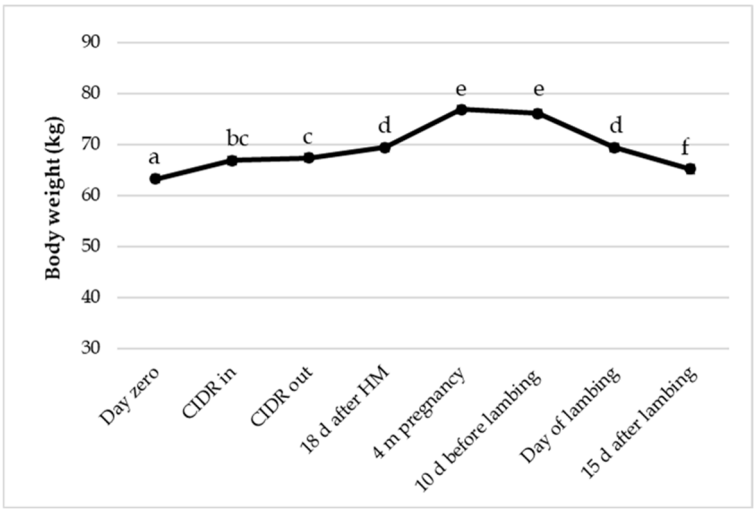

Figure 4.1 Effect of physiological conditions on body weight (kg) of ewes

4.3.2 Effect of ß- carotene supplementation, physiological condition and age on oxidative status of ewes

Our results indicated that ß- carotene supplementation and the physiological condition of ewes, as well as the interaction between both, significantly influenced ($p < 0.001$) their oxidative status. Figure 4.2 illustrates the interaction effect of ß-carotene supplementation and physiological condition on the oxidative status of ewes. The results showed that pre or during pregnancy, ß-carotene supplementation did not affect the oxidative status of the ewes ($p > 0.05$). At parturition, however, the group supplemented with 100 mg and 75 mg ß- carotene showed higher ($P < 0.001$) oxidative status than the control group (Figure 4.2). However, the observed significant differences faded 15 days later after lambing. The oxidative status of the group supplemented with 50 mg ß- carotene did not differ significantly from their counterparts at parturition. Moreover, oxidative status on the day of parturition was significantly higher than that of pre-pregnant and pregnant ewes in groups supplemented with 100 mg and 75 mg ß-

carotene. Then, 15 days later after lambing the oxidative status of both groups declined back to levels similar to those during pregnancy or pre-pregnancy ($p > 0.05$). The oxidative status for control and 50 mg ß-carotene groups did not significantly change during the various physiological conditions. Researchers have reported ewes to be prone to oxidative stress pre, during and post-parturition (Mousaie et al. 2017, Dunière et al. 2021, Moradi et al. 2021, Yaqub et al. 2021). Our study assessed plasma oxidative status by measuring hydroperoxides, which are intermediate products resulting from the oxidation of lipids, peptides, and amino acids. Interestingly, as shown, supplementation of ewes with 100 mg and 75 mg ß-carotene increased the hydroperoxides levels in the plasma. Although ß-carotene's antioxidant properties are to quench singlet oxygen, scavenge oxyradicals, and terminate other free radical reactions, it is evident that its overall physiological action is complex (Black 2002). Under oxidative stress conditions, biochemical studies have shown that carotenoids may act as a pro-oxidant (Zhang and Omaye 2001, Alija et al. 2005, Siems et al. 2005). When carotenoids are exposed to reactive species under mild oxidative stress, products with high molecular weight, such as apo-carotenals, will be formed (Ribeiro et al. 2018). The problem arises when these products are further oxidized into short-chain breakdown products, depending on the type and concentration of the oxidant (Siems et al. 2005, Ribeiro et al. 2018). The elevated levels of hydroperoxides observed with higher ß-carotene supplementation (75 mg and 100 mg/day) could be related to the accumulation of these broken-down products due to parturition-induced oxidative stress. In this context, the results may indicate that parturition is the most oxidatively stressed condition during the female reproduction cycle. Our results indicate that ß-carotene may work as pro-oxidants under oxidatively stressed conditions, especially with high levels of supplementation. As far as we know, this is the first in-vivo study in ruminants that shows ß-carotene may work as a prooxidant under high oxidative stress conditions.

It is crucial noting that the animals in this study were grazing, meaning they might have received a pasture-dependent antioxidant combination. The ß-carotene in the 100 mg and 75 mg groups might have acted as the first line of defence, interacting excessively with the oxidants and shielding the rest of the antioxidants, resulting in a higher concentration of hydroperoxides than in the control or 50 mg groups. The control group showed stable oxidative status even under the most oxidatively stressful situation (parturition). According to later assumptions, the combination of antioxidants may be able to block the oxidative stress effects without raising plasma hydroperoxide concentrations.

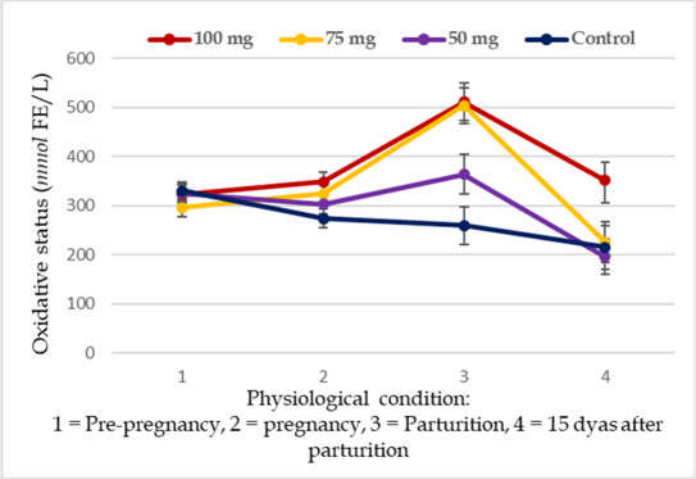

Figure 4.2: Effect of interaction between physiological condition and ß-carotene supplementation on oxidative status of ewes

Supplementation with ß-carotene did not affect (p = 0.353) the oxidative status of the ewes during oestrus synchronization. The oxidative status means were 333 ± 16.6, 291 ± 16.8, 317 ± 16.8 and 316 ± 16.8 *mmol* FE/L for 100 mg, 75 mg, 50 mg and control groups, respectively. However, the oxidative status of the ewes was significantly affected (p = 0.002) by oestrous synchronization (Figure 4.3). Compared to CIDR insertion (285 ± 18.1 *mmol* FE/L), the removal showed a non-significant increase (341 ± 18.1 *mmol* FE/L) in the oxidative status of the ewes (Figure 4.3). After maintaining this oxidative status for 48 h (340 ± 20.9 *mmol* FE/L), it significantly declined at 72 h (259 ± 18.4 *mmol* FE/L) to a level similar to that

before the CIDR removal. Our results suggest that oxidative status may slightly increase (hydroperoxides increase) during the 72 hours after CIDR removal (Figure 4.3). (Salinas-Rios et al. 2016), reported similar results in terms of lipid peroxidation, where CIDR removal caused a numerical non-significant increase in peroxidation and then declined gradually thereafter. At CIDR removal, ewes in this study were injected with eCG. With its FSH-like activity, the eCG may initiate preovulatory events by increasing endogenous gonadotrophin levels (Abecia et al. 2012). The slight increase in oxidative status following CIDR removal could be due to the hormonal changes caused by CIDR removal and eCG injections. These changes may increase metabolic activity and thus oxidative status during this period of oestrous synchronization. The slight increase in oxidative status may also be partially due to vaginal irritation caused by the removal of CIDR (Kuru et al. 2018).

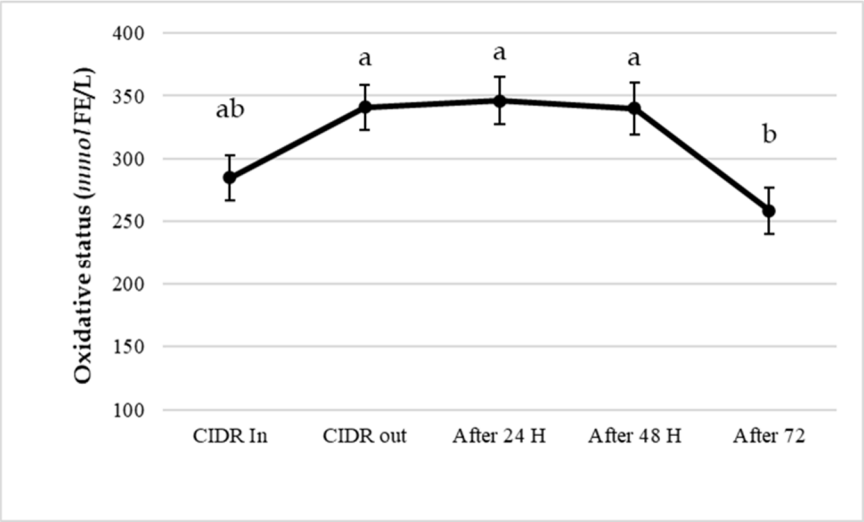

Figure 4.3: Effect of oestrous synchronization on the oxidative status of ewes

Although it appears that old ewes had a higher value of oxidative status than young ones (332 ± 11.9 and 312 ± 12.5 mmol FE/L, respectively), our results showed no significant difference between the two groups (p = 0.213). Martino et al. (Martino et al. 2012) reported that reactive oxygen species were significantly higher in oocytes from aged donors as compared to those from young ones. In their study, Martino et al. used older sheep (young: 2-5 y and old:

7-10 y) than those in our study (young: 1-3 y and old: 4-6 y), which may explain the difference in results.

4.3.3 Effect of ß-carotene supplementation, time of sampling (physiological condition) and age of animals on progesterone concentration

Supplementation with ß-carotene did not affect plasma progesterone concentration P4 ($p = 0.506$). The average progesterone concentration means were 59.1 ± 5.91, 51.2 ± 6.85, 62.9 ± 5.91 and 64.3 ± 5.91 *ng*/ml for 100 mg, 75 mg, 50 mg and control group, respectively. Our results are in line with the previous studies in sheep (Smith et al. 1976) and cows (Kaewlamun et al. 2011, Hye et al. 2020), in which plasma progesterone was not affected by ß-carotene supplementation.

Multiple factors influence the development, regression, and secretory activities of the corpus luteum (Hye et al. 2020). Therefore, supplementing with ß-carotene alone may not be sufficient to promote luteal development and progesterone production (Hye et al. 2020). Nevertheless, other studies indicated that ß-carotene supplementation increased progesterone concentration in cows (Çelik et al. 2009) and goats (Arellano-Rodriguez et al. 2009, Gore and Lehloenya 2020). The differences between studies might be due to the season, species, breed, age, ß-carotene content in the feed, and level of ß-carotene supplementation used. The time of sampling ($p < 0.001$) and age ($p = 0.0137$), as well as the interaction between both ($p = 0.037$), significantly influenced the progesterone concentration in the ewes. Figure 4.4 shows the interaction between time and age on progesterone concentration in ewes. The interaction results show a significant difference between the old and young ewes on day zero (Figure 4.4). This difference, however, became non-significant on the day of mating and 12 days after mating (Figure 4.4). A higher level of progesterone in old ewes than in younger ewes at day zero may be the result of an active corpus luteum, whose activity was neutralized later by CIDR of

oestrous synchronization. Similar progesterone concentrations in young and old ewes seen in current and previous studies (Smith et al., 1976, Michels et al., 1998) may reflect similar luteal function in young and old sheep. The results show that old and young ewes had higher progesterone concentrations 12 days after mating than they did on the day of mating (Figure 4.4). The increased progesterone concentration 12 days after mating in both old and young ewes is probably due to the formation of the corpus luteum.

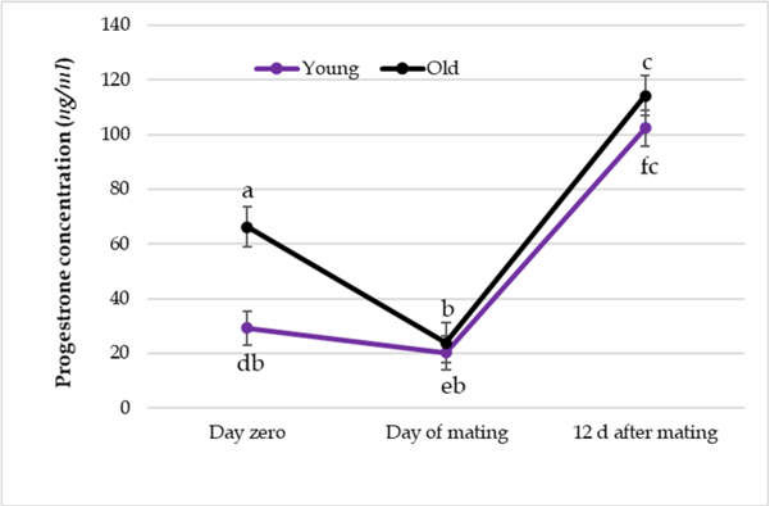

Figure 4.4 Effect of the interaction between age and sampling time on the progesterone concentration in ewes

4.3.4 Effect of ß-carotene supplementation, physiological condition and age of animals on estrogen concentration

Supplementation with ß-carotene tended to affect estrogen concentration of ewes (p = 0.079). The average estrogen concentration means were 0.57 ± 0.06, 0.43 ± 0.07, 0.69 ± 0.06 and 0.62 ± 0.06 ng/ml for 100 mg, 75 mg, 50 mg and control group, respectively. Celic et al. (Çelik et al. 2009) studied the effect of ß-carotene injection (1 mg/kg) on repeat breeder cows. They performed an Ovsynch program with two injections of GnRH (10µg), 7 d before and 48 h after the injection of PGF2α (25 mg). Our results agree with their findings where ß-carotene injection did not affect serum estrogen levels on the day of the first GnRH injection or the day

of the PGF2α injection. However, they observed an increase in estrogen level on the day of the second GnRH injection. Their results may indicate that the ß-carotene effect on estrogen was only observed during the late proestrus or oestrous stage when estrogen usually peaks. Those later periods were not included in our study as estrogen concentration was measured at the very beginning of proestrus (at CIDR removal). A non-significant effect of ß-carotene supplementation on estrogen concentration was also observed in goats (Gore and Lehloenya 2020). Although with a tendency (p = 0.083) of high estrogen concentration on day zero (0.61 ± 0.04) did not differ significantly from that on the day of CIDR removal (0.54 ± 0.04). The concentration of estrogen in older ewes (0.61± 0.05) did not also differ significantly (p = 0.259) from that of young ewes (0.54 ± 0.04). The observed lack of significant effect of age on estrogen concentration in ewes, agree with earlier reports (Smith et al. 1976, Gonzalez-Bulnes et al. 2004).

4.3.5 Effect of ß-carotene supplementation on the birth weight of lambs

The supplementation of ewes with ß-carotene did not affect the birth weight of their lambs (p = 0.750). The average birth weight means were: 3.34 ± 0.17, 3.20 ± 0.16, 3.12 ± 0.17 and 3.26 ± 0.17 kg for 100 mg, 75 mg, 50 mg and control group, respectively. However, lambs from ewes supplemented with ß-carotene tended to have higher weight at 15 days old *(p = 0.080)*: 7.04 ± 0.29, 6.83 ± 0.26, 6.31 ± 0.28 and 6.28 ± 0.28 kg for 100 mg, 75 mg, 50 mg and control group, respectively. It has been reported that ß-carotene supplementation did not influence the birth weight of goats kids (Gore 2016) and calves (Kaewlamun 2010) but it was reported to have heavier litters in gilts at birth and weaning (Brief and Chew 1985). As shown in Figure 4.5, the litter size significantly affected the weight of the lambs at birth and 15 days old (p < 0.001). Single lambs were the heaviest both at birth and 15 days old compared to twins, triplets or quadruplets (Figure 4.5). At birth and 15 days old, twins were heavier than triplets

and quadruplets (Figure 4.5). No significant difference in weight was observed between triplets and quadruplets at birth or 15 days old (Figure 4.5). Although the total birth weight of twins or more is reported to be higher than that of a single lamb (Sarvinda et al. 2022), the individual lamb of twins, as demonstrated, has a lower body weight compared to that of a single born. Our results may indicate that the difference in weight at birth between singletons and twins may persist as the lambs grow. In their study, Corner et al. reported that singletons were heavier ($P < 0.05$) than twin lambs at birth, 42 and 94 days old (Corner et al. 2007). The persistence of the difference may be due to competition for dam's milk in the case of twins, as opposed to no competition in the case of singletons (Boujenane and Diallo 2017).

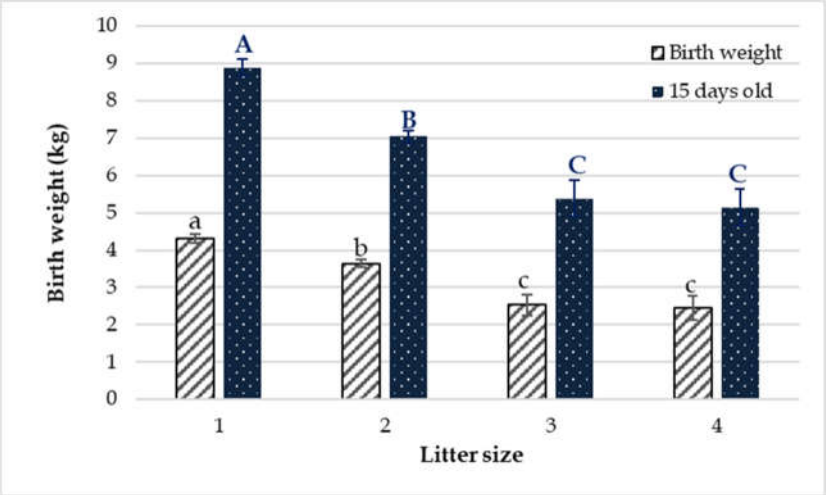

Figure 4.5 Effect of litter size on body weight (kg) of lambs at birth and 15 days old

The results showed a tendency for the male lambs to be heavier than the female ones at birth ($p < 0.072$) and 15 days old ($p < 0.074$). At birth, the weight means were: 3.33 ± 0.13 and 3.13 ± 0.13 kg, while at 15 d old were: 6.82 ± 0.21 and 6.40 ± 0.22 kg for male and female lambs, respectively. It was documented in many studies that male lambs are born heavier and may grow faster than females (Morel et al. 2008, Boujenane and Diallo 2017). However, in some studies, the effect of sex on the birthweight of lambs was not detectable (Abdullah 2010, Simeonov et al. 2014).

4.3.6 Effect of carotene supplementation on the survival rate of lambs

Our results showed that ß-carotene did not affect the survival rate of the lambs (p = 0.298) at 15 days old. The survival rates were 80.0, 88.9, 92.9, and 90.5 % for the 100 mg, 75 mg, 50 mg, and control groups, respectively. The results suggest that ß-carotene supplementation does not affect lamb survival. According to our knowledge, no such investigation has been conducted in ewes. Nevertheless, unlike in the current study, a positive effect on the survival of calves was reported in ß-carotene injected cows (Zubova et al. 2021). The differences between studies might be due to species, feed ß-carotene content, and level of ß-carotene supplementation used. We found that the litter size significantly affected the survival rates of the lambs ($p < 0.001$). Figure 4.6 shows the effect of litter size on the survival rates of the lambs. The survival rates of lambs from litter sizes of one or two were significantly higher than those of three or four (Figure 4.6). The effect of the litter size on the survival rates of lambs may be closely related to the effect of the litter size on the birth weight and weight increase in the lambs (Morris et al. 1999, Corner et al. 2007). As demonstrated previously (Figure 4.6) lambs from litter sizes three and four were significantly lighter than those of one and two, which may result in a significantly less survival rate (Figure 4.6). There was a tendency for survival rate at 15 days to be lower in male lambs (83.7%) than in female lambs (92.4%), but this was not statistically significant (p = 0.084). The results of female lambs having higher survival rates than male lambs were documented elsewhere (Lax and Newton 1965, Hight and Jury 1970, Zapasnikienė 2002, Binabaj et al. 2013, Tesema et al. 2020).

Several reasons for the higher survival rate of female lambs are suggested, including unidentified sex-linked genetic determinants (Mandal et al. 2007). In addition to high birthweight-caused dystocia and consequent dam death, which may result in lamb starvation (Smith 1977, Binabaj et al. 2013).

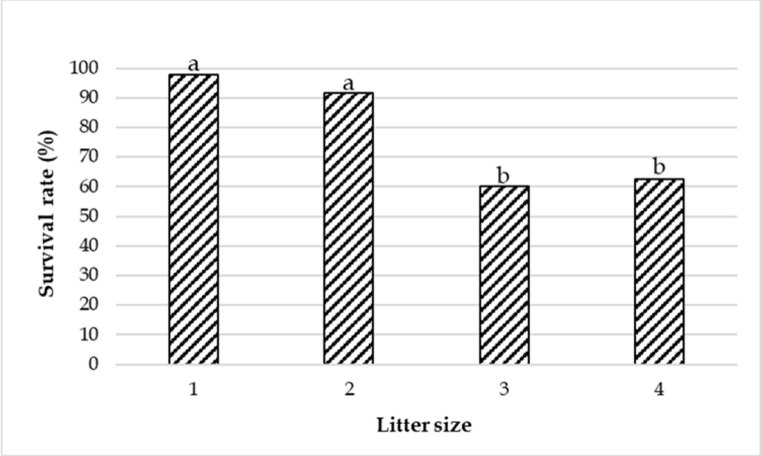

Figure 4.6: Effect of litter size on survival rate (kg) of lambs up to 15 days old

4.3.7 Effect of ß-carotene supplementation and age on the litter size of lambs

The effect of ß-carotene supplementation on litter size frequencies is presented in Figure 4.7. The supplementation of the ewes with ß-carotene did not affect the litter sizes (p = 0.36). However, quadruplets were found in the 100 mg (4.2%) and 75 mg ß-carotene groups (11.1%), but not in the control or 50 mg ß-carotene groups (Figure 4.7). The nonsignificant effect of ß-carotene and vitamin E supplementation on the litter size of ewes was reported elsewhere (OZMEN et al. 2022). On the other hand no significant effect was reported in sows of third or higher parity, however, in second parity, ß-carotene treatment increased the total number of piglets per litter and the number of piglets born alive (Krammer and Aurich 2010).

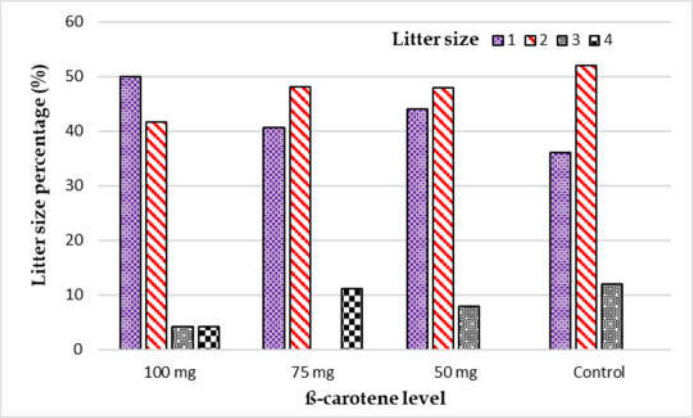

Figure 4.7: Effect of β-carotene supplementation on the frequencies of singles, twins, triplets and quadruplets in merino ewes

We found that age did not affect the litter size in ewes (p = 0.392). Frequencies of singles, twins, triplets and quadruplets for young ewes were: 47.8, 41.3, 4.3 and 6.5 %, while for old ewes were: 38.2, 52.7, 7.3 and 1.8 %, respectively. Similar findings of the nonsignificant effect of ewe age on litter size have been reported by Vanli and Ozsoy (Vanli and Ozsoy 1988) and by Fogarty et al (Fogarty et al. 1992), while Said et al (Said et al. 1999) and Shorten et al (Shorten et al. 2013) reported significant effects.

Table 4.1 shows the effect of ß-carotene supplementation on oestrus parameters, conception rate, and corpus luteum size in ewes. Supplemental ß-carotene had no effect on oestrus response (p = 0.416), oestrus duration (p = 0.900), oestrus onset (p = 0.959), conception rate (p = 0.375) and corpus luteum size (p = 0.935). Our findings are similar to those found in goats (Gore and Lehloenya 2020) and cattle (Wang et al. 1987, Wang et al. 1988, Hye et al. 2020), where ß-carotene supplementation did not affect the oestrous response, onset, duration, or conception rate. No effect on the corpus luteum size was also reported in cows (Hye et al. 2020). In disagreement with the current study, β-carotene supplementation increased oestrous response in ewes (Salem et al. 2015) and the conception rate in cows (Aréchiga et al. 1998, Bhateshwar et al. 2021).

Table 4.1: Effect of β-carotene supplementation on oestrous response, oestrous duration, oestrous onset, conception rate and corpus luteum size at 12 d after hand mating (Mean ± SE)

Treatments	Oestrus response (%)	Oestrus onset (hrs)	Oestrus duration (hrs)	Conception rate (%)	Corpus luteum size (mm)
100 mg	100	19.4 ± 1.9	43.6 ± 2.7	95.8	9.9 ± 0.8
75 mg	96.2	20.0 ± 1.8	45.3 ± 2.6	100	9.2 ± 0.8
50 mg	100	18.2 ± 1.8	44.4 ± 2.6	100	9.9 ± 0.8
Control	100	18.7 ± 1.9	45.4 ± 2.6	100	9.8 ± 1.0
p-value	0.416	0.900	0.959	0.375	0.935

The effect of age on oestrus parameters, conception rate and corpus luteum size is presented in Table 4.2. Although age did not affect the oestrous response, conception rate and corpus luteum size, older ewes significantly (p < 0.001) showed earlier onset and longer

duration of the oestrus than the younger ewes (Table 4.2). The results indicate that older ewes may exhibit more intense oestrous symptoms than younger ones. This indication was observed elsewhere (Simitzis et al. 2006, Pascual-Cordova et al. 2009). No significant effect of age on corpus luteum size was also reported (Berlinguer et al. 2012). In disagreement with the present results, Berlinguer et al. reported no effect of age on oestrous duration (Berlinguer et al. 2012). However, Berlinguer et al. (Berlinguer et al. 2012) compared adults (4-6) and very old ewes (12-14 y), while in this study, we compared young (1-3 y) and old ewes (4-6 y), which may explain the differences in findings.

Table 4.2 Effect of age on oestrous response, oestrous duration, oestrous onset, conception rate and corpus luteum size at 12 d after hand mating (Mean ± SE)

Treatments	Oestrus response (%)	Oestrus onset (hrs)	Oestrus duration (hrs)	Conception rate (%)	Corpus luteum size (mm)
Young	97.8	23.2 ± 1.4	38.2 ± 2.0	97.8	10.0 ± 0.6
Old	100	15.0 ± 1.2	51.2 ± 1.8	100	09.4 ± 0.6
p-value	0.273	<0.001	<0.001	0.277	0.553

4.4 Conclusions

This study is the first to report that, in grazing sheep, supplemental ß-carotene higher than 75 mg/day may act as a pro-oxidant during stressful conditions, such as parturition. Therefore, the supplementation of ß-carotene may not be recommended during the parturition time. However, although the supplementation of the ewes with ß-carotene did not affect the litter sizes, quadruplets were observed only in the 100 mg (4.2%) and 75 mg ß-carotene groups (11.1%). This observation might require further investigation on the effect of ß -carotene supplementation before and during breeding, especially in herds with low fertility. The survival rate of singles and twins is higher than that of triplets and quadruplets. The study indicates that supplementation of ß-carotene may not be of benefit to the reproduction and reproduction

performance of healthy grazing ewes. Finally, the older ewes seem to have earlier oestrus with a longer duration than the younger ones.

CHAPTER FIVE

The effect of β-carotene supplementation below or above requirement on the production and reproduction performance of the female sheep and goats - A review

Scientific paper prepared to be submitted for publication

Abstract

The high rate of metabolic activity during breeding, pregnancy, parturition and lactation may lead to oxidative stress in sheep and goats. Antioxidant supplementation is proposed as a therapy to overcome the resultant stress. In this paper, we reviewed the supplementation effect of β-carotene, one of those antioxidants, on the production and reproduction performance of sheep and goats. Data were extracted from peer-reviewed articles that were searched in Web of Science, Google Scholar and Scopus databases. Thirteen peer-reviewed published articles were found on the subject under review. We estimated the animal requirements for β-carotene, the amount of β-carotene provided to the animal in the diet and the amount of β-carotene supplementation used in each article. Those estimations were done to indicate whether the diet fulfils the animal requirements for β-carotene or not. The review showed that β-carotene supplementation enhances the production and reproduction performance of the sheep when fed on a carotene-deficient diet. β-carotene supplementation may increase the levels of some hormones such as progesterone in sheep and goats when fed on a diet that fulfils their requirements of β-carotene. However, supplementation of β-carotene above the requirements does not seem to affect the production and reproduction parameters such as body weight, milk production, oestrous parameters, conception rate, pregnancy rate, and litter size.

Keywords: β-carotene, small ruminants, ewe, doe, reproduction, progesterone, litter size, oxidative stress.

5.1 Introduction

Antioxidant supplementation is used as a therapy for oxidative stress (Lykkesfeldt and Svendsen 2007). So far thirteen articles have been published on the effect of β-carotene supplementation used as a tool to manipulate the production and reproduction performance of goats and sheep, however, the findings are inconsistent in terms of its effect on studied production and reproduction parameters. Some authors reported a positive impact for β-carotene supplementations (Peirce 1954, Salem et al. 2015), while others reported no effect on the production and reproduction performance (Brozos et al. 2007, Meza-Herrera et al. 2011, Köse et al. 2013, Meza-Herrera et al. 2014, Gore 2016, Meza-Herrera et al. 2017). Such inconsistency, may usually be linked to the variation in the amount of β-carotene supplemented, the amount of β-carotene in the animal diet and to the difference in the animal requirement for β-carotene. Thus, this review was undertaken with an aim to analyze available literature in order to determine the effect of β-carotene supplementation on the production and reproduction performance of sheep and goats considering the amount of β-carotene supplemented, the amount of β-carotene provided by the animal diet and the animal requirements for β-carotene.

5.2 Methods

Peer-reviewed articles for the subject under investigation were searched in Web of Science, Google Scholar and Scopus databases. Thirteen peer-reviewed published articles were found on the subject under review. We estimated the animal requirement for β-carotene, the amount of β-carotene provided to the animal in the diet and the amount of β-carotene supplementation used in each article. The requirements were calculated according to NRC (NRC 2007) as μg β-carotene per kg body weight per day (μg/kg BW/d). In case was not reported, the amount of β-carotene provided to the animal in the basic diet was estimated (μg/kg BW/d) from the primary sources of β-carotene in that diet. The estimation was done to indicate

whether the diet fulfils the animal requirements for β-carotene or not. In studies number 6-13 (Table 5.1), the percentage of the different components in the diet was not mentioned. However, it was reported that the diet was based on alfalfa hay, which make up at least 50% of the diet and from this β-carotene provided was calculated. The levels of β-carotene supplementation (µg/kg BW/d) were calculated by dividing the daily supplementation of β-carotene (µg) by the animal weight (kg).

5.3 Oxidative stress

Oxidants such as the reactive oxygen species (ROS) are involved in enzymatic reactions, cell energy production, signal transduction, gene expression, and the antimicrobial action of neutrophils and macrophages (Bayr 2005, Valko et al. 2007). Nevertheless, when the oxidants exceed the antioxidant capacity of the body, ROS can damage DNA, protein, lipid and cell components as well as alter the reduction-oxidation state (Ames et al. 1993, Miller et al. 1993, Bayr 2005), and interfere with metabolic processes (Miller et al. 1993). The oxidants exceed the antioxidants during the pathophysiological conditions (Sies 1997) and also during normal conditions such as breeding, pregnancy, parturition and lactation. These conditions may lead to oxidative stress as in cows (Drackley 1999, Castillo et al. 2005), goats (Di Trana et al. 2006, Celi et al. 2008, Jozwik et al. 2010, Zobel et al. 2015) and sheep (Kamiloğlu et al. 2006, Ognik et al. 2015, Lotfollahzadeh et al. 2016). Oxidative stress reduces litter size (Stier et al. 2012), leads to granulosa cell apoptosis (Tilly and Tilly 1995), endometriosis, polycystic ovary syndrome, unexplained infertility, spontaneous abortion, recurrent pregnancy loss and preeclampsia (Agarwal et al. 2012).

Oxidants can add oxygen, or remove hydrogen or an electron from the target molecule, leaving an unpaired electron in the molecule's outer orbit and thus making it unstable (Lykkesfeldt and Svendsen 2007). The antioxidants are the only substances that can delay or

inhibit the process of oxidation, e.g. by donating electrons to the oxidants (Halliwell and Gutteridge 1989). Thus antioxidants supplementation can prevent ROS-mediated damage (Bayr 2005), optimize animal performance and maintain proper oxidant/antioxidant balance (Miller et al. 1993, Celi 2011). The antioxidants supplementation can involve enzymes and low molecular mass substances such as tocopherols and carotenes in the lipid phase and ascorbate, glutathione and others in the aqueous phase (Sies 1997).

5.4 β-carotene

5.4.1 β-carotene functions

β-carotene is a red-orange pigment and one of a large group called carotenoids (Smith 1998). In animals, β-carotene functions mainly as pro-vitamin A (Smith 1998, Nagarajan et al. 2017) and as a scavenger of free radicals, especially against the singlet state oxygen (Stahl and Sies 2005). Carotenoids with one or two beta-ionone rings at the end of their structure are called provitamin A carotenoids such as β-carotene, which has two rings (Nagarajan et al. 2017). Vitamin A can be sourced from the animal as retinoids or in the form of pro-vitamin A carotenoids such as β-carotene. However, β-carotene as a source of vitamin A is safer than retinoid. Under the condition of vitamin A adequacy, the retinoic acid suppresses the enzyme (β-carotene oxygenase) that converts β-carotene into vitamin A, in the intestinal mucosa, liver, and corpus luteum (Combs Jr and McClung 2017, Nagarajan et al. 2017). The importance of β-Carotene as a source of vitamin A in animal species was reviewed by Green and Fascetti (Green and Fascetti 2016). β-carotene scavenges peroxyl radicals and further decay to generate non-radical products and may terminate radical reactions by binding to the attacking free radicals (Paiva and Russell 1999). β-carotene is lipophilic; scavenges radicals within the lipophilic compartment such as the cell membrane even more effectively than Vitamin E (Niki et al. 1995). However, some authors consider the β-carotene effects to come from its impact

on biochemical systems and not from its antioxidant properties (Pryor et al. 2000). They argue that ß-carotene does not have a system that can donate a hydrogen atom or an electron to stabilize the oxidants. For example, both vitamin E and C have a hydroxyl group that is attached to an aromatic system, while ß-carotene has only a hydrocarbon compound (Pryor et al. 2000).

5.4.2 β-carotene requirements and toxicity

Animals are unable to synthesize carotenoids and obtain them from their diet (Britton and Khachik 2009, Kopsell and Kopsell 2010, Desmarchelier and Borel 2017). According to the National Research Council (NRC 2007), sheep and goats requirements for vitamin A during breeding and early gestation are equal to 157 µg of β-carotene/kg BW/day and 267 µg of β-carotene/kg BW/day during late pregnancy and lactation. The subtropical grasses meet the requirements of grazing livestock for β-carotene except for a few months through the year (Arizmendi-Maldonado et al. 2003). To our knowledge, there is no study on the toxicity of β-carotene in ruminants. Nevertheless, the treatment of high doses (approximately 180 mg of β-carotene/day) had no side effects in humans (Bendich 2001), except for hypercarotenemia in some subjects (Diplock 1995). Supplementation of experimental animals with about 17 times their requirements of β-carotene (Fiorelli et al. 2014) and up to 1000 mg/day of β-carotene per kg body weight showed no signs of toxicity (Woutersen et al. 1999). However, at the cellular level, β-carotene at high concentrations acts as a prooxidant and may lead to healthy cells exposed to oxidative stress to neoplastic transformation (Palozza et al. 2001). Because of the oxidative attack, carotenoid breakdown highly reactive aldehydes and epoxides that inhibit the respiration of the mitochondria, reduce protein sulfhydryl content, decrease GSH levels and redox state, and elevate the accumulation of malondialdehyde (Siems et al. 2009).

5.5 The effect of β-carotene supplementation below or above the requirement on the production and reproduction performance of sheep and goats

Peirce (Peirce 1954) conducted two experiments on the effect of a ß-carotene deficient diet on sheep performance. In the first experiment, sheep were fed on a ß-carotene deficient diet (7-17 µg/kg BW/day) before and during the mating period. Then during the gestation period, the sheep were fed on a diet with different levels of ß-carotene (50, 100 and 150 µg/kg BW/day). According to the NRC (227.5 µg/kg BW/day), those diets are ß-carotene deficient. The study reported cases of night blindness, loss of appetite and some of the experimental animals died or became very weak when fed those ß-carotene deficient diets. In the second experiment (Peirce 1954) the sheep fed on diets with different levels of ß-carotene (≈7, 25, 50, 75 and 100 µg/kg BW/day) before and during mating and gestation periods (Table 5.1). The study reported the lambing performance for each diet at least during two reproduction cycles. Survival rates of the lambs calculated from the study for the different diets were 50, 48, 65, 66.7 and 62.2 % for 7, 25, 50, 75 and 100 µg/kg BW/day, respectively during the first week (Table 5.1). Although the diet used in this second experiment is also ß-carotene deficient according to the NRC recommendation and the fact that the study was challenged with a lot of complications, the results indicated to some degree higher survival rates for the lambs, as the level of β-carotene increases. Salem *et al* (Salem et al. 2015) reported that injection of lambs with 100 µg β-carotene/kg BW twice per week increases oestrous cases and estradiol-17β concentrations at puberty and post-puberty oestrus. No green forages were offered, which indicates that the diet did not fulfil the lamb requirement of β-carotene (Table 5.1). The results of both studies by Salem *et* al (Salem et al. 2015) and Peirce (Peirce 1954) indicate that β-carotene supplementation may enhance the production and reproduction performance of the sheep fed on a carotene-deficient diet.

Brozos *et al* (Brozos et al. 2007) found that supplementation of 1644 µg β-carotene/kg BW/day does not affect body weight, luteinizing hormone (LH) concentration after oestrous synchronization and milk yield of ewes fed on a diet that fulfils their requirements of vitamin A and β-carotene (Table 5.1). Though, no details were given regarding the diet used, Köse *et al* (Köse et al. 2013) reported that injection of ewes with 1000 µg β-carotene/kg BW/day on the day of sponge withdrawal did not affect the oestrous response, pregnancy rate, lambing rate and litter size. Gore (Gore 2016) supplemented goats with 2000 µg β-carotene/kg BW/day for 60 days starting 28 days before oestrous synchronization and with 1000 µg β-carotene/kg BW/day from drying off until kidding (~ 60 days). The β-carotene supplementation did not affect milk production, body weight, oestrous parameters, oestradiol-17β concentration, number of follicles, size of largest follicle and CL, gestation length, litter size and birth weight of the kids. However, the supplementation increased plasma progesterone (P4) concentration and glutathione peroxidase activity. The diet used in the study fulfiled the goat requirements for β-carotene (Table 5.1). A number of researchers reported a tendency of increase but not statistically significant ($p > 0.05$) in total follicles, corpus luteum number, total ovarian activity (Arellano-Rodriguez et al. 2007, Arellano-Rodriguez et al. 2009), and serum progesterone (P4) concentration in goats (Arellano-Rodriguez et al. 2009). The goats were supplemented with approximately 1116 µg β-carotene/kg BW/day (Table 5.1). A similar non-significant trend ($p > 0.05$) of increase in total follicles number, total corpus luteum number and total ovarian activity was reported again by Meza-Herrera et al and López-Flores et al (Meza-Herrera et al. 2013, López-Flores et al. 2018) in addition to a decrease in the growth hormone (López-Flores et al. 2018). An increase in the ovulation rate combined with lower LH concentrations was also reported (Meza-Herrera et al. 2013). The goats in the latter two studies were supplemented with approximately 1089 µg β-carotene/kg BW/day (Table 5.1). The diet used by the

researchers fulfilled the goats requirements for β-carotene (Table 5.1). It is reported (Meza-Herrera et al. 2011, Meza-Herrera et al. 2014, Meza-Herrera et al. 2017) that supplementation of 2890 µg β-carotene/kg BW/day had no effect on the percentage of goats depicting puberty or on their live weight. Nevertheless, the supplementation had increased serum insulin concentration (Meza-Herrera et al. 2011). The diet used by the research fulfilled the requirement of β-carotene (Table 5.1). On this basis thereof, β-carotene supplementation may increase the levels of some hormones such as progesterone in the blood of sheep and goats fed on diets that fulfil their requirements of β-carotene. However, such supplementation above their β-carotene requirements does not appear to affect their production and reproduction parameters such as body weight, milk production, oestrous parameters, conception rate, pregnancy rate, and litter size.

Table 5.1 A list of peer-reviewed published articles that show the level of β-carotene supplemented, the level of β-carotene offered already in the diet and the animal requirements of β-carotene used in each study

NO	References	Species	The physiological condition	β-carotene requirement (µg/kg BW/day)*	Type of the diet#	β-carotene in the diet (µg/kg BW/day)	β-carotene supplementation (µg/kg BW/day)
1	(Peirce 1954)	Sheep	Pregnancy	227.5	Chaffed wheaten straw, Linseed meal, Wheat and barley	7-8	25, 50, 75 and 100
2	(Salem et al. 2015)	Sheep	Breeding	157	Concentrate	Not mentioned	100 (twice/ week)
3	(Brozos et al. 2007)	Sheep	Lactation	267	Concentrate, alfalfa hay and wheat straw	>348,97	1644.74
4	(Köse et al. 2013)	Sheep	Breeding	157	Not mentioned	Not mentioned	1000
5	(Gore 2016)	Goats	Breeding	157	Total mixed ration (TMR)	>676.6	2000
			Pregnancy	227.5	Total mixed ration (TMR)	>676.6	1000
6	(Arellano-Rodriguez et al. 2007)	Goats	Breeding	157	Alfalfa hay, corn silage and corn grain	>598.21	1116
7	(Arellano-Rodriguez et al. 2009)	Goats	Breeding	157	Alfalfa hay, corn silage and corn grain	>598.21	1116
8	(Meza-Herrera et al. 2013)	Goats	Breeding	157	Alfalfa hay, corn silage and corn grain	>601.31	1089
9	(López-Flores et al. 2018)	Goats	Breeding	157	Alfalfa hay, corn silage and corn grain	>601.31	1089
10	(Meza-Herrera et al. 2013)	Goats	Breeding	157	Alfalfa hay, corn silage and corn grain	>601.31	1089
11	(Meza-Herrera et al. 2011)	Goats	Prepubertal (early maturing)	500	Alfalfa hay, corn silage and corn grain	>774.57	2890
12	(Meza-Herrera et al. 2014)	Goats	Prepubertal (early maturing)	500	Alfalfa hay, corn silage and corn grain	>774.57	2890
13	(Meza-Herrera et al. 2017)	Goats	Prepubertal (early maturing)	500	Alfalfa hay, corn silage and corn grain	>774.57	2890

*The requirements were calculated according to the (NRC 2007).

#The level of β-carotene in alfalfa hay (1 kg = 20.3 mg) where estimated according to Charkey *et al* (Charkey et al. 1961)

5.6 Conclusions and recommendations

In conclusion, β-carotene supplementation enhances the production and reproduction performance of the female sheep fed on a carotene-deficient diet. Supplemental β-carotene may increase the levels of some hormones such as progesterone in the blood of sheep and goats fed on diets that fulfil their requirements of β-carotene. However, such supplementation above their β-carotene requirements does not appear to significantly affect their production and reproduction parameters such as body weight, milk production, oestrus parameters, conception rate, pregnancy rate, and litter size. In future studies, it may be advisable to determine the diet β-carotene content and to plan experiments using either a β-carotene deficient diet or a diet that fulfils the animal requirements of β-carotene.

CHAPTER SIX

Conclusions, Recommendations, Further Research and Critical Evaluation

6.1 Conclusions

Natural antioxidants in animal nutrition have recently gained attention due to consumer safety concerns. The study concluded that tannin extract is a promising feed supplement for ruminants due to its antioxidant activity and other co-benefits. The results suggest that encapsulating TE provides maximum benefit from the TE supplement (e.g., sperm quality in sheep). One of the key issues limiting tannin extract use in ruminant feed is its negative effect on feed intake and digestion. The study demonstrated based on a meta-analysis that tannin extract should not exceed 10 g/kg DM in cattle and 2.3 g /kg DM in sheep as higher levels may drastically affect the nutrient digestibility. As a result of knowing those threshold levels, TE inclusion in ruminant feed (sheep and cattle) can be used for a multitude of applications and co-benefits without compromising the animal performance. β-carotene supplementation to fulfil the requirement is a crucial element to enhance the production and reproduction of female sheep fed on a β-carotene diet. Nevertheless, supplementing sheep above their β-carotene requirements does not seem to significantly affect their production and reproduction parameters such as body weight, milk production, oestrous parameters, conception rate, pregnancy rate, and litter size. Moreover, this study is the first to report that, in grazing sheep, supplemental ß-carotene higher than 75 mg/day may act as a pro-oxidant during stressful conditions, such as parturition. Therefore, the supplementation of sheep with β-carotene above their requirement may not be recommended. This study paved the way for the optimal use of tannin extract and ß-carotene as antioxidant supplements for ruminants in general and sheep in particular.

6.2 Recommendations

- For various applications and co-benefits, tannin extract inclusion in ruminant feed should not exceed 10 g/kg DM in cattle and 23 g/kg in sheep.
- Encapsulation of tannin extract can be recommended to maximise the benefits of TE supplementation.
- Supplemental ß-carotene higher than 75 mg/day during stressful conditions, such as parturition may not be recommended.
- Quadruplets and triplets need extra care from farmers since they are less likely to survive than singles during their early days of life.
- In future studies, it might be advisable to determine the diet β-carotene content and plan experiments that either use a β-carotene deficient diet or a diet that meets the animal requirements for β-carotene.

6.3 Areas for further research

Future studies could encompass the following suggested topics:

- Effects of supplementation with levels higher than 0.06 g/kg BW/d of encapsulated tannin extract and lower than the threshold on oxidative status and reproduction performance in rams.
- Further studies to elucidate the mechanisms of action that condensed tannin extract has on semen quality and quantity are warranted.
- Testicular and or semen oxidative status following supplementation with condensed tannin extract (e.g., black wattle TE).
- Even though in this study the supplementation of ewes with ß-carotene did not affect litter sizes, quadruplets were only observed in the 100 mg (4.2%) and 75 mg (11.1%) groups. This observation might call for further studies on the effect of β-carotene supplementation before and during breeding, especially in herds with low fertility.

- Comparison of ß-carotene behaviour in sheep, goats, and cattle in terms of antioxidant activity, bioavailability, and metabolism.

6.4 Critical Evaluation

Study 1 succeeded in measuring the plasma oxidative status in the animals by adapting an assay recently used in humans (Mehdi and Rizvi 2013). Nonetheless, there was a chance to get more information if the oxidative status was also measured for semen and/or testicular tissue. The result of study 1 showed no significant difference in semen quality and oxidative status of the rams between the control group and the tannin-treated groups. The lack of significance could be due to the levels of tannin extracts used being very small (0.03 and 0.06 g/kg BW/day).

In study 2, The primary limitation of using P-values in model selection is that they are sensitive to sample size. A large sample size may produce highly significant p-values for trivial effects, while a small sample size can result in nonsignificant p-values for important and strong effects (Wang and Long 2022). Therefore, a reasonable number of observations for each parameter under investigation must be provided. This presented a challenge in this study to account for the physiological status of the animals (lambs, heifers, cows, steers, rams, wethers, lactating ewes and cows ..etc). By dividing the data into such groups, the number of observations per group will be very small. As a result, one of the limitations of this meta-analysis is that it didn't account for the physiological status of the animals. Instead, animals were divided into broader categories based on their growth stage: growing and mature. Not accounting for the physiological status necessitated excluding lactating cows and ewes from the data of N balance and weight gain due to their milk production.

The backwards-stepwise process starts with all potential predictors and then eliminates the least statistically significant potential predictor, one by one. When each predictor remaining in the equation is statistically significant, the elimination process ends. If there are many

potential predictors, backward elimination is challenging and impossible if there are more potential predictors than observations (Smith 2018). Therefore potential predictors need to be thought of deeply and should be derived from good theory (Anderson and Burnham 2004). In this meta-analysis, seven potential predictors were derived and subjected to a backward stepwise procedure. The maximum number of observations per stepwise procedure was 63 in the OMD response data set, while the minimum was 32 in the weight gain response data set. Accordingly, in this study, there was no challenge associated with large numbers of predictors for the stepwise procedure. However, statisticians recommend having at least 10 observations per variable (potential predictor). Therefore, the number of observations required for the full-fit models might be one of the limitations of this study, especially for ADFD and WG models. Finally, the largest concern regarding using stepwise methods for variable selection is the fact that it generates biased regression coefficients, confidence intervals and p-values (Harrell 2015). Due to this bias, it is believed that the stepwise selection may delete some of the important predictors (Harrell 2015). However, The full model may require more predictors than the researchers or the stakeholders care to collect in future samples (Harrell 2015). It also requires predicted values to be conditional on all of the predictors (Harrell 2015). Therefore, using a model with all potential predictors limits the generalization ability of predictive models; overfitting (Olusegun et al. 2015). Nonetheless, a full model fit is considered to provide accurate standard errors, error mean square, and P-values (Harrell 2015). In this study using the full fit models with all potential predictors- as a reference, we found that all the significant predictors in the full fit were selected by the stepwise selection, those composed 77% of the selected variables. Those with a trend ($P < 0.1$) were also selected and composed 5% of the selected variables. Therefore, we believe the bias associated with backward stepwise selection for variables might be minimum in this study. However, the use of backward stepwise selection is considered one of the limitations of this study. Indeed, the meta-analysis performed in this

study paved the way to define the threshold levels for tannin extract in the ruminant feed, providing a quantitative data that is very useful for researchers and stockholders.

Due to the limited number of studies done on sheep and goats a systematic review (chapter five) approach was used to answer the question regarding the benefit of supplemental β-carotene when the animals fed on a diet below or above their requirement of β-carotene (NRC 2007). In the future, with the expected accumulation of studies related to the subject, a meta-analysis approach might be better in answering this question.

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