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# Effect of condensed tannin and fresh forage diets on the formation of indole and skatole in the rumen and on the pastoral odour and flavour of sheep meat

A thesis presented in partial fulfilment of the requirements for the degree of

## **Doctor of Philosophy**

in Animal Science

at Massey University Palmerston North New Zealand

Nicola Maria Schreurs 2006

## DECLARATION

This is to certify that the research carried out for this Doctoral thesis was completed by the candidate (Nicola Maria Schreurs) whilst a postgraduate student in the Institute of Veterinary, Animal and Biomedical Sciences, Massey University, Palmerston North, New Zealand.

It is confirmed that the candidate has pursued the course of this study in accordance with the requirements of the Massey University regulations. I hereby affirm that the content of this thesis is the original research conducted by the candidate and has not been used in part or whole for any other qualification. I certify to the best of my knowledge that all references to previous work are included in the reference sections and any assistance received has been acknowledged.

All ethical requirements applicable to this study have been complied with as required by the legislation and have been approved by the Animal Ethics Committees of AgResearch Limited and Massey University. The applicable ethical authorisation codes are: 37/01, 17/02, 33/03-03 (AgResearch Grasslands Animal Ethics Committee) and \$2/115 (Massey University Animal Ethics Committee).

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## This thesis is dedicated to Thomas

For my clever Thomas... Who knew when to be silent and then what to say Who knew to provide comfort and when it was best to stay away "Dear sir or madam, will you read my book? It took me years to write, will you take a look?"

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Paperback writer The Beatles

## ABSTRACT

Flavour is a factor that has a large influence on meat quality. Pastoral flavour that results from the grazing of pasture is an undesirable characteristic of meat flavour for consumers more accustomed to meat produced by grain and concentrate feeding systems. In New Zealand there is a reliance on grazing systems for sheep meat production, however the resulting meat flavour is one factor that impedes the increase of sheep meat exports to discerning markets.

Correlation of chemical analyses to sensory evaluations of sheep meat has identified that a high concentration of indole and skatole in the fat is associated with pastoral flavours. Indole and skatole are formed in the rumen from the microbial fermentation of tryptophan. New Zealand pasture is high in protein, which is both highly soluble and rapidly degraded in the rumen. As such, pasture diets provide a rich and ready source of tryptophan for the formation of indole and skatole in the rumen. Condensed tannins are known to slow the degradation of protein in the rumen. Therefore, one of the objectives of this study was to establish if dietary condensed tannin can reduce the ruminal biogenesis of indole and skatole and consequently, ameliorate pastoral flavour in sheep meat.

White clover usually comprises up to 30% of the botanical composition of pastures in New Zealand, is highly degradable in the rumen and likely to result in a high availability of tryptophan in the rumen for conversion to indole and skatole. Therefore, another objective of this study was to determine if feeding white clover gave a significant increase in the formation of indole and skatole in the rumen compared to perennial ryegrass and if this has an effect on pastoral flavour in meat.

These hypotheses were tested using a series of *in vitro* rumen fermentations that incorporated the use of fresh forages (Chapter 3 and 6). *In vivo* experiments were utilised to assess rumen formation of indole and skatole with different forages (Chapter 4) and to assess effects of dietary condensed tannin (CT; Chapter 5 and 7). Meat and fat samples from lambs used in Chapters 5 and 7 underwent sensory evaluation to determine if forage or CT treatments were having an effect on the fat odour or meat flavour.

From the *in vitro* and *in vivo* experiments of (Chapter 3, 4, 6 and 7) it was calculated that the formation of indole and skatole with perennial ryegrass is generally only 6-41% of that formed with white clover. A higher concentration of indole and skatole was also observed in the blood plasma of lambs that were fed white clover compared to those that were fed perennial ryegrass (Chapter 7) and white clover gave an overall more intense flavour in the meat.

Comparison of forages fermented *in vitro* (Chapter 3) showed that with forage legumes of a higher CT concentration, such as *Lotus pedunculatus* (98 gCT kg<sup>-1</sup> DM) and *Dorycnium rectum* (122 gCT kg<sup>-1</sup> DM), the indole and skatole formed was only 7-21% of that formed with white clover. With forages of an intermediate CT concentration such as sulla *(Hedysarum coronarium)* and *Lotus corniculatus* the indole and skatole concentration formed was 53-68% of that of white clover. From *in vitro* rumen fermentation of mixtures of white clover and *Lotus pedunculatus* it was concluded that the CT in *Lotus pedunculatus* was not reacting with the protein in white clover. Fermentation of fresh white clover in the presence of an increasing concentration of added CT extract showed that at a higher CT, indole and skatole formation were reduced to low levels. It was inferred that this was due to optimal protein binding and the availability of free condensed tannin to bind other sources of protein, including rumen microbes. However, *in vivo* dosing with a CT extract resulted in only a small reduction in rumen indole and skatole concentration. This indicated that when CT was dosed into the *in vivo* rumen of lambs fed fresh forages the CT probably passed from the rumen before adequate protein release from the forage had taken place. Thus, in the grazing situation it will be optimal to provide CT *in planta* to maximise protein binding and this, in combination with the high CT concentration needed (approximately 80 gCT kg<sup>-1</sup> DM), makes *Lotus pedunculatus* or *Dorycnium rectum* the prime candidates for further grazing trials into pastoral flavour amelioration using CT forages.

Grazing *Lotus corniculatus* (40 gCT kg<sup>-1</sup> DM) in a field experiment resulted in a lower rumen and blood plasma and fat concentration of indole and skatole in comparison to the grazing of perennial ryegrass/white clover pasture. However, a change in the pastoral odour of the fat was not perceived by the sensory panel when comparing fat samples from lambs that had grazed *Lotus corniculatus* and perennial ryegrass/white clover pasture. The concentration of skatole in the body fat was less variable in the lambs that had grazed *Lotus corniculatus* and resulted in no lambs with a high outlying concentration (>100 ng g<sup>-1</sup>) of indole and skatole. This finding holds some potential for reducing pastoral flavour for consumers sensitive to high indole and skatole concentration in the fat. When condensed tannin was dosed to lambs that were fed white clover or perennial ryegrass in the form of a grape seed extract the intermittent supply of CT slightly reduced indole and skatole formation in the rumen and reduced the plasma concentration of indole and skatole. Flavour assessment of meat from the lambs fed white clover or perennial ryegrass with or without CT suggested that CT reduced the intensity of pastoral flavours. However, there were minimal effects on indole and skatole concentration in the body fat. It was possible that other pastoral flavour related compounds derived from the degradation of amino acids, in addition to indole and skatole that were measured, were having an effect on the meat flavour.

It was concluded that dietary condensed tannin is able to reduce the formation of indole and skatole in the rumen and can alter the sensory attributes of sheep meat including reducing pastoral flavours. A higher CT concentration present within the forage plant (approximately 80 g kg<sup>-1</sup> DM) will be best to minimise indole and skatole formation in the rumen and reduce pastoral flavours in the meat. Further research is required to confirm this in the grazing situation. Feeding white clover results in a greater rumen biogenesis of indole and skatole compared to perennial ryegrass and therefore, may be the primary contributor to pastoral flavours when ruminants graze conventional pastures. Further research is required to evaluate the flavour attributes that result from feeding white clover to meat producing ruminants in the New Zealand grazing situation.

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# TABLE OF CONTENTS

ABSTRACT	i
ACKNOWLEDGEMENTS	v
TABLE OF CONTENTS	vii
LIST OF TABLES	xiii
LIST OF FIGURES	xvii
LIST OF ABBREVIATIONS	xxi
GENERAL INTRODUCTION	xxiii
CHAPTER 1: LITERATURE REVIEW	1
1.1. Introduction	2
1.2. Flavour of Meat Products from Ruminants	5
1.2.1. Definition of flavour and flavour perception	5
1.2.1.1. Olfactory perception	5
1.2.1.2. Quantitative and qualitative aspects	7
1.2.2. Chemical components of meat flavour	8
1.2.2.1. Volatile compounds of meat flavour and aroma	9
1.2.2.2. Effect of lipids on meat flavour	10
1.2.3. Factors affecting meat flavour	11
1.2.3.1. Differences hetween species.	
1.2.3.2. Breed/Genetic differences	12
1.2.3.3. Differences between sex and castration classes	13
1.2.3.4. Changes with age of animal.	13
1.2.3.5. Changes with liveweight and fat cover	14
1.2.3.6. Pre-slaughter effects	14
1.2.3.7 Cooking effects	14
1.2.3.8. Animal nutrition and diet on meat flavour.	
1.3. Pastoral Flavours of Ruminant Meat	17
1.3.1. Grain vs. forage feeding on meat flavour	
1.3.2 Elayour perception of meat from animals fed nasture	18
1.3.2.1 Flavour description of meat from pasture and grain-fed animals	18
1 3 2 2 Grass vs. Legume flavours	21
1 3 2 3 Causative volatile agents for nastoral flavours	22
1.3.3. The influence of skatole and indole on pastoral flavour	24
1331 Detection thresholds for skatole and indole	26
14 Formation of Skatole and Indole by Ruminants	28
1.4.1. Structures and properties of indolic compounds	28
1 4 1 1 Indole and skatole chemical structure	28
1412 Chemical physical and biological properties of indoles	20
1 4 1 3 Relation of chemical/physical properties to adour/sensory characteristics	20
1.4.2 Rumen protein digestion and degradation	30
1.4.2.1 Formation of free amino acids in the rumen from dietary protein	30
1.4.2.2. Amino acid utilisation and degradation by microbes in the rumen	31
1.4.3 Formation of Skatole and Indole from Tryptonban in the Rumen	32
1.4.3.1 Determination of truntonhan as the precursor for indole and skatole	32
1.4.3.2. Elucidating the formation pathway of skatole and indole in the rumen	32
1.4.4 Microbes involved in formation of indolic compounds	34
1 4 4 1 Lactobacillus so strain 11201	35
1 4 4 2 Racteria and protozoa interactions	
1 4 4 3 Rumen conditions for indole and skatole formation by microhes	36
1.5 Absorption and Metabolism of Indole and Skatole	
1.5.1 Absorption of indoles in the ruminant	38
1.5.1.1 Absorption from the digestive tract	28
1512 Absorption from the blood	10
1.5.2 Metabolism and excretion of skatole and indole in the ruminant	40
1.2.2.1.2.2.1.2.1.2.1.2.2.2.2.2.2.2.2.2	

1.5.2.2. Urinary metabolites	41
1.5.2.3. Pathways of skatole metabolism	
1.5.2.4. Role of the liver and P450 cytochrome	
1.5.3. Relationship of liver metabolism and deposition of indoles in the fat	47
1.6. Changing Meat Flavour by Controlling Skatole and Indole Formation and Met	abolism in
Ruminants	49
1.6.1. lonophore antibiotics	49
1.6.1.1. Effect of ionophore antibiotics on skatole concentration in the rumen	
1.6.1.2. Mode of action	50
1.6.2. Microbial manipulations	50
1.6.2.1. Defaunation to decrease degradation of protein in rumen	51
1.6.2.2. Defaunation to reduce conversion of tryptophan to skatole and indole	51
1.6.3. Animal Factors	
1.6.3.1. Animal variation and selection	
1.6.3.2. Improving liver metabolism	53
1.6.4. Feeding Regimes	
1.6.4.1. Grain and concentrate diets	54
1.6.4.2. Increased readily fermentable carbohydrate	
1.6.6. Reducing rumen protein degradation using condensed tannins	
1.7. Condensed Tannins	
1.7.1. Prevalence and chemical structure of condensed tannins	
1.7.1.1. Prevalence of condensed tannins in plants	60
1.7.1.2. The condensed tannin monomers	
1.7.1.3. Stereochemistry of monomers	60
1.7.1.4. Interflavonoid linkage	61
1.7.1.5. Procyanidin to prodelphinidin ratio	61
1.7.2. Chemical properties and protein binding activity of condensed tannins	
1.7.2.1. Molecular weight	62
1.7.2.2. Binding of condensed tannin to protein to form complexes	63
1.7.2.3. pH dependent activity	64
1.7.2.4. Effect of polyethylene glycol	64
1.7.3. Condensed tannins and the ruminant	64
1.7.3.1. Reduced protein degradation in the rumen	65
1.7.3.2. Anti-microbial effects	66
1.7.3.3. Increased amino acid absorption in intestines	
1.7.3.4. Digestion and absorption of condensed tannins by the ruminant	67
1.7.3.5. Detrimental effects of condensed tannins in ruminant nutrition	
1.7.4. Effects of condensed tannin on rumen skatole and indole formation	67
1.7.4.1. Condensed tannin forages and indoles in milk and fat	68
1.8. Conclusions and Hypotheses.	70
1.9. References	75

I

CHAPTER 2: MATERIALS AND METHODS	92
2.1. Introduction	93
2.2. Measurement of Indoles in Rumen Fluid by Solid Phase Extraction and High I	Performance
Liquid Chromatography	95
2.2.1. Solid phase extraction	95
2.2.2. High Performance Liquid Chromatography (HPLC)	100
2.2.3. Quality criteria of determinations of indoles in rumen fluid	
2.3. Measurement of Indole and Skatole in Plasma by Ether Extraction and High I	Performance
Liquid Chromatography	
2.3.1. Calibration	
2.3.2. Extraction procedure	105
2.3.3. High Performance Liquid Chromatography (HPLC)	106
2.3.4. Quality criteria of determinations of indoles in plasma	106
2.4. Measurement of Indole and Skatole in Fat by Simultaneous Distillation Extract	ion and Gas
Chromatography/Mass Spectrometry	107

2.4.1. Simultaneous distillation extraction	107
2.4.2. Gas chromatography/ mass spectrometry	107
2.4.3. Quality criteria of determinations of indoles in fat	108
2.5. Flavour and Odour Panels	109
2.5.1. Preparation of fat for odour assessment	109
2.5.2. Preparation of meat samples for flavour assessment	
2.5.3. Group sessions	110
2.5.4. Individual assessment for odour or flavour scoring	
2.6. In Vitro Forage Fermentation Incubations	
2.6.1. Forage preparation	
2.6. In Vitro Forage Fermentation Incubations         2.6.1. Forage preparation         2.6.2. Preparation of incubation bottles	
<ul> <li>2.6. In Vitro Forage Fermentation Incubations</li> <li>2.6.1. Forage preparation</li> <li>2.6.2. Preparation of incubation bottles</li> <li>2.6.3. Rumen fluid inoculate</li> </ul>	
<ul> <li>2.6. In Vitro Forage Fermentation Incubations</li> <li>2.6.1. Forage preparation</li> <li>2.6.2. Preparation of incubation bottles</li> <li>2.6.3. Rumen fluid inoculate</li> <li>2.6.4. Sampling protocol and timing</li> </ul>	
<ul> <li>2.6. In Vitro Forage Fermentation Incubations</li> <li>2.6.1. Forage preparation</li> <li>2.6.2. Preparation of incubation bottles</li> <li>2.6.3. Rumen fluid inoculate</li> <li>2.6.4. Sampling protocol and timing</li> <li>2.7. Chemicals and Compounds</li> </ul>	
<ul> <li>2.6. In Vitro Forage Fermentation Incubations</li> <li>2.6.1. Forage preparation</li> <li>2.6.2. Preparation of incubation bottles</li> <li>2.6.3. Rumen fluid inoculate</li> <li>2.6.4. Sampling protocol and timing</li> <li>2.7. Chemicals and Compounds</li> <li>2.8. Discussion</li> </ul>	

# 

INDOLL AND SKATOLL IN IN VITRO ROMENT LEMILITATIONS	
3.1. Abstract	121
3.2. Introduction	122
3.3. Materials and Methods	124
3.3.1. Experimental design	124
3.3.2. Forage preparation	125
3.3.2.1. Experiment 1: Varied forage condensed tannin concentration	125
3.3.2.2. Experiment 2: Forage maturity	125
3.3.2.3. Experiment 3: Nitrogen fertiliser application	126
3.3.2.4. Post-harvest storage and forage analyses	126
3.3.3. Rumen fluid inoculate	127
3.3.4. In vitro rumen incubation method	127
3.3.5. Sampling and metabolite analysis	128
3.3.6. Statistical analysis	129
3.4. Results	130
3.4.1. Experiment 1: Varied forage condensed tannin concentration	130
3.4.1.1. Nutrient composition of the forages	130
3.4.1.2. Metabolite formation	132
3.4.1.3. Addition of polyethylene glycol to incubations	136
3.4.2. Experiment 2: Forage maturity	136
3.4.2.1. Nutrient composition of the forages	136
3.4.2.2. Metabolite formation	137
3.4.3. Experiment 3: Nitrogen fertiliser application	140
3.4.3.1. Nutrient composition of the forages	140
3.4.3.2. Metabolite formation	140
3.4.4. Indole, skatole, ammonia and branched-chain VFA correlations	141
3.5. Discussion	143
3.6. Acknowledgements	149
3.7. References	150

CHAPTER 4: CONCENTRATION OF INDOLES AND OTHER RUMEN ME	TABOLITES IN
SHEEP AFTER A MEAL OF FRESH WHITE CLOVER, PERENNIAL RYEGR	ASS OR LOTUS
CORNICULATUS AND THE APPEARANCE OF INDOLE IN THE BLOOD	155
4.1. Abstract	
4.2. Introduction	
4.3. Materials and Methods	
4.3.1. Experimental design	

4.3.2. Animal housing and care	157
4.3.3. Experiment 1: Profile of rumen metabolites when feeding fresh forages	158
4.3.3.1. Forages and Feeding	158
4.3.3.2. Rumen Pool Size	159
4.3.3.3. Rumen fluid sampling and analysis	159
4.3.4. Experiment 2: Comparison of blood plasma and rumen fluid indole and skatole	
4.3.4.1. Forages and Feeding	
4.3.4.2. Rumen fluid sampling and analysis	
4.3.4.3. Blood sampling and plasma analysis	
4.3.5. Statistical analyses	
4.4. Results	
4.4.1. Composition of diets and intakes	
4.4.2. Rumen pools	
4.4.3. Skatole and indole production in the rumen	
4.4.4. Total nitrogen, soluble nitrogen and ammonia	
4.4.5. Volatile fatty acids	
4.4.6. pH	
4.4.7. Plasma and rumen comparison	
4.5. Discussion	
4.6. Acknowledgements	
4.7. References	

#### CHAPTER 5: SKATOLE AND INDOLE CONCENTRATION AND THE ODOUR OF FAT FROM LAMBS THAT HAD GRAZED PERENNIAL RYEGRASS/WHITE CLOVER PASTURE 5.4.1. Experiment 1: Comparison of rumen fluid obtained from stomach lavage and fistula ....... 198

#### 

6.2. Introduction	.223
6.3. Materials and Methods	.225
6.3.1. Experimental design	.225
6.3.2. Forage preparation	. 226
6.3.3. Extraction of CT from Lotus pedunculatus.	.226
6.3.4. Rumen fluid inoculate	.227
6.3.5. In vitro rumen incubation method.	.227
6.3.6. Sampling and metabolite analysis	.228
6.3.7. Statistical analysis	. 229
6.4. Results	.231
6.4.1. Nutrient composition of the forages	.231
6.4.2. Experiment 1: Metabolite formation when incubating ratios of white clover and Lotus	
pedunculatus	.231
6.4.3. Addition of polyethylene glycol to incubations with ratios of white clover and Lotus	
pedunculatus	.232
6.4.4. Experiment 2: Metabolite formation when incubating white clover with a CT extract from <i>pedunculatus</i>	Lotus .232
6.4.5. Experiment 3: Metabolite formation when incubating white clover and perennial ryegrass	with a
CT extract from grape seed	.237
6.4.6. Experiment 4: Skatole and indole formation when adding tryptophan and PEG to incubation	ons
with Lotus pedunculatus	.240
6.5. Discussion	.245
6.6. Acknowledgements	.250
6.7. References	.251

CHAPTER 7: THE EFFECT OF SUPPLEMENTATION OF A WHITE C	LOVER OR
PERENNIAL RYEGRASS DIET WITH GRAPE SEED EXTRACT ON INDOLE AN	<b>NS SKATOLE</b>
METABOLISM AND THE SENSORY CHARACTERISTICS OF LAMB	255
7.1. Abstract	
7.2. Introduction	
7.3. Materials and Methods	
7.3.1. Experimental design	258
7.3.1.1. Experiment 1: Indole and skatole in the fat of pre- and post-chilled carcasses	
7.3.1.2. Experiment 2: Sensory aspects of meat from lambs fed white clover or perent	ial ryegrass
and supplemented with a grape seed extract	
7.3.2. Animal housing and management	
7.3.3. Feeding and dosing the grape seed extract	259
7.3.4. Animal measurements	
7.3.5. Sampling	
7.3.6. Laboratory methods	
7.3.6.1. Rumen fluid analysis for indoles, volatile fatty acids and ammonia	
7.3.6.2. Plasma analysis for indoles, ammonia and urea	
7.3.6.3. Fat analysis for indoles	
7.3.7. Odour assessment of fat by sensory panel	
7.3.8. Flavour assessment of minced meat by sensory panel	
7.3.9. Statistical analysis	
7.4. Results	
7.4.1. Experiment 1: Indole and skatole in the fat of the pre- and post-chilled carcass	
7.4.2. Experiment 2: Sensory aspects of meat from lambs fed white clover or perennial r	yegrass and
supplemented with a grape seed extract	
7.4.2.1. Composition of diets and intakes	
7.4.2.2. Ammonia and VFA in rumen fluid prior to slaughter	
7.4.2.3. Rumen indole and skatole	
7.4.2.4. Plasma indole and skatole	
7.4.2.5. Plasma ammonia and urea	
7.4.2.6. Live weight, live weight gain, carcass weight and fatness values	
7.4.2.7. Indole and skatole concentration in the fat	

7.7.2.8. Odour of fat and flavour of meat	
7.5. Discussion	
7.6. Acknowledgements	
7.7. References	288

CHAPTER 8: GENERAL DISCUSSION	293
8.1. Introduction	291
8.2. Methodological Considerations	295
8.2.1. Experimental design	295
8.2.2. In vitro rumen fermentations	296
8.2.3. Animal numbers	297
8.2.4. Single time-point sampling of dynamic biological systems	297
8.2.5. Accuracy and precision of analyses	298
8.2.6. Sensory panels	298
8.3. The Effect of Different Forages on Indole and Skatole Formation in the Rumen	300
8.3.1. Differences in the formation of indole and skatole between forages	300
8.3.2. Forage-based factors that influence indole and skatole formation	300
8.3.3. White clover vs. perennial ryegrass effects on meat flavour	303
8.4. What Role do Condensed Tannins have in Lowering Indole and Skatole Formati	on and
Ameliorating Pastoral Flavour?	304
8.4.1. Concentration of condensed tannin (CT)	304
8.4.2. Endogenous vs. exogenous plant CT	305
8.4.2. Effect of CT on the indole and skatole concentration in the blood and fat and the pastoral	odour
and flavour	306
8.4.3. Dietary CT and its effect on pastoral odour and flavour in sheep meat	308
8.5. Conclusions and Future Research	311
8.5.1. Major conclusions of this thesis	311
8.5.2. Future research	312
8.6. References	316

## LIST OF TABLES

#### Chapter 1:

Table 1.3. Composition of pasture compared to concentrates (g kg<sup>-1</sup>) and the ratio of nitrogen and carbohydrates (adapted from Young *et al.*, 2003).....pg 55

#### Chapter 2:

Table 2.1. Accuracy and precision of indole and skatole determination in rumen fluidpg 101
Table 2.2. Volumes of standards used to create calibration standards and the concentration of 2-         methylindole, indole and skatole in the calibration standards
Table 2.3. Response factors and r-squared values for the calibration curves obtained for indole and skatole
Table 2.4. Precision and accuracy of indole and skatole determination in plasmapg 105
Table 2.5. Descriptors/definitions associated with the categorical odour/flavour attributes used in sensory analysis
Table 2.6. Chemicals and compounds used in methods outlined in this Materials and Methods section

#### Chapter 3;

Table 3.1. Experiment 1. Nutrient composition of the minced forages used in the <i>in vitro</i> incubations
Table 3.2. Experiment 1. Concentration of indole and skatole, ammonia and volatile fatty acids (VFA) in the media after 10 hours of incubating seven forages in the absence of polyethylene glycol
Table 3.3. Experiment 2. Nutrient composition of foliage from young and mature white clover, perennial ryegrass, and <i>Lotus corniculatus</i> . The foliage was minced and used in the <i>in vitro</i> incubations

#### Chapter 4:

Table 4.3. Experiment 1. Maximum rumen concentration of indolic compounds, ammonia and volatile fatty acids (VFA) adjusted for intake of crude protein (CPI) or dry matter (DMI) when feeding white clover, perennial ryegrass or *Lotus corniculatus*. Also included for comparison is minimum rumen pH......pg 169

Table 4.4. Experiment 1. Time taken after the start of feeding to reach maximum rumen concentration of indolic compounds, ammonia, volatile fatty acids (VFA) when feeding white clover, perennial ryegrass or *Lotus corniculatus*. Also included for comparison is time taken to reach minimum rumen pH......pg 170

#### Chapter 5:

Table 5.2. Experiment 2. Mean concentration of metabolites and the proportion of volatile fatty acids from the rumen fluid of lambs after 111 days of grazing perennial ryegrass/white clover pasture (PRG/WC; *Lolium perenne/Trifolium repens*; n=12) or *Lotus corniculatus* L.( cv. Grasslands Goldie; n=12) in the summer of 2002/2003......pg 204

Table 5.3. Experiment 2. Mean, range and absolute value of residuals (z; a measure of variance) of indole and skatole concentration observed in the rumen and plasma prior to slaughter (after 111 days grazing) and in the tail-stub fat of lambs that grazed perennial ryegrass/white clover

pasture (PRG/WC; Lolium perenne/Trifolium repens) or Lotus corniculatus (cv. Grasslands Goldie)......pg 209

Table 5.4. Experiment 2. Least squares (LS) mean odour scores of inter-muscular fat from lambs slaughtered prior to being allocated to treatments (n=9) or after 111 days of grazing perennial ryegrass/white clover pasture (PRG/WC; *Lolium perenne/Trifolium repens*; n=9) or *Lotus corniculatus* (cv. Grasslands Goldie; n=9) in the summer of 2002/2003......pg 211

#### Chapter 6:

Table 6.3. Experiment 2. Mean concentration of indole and skatole, ammonia and volatile fatty acids (VFA) in the media after 10 hours of incubating white clover with condensed tannins (CT) extracted from Lotus pedunculatus added at 0-80 g kg<sup>-1</sup> of the dry matter content of the forages incubations. the square added to Results are least means of four replicates..... .....pg 238

#### Chapter 7:

Table 7.6. Experiment 2. Mean odour scores of inter-muscular fat from lambs fed white clover

#### Chapter 8:

Table 8.1. Influence of different forages on the odour and flavour of lamb meatpg 294
Table 8.2. Crossover design applicable to Experiment 1 of Chapter 4 when feeding White clover         (WC), Perennial ryegrass (PRG) and Lotus corniculatus (LC)pg 296
Table 8.3. Influence of different forages and condensed tannin (CT) on the formation of indole         and skatole in the rumen relative to control treatments         pg 302
Table 8.4. Effect of dietary condensed tannins on the indole and skatole concentration (parts perbillion; ppb) in the blood plasma and body fatpg 308

## LIST OF FIGURES

#### Chapter 1:

Figure 1.1. Odour receptor in nasal epithelium showing the pathway of activated and effector proteins to open membrane channels prompting an action potential (Liang and Jinks 1996).....pg 7

Figure 1.2. Intensities (scale: 0 = none, 10 = very high) of some flavour descriptors in meat from pasture- and concentrate-finished cattle. (Priolo et al., 2001).....pg 21

Figure 1.3. Principal components analysis of correlations between frequency of panellist's comments and relative concentrations of 18 odorous compounds in the headspace of subcutaneous fat from lambs 132 days old (Young et al., 2003).....pg 25

Figure 1.4. Chemical structures of indole and skatole.....pg 27

Figure 1.5. Pathway of indole and skatole formation from tryptophan in the rumen (from Deslandes *et al.*, 2001).....pg 33

Figure 1.6. Concentration of skatole in rumen fluid and jugular blood plasma of a cow over a 96-hour period after an intraruminal dose of L-tryptophan at time 0 (0.35g kg<sup>-1</sup> of body weight; from Yokoyama *et al.*, 1975).....pg 38

Figure 1.7. Pathways of skatole metabolism (from Bæk et al., 1997).....pg 43

Figure 1.8. Outline of the two phases involved in the metabolic pathway of indole and skatole in the ruminant.....pg 44

Figure 1.9. Catalytic cycle of cytochrome P450 in monooxygenase reactions (from Stryer 1995).....pg 47

Figure 1.10. The condensed tannin monomer. When R1 is H, the condensed tannin is an (epi)catechin polymer (procyanidin) and when R1 is OH, the condensed tannin is an (epi)gallocatechin polymer (prodelphinidin) (from Barry and McNabb 1999).....pg 61

#### Chapter 2:

Figure 2 2/3. Co Analyte	2.1. Ste olumn e elutio	eps involve conditionin	d in t g an	he solid phase d equilibratio	extraction, 4. Sa	on (SPE mple lo	) method: bading, 5.	1. sample pre- Interference c	ireatment, lution, 6.
Figure Techno	2.2. logy)	Structure	of	ISOLUTE®	ENV+	resin	(Source:	International	Sorbent
<b>F</b> '					1.	10.01	UTE® END		1.

Figure 2.3. Retention and elution interactions between ISOLUTE<sup>®</sup> ENV+ resin and the analyte (Source: International Sorbent Technology)......pg 99

#### Chapter 3:

Figure 3.1. Experiment 1. Indole formation *in vitro* when incubating seven different forages in the presence ( $\Box$ ) and absence ( $\bullet$ ) of polyethylene glycol (PEG). PEG inhibits the activity of condensed tannins. Results are the least square means of four replicates and the error bars are the standard error of the mean. \*, P<0.05; \*\*, P<0.01; \*\*\*, P<0.001......pg 134

Figure 3.2. Experiment 1. Skatole formation *in vitro* when incubating seven different forages in the presence ( $\Box$ ) and absence ( $\bullet$ ) of polyethylene glycol (PEG). PEG inhibits the activity of condensed tannins. Results are the least square means of four replicates and the error bars are the standard error of the mean. \*, P<0.05; \*\*, P<0.01; \*\*\*, P<0.001......pg 135

#### Chapter 4:

Figure 4.1. Experiment 1. Mean concentration of indole adjusted for crude protein intake (CPI) in the rumen of sheep (n=6) fed white clover  $(\triangle)$ , perennial ryegrass  $(\bullet)$  or Lotus corniculatus  $(\Box)$ . Error bars shown are the SEM and the double-ended arrow indicates the time over which feeding took place.....pg 168 Figure 4.2. Experiment 1. Mean concentration of skatole adjusted for crude protein intake (CPI) in the rumen of sheep (n=6) fed white clover  $(\triangle)$ , perennial ryegrass  $(\bullet)$  or Lotus corniculatus (D). Error bars shown are the SEM and double-ended arrow indicates the time over which feeding took place.....pg 168 Figure 4.3. Experiment 1. Least squares means of total nitrogen concentration per kg crude protein intake (CP1) in the rumen of sheep (n=4) fed white clover ( $\triangle$ ), perennial ryegrass (•) or Lotus corniculatus ( $\Box$ ). SEM = 1.47 g g<sup>-1</sup> per kg CPl. Double-ended arrow indicates the time over which feeding took place.....pg 171 Figure 4.4. Experiment 1. Least squares means of soluble nitrogen concentration per kg crude protein intake (CPI) in the rumen of sheep (n=4) fed white clover  $(\triangle)$ , perennial rycgrass  $(\bullet)$  or Lotus corniculatus ( $\Box$ ). SEM = 50.6 mg g<sup>-1</sup> per kg CP1. Double-ended arrow indicates the time over which feeding took place.....pg 171 Figure 4.5. Experiment 2. Mean concentration indole in the rumen fluid (•) and blood plasma ()) of sheep fed perennial ryegrass. Rumen concentration has been adjusted for crude protein intake (CPI). Error bars indicate the range and double-ended arrow indicates the period over which feeding took place.....pg 174 Figure 4.6. Experiment 2. Mean concentration of skatole in the rumen fluid (•) and blood plasma  $(\Box)$  of sheep fed perennial ryegrass. Rumen concentration has been adjusted for crude protein intake (CPI). Error bars indicate the range and double-ended arrow indicates the period over which feeding took place......pg 174

#### Chapter 5:

#### Chapter 6:

#### Chapter 7:

Figure 7.2. Experiment 2. Indole (A) and skatole (B) concentration in the rumen fluid over a 9 week treatment period for lambs fed white clover with ( $\blacksquare$ ) and without ( $\Box$ ) a supplement of grape seed extract or perennial ryegrass with ( $\bullet$ ) and without ( $\circ$ ) a grape seed extract supplement. Vertical bars represent the 95% confidence interval......pg 272

#### Chapter 8:

Figure 8.1. Suggested rumen microbial metabolism of plant protein to compounds implicated in the cause of pastoral odour and flavour in the meat of sheep grazing fresh forage diets. Sites of inhibition by condensed tannin (CT) indicated. This figure created using data from: Wong et al. (1975); Ha and Lindsay (1991); Young et al. (1997, 2003); Le et al. (2005)......pg 310

## ABBREVIATIONS

2MI	2-methylindole
ABPE	acute bovine pulmonary emphysema
ATP	adenosine triphosphate
BCVFA	branched-chain volatile fatty acids
BD	Broadleaf dock
BS	equilibrium buffer solution
°C	degrees Celsius
С	carbon
СНО	carbohydrate
cm	centimetre
CO <sub>2</sub>	carbon dioxide
СР	crude protein
СРІ	crude protein intake
СТ	Condensed tannin
cv.	cultivar
<b>d</b> <sub>3</sub>	dueterated
DM	dry matter
DMACA-HCl	4-dimethylaminocinnamaldehyde-hydrocholoric acid
DMI	dry matter intake
DR	Dorycnium rectum
EU	European Union
FAD	flavin adenine dinucleotide
Fe	iron
FIA	flow injection analysis
FMN	flavin mononucleotide
g	gram
g	centrifugal force (multiples of earth's gravitational force)
GC/MS	gas chromatography/mass spectrometry
GCO	gas chromatography-olfactory
GSE	grape seed extract (a source of condensed tannin)
HCI	hydrochloric acid
HPLC	high performance liquid chromatography
ID	internal diameter
iu	international units
kg	kilogram
L	litre
LC	Lotus corniculatus
LP	Lotus pedunculatus
M	molar
m <sup>2</sup>	metre squared
ME	metabolisable energy
МеОН	methanol
mg	milligrams
MJ	megajoules
mL	millilitre
mm	millimetre

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mmol	millimole
MW	molecular weight
N	nitrogen
n	number (replicates)
NADPH	nicotinamide adenine dinucleotide phosphate
ND	not determined
NDF	neutral detergent fibre
ng	nanogram
NIRS	near infra-red reflectance spectrometry
nm	nanometre
NZ	New Zealand
OMD	organic matter digestibility
РС	procyanidin
PD	prodelphinidin
PEG	polyethylene glycol
рН	power of hydrogen
ppb	parts per billion
ppm	parts per million
PRG	Perennial ryegrass
R	registered
SL	Sulla
SD	standard deviation
SDE	simultaneous distillation extraction
SEM	standard error of the mean
sp.	species (singular)
SPE	solid phase extraction
spp.	species (plural)
SSS	soluble sugars and starch
USA	United States of America
V	volts
v/v	volume by volume
VFA	volatile fatty acids
W	watt
w/w	weight by weight
WC	White clover
μg	microgram
μL	microlitre
μm	micrometre (micron)

## **GENERAL INTRODUCTION**

#### Background

The export of sheep meat is of major economic importance to New Zealand. Approximately 350,000 tonnes of sheep meat worth \$NZ 2.2 billion is exported on an annual basis. This contributes 45% of the total meat exports or 20% of the total exports from the New Zealand pastoral sector. New Zealand contributes to only 44% of the total world exports of sheep meat indicating that there is room to increase export volumes. Export volumes of sheep meat to some markets are particularly low due to low demand. Lamb exported to the United Kingdom represents 24% of total lamb exports from New Zealand while lamb exported to Japan accounts for only 2%. Low demand is partially a consequence of pastoral flavours reducing the quality and desirability of sheep meat in these specific markets. Pastoral flavour is a characteristic of meat and milk from animals (ovine and bovine) raised on pasture and is considered an undesirable attribute to those consumers accustomed to meat or milk from animals raised on grain or concentrate diets.

In New Zealand there is a reliance on the year-round outdoor grazing of pasture (comprised of approximately 80% perennial ryegrass and 20% white clover) to maintain profit margins, as the high cost of grain and concentrates make them uneconomical as animal feeds under New Zealand conditions. Reliance on pasture-finishing means that sheep meat is discounted in specific markets (e.g., Japan) due to perceived differences in quality (including the flavour). Sheep meat from New Zealand is therefore, traded as a commodity item in these specific markets and it does not obtain the economic opportunities of a high-value product. Ameliorating pastoral flavours in sheep meat

would contribute towards improving New Zealand sheep meat quality for selective markets and endeavours to make sheep meat a product of higher value thus, providing the potential to increase export volumes to specific markets and improve economic returns.

#### **Objectives:**

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Pastoral flavours have been attributed to flavour chemicals in the meat, particularly indole and skatole, that are formed in the rumen from feeding some fresh forages. To fit with meat production from grazing systems, it may be possible to identify forage species that form less indole and skatole in the rumen and therefore, reduce pastoral flavours in the meat. Previous studies have identified that feeding legumes results in a more intense sheep meat flavour, indicating that the white clover component of conventional New Zealand pastures maybe responsible for pastoral flavours in sheep meat.

This study aims to investigate alternative forages for their ability to lower the formation of indole and skatole in the rumen and considers incorporating condensed tannin (CT) into the ruminant diet to ameliorate pastoral flavours in the meat. Therefore, the specific objectives of this thesis are:

- Establish if dietary condensed tannin lowers the formation of indole and skatole in the rumen to minimise the indole and skatole concentration in the meat thus, minimising pastoral flavour.
- Establish if white clover results in a greater formation of indole and skatole in the rumen compared to perennial ryegrass and results in more intense pastoral flavour in the meat.

#### Thesis format:

Chapter 1 is a review of the literature surrounding pastoral flavour associated with indole and skatole in the meat. It covers aspects of flavour differences between diets for ruminants, the formation and metabolism of indole and skatole and methods to reduce indole and skatole formation. Chapter 2 outlines some of the experimental methods used in this work.

Five experimental chapters have been incorporated into this thesis.

- Chapters 3 and 4 cover experiments that look at the formation of indole and skatole in the rumen with different forages using both *in vitro* and *in vivo* techniques.
- Chapter 5 examines the grazing of conventional pasture in comparison to the CTcontaining *Lotus corniculatus* and the effect this has on rumen, blood plasma and fat concentration of indole and skatole and also incorporates sensory evaluation of the fat.
- Chapter 6 reports the results from *in vitro* rumen fermentations that were used to understand the role of condensed tannin to lower the formation of indole and skatole.
- Chapter 7 investigates rumen, blood plasma and fat concentration of indole and skatole when feeding lambs white clover or perennial ryegrass supplemented with or without a grape seed extract (source of condensed tannin). Fat and meat samples from this experiment were used in sensory evaluation of pastoral flavours.

The results of the experimental chapters are summarised and compared in a General Discussion in Chapter 8.

## **CHAPTER 1**

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Literature Review

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## **1.1. INTRODUCTION**

The role of meat in the human diet is a very broad subject and addresses many issues including those of nutrition, sociality, economics and enjoyment. Meat is an important source of nutrients but most people will eat meat because they enjoy the characteristic aroma, flavour and texture that meat provides (Farmer 1994). Therefore, consumer decisions to purchase meat products will be partly based on the perceived aromas and flavours of the meat. Consequently, consumer demand for meat products will be affected by quality parameters such as meat flavour. (Rubino *et al.*, 1999; Moloney *et al.*, 2001).

New Zealand does not have a predominant domestic market for sheep meat, exporting approximately 87% of the sheep meat that is produced (Boutonnet 1999). Export markets are therefore, necessary for the survival of the sheep meat industry (Boutonnet 1999). New Zealand is the world's largest exporter of sheep meat and the acceptance of sheep meat is an important economic issue for New Zealand (Prescott *et al.*, 2001). New Zealand as a meat exporting country has to adapt its products or implement selective trading procedures to provide consumer-acceptable products for each of its markets (Boutonnet 1999). Meat flavour and aroma is one aspect that needs to be considered when wanting to take advantage of export markets.

New Zealand's export meat markets (*Table 1.1*) are dominated by the European market for lamb and the North American market for beef. Exports of New Zealand sheep meat to Asia are low compared to the export quantities of sheep meat to other regions (*Table 1.1*). This reflects a low consumption of New Zealand's sheep meat in Asian countries,

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which can be partly attributed to a poor acceptability of the meat's odour and flavour. Flavour is also one reason for the low levels of lamb consumption by Americans and young Europeans (Rubino *et al.*, 1999; Field *et al.*, 1983). The Asian markets, specifically the Japanese and increasingly affluent Chinese markets, represent a significant opportunity for New Zealand meat exporters to obtain new markets provided the source of sensory factors that have negative impacts on consumer acceptability can be identified and modified (Prescott *et al.*, 2001).

Table 1.1. Total global meat shipments and breakdown of meat shipments by destination (tonnes of product weight) for the New Zealand meat industry for the 2002/2003 season (New Zealand Meat Board 2003).

	Lamb	Mutton	Beef & Veal	Other
Total Global Shipments	316,697	60,641	410,283	3,909
Shipment destination:				
- United Kingdom	76,209	13,935	503	31
- Rest of EU	87,579	16,891	1,795	48
- Middle East	22,338	4,022	3,224	397
- Japan	7,894	1,862	19,253	488
- Rest of Asia	39,067	12,723	94,281	1,324
- North America	46,931	4,175	279,078	665
- Pacific (excluding NZ)	20,612	3,294	12,548	437

Pasture diets comprising of approximately 80% perennial ryegrass (*Lolium perenne*) and 20% white clover (*Trifolium repens*) have been developed to maximise year-round ruminant production under grazing in New Zealand. Pasture is an inexpensive source of nutrients for production purposes with low production costs. This allows a sufficient margin to be made by producers with current meat prices (Boutonnet 1999). Poor

acceptability of the flavour of sheep meat from New Zealand has been partly attributed to "pastoral" or "oceanic" flavours that are derived from grazing pasture (Young *et al.*, 2003).

Perennial ryegrass/white clover pasture diets are relatively rich in soluble protein (Ulyatt 1981) and rumen microbes breakdown approximately 70% of that protein to yield amino acids (Waghorn and Barry 1987). A consequence of high protein concentration in plants, and a high degradability of that protein, is an excess of amino acids in the rumen when animals consume pasture. Excess amino acids are broken down into ammonia and other products. A proportion of the total tryptophan concentration in the rumen is degraded by rumen microorganisms to indolic compounds such as indole and skatole. Some of the skatole and indole is deposited in the fat of the ruminant, including in the meat fat (Prescott *et al.*, 2001). These indolic compounds have been identified as a possible cause of pastoral flavour in meat (Young *et al.*, 2003). Reducing skatole and indole levels in meat produced from ruminants grazing fresh pasture diets may therefore, improve the acceptability of sheep meat in some markets (Prescott *et al.*, 2001).

## **1.2. FLAVOUR OF MEAT PRODUCTS FROM RUMINANTS**

Consumer research has indicated that tenderness and flavour are amoung the most important elements of the eating quality of meat (Moloney *et al.*, 2001). Flavour is an important aspect for the overall acceptability of meat products and is frequently considered the most important palatability characteristic of cooked sheep meat. A consumer may accept or reject a piece of lamb based solely on its odour and flavour, while beef acceptability will also be based on tenderness, and pork based on juiciness (Batcher *et al.*, 1969; Ford and Park 1980).

The flavours of products derived from ruminants have been classified as normal flavours associated with the product including characteristic "species" flavours, "off"-flavours which develop during storage or undesirable "foreign"-flavours or "taints" (Ford and Park 1980). Foreign-flavours are most often influenced by animal diet (Purchas 1989).

#### 1.2.1. Definition of flavour and flavour perception

Flavour has been defined as the psychological interpretation of a physiological response to a physical stimulus. Thus, the perception of flavour integrates the separate sensations of smell, taste and touch (Noble 1996). Cooked meat flavour is influenced by compounds contributing to the sensation of taste but the volatile compounds formed during cooking determine aroma attributes and contribute most to the characteristic flavour of meat (Mottram 1998).

#### 1.2.1.1. Olfactory perception

Both taste and olfactory are involved in the sensation of flavour however, flavours are

largely a consequence of volatile chemicals that are perceived in the olfactory epithelium at the back of the nose. They are different from taste chemicals that are detected by receptors on the tongue to give the five main taste sensations – sweet, sour, bitter, salt and umami (Farmer 1994).

Taste chemicals are perceived similarly among differing countries and ethnic backgrounds (Winger and Hagyard 1993). Meat odour and flavour chemicals will be perceived differently between countries and ethnicities (Rubino *et al.*, 1999). Genetic variation has been implicated for the disparity in the determination of some flavour compounds (Beidler 1954). However; differences in odour and flavour perception are more likely to be a consequence of meat consumption history and people in different cultures becoming accustomed to different flavours over time (Prescott *et al.*, 2001). Olfactory senses are generally considered more sensitive than taste senses, with the response to odour being 10,000 times more sensitive than that of taste (Lawrie 1998).

Volatile odour chemicals reach the receptors in the nasal epithelium through the nose or through the posterior nares at the back of the nose and throat when food is chewed (Farmer 1994). Binding of the odourant to the olfactory receptor causes a conformational change in the receptor and the linked G-protein becomes activated. The activated G-protein diffuses laterally away from the receptor until it contacts and activates an effector protein. This in turn opens membrane channels allowing the exchange of ions initiating an action potential and consequently a nerve impulse that is transmitted to the brain (*Figure 1.1*; Laing and Jinks 1996; Bell 1996).


Figure 1.1. Odour receptor in nasal epithelium showing the pathway of activated and effector proteins to open membrane channels prompting an action potential (Liang and Jinks 1996).

## 1.2.1.2. Quantitative and qualitative aspects

Whether a compound has an impact on flavour or odour is dependent on its concentration and detection threshold. A detection threshold is a measure of how sensitive the human nose is to a particular compound (Farmer 1994). Chemical compounds have different threshold concentrations at which they are detected or recognised. Some highly volatile compounds need only be present in very low concentrations to have a significant flavour or odour effect. Furthermore, detection thresholds for a particular compound will vary from person to person and be dependent on the person's ethnicity and culture.

Volatile components of cooked meat are present at concentrations in the part per million (ppm) or part per billion (ppb) ranges. Therefore, in a meat odour or flavour profile the concentration of a compound in relation to its detection threshold is important. Only compounds with a low odour threshold are likely to contribute to meat flavour (Mottram 1992). If a compound exceeds its threshold value, it may provide an important

contribution to meat flavour. However, at concentrations much higher than threshold then, it may impart foreign-flavours (Wasserman 1979).

Compared to the total number of volatile compounds identified in meat, only a small fraction has been reported to possess meaty aroma characteristics. The meaty aroma compounds tend to be sulphur-containing and exhibit pleasant aromas at concentrations present in meat however, at high levels the odour is objectionable. This indicates the necessity to evaluate not only qualitative aspects of meat flavour but also the quantitative aspects (Shahidi 1994).

# 1.2.2. Chemical components of meat flavour

Meat flavour related chemicals are comprised of low-molecular weight volatile compounds with odour properties that are detected by receptors in the nasal epithelium and non-volatile compounds with taste and tactile properties that are detected by receptors on the tongue (Dwivedi 1975, Farmer 1994, Shahidi 1994).

Meat flavour is thermally-derived as raw meat has little aroma and only a blood-like taste whereas cooking develops the flavour of meat (Moody 1983; Mottram 1994). Volatiles will be formed during cooking as the product of reactions of non-volatile precursors (Dwivedi 1975) as well as from the release of volatiles already present within the meat tissue. Volatiles trapped in the meat and particularly in the fat are likely to contribute to undesirable foreign-flavours (Shahidi 1994).

An obvious fact of cooked meat from different species is that each type of meat has its

own characteristic flavour. All types of meat consist of broadly similar chemical components whether it is lamb, beef or chicken, therefore differences in meat flavour must arise from significant differences in the proportion of compounds that are present or in the way they interact. No single compound is likely to characterise overall meat flavour but more likely a subtle quantitative balance of various components (Dwivedi 1975).

## 1.2.2.1. Volatile compounds of meat flavour and aroma.

Over 1000 volatile compounds have been associated with meat flavour (Mottram 1998) and these compounds are representative of most classes of organic compounds (Shahidi 1994). Some of these volatiles will contribute to desirable "meaty" flavours while others will contribute to other characteristic flavours such as those associated with species-specific flavours, off-flavours or undesirable foreign-flavours. Hornstein *et al.* (1960) identified that a highly volatile fraction from cooked beef resulted in disagreeable aromas while a less volatile fraction resulted in a pleasant meaty aroma.

Volatile flavour compounds from cooked meat can be divided into three groups, those formed from lipid oxidation, those formed from the Maillard reaction and those already present in the meat fat. Compounds from lipid oxidation include straight chain aldehydes, ketones, hydrocarbons, alcohols and alkylfurans. Volatile compounds formed from the Maillard reaction include heterocyclic nitrogen and sulphur compounds (Elmore *et al.*, 2000). Other numerous volatile compounds, already present in meat fat, are released on cooking and include compounds such as the branched-chain fatty acids that contribute to species-specific flavours or those such as the indoles and phenols that

arise from the type of feed consumed by the animal.

# 1.2.2.2. Effect of lipids on meat flavour

Lipids play an important role in the overall flavour of meat (Shahidi 1994). The lean meat is responsible for a basic meaty flavour common to all species while the lipid fraction of meat, which includes adipose tissue and interstitial fat, tends to provide the volatile compounds that give meat its characteristic flavour (Hornstein and Crowe 1963; Pearson *et al.*, 1973; Brennard and Lindsay 1982; Moody 1983; Mottram 1992; Mottram 1994).

Fat influences meat flavour in three main ways. Firstly, the lipid components will undergo reactions during cooking to form flavour volatiles. Oxidation of unsaturated fatty acids results in the formation of carbonyl compounds that are present in organoleptically significant amounts. Furthermore, the compounds formed from the lipid during cooking may react with compounds produced in the lean meat to form further flavour volatiles (Dwivedi 1975).

Secondly, lipid depots may act as a solvent, trapping aroma components obtained either, from extraneous sources or as part of the flavour forming reactions (Mottram 1992). Thus, the lipid serves as a depot for fat-soluble compounds that strongly affect flavour and volatise on heating (Moody 1983). Flavour differences in beef from steers finished on grain and grass diets were not apparent in lean meat but were detected when 20% fat was added to the lean muscle (Maruri and Larick 1992). These pastoral flavours are likely to arise from volatiles solubilised in the fat but produced elsewhere because of

feeding forages (Bailey et al., 1994).

In addition, fatty acids will directly contribute to meat flavour. Volatile free fatty acids are key flavour compounds in many meat products. In some cases, they provide desirable characteristics or background flavour notes to products, but in other cases, they can contribute to less desirable flavours (Brennard *et al.*, 1989). Branched-chain fatty acids, and in particular, 4-methyloctanoic and 4-methylnonanoic acids have been identified in cooking volatiles in sufficient quantities to provide the characteristic odour of sheep meat (Wong *et al.*, 1975; Young and Braggins 1996; Rousset-Akrim *et al.*, 1997; Mottram 1998).

# 1.2.3. Factors affecting meat flavour

Flavour is one of the most important variants in the overall acceptability of meat (Dransfield *et al.*, 1982). Influences upon the animal, carcass and meat cuts throughout the production process can all affect the meat flavour. The potential meat flavour will be influenced by factors directly linked to the animal (genotype, age and sex) and factors external to the animal (diet, pre-slaughter procedures and environment; Priolo *et al.*, 2001). Post-slaughter factors such as storage and cooking method, including the addition of ingredients during cooking, will considerably influence the final meat flavour experience.

# 1.2.3.1. Differences between species

Meat flavour has high species specificity, for example, sheep-meat flavour is different to that of beef (Priolo *et al.*, 2001). Species-specific flavours are inherent to the type of animal from which the meat comes from, unlike flavours that arise from differences in the animal's environment; for example flavours that arise from differences in the type of feed consumed by the animal.

Differences in the flavour of meat resulting from species differences seem to arise from the fat or lipid proportion of the meat (Purchas 1993). Characteristic sheep meat flavours have been attributed to the branched-chain fatty acids (Wong *et al.*, 1975; Young and Braggins 1996; Rousset-Akrim *et al.*, 1997; Mottram 1998) although, many other compounds have been identified as possible contributors to sheep meat odour and flavour (Young *et al.*, 1994).

# 1.2.3.2. Breed/Genetic differences

Studies investigating the effect of sheep breed on meat flavour have been contradictory (Crouse 1983). Where age and level of fatness were constant, there were no flavour differences amoung breeds. In contrast, Elmore *et al.* (2000) found that aroma composition was affected by breed with the concentration of 54 compounds identified as being significantly different in meat samples obtained from Suffolk compared to Soay breeds. Furthermore, it has been noted that sire has an influence on beef flavour (Purchas 1993).

Some fine-woolled breeds of sheep produce meat with more intense mutton flavour than coarse wool breeds (Cramer *et al.*, 1970) but a study using Merinos (a fine wool breed) contradicted this although, the abnormal muscle pH may have affected meat flavour in that study (Young *et al.*, 1993). The Merino meat fat did have a more oily consistency, which is related to fatty acid composition, and variations in fatty acid composition of

meat fat are often implicated in odour and flavour differences (Young et al., 1994).

# 1.2.3.3. Differences between sexes

Studies looking at the flavour differences between sexes have been inconsistent and it is probable that the results were confounded by factors such as animal age, weight or fat content (Crouse 1983). A common complaint from the consumption of pork from boars is a problem called boar taint. The problem is thought to result from the production of a pheromonal testicular steroid called androstenone which interferes with the metabolism of skatole that is formed in the gut (Annor-Frempong *et al.*, 1997). Comparable meat flavour problems have not been found in rams or bulls (Purchas 1993). Overall, there seem to be insignificant differences in flavour and odour between ram, wether and ewe meat (Young *et al.*, 1994).

### 1.2.3.4. Changes with age of animal

Generally, there is a belief that as an animal grows older their meat becomes more strongly flavoured (Sink and Caporaso 1977). It has been noted that there is a much stronger odour and flavour to meat from ewes when compared to that of meat from lambs (Young *et al.*, 1994). Calves up to 11 months old are said to lack the typical flavour quality and intensity of beef (Dwivedi 1975).

Changes in animal metabolism and changes in muscle composition are likely to be involved with meat flavour differences in animals at different ages (Dwivedi 1975). As sheep age the fatty acid composition of fat depots changes and generally the fats become more saturated. It has also been suggested that the content of branched-chain fatty acids in adipose tissue changes with age (Young *et al.*, 1994).

### 1.2.3.5. Changes with liveweight and fat cover

As an animal gets older it will also tend to get heavier and the fat content of the meat tends to increase. Fat content contributes to the flavour of meat and therefore, as the animal ages it is unclear as to whether flavour differences are due to age, weight or fatness. There have been implications that heavier rams produce meat that has a more intense flavour (Crouse *et al.*, 1981) while Field *et al.* (1983) concluded that fatness had no significant effect on sheep meat flavour.

### 1.2.3.6. Pre-slaughter effects

In the post-mortem animal, all muscles convert their stores of glycogen into lactic acid, causing the pH to fall to a value of approximately 5.5. If the glycogen store is lower than 0.6% the ultimate pH will be higher, in the range of 5.5-7.0 (Dwivedi 1975). Studies have shown that the flavour of high pH beef is generally less acceptable and there is less flavour strength (Purchas *et al.*, 1986; Dransfield *et al.*, 1982). Lamb has shown similar sensory results with overall odour and flavour decreasing as meat pH increased (Braggins 1996). Stress or excessive muscular activity prior to slaughter can lower glycogen stores and lead to meat that has a high pH.

Lamb flavour was not affected by length of time off feed prior to slaughter, but odour intensity was significantly higher in lambs that had been stood off pasture for only one hour compared to 24 hours before slaughter (Czochanska *et al.*, 1970).

# 1.2.3.7. Cooking effects

The flavour of cooked meat depends on the method of cooking. Different cooking methods in terms of the conditions of temperature, water content and reactant

concentrations, will favour different reaction processes during cooking (Mottram 1992). Thus, cooking method will alter the total amount and composition of volatiles liberated during cooking (Ford and Park 1980).

Sheep meat is prepared for the table by methods that vary from culture to culture (Young *et al.*, 1994). Often there is the use of herbs and sauces during or after cooking and it is believed that these act as antioxidants to modify oxidation reactions to reduce cooking odours or alternatively, to mask odours and flavours and add new desirable flavours (Young *et al.*, 1994).

# 1.2.3.8. Animal nutrition and diet on meat flavour

Of all the animal and environmental factors that can influence meat flavour of a particular animal, diet appears to have the most influence (Duckett and Kuber 2001). There can be both direct and indirect effects of nutrition on meat flavour. Direct effects of nutrition result in specific components from the diet ending up in the meat and affecting flavour. An indirect effect occurs because of animal factors such as weight, growth rate or level of fatness that in turn affect the flavour of meat (Purchas 1993).

The diet of the animal is more likely to influence foreign-flavours in the meat. For example, Bailey *et al.* (1994) showed that feeding forages increased the intensity of grassy flavours in lamb compared to feeding a corn-based grain diet, while the same diets had no effect on the intensity of lamby flavour. However, grain diets have been shown to elevate branch chain fatty acid concentration in lamb fat (Young *et al.*, 2003) and branch chain fatty acids are said to influence the characteristic odour and flavour of

sheep meat (Wong *et al.*, 1975; Young and Braggins 1996; Rousset-Akrim *et al.*, 1997; Mottram 1998).

One of the most noticeable meat flavour differences due to diet is that of ruminant meat from animals raised on forages in comparison to those raised on grain or concentrates. Beef from steers raised on pasture is considered undesirable in some markets compared to beef from animals on a concentrate diet (Young *et al.*, 1999). Pasture-raised lamb had a more intense barnyard and faecal flavour and odour compared to lamb that had been produced with grain feeding (Young *et al.*, 2003).

The group of flavours used to describe the flavour of meat from animals raised on pasture have collectively been called "pastoral" flavours. The following section reviews the research that has investigated the perception of pastoral flavours and the chemistry behind these flavours.

# **1.3. PASTORAL FLAVOURS OF RUMINANT MEAT**

## 1.3.1. Grain vs. forage feeding on meat flavour

Flavour differences between the meat of animals finished on pasture compared to those finished on grain have been well documented for cattle (Bowling *et al.*, 1977; 1978; Melton *et al.*, 1982a,b; Larick *et al.*, 1987; Larick and Turner 1990; Maruri and Larick 1992; Schroeder *et al.*, 1980; Young *et al.*, 1999) and sheep (Paul *et al.*, 1964; Bailey *et al.*, 1994; Rousset-Akrim *et al.*, 1997; Young *et al.*, 2003; Duckett and Kuber 2001). Trials comparing grain and forage feeding prior to slaughter are summarised in *Table 1.2*.

In *Table 1.2* the majority of studies found that finishing ruminants on pasture or fresh forages gave a less desirable meat flavour than finishing on grain-based diets. The rejection of meat from ruminants finished on forage or pasture is generally a consequence of the consumer being accustomed to the flavour of meat from animals raised on concentrate- or total mixed ration-based diets. Most studies looking at the acceptability of meat flavour from forage or grain fed animals have been undertaken in the United States of America (USA). Similar tests carried out in countries where consumers are accustomed to meat from animals raised on pasture may have given different results (Priolo *et al.*, 2001).

The comparison of the results from meat flavour studies are often confounded by animals being different ages at slaughter, and having different live weights, fat cover or marbling. However, the large number of studies that found differences in flavour between forage and grain feeding indicates that the effect of diet on meat flavour is real and that there is a direct effect of pasture feeding leading to an objectionable flavour for those consumers accustomed to meat from concentrate-based production systems.

# 1.3.2. Flavour perception of meat from animals fed pasture

Meat from grain-fed sheep has a different odour and flavour profile compared to that from pasture-fed sheep (Young *et al.*, 1994; Young *et al.*, 2003). Meat from barley-fed lambs has been described as "bland" by a taste panel in a study that considered pastureraised lamb meat to have a "strong" flavour (Locker 1980).

## 1.3.2.1. Flavour description of meat from pasture and grain-fed animals

An American study using beef from grass-fed animals in comparison to animals on a limited grain ration where liveweight gains were similar for both groups, found that flavour was less desirable with beef from grass-fed animals. Undesirable flavours described as "grassy", "milky" and "fishy" were deemed to be responsible for the lower flavour score (Brown *et al.*, 1979).

Sensory panels have described beef from grass-fed cattle as having "pastoral" or "grassy" (Berry *et al.*, 1980; Larick *et al.*, 1987), and "dairy" flavours (Melton *et al.*, 1982a,b). Flavour descriptors such as "barnyard", "animal", "shcepy", "milky" "faecal" and "grassy" are more intense with pasture-finished lambs (Rousset-Akrim *et al.*, 1997; Priolo *et al.*, 2001; Young *et al.*, 2003; *Figure 1.2*). Such descriptors are collectively called "pastoral" flavours because of the production systems from which they originate.

Table 1.2. Summary of studies	comparing flavou	rs and odours (	of meat and/or	fat from anima	als fed forage∙	• or grain-based	diets prior to
slaughter.							

Author/year	Country	Meat	Forage Diet	Grain Diet	Major flavour findings of study
Bowling <i>et al.</i> , (1977)	USA	Beef	Unspecified	Unspecified	Forage-finished beef had a less desirable flavour than grain-finished beef
Bowling et al., (1978)	USA	Beef	Bermuda or Sudan grass	Unspecified grain diet after grass	Animals finished on grass had less desirable flavour than those animals finished on the grain.
Brown et al., (1979)	USA	Beef	Orchard grass/ Kentucky fescue/ Ladino clover	Corn-based grain ration	Beef from grass-fed animals was less desirable than grain-fed beef. Flavours described as grassy, milky, fishy responsible for the lower desirability
Westerling & Hendrick (1979)	USA	Beef	Fescue pasture for 180 days	Corn based concentrate after pasture	Animals slaughtered straight off pasture had less desirable meat flavour compared to those changed to concentrate
Schroeder et al., (1980)	USA	Beef	Native range or forage sorghum	Corn based concentrate	Finishing cattle on forage sorghum or native range gave a less desirable flavour compared to finishing on concentrate.
Melton et al., (1982a)	USA	Beef	Orchard grass/ Kentucky fescue/ Ladino clover	Corn based grain diet (ad libitum)	Beef from grass-fed animals was undesirable due to high intensities for dairy flavours compared to the grain diet.
Melton et al., (1982b)	USA	Beef	Orchard grass/ Kentucky fescue/ Ladino clover.	Corn diet after grass.	Increased time feeding grain made undesirable flavours associated with feeding grass less intense.
Larick <i>et al.</i> , (1987)	USA	Beef	Tall fescue. Bromegrass/ red clover or orchard grass	Corn diet after grass.	Beef from steers slaughtered directly off pasture had an intense, undesirable grassy flavour which lost intensity as time feeding corn prior to slaughter increased.
Muir <i>et al.,</i> (1998)	NZ	Beef	Perennial ryegrass/ White clover/Subterranean pasture	70% maize grain, 30% pasture silage	No difference between grass- and grain-finished beef.

#### CHAPTER 1: Literature Review

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#### Table 1.2. continued...

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Young et al., (1999)	NZ	Beef	Perennial ryegrass/ White clover pasture	Maize-based concentrate	Mince from steers finished on forage was unfavourable compared to meat from steers finished on the concentrate.	
Paul <i>et al.</i> , (1964)	USA	Lamb	Trefoil/Ladino clover	Barley and alfalfa hay	Trefoil/ladino pasture fed 60 days before slaughter gave less acceptable lamb flavour than the barley/alfalfa diet	
Batcher et al., (1969)	USA	Lamb	Unspecified Unspecified		No differences in meat flavour were found between forage and grain feeding regimes	
Bailey <i>et al.</i> , (1994)	NZ & USA	Lamb	White clover, Lucerne, Lotus corniculatus, Perennial ryegrass, Radish, Cowpea, Sudan grass	Corn-based diets	Finishing animals on corn gave milder meat than forages. Radish and lotus gave milder meat than other forages. Grain diets reduced grassy flavours compared to forage diets.	
Rousset-Akrim et al., (1997)	France & NZ	Lamb	Perennial ryegrass/ White clover pasture	Maize-based concentrate	Sheep & animal flavours were more intense with lamb from animals fed pasture than concentrate	
Hopkins <i>et al.</i> , (2001)	Australia	Lamb	Annual ryegrass/ Subterranean clover pasture	Supplement of oats & sunflower seed with pasture	Supplementing pasture with oats and sunflower meal resulted stronger flavour and aromas and less acceptable meat flavour	
Young et al., (2003)	NZ	Lamb	Perennial ryegrass/ White clover pasture	Maize based concentrate	Meat from lambs raised on pasture had more intense barnyard, sheep and faecal flavours and odours.	

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Abbreviations: USA - United States of America, NZ - New Zealand

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Figure 1.2. Intensities (scale: 0 = none, 10 = very high) of some flavour descriptors in meat from pasture- and concentrate-finished cattle. (Priolo *et al.*, 2001).

# 1.3.2.2. Grass vs. legume flavours

New Zealand's temperate climate means that domestic ruminants can be raised and finished on a pasture-based production system all year round (Daly *et al.*, 1999). Grasses and legumes are the types of forages that are predominately used in pasture-based production systems. In New Zealand, pastures are traditionally perennial ryegrass-based with a variable (0-30%) white clover component. The white clover component in New Zealand pastures can vary due to seasonal and regional changes but may also differ from farm-to-farm because of differences in pasture management. On average, it is considered that the botanical composition of New Zeland pasture is 80% perennial ryegrass and 20% white clover (Waghorn and Barry 1987).

Meat odour and flavour can be different because of feeding legume compared to grass diets (Young *et al.*, 1994). Meat from sheep fed clover alone had a significantly more intense flavour and odour than meat from grass-fed sheep and this is likely to have been due to a foreign-flavour note (Cramer *et al.*, 1967; Shorland *et al.*, 1970; Fraser *et al.*, 1996). Purchas *et al.*, (1986) found meat from clover-fed lamb resulted in a higher number of taste panel members considering stronger flavours to be present compared to perennial ryegrass. Lucerne pastures have also proven to give more intense, unacceptable "sharp" and "sickly" flavour in lamb compared to grass pastures (Park *et al.*, 1975). This indicates that undesirable meat flavours that arise because of grazing pasture may be intensified by the pasture's clover or legume component.

# 1.3.2.3. Causative volatile agents for pastoral flavours

Some consumers have clearly perceived that meat from pasture-raised animals has an undesirable flavour. It is likely that specific components of the diet are responsible for producing compounds that are deposited in the fat and cause flavours and odours associated with pastoral flavours. Dietary components can have a direct flavour effect. However, in the ruminant it is more likely that compounds with organoleptic properties are formed in the rumen from dietary components (Keen 1998).

Diet affects fatty acid composition and fatty acids are precursors for many flavour compounds (Melton 1983). Melton *et al.* (1982a) attributed undesirable flavours in beef from animals finished on grass to fatty acids and in particular, tetradecenoic (C14:1), octadecanoic (C18:0), octadecenoic (C18:1) and octadecatrienoic (C18:3) fatty acids. However, Purchas *et al.* (1986) found that no single fatty acid could account for the variation in flavour characteristics for lambs fed various legumes and perennial ryegrass.

Medium chain fatty acids were present at higher concentrations in the volatiles of lambs fed lucerne, clover, lotus or ryegrass than those fed corn (Bailey *et al.*, 1994). Suzuki and Bailey (1985) also found that fat from lambs fed clover, compared to lambs fed maize, had a higher volatile concentration of medium-chain fatty acids as well as 2,3-octanedione and diterpenes.

Larick *et al.* (1987) carried out chemical analyses on subcutaneous fat from cattle that were fed on grain for increasing amounts of time after feeding on grass. They found a decrease in high molecular weight alkanes, heptanal, 2-decenal, 2,3-octanedione, 3hydroxyoctan-2-one,  $\delta$ -dodecalactone and diterpenoids with increased time that animals were fed grain. Diterpenoids have been considered as one cause of undesirable flavour in forage-finished beef (Maruri and Larick 1992). Pastoral flavour has been attributed to methylphenols that have been identified in higher concentrations in pasture-fed beef (Ha and Lindsay 1991) and lamb (Young *et al.*, 1997).

Young et al. (1997) found that not only 2,3-octanedione, phenols and diterpenoids were

higher in rendered fat from lambs fed pasture but, also skatole (3-methylindole) was especially found to be significantly higher in the fat from pasture-fed lambs compared to fat from lambs fed a maize-based grain diet. Furthermore, skatole concentration was found to be significantly higher in brisket fat of steers finished on pasture compared to a maize based concentrate diet (Lane and Fraser 1999). While some of these pastureassociated fat volatiles have potent organoleptic properties in the above studies, no direct evidence of the sensory impact of individual compounds was provided.

# 1.3.3. The influence of skatole and indole on pastoral flavour

Skatole (3-methylindole) is a nauseating faecal-smelling compound. It is a component of boar taint (Hansson *et al.*, 1980) and weed-taint in milk of cows fed *Lepidium* spp. (Park 1969). Skatole and indole have also been identified with pastoral flavours in dairy products (Keen 1998) and isolated as part of the aroma bearing material from cheese (Kowalewska *et al.*, 1985). Skatole and indole therefore, seem to be likely candidates for contributing to pastoral flavours in meat from ruminants.

When a faecal-like taint was noted in beef, laboratory tests by Empey and Montgomery (1959) found this taint to resemble that detected when adding skatole to beef samples. Young and Braggins (1996) and Young *et al.* (1997) reported that the presence of skatole was highly correlated with "animal" and "rancid" flavour in meat from lambs raised on pasture. Meat from Romney lambs that had been grazing ryegrass/clover pasture was found to contain a significantly higher concentration of skatole while skatole was only present at basal levels in meat from lambs fed a concentrate diet (Young *et al.*, 2003).

The study by Young *et al.* (2003) provided direct evidence of a sensory effect of skatole in the fat of pasture-raised lambs by means of a person sniffing the chemical components of the fat after they were separated and eluted off a gas chromatographic column (gas chromatography-olfactometry; GCO). While a number of odourous compounds were detected, skatole was the only compound to give a distinctive faecal odour. Principal components analysis of flavour and odour perception by panel assessment and electronic nose showed that the pastoral flavour descriptors "faecal" and "barnyard" were associated with the presence of skatole, and the related compound indole, in meat from pasture-raised lambs. (Young *et al.*, 2003; *Figure 1.3*). Bendall (2001) carried out GCO analysis on milk from cows fed pasture or concentrate and found that indole and skatole were two factors that determined the aroma of milk from pasture-fed cows.



Figure 1.3. Principal components analysis of correlations between frequency of panellist's comments and relative concentrations of 18 odorous compounds in the headspace of subcutaneous fat from lambs 132 days old (Young *et al.*, 2003).

## 1.3.3.1. Detection thresholds for skatole and indole

Detection threshold values are the concentration at which a compound needs to be present in a meat or fat sample before it is detected as a flavour or odour. The concentration of a compound, below the detection threshold will not be detected by sensory analysis. The detection threshold varies for different compounds.

The sensory qualities of indole and skatole were first investigated in pork in relation to boar taint. These investigations usually took into consideration the sex hormone androstenone. Interactions of skatole with androstenone have been noted as enhancing the sensory impression of boar taint (Hansson *et al.*, 1980) thus, using skatole alone for the detection of boar taint is not possible. However, boar taint related studies have determined the threshold value for skatole in fat to be in the range of 0.09- 0.25 parts per million (ppm; Garcia-Regueiro and Diaz 1989, Moss *et al.*, 1992, Babol *et al.*, 2002). A study using a trained panel found the detection threshold for skatole to be lower at 0.008-0.06 ppm (Annor-Frempong *et al.*, 1997).

Organoleptic studies of milk fat have found the detection threshold values are between 0.6 and 0.9 ppm for indoles (Cant and Walker 1980). Early studies suggested that the indole and skatole content of normal (non-tainted) milk fat from cows and goats ranged from 0.05 to 0.3 ppm. *Lepidium*-tainted butterfat was shown to yield 0.5 ppm of skatole and 0.3 ppm of indole (Park 1969).

It has been shown that skatole and indole concentration in the meat/fat of ruminants is dependent on diet. This is consistent with skatole and indole being formed in the rumen

from dietary tryptophan, as it is likely that diet will affect the amount of skatole and indole that is formed. The amount of skatole and indole deposited in tissues will also be influenced by the efficiency with which the liver metabolises and clears the molecules from the body.

# **1.4. FORMATION OF SKATOLE AND INDOLE BY RUMINANTS**

Much of the research that looked at the formation of indoles from tryptophan in the rumen was conducted in the 1970's and early 1980's in association with pulmonary emphysema and edema in ruminants. This research was able to elucidate the formation pathway of skatole and indole in the rumen.

# 1.4.1. Structures and properties of indolic compounds

Flavour and odour compounds are attributed as being low molecular weight, volatile compounds. Such attributes originate from their chemical structure, as the chemical structure of any molecule will determine its molecular weight, polarity, volatility and other characteristics that influence chemical and physical properties.

# 1.4.1.1. Indole and skatole chemical structure

Indoles are biologically important heterocyclic aromatic compounds. Indole and skatole (3-methylindole) are fused-ring heterocyclic amines that consist of a benzene ring fused to the C2-C3 bond of pyrrole (Hart *et al.*, 2002; *Figure 1.4*).



Figure 1.4. Chemical structures of indole and skatole.

## 1.4.1.2. Chemical, physical and biological properties of indoles

The molecular weight of indole and skatole are 117.2 and 131.1, respectively. The boiling point of indole is 254°C while it is 265°C for skatole. In the solid-state skatole and indole are white crystalline compounds. Skatole is a volatile compound that is soluble in fat, hot water, alcohol, benzene, chloroform and ether (Deslandes *et al.*, 2001). Indoles, as a class of chemicals, are very easily oxidised. For example, the oxidation potential of skatole is estimated to be 1.07V (Yost 1989).

Indolic compounds including skatole are classed as nonpolar, lipophilic compounds that are capable of moving between fatty acid chains (Bray and Emmerson 1994). These chemical properties of indolic compounds give them the ability to disrupt biological membranes (Bray *et al.*, 1975). However, skatole-induced pneumotoxicity cannot be attributed to the direct effect of this indolic compound disrupting the lung membrane, as this does not account for organ and cell type specificity (Bray and Emmerson 1994) plus other indoles are unable to induce lung damage (Hammond *et al.*, 1980a, Carlson and Bray 1983). It is likely that toxicity involves the formation of reactive intermediates in specific tissues (Bray and Emmerson 1994).

## 1.4.1.3. Relation of chemical/physical properties to odour/sensory characteristics

In order for a substance to have odour and sensory characteristics, it needs to conform to certain chemical and physical properties. To create an odour impression the odourous molecule must traverse the nasal cavity to the olfactory receptor, which implies that a reasonable degree of volatility is required. Odour substances also need to pass through the mucous surrounding the nasal epithelium and the fat-layer of nerve cells to reach the receptors. This requires that the odourants have the capability to be both water- and fat-

soluble (Fisher and Scott 1997). Polarity of the molecules also has an influence on olfactory perception, as highly polar compounds are water-soluble but not fat-soluble. Indole and skatole fulfil all these preconditions necessary to create an odour impression, as they are fat soluble and soluble in water, highly volatile and non-polar compounds.

## 1.4.2. Rumen protein digestion and degradation

Indole and skatole are formed in the rumen from the microbial fermentation of tryptophan. Tryptophan is an essential amino acid that is derived from dietary and microbial protein. Approximately 1.2-2.3% of the amino acids in plant leaf protein is tryptophan (Allison 1973) while 0.6-5% of microbial protein consists of tryptophan (Anderson *et al.*, 1958).

## 1.4.2.1. Formation of free amino acids in the rumen from dietary protein

The digestion of forage protein in the rumen can be attributed to the combined processes of solubilisation and degradation. Solubilisation can be defined as the release of protein from plant cells into the rumen environment during chewing and is an important prerequisite for degradation (Nugent *et al.*, 1983, Min *et al.*, 2000). Solubility is an inherent characteristic of proteins (Chalupa 1975) and contributes to differences in rumen degradation of proteins from different feeds. Degradation of protein in the rumen is a consequence of the combined action of microbial and plant proteolysis (Kingston-Smith and Theodorou 2000), resulting in the formation of peptides and amino acids. Further action by microbial deaminases results in the formation of ammonia from peptides and amino acids. Protein degradation in this way involves two steps; firstly, there is the hydrolysis of peptide bonds (proteolysis) to produce peptides and amino acids and secondly, there is the deamination and final catabolism of amino acids (Mackie and White 1990, Cotta and Russell 1996). In spite of the proteolytic capabilities of rumen microbes and plant proteases, some dietary protein can resist degradation and bypass the rumen (Chalupa 1975).

# 1.4.2.2. Amino acid utilisation and degradation by microbes in the rumen

Following proteolysis, liberated peptides or amino acids may leave the rumen, be utilised for microbial growth or be degraded to ammonia (Mackie and White 1990, Nolan 1993). As well as ammonia, other products are formed from the microbial deamination of amino acids. In the case of the amino acid tryptophan, indolic compounds are formed (Deslandes *et al.*, 2001).

In the rumen, protein from fresh forage diets, such as perennial ryegrass/white clover pasture, is rapidly degraded, while the structural carbohydrate is relatively slowly degraded (Dove 1996). The result is a lack of energy for microbial protein synthesis to fully utilise the peptides, amino acids and ammonia that are released from the degradation of plant protein. Consequently, feeding perennial ryegrass/white clover pasture is likely to result in greater availability of tryptophan in the rumen for deamination and decarboxylation by rumen microbes to form indole and skatole (Carlson and Yost 1989; Russell *et al.*, 1992; Young *et al.*, 2003). Hence the higher skatole concentration in fat from steers and lambs finished on pasture (perennial ryegrass/white clover) compared to those fed grain has been related to the greater solubility and rapid degradation of plant protein making more tryptophan available in the rumen (Young and Baumeister 1999; Young *et al.*, 2003).

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## 1.4.3. Formation of skatole and indole from tryptophan in the rumen

Original studies into the formation of skatole and indole in the rumen were in relation to the occurrence of pulmonary edema and emphysema when animals were let out onto lush pasture after feedlot (Carlson and Breeze 1984). Once it was realised that indole and skatole themselves were not present in the plant material, investigations turned to finding the precursors in the plant that were likely to be modified to indole and skatole in the digestive tract.

# 1.4.3.1. Determination of tryptophan as the precursor for indole and skatole

Studies were able to show that intraruminal doses of tryptophan caused interstitial pulmonary emphysema and edema in cattle (called Acute Bovine Pulmonary emphysema, ABPE) within 1 to 5 days after dosing but, this disease did not occur when tryptophan was dosed intravenously or post-ruminally (Carlson *et al.*, 1968). Lewis and Emery (1962a, b) were able to show that adding tryptophan to the rumen of a steer and to rumen fluid incubated *in vitro*, did in fact produce indole and skatole. Further investigation showed that when skatole was dosed into the rumen or intravenously it was followed by the onset of ABPE. This showed conclusively that skatole was the causative agent for ABPE (Carlson *et al.*, 1972; Carlson *et al.*, 1975; Dickinson *et al.*, 1976). Once it had been established that tryptophan was the precursor for indole and skatole and skatole formation in the rumen, studies commenced to investigate the pathway of skatole and indole formation from tryptophan to provide information that could be used to control ABPE.

# 1.4.3.2. Elucidating the formation pathway of skatole and indole in the rumen Initial studies using an artificial rumen and $[^{14}C]$ -labelled L-tryptophan showed that

after 1 hour of incubation, 3% of the label was recovered as indole acetic acid (Yang and Carlson 1972). *In vitro* incubations inoculated with rumen fluid and with added [<sup>14</sup>C]-labelled L-tryptophan found indole, indole acetic acid and skatole to be the major metabolites of tryptophan (Yokoyama and Carlson 1974).

Further studies using [<sup>14</sup>C]-labelled tryptophan found 39% of the tryptophan was converted to skatole, 7% into indole and 4% into indole acetic acid. With the incubation of indole acetic acid, 38% of it was converted to skatole but no indole was formed. This indicated that indole acetic acid was an intermediate in the formation of skatole but not indole (Yokoyama and Carlson 1974). Furthermore, Okuuchi *et al.*, (1993a) showed that indole acetic acid added to bacterial incubations gave an increase in the formation of skatole. The formation of skatole with the [<sup>14</sup>C] label located on the methyl carbon of skatole from indole acetic acid suggested a decarboxylation reaction (Yokoyama and Carlson 1974). Indole appears to be formed independently of skatole in the rumen as reductions in skatole formation give varied responses with indole formation (Hammond and Carlson 1980).

Yokoyama and Carlson (1974) were able to conclude the route by which skatole is formed from L-tryptophan is a two-step process involving the initial formation of indole acetic acid with subsequent decarboxylation to skatole (*Figure 1.5*). Indole is formed in a one-step process from tryptophan (Deslandes *et al.*, 2001). Studies with anaerobic microorganisms from the intestine indicate that the microorganisms transaminate tryptophan with alpha-ketoglutarate to indolepyruvate and this is decarboxylated to form indole acetic acid (Chung *et al.*, 1975; *Figure 1.5*).



Figure 1.5. Pathway of indole and skatole formation from tryptophan in the rumen (from Deslandes *et al.*, 2001).

# 1.4.4. Microbes involved in formation of indolic compounds

Studies incubating tryptophan with washed suspensions of ruminal microorganisms revealed that indole and indole acetic acid are formed by a number of rumen microorganisms (Yokoyama and Carlson 1974; Carlson and Breeze 1984) including several species of *Clostridia*, which are able to utilise amino acids as a source of carbon and energy for growth (Elsden *et al.* 1976). Skatole formation however, has only been reported for five known species of bacteria to date. These being; *Lactobacillus* sp. Strain 11201, *Clostridium scatologenes, Clostridium nauseum, Rhizobium* sp. and *Pseudomonas* sp. (Honeyfield and Carlson 1990; Deslandes *et al.*, 2001). Currently, *Lactobacillus* sp. Strain 11201 has been the only bacteria identified with skatole production in the rumen. Studies are currently being undertaken to determine if other

rumen bacteria are capable of converting tryptophan to skatole in sheep grazing New Zealand pastures.

# 1.4.4.1. Lactobacillus sp. strain 11201.

Based on physiological, biochemical and metabolic characteristics *Lactobacillus* sp strain 11201 was identified as a bacterium responsible for the formation of skatole in the rumen (Yokoyama *et al.*, 1977; Honeyfield and Carlson 1990). Although this strain produces skatole from the decarboxylation of indole-3-acetic acid, it cannot convert Ltryptophan to indole, indole acetic acid or skatole. This shows that more than one bacterial species is involved in the production of skatole from tryptophan (Yokoyama *et al.*, 1977; Yokoyama and Carlson 1981; Honeyfield and Carlson 1990).

The difficulty in isolating this rumen bacterium suggests that it is not predominant in the rumen and the fact that it does not produce skatole in a controlled medium indicates that skatole formation is not its constitutive function. It is an obligate anaerobe with slow growth. The bacterium is gram-positive, non-spore forming, non-motile and does not produce gas. It forms short rods  $(1.9 \times 0.7 \mu m)$  irregular and pleomorphic in shape that occur in singles, pairs and short chains of four to six cells. This strain also forms paracresol (Yokoyama *et al.*, 1977; Deslandes *et al.*, 2001). The skatole-forming enzyme was found to be associated with the *Lactobacillus* bacterial cells or cell debris and thus, is not a cytosolic enzyme (Honeyfield and Carlson 1990).

### 1.4.4.2. Bacteria and protozoa interactions.

Incubations with only rumen protozoa do not produce skatole from tryptophan (Onodera *et al.*, 1992; Okuuchi *et al.*, 1993a; Mohammed *et al.*, 2003). Skatole production was 3-

5 times higher with a mixed microbial suspension (bacteria plus 4% protozoa) than bacterial-only suspensions, even though no skatole was produced by protozoa alone (Onodera *et al.*, 1992; Mohammed *et al.*, 2003). This suggests an interaction of the bacteria and protozoa. Protozoal-only incubations produce indole equivalent to the amount of tryptophan degraded indicating that indole is the principal metabolite resulting from tryptophan metabolism in rumen protozoa (Onodera *et al.*, 1992). However, Mohammed *et al.* (2003) indicated that protozoa were also capable of producing indole acetic acid.

Okuuchi *et al.* (1993a) concluded that in incubations containing only bacteria, that tryptophan did not get converted to indole acetic acid very quickly resulting in the accumulation of indole pyruvate, which is an inhibitor of the formation of skatole. In mixed suspensions containing bacteria and protozoa, tryptophan was rapidly converted to indole acetic acid. Protozoa have been shown to have strong tryptophan aminotransferase activities, producing substrate for the formation of skatole by bacteria (Okuuchi *et al.* 1993a).

# 1.4.4.3. Rumen conditions for indole and skatole formation by microbes.

Honeyfield and Carlson (1990) showed that bacterial formation of skatole from indole acetic acid was maximal at pH 7.5. Maximal skatole production occurred in rumen conditions where pH is neutral while indole production was not affected by pH in the range of 4.5-8.0. Lowering pH decreased skatole production due to a general decrease in microbial activity in the rumen (Hammond *et al.*, 1984).

The microorganisms involved in the conversion of tryptophan to skatole in the rumen seem to be resilient to deprivation of energy in the nutritive medium. Decreasing the concentration of glucose in rumen incubations resulted in a progressive increase in the conversion of [<sup>14</sup>C]-tryptophan to [<sup>14</sup>C]-skatole (Yokoyama and Carlson 1974). Short-term starvation may alter the microbial population in the rumen inhibiting the growth of one type of organism, but not others. Consumption of feeds high in tryptophan following a period of feed deprivation may enhance or amplify the production of skatole from the resilient microorganisms (Bray and Emmerson 1994).

# **1.5. ABSORPTION AND METABOLISM OF INDOLE AND SKATOLE**

Accumulation of indoles in the adipose tissue is not only dependent on the concentration of indoles formed in the digestive tract but, is also influenced by absorption from the gut, removal of indole from the body by metabolism in the liver and other tissues, and urinary excretion (Deslandes *et al.*, 2001).

# 1.5.1. Absorption of indoles in the ruminant.

Indoles that are formed in the rumen are absorbed from the digestive tract into the portal blood stream. The portal blood goes to the liver and after passage through the liver, the blood enters the general circulation of the body. A portion of the indoles in the blood will be metabolised by the liver on passage through this organ, with any indoles that bypass the liver being available for absorption by other tissues in the body (Babol *et al.*, 1998a).

# 1.5.1.1. Absorption from the digestive tract.

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Studies investigating the appearance of indolic compounds in the blood after direct rumen administration (Carlson *et al.*, 1975) or with the administration of tryptophan (Yokoyama *et al.*, 1975; Hammond *et al.*, 1984) indicate that the absorption of skatole and indole from the rumen is relatively rapid. In goats with both rumen cannula the half-life of skatole in the rumen was estimated as 2.5 hours (Hammond *et al.*, 1983).

Rumen and blood plasma concentration of indole and skatole in steers dosed with tryptophan were highest at 12-24 hours after dosing (Yokoyama *et al.*, 1975; Hammond *et al.*, 1984; *Figure 1.6*). The portal blood concentration of skatole and indole were shown to be greater than the arterial concentration for up to 48 hours after dosing,

indole in ruminal fluid and blood plasma and accounted for nearly 50% of the tryptophan that was dosed (Hammond *et al.*, 1983).

Skatole and indole that passes into the duodenum accounts for only 1.1% of the tryptophan consumed by the animal (Hammond *et al.*, 1984b). It is known that indole and skatole are terminal degradation products of rumen microbes so; the disappearance of indolic compounds from the gut can be attributed to their absorption (Deslandes *et al.*, 2001).



Figure 1.6. Concentration of skatole in rumen fluid and jugular blood plasma of a cow over a 96-hour period after an intraruminal dose of L-tryptophan at time 0 (0.35g kg<sup>-1</sup> of body weight; from Yokoyama *et al.*, 1975).

## 1.5.1.2. Absorption from the blood.

The concentration of skatole in the blood plasma is likely to reflect the skatole enroute to excretion or body deposition. Jugular infusion of skatole resulted in a low blood plasma concentration suggesting rapid clearance of skatole from the blood plasma (Hammond *et al.*, 1979b). The half-life of skatole in the blood has been estimated at 20-25 minutes (Bray and Carlson 1979, Bradley and Carlson 1982). Almost all of the skatole infused into the jugular vein of goats was metabolised and excreted in the urine within 72 hours (Hammond *et al.*, 1979b). This indicates that skatole is rapidly absorbed but does not accumulate in the blood and therefore, is rapidly excreted or absorbed by body tissues (Carlson and Dickinson 1978; Yokoyama *et al.*, 1975; Bray and Carlson 1979; Bradley and Carlson 1982).

# 1.5.2. Metabolism and excretion of skatole and indole in the ruminant

Metabolism of lipophilic, non-polar indolic compounds to more polar forms is essential for their detoxification and subsequent excretion (Bray and Emmerson 1994). The indole and skatole produced in the rumen is metabolised in the liver with the products of metabolism excreted in the urine.

# 1.5.2.1. A background in lung toxicity and boar taint

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Much of the literature investigating the metabolism of skatole has been focussed on determining the mechanisms of pulmonary selectivity and the precise chemical transformations of skatole that lead to lung injury associated with pulmonary edema and emphysema (Yost 1989). Although much of the research emphasis with ruminants has been on the metabolism of skatole in the lungs, there have been investigations into the excretion of indole metabolites in the urine and the formation of these excretory

products in the body. This gives clues to the possible mechanism by which skatole is metabolised and excreted.

Skatole formed by hindgut fermentation in pigs has been found to contribute to boar taint and the level of skatole that ends up in the adipose tissue has been linked to the effectiveness of the liver to clear skatole from the body (Squires and Lundstrom 1997). Consequently, much of the research into liver metabolism of skatole has focused on the pig. Due to its toxic effects in the lungs and its role in boar taint, skatole has been the primary compound of interest when studying indole metabolism and consequently, skatole is the main compound discussed in this review in terms of metabolism and body clearance of indoles in the ruminant.

# 1.5.2.2. Urinary metabolites

The earliest research into the metabolism of skatole in ruminants looked at the appearance of skatole metabolites in the urine. Ten distinct urinary metabolites but, not skatole itself, were detected in the urine of goats that had been infused with <sup>14</sup>C-skatole. The major urinary metabolite in goats appeared to be 3-methyloxindole or related derivatives as 60% of the radioactivity was associated with these products in the urine. Also present in the urine at a high concentration was indole-3-carboxylic acid and its conjugates (Hammond *et al.*, 1979b). In pigs, 3-hydroxy-3-methyloxindole was identified as a metabolite produced by liver microsomes (Babol *et al.*, 1998b) and a conjugate of this compound was found in the urine of goats (Smith *et al.*, 1993).

Formation of mercapturic acid conjugates was the major metabolic pathway in pigs as

the mercapturate adduct, 3-[(*N*-Acetylcysteine-*S*-yl)methyl]indole was the main metabolite found in the urine of pigs (Bæk *et al.*, 1997). The mercapturate adduct has also been identified at a low concentration in the urine of goats and mice (Skiles *et al.*, 1991).

In studies where radioactive tryptophan was dosed to cattle, the first detectable radioactivity appeared in the urine after 2-3 hours. Radioactivity continued to be excreted in the urine until 42-60 hours after dosing. The formation of indole derivates that are excreted in the urine is important for detoxifying high doses of tryptophan in the ruminant, as 77.8% of the radioactivity from tryptophan dosed to cattle was detected as indole derivatives in the urine (Yang and Carlson 1972).

# 1.5.2.3. Pathways of skatole metabolism

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When goats were infused with [<sup>14</sup>C]-labelled skatole into the rumen, less than 1% of the label appeared in expired air and in the faeces. The skatole did not accumulate in the liver, lungs or kidneys. However, radioactivity from labelled metabolites was present in these tissues after 30 minutes. Therefore, skatole was rapidly metabolised and excreted in the urine and did not accumulate at a high concentration in the tissues of the organs associated with skatole metabolism (Hammond *et al.*, 1979b).

Skatole can be metabolised by several pathways (*Figure 1.7*). The metabolites of skatole identified in mammals are 3-methyloxindole and its derivatives, the ring-hydroxylated sulphate conjugates, 3-methyl oxidation products such as indole-3-carbinol and indole-3-carboxylic acid and the pyrrole ring opened products 2-
formamidoacetophenone and 2-aminoacetophenone and 3-methyloxindole (Skiles *et al.*, 1989; Bæk *et al.*, 1997; *Figure 1.7*). The pathway for the metabolism of skatole appears to be species dependent. As an example, the major metabolite found in the urine of goats (a ruminant) is 3-methyloxindole (and its derivatives) while, 6-sulphatoxyskatole is present in the urine of humans but has not been detected in goats, mice or rats (Bæk *et al.*, 1997).



Figure 1.7. Pathways of skatole metabolism (from Bæk et al., 1997).

The major skatole metabolic pathway in the ruminant occurs in two phases. The first phase is the oxidation of skatole, which is catalysed by the P450 cytochrome in the liver. Oxidation results in the formation of methyloxyindole and hydroxylated metabolites (*Figure 1.8*). The second phase involves the conjugation of oxidation products to glucuronic acid, glutathione and sulphate (Smith *et al.*, 1993). The conjugation reactions are catalysed by transferases (Babol *et al.*, 1998a). The effect of conjugation is to decrease the biological activity and increase the water solubility of the substance thus, facilitating its excretion from the body in the urine.



Figure 1.8. Outline of the two phases involved in the metabolic pathway of indole and skatole in the ruminant

## 1.5.2.4. Role of the liver and P450 cytochrome

Early evidence suggested that mixed function oxidases are involved in the metabolism of skatole in various tissues throughout the body (Bray and Carlson 1979). Monooxygenase activity associated with P450 in the lung results in the formation of intermediates from skatole. These intermediates covalently bind to tissue macromolecules in the lung and cause cellular damage associated with pulmonary emphysema and oedema (Nocerini *et al.*, 1983). However, binding of 3-methylindole or its intermediates to hepatic tissues does not cause hepatotoxicity and this is likely to be due to differences in the detoxification of reactive metabolites in different organs (Thornton-Manning *et al.*, 1993).

Cytochrome P450 refers to a group of hemoproteins responsible mainly for oxidative metabolism of a large number of small molecular weight, usually non-water soluble, lipophilic compounds. The P450 cytochrome facilitates the clearance of skatole by forming intermediates in skatole metabolism which then go on to be conjugated to sulphate or glucuronic acid (Babol *et al.*, 1998a). Skatole fits well into the class of compound biodegraded by cytochrome P450 and studies have shown that the cytochrome P450 system is involved in the metabolism of skatole (Yost 1989; Skiles *et al.*, 1989; Thornton-Manning *et al.*, 1993).

The P450 cytochrome system is found embedded in the membranous endoplasmic reticulum of most eukaryotic cell types (Peterson and Prough 1986) although the liver appears to be the primary site for the metabolism of skatole (Babol *et al.*, 1998b). There are a great number of P450 isozymes in the mammalian hepatic P450 family but even

before the discovery and isolation of multiple forms of hepatic cytochrome P450 the broad and overlapping substrate specificity of these enzymes was recognised in microsomal preparations (Miwa and Lu 1986).

Cytochrome P4502E1 is considered as the predominant cytochrome for the metabolism of skatole in the liver of pigs (Doran *et al.*, 2002) although the use of specific P450 cytochrome inhibitors indicated that P4502A6 has a more relevant role in skatole metabolism (Diaz and Squires 2000). Furthermore, Thornton-Manning *et al.* (1993) reported that eight other forms of P450 can metabolise skatole. A comparison of the metabolic activity of P4501A1 in humans and rats revealed that the two cytochromes have similar abilities to metabolise heterocyclic amines (Kanazawa *et al.*, 1999). The P450 cytochrome involved in the liver metabolism of skatole in ruminants has not yet been identified.

The monooxygenase reaction catalysed by cytochrome P450 requires the input of two electrons. In mammalian systems, these two electrons are derived from NADPH, which are introduced to the P450 cytochrome in two sequential one-electron steps. The electron chain that feeds electrons to the cytochrome P450 consists of a complex flavoprotein. Microsomal preparations have indicated that NADPH-cytochrome *c* reductase feeds electrons from NADPH to P450 cytochromes. NADPH-cytochrome *c* has both flavin adenine dinucleotide (FAD) and flavin mononucleotide (FMN) prosthetic groups. The initial electron acceptor from NADPH is FAD while FMN serves to reduce the P450 cytochrome. (Peterson and Prough 1986).

P450 functions in a cyclic manner beginning with a substrate-binding and electron acceptance step that results in the conversion of the heme iron from the ferric form (Fe3+, low-spin) to the ferrous form (Fe2+, high-spin). Oxygen is then bound to the heme followed by the acceptance of a second electron. One of the bound oxygen atoms is then reduced to water and a hydrogen atom is abstracted from the substrate to form a free radical of the substrate. The free radical then acquires the second oxygen atom to form the hydroxylated product (Ortiz de Montellano 1986, Stryer 1995, *Figure 1.9*).



Figure 1.9. Catalytic cycle of cytochrome P450 in monooxygenase reactions (from Stryer 1995).

# 1.5.3. Relationship of liver metabolism and deposition of indoles in the fat

A high concentration of indoles in the serum of blood collected during exsanguination corresponded to a high concentration of indoles in the subcutaneous fat of pigs (Tuomala *et al.*, 1996). The skatole that remains in the blood after absorption from the gut and liver metabolism was found to be quickly deposited in fatty tissue (Rius and Garcia-Regueiro 2001).

The liver is the primary site for the metabolism of skatole and the concentration of skatole in the blood above the liver's P450 cytochrome capacity will bypass liver metabolism and enter the general circulation to be available for deposition in the body fat. Therefore, liver enzyme activities are likely to have a dramatic effect on skatole concentration in fat depots (Babol *et al.*, 1998a). The *in* vivo contribution of the P450 cytochrome depends on the intrinsic metabolic activity of the enzyme and its abundance (Diaz and Squires 2000).

Cytochrome P450 enzymes are inducible by a variety of dietary factors and a number of chemical reagents including indolic compounds (Yang *et al.*, 1992). However, injecting skatole into pigs on a daily basis (1mg kg liveweight, intramuscularly) did not affect the rate of oxidation or conjugation reactions (Babol and Squires 1999). The rate of microsomal skatole disappearance is proportional to the P4502E1 content of the microsomes (Doran *et al.*, 2002) and high levels of cytochrome P4502E1 in the liver of pigs were correlated to low levels of skatole in the body fat depots. This is presumably due to the rapid metabolism and clearance of skatole by P4502E1 (Squires and Lundstrom 1997, Doran *et al.*, 2002).

# **1.6. CHANGING MEAT FLAVOUR BY CONTROLLING SKATOLE** AND INDOLE FORMATION AND METABOLISM IN RUMINANTS

Previous interest in reducing the formation of skatole in the ruminant has centred on trying to control pulmonary emphysema and oedema (Hammond *et al.*, 1978; Hammond *et al.*, 1980b), while the pig has been the model for looking at liver metabolism of skatole and deposition in body fat in relation to boar taint (Lundstrom *et al.*, 1994; Squires and Lundström 1997). The research that has been done on skatole mitigation in ruminant and monogastric animals can be applied to control the formation and metabolism of skatole and indole to ameliorate pastoral flavours in the meat of ruminants.

## 1.6.1. Ionophore antibiotics

Hammond and Carlson (1980) screened a wide range of compounds to test their ability to reduce indole and skatole formation during *in vitro* rumen fermentations. The compounds they used included deaminase and methane inhibitors, decarboxylase inhibitors, ionophore antibiotics, general antibiotics (such as penicillin) and other biologically-active compounds. The study concluded that ionophore antibiotics were the most effective at reducing the formation of skatole.

#### 1.6.1.1. Effect of ionophore antibiotics on skatole concentration in the rumen.

Skatole production was reduced by more than 80% *in vitro* when ionophores were incubated in rumen fluid (Hammond and Carlson 1980). *In vivo* trials using monensin (Hammond *et al.*, 1978, 1980b; Carlson *et al.*, 1983; Honeyfield *et al.*, 1985) and lasalocid (Nocerini *et al.*, 1985) have also shown reduced formation of skatole in the rumen. Monensin decreased rumen skatole concentration by approximately half when

given to cows on pasture compared to cows not supplemented with monensin (Carlson *et al.*, 1983; Potchoiba *et al.*, 1992). However, the effects of monensin are generally short term and in animals dosed with monensin, the rumen skatole concentration was not different to that of untreated animals approximately 10 days after its administration (Carlson *et al.*, 1983).

## 1.6.1.2. Mode of action

The mode by which monensin has its effects in the rumen are still unclear however, it is believed that monensin inhibits the growth of gram positive deaminating ruminal bacteria (Potchoiba *et al.*, 1992) or possibly alters rumen fermentation by changing the metabolism of rumen microorganisms (Joblin 1993).

Incubating ionophore antibiotics in the presence of either tryptophan or indole acetic acid suggests the inhibitory effect of ionophores is on the process of tryptophan conversion to indole acetic acid and of indole acetic acid to skatole (Hammond and Carlson 1980). However, the use of monensin also increased the conversion of tryptophan to indole (Hammond and Carlson 1980). In terms of meat flavour, this would not be ideal, as both skatole and indole have been linked to pastoral flavour (Young *et al.*, 2003).

## 1.6.2. Microbial manipulations

The rumen environment contains bacteria, protozoa, fungi and other micro-organisms that influence the degradation of feed protein and availability of protein to the ruminant. Furthermore, bacteria are involved in the formation of skatole and indole from tryptophan. Ionophore antibiotics can have an antimicrobial effect that results in a decrease in skatole and indole concentration in the rumen. However, other microbial manipulations could possibly be used to decrease the formation of indolic compounds from protein in the rumen.

## 1.6.2.1. Defaunation to decrease degradation of protein in rumen

Improved production in defaunated sheep has been attributed to a net decrease in protein degradation and an increased flow of feed and bacterial nitrogen into the duodenum (Bird and Leng 1984; Ushida and Jouany 1985; Hsu *et al.*, 1991). Defaunation is also associated with a decrease in rumen ammonia concentration (Hsu *et al.*, 1991).

Protozoa aid in the degradation of plant cell walls and make intracellular plant proteins more accessible to bacterial enzyme degradation. When protozoa are removed from the rumen, protein degradability and the rate of protein degradation are lower compared to the faunated rumen (Ushida and Jouany 1985). Furthermore, protozoa have also been shown to liberate endogenous tryptophan while bacteria incorporate tryptophan into microbial protein (Mohammed *et al.*, 2003). It is likely that defaunation will decrease the amount of protein that is degraded to amino acids and decrease the amount of tryptophan available to be converted to skatole and indole.

## 1.6.2.2. Defaunation to reduce conversion of tryptophan to skatole and indole

When a mix of rumen bacteria and protozoa were incubated with tryptophan, more skatole was formed than in incubations containing only bacteria even though incubations with only protozoa produced no skatole (Onodera *et al.*, 1992; Okuuchi *et al.*, 1993a,b; Mohammed *et al.*, 2003). This suggested an interaction between rumen

protozoa and bacteria that has been attributed to the active deaminase activity of ciliate protozoa (Wallace *et al.*, 1987). Thus, the *in vitro* studies indicate that removing the protozoa from the rumen environment is likely to reduce the amount of skatole and indole formed from tryptophan; however to date, no *in vivo* studies have been undertaken using defaunated animals to test this theory.

## 1.6.3. Animal Factors

Comparison of *in vivo* ruminal skatole levels in individual cows has suggested that some cows are more resistant to skatole induced pulmonary emphysema and oedema. Factors such as genetics, nutritional status or metabolic differences in detoxification may be involved (Honeyfield *et al.*, 1985).

## 1.6.3.1. Animal variation and selection

Animal-to animal variation is substantial for the concentration of indoles in the body fat when animals are fed the same diet (Young *et al.*, 2003). Skatole levels in the back fat of pigs vary widely amoung different groups of pigs and this has been attributed to diet and environmental factors. However, differences in back fat skatole concentration between individual pigs under the same management and environmental conditions has been proposed to be due to genetic factors (Squires and Lundström 1997).

Higher skatole concentration in the back-fat of some swine maybe indicative of differences in the metabolism of skatole, as Diaz *et al.*, (1999) showed large interindividual differences in the rate of production of phase one metabolites and attributed this to differences in cytochrome P450 enzyme activity. It is well established that genetic factors influence the expression of P450 in humans and rodents, and genetic differences in P450 expression (referred to as polymorphisms) have been discovered in humans via clinical pharmacological approaches such as metabolic profiles of drugs in population studies (Nhamburo *et al.*, 1990). In pigs, wild-type breeds have a higher skatole concentration in the back fat compared to the Yorkshire breed (Lundström *et al.*, 1994). High variability in the P450 cytochrome content and activity in pig hepatic tissue could be due to genetic polymorphisms and could explain the variability in the back fat concentration of skatole when pigs are managed under similar conditions (Diaz and Squires 2000).

## 1.6.3.2. Improving liver metabolism

It was reasoned that selection for faster growth and decreased back fat depth in Yorkshire pigs had resulted in a higher expression of cytochrome P450 enzymes in the liver and hence, higher skatole clearance from the body (Lundström *et al.*, 1994; Squires and Lundström 1997). In agreement with this theory, the rate of skatole disappearance was proportional to the P450 content in the microsomes (Doran *et al.*, 2002) and a low concentration of skatole in the back-fat of pigs has been correlated to high content of cytochrome P450 in the liver (Babol *et al.*, 1998b). This suggests that there is the possibility of using genetic selection to improve liver metabolism and lower the concentration of indolic compounds in meat.

Cytochrome P450 activity can be induced in the liver by a number of chemical agents (Rahden-Staron *et al.*, 2001; Ueng *et al.*, 2001; Madan *et al.*, 2003). However, the studies on P450 inducers to date, have largely been tested with hepatocytes or microsomal preparations from monogastric species and have not investigated those

P450 cytochromes involved with indole and skatole metabolism in the liver of ruminants. It may be possible to find an inducer of liver P450 in ruminants to improve the metabolism of indole and skatole.

## 1.6.4. Feeding Regimes

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Since pastoral flavours originate from the type of feed consumed by ruminants, a logical step to improve meat flavour would be to implement changes in the diet that would lower skatole and indole production in the rumen and thus, improve meat flavour.

# 1.6.4.1. Grain and concentrate diets

The majority of the literature on this subject suggests that the flavour of meat from animals fed grain is preferential to the flavour of meat from animals fed on forage (*Section 1.3.1*). Thus, the simplest way to improve meat flavour would be to feed grain or concentrate diets. This was confirmed by Young *et al.* (2003) who found a very low concentration of indolic compounds in the fat of lambs that had been fed a maize-based diet and this was related to a lower nitrogen to soluble carbohydrate ratio (*Table 1.3*) and a slower rate of protein degradation.

It has been recommended that a grain diet fed for 90-100 days before slaughter removes the effect of feeding pasture on meat flavour and significantly improves the consumer acceptability of the flavour of beef and sheep meat (Melton 1983). Furthermore, Larick *et al.* (1987) found a significant decrease in grassy flavour intensity in beef after 84 days on a concentrate diet following a change in diet from pasture.

As simple as this solution seems, it is not practical or economical in New Zealand. In

temperate regions of the world, grazing pasture confers a higher profit margin, as pasture is a cheap source of feed compared to grain (Boutonnet 1999). In practical terms, traditional grazing systems are not set up to implement intensive grain feeding to improve meat flavour. In a country like New Zealand, there is the further problem that the production of grain for animal feed is minimal and occurs in selective regions, which adds price and logistical problems for meat producers considering the use of grain feeding.

## 1.6.4.2. Increased readily fermentable carbohydrate

High skatole and indole concentrations in the rumen when animals are fed fresh forages has been linked to a high concentration of protein in the forage relative to the readily fermentable (or soluble) carbohydrate (*Table 1.3*) leading to inefficient use of tryptophan in the rumen (Young *et al.*, 2003). Protein degradation is rapid while fibre, the principal carbohydrate of plant material, is broken down slowly in the rumen. Increasing the readily fermentable carbohydrate content of the diet would provide a rapid source of energy to improve the incorporation of amino acids and ammonia into microbial protein and reduce the amount of tryptophan that was converted to skatole and indole.

Table 1.3.	Composition	of pasture	compared	to conc	entrates	(g	kg <sup>-1</sup> )	and	the	ratio	of
nitrogen an	id carbohydra	ites (adapte	d from You	ng et <b>a</b> l.,	, 2003).						

	Crude Protein (N)	Soluble carbohydrate (CHO)	Acid detergent fibre	Neutral detergent fibre	Ratio N/CHO
Summer pasture	136	138	269	445	1.0
Autumn pasture	208	114	232	407	1.8
Lucerne-based concentrate	166	215	ND	ND	0.8
Maize-based concentrate	128	370	ND	ND	0.4

Increasing the readily fermentable carbohydrate content of *in vitro* incubations, by adding starch or glucose, reduced the formation of skatole and indole from tryptophan by 2-4 fold (Mohammed *et al.*, 2003). It is likely that the increased readily fermentable carbohydrate content provided more energy to microbes allowing more tryptophan to be incorporated into microbial protein. In a study by Kim *et al.*, (1999), it was shown that infusing a readily fermentable carbohydrate (maltodextrin) into the rumen of dairy cows, in synchrony with ruminal nitrogen release, increased microbial protein synthesis and reduced rumen ammonia concentration.

## 1.6.6. Reducing rumen protein degradation using condensed tannins

With the grazing of pasture, the rapid breakdown of plant protein in the rumen results in the rapid release of peptides and amino acids into the rumen environment. Amino acids are broken down to form ammonia, which is absorbed across the rumen wall, converted to urea in the liver, and then excreted in the urine (MacRae and Uylatt 1974; Uylatt and MacRae 1974; Waghorn and Barry 1987).

A central theorem in improving protein utilisation and animal production in the grazing ruminant is to increase the amount of rumen by-pass protein so more protein reaches the small intestine and less is lost as ammonia absorbed through the rumen wall (Barry 1981). If this can be achieved, this could also reduce skatole and indole formation in the rumen and improve meat flavour, since skatole and indole formation has been linked to rapid protein degradation in the rumen.

Condensed tannins are phenolic compounds found in a variety of legume forages

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(Terrill *et al.*, 1992). Condensed tannins can confer nutritional advantages to ruminants by reducing protein degradation in the rumen and increasing the flow of protein and essential amino acids to the intestine (McNabb *et al.*, 1996). Therefore, it is likely that dietary condensed tannin has the potential to reduce the amount of skatole and indole formed in the rumen and deposited in the meat, which would in turn improve meat flavour. This hypothesis has not yet been tested.

# **1.7. CONDENSED TANNINS**

Tannins are naturally occurring plant phenols and are classified secondary metabolites in plants. Tannins are broadly divisible into two major groups, the hydrolysable tannins (polyesters based on gallic and ellagic acid) and polymeric proanthocyanidins which are commonly called condensed tannins (Haslam 1989; Mangan 1988; Tanner *et al.*, 2000). Condensed tannins are largely responsible for the range of reactions normally attributed to tannins (Haslam 1989).

# 1.7.1. Prevalence and chemical structure of condensed tannins

Condensed tannins are widespread in woody plants, but are also found in many forage plants. There is a great diversity in the molecular structure of CT. This is a consequence of differences in the hydroxylation of flavan-3-ol units, stereochemistry, interflavanoid bonds, ratio of procyanidin to prodelphinidin units and the number of flavan-3-ol units in the condensed tannin polymer (Foo *et al.*, 1982; Williams *et al.*, 1983; Haslam 1989; McNabb *et al.*, 2000).

#### CHAPTER 1: Literature Review

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	CT concentration (g kg <sup>-1</sup> DM)			• •	Predominate	Mean	<b>B</b> <i>t</i> =			
Plant species .	Free	Protein- bound	Fibre- bound	Total	<ul> <li>Assay type</li> </ul>	monomer <sup>2</sup>	weight	Reference		
Perennial ryegrass (Lolium perenne)	0.8	0.5	0.5	1.8	Butanol-HCI	PC & PD		Jackson <i>et al.</i> , 1996		
White clover (Trifolium repens)	ND1	ND1	ND <sup>1</sup>	0.6	DMACA-HCl3	PD	·	Li et al., 1996		
Lucerne (Medicago sativa)	0	0.5	0	0.5	Butanol-HCI	PD	-	Jackson et al., 1996		
Birdsfoot trefoil (Lotus corniculatus)	36	9	2	47	Butanol-HCI	PC	2500-2700	Jackson et al., 1996; Meagher et al., 2004		
Big trefoil (Lotus pedunculatus)	61	14	1	77	Butanol-HCI	PD	5200-5500	Terrill et al., 1992; Meagher et al., 2004		
Sulla (Hedysarum coronarium)	33	9	3	45	Butanol-HCl	PD	-	Terrill et al., 1992		
Canary clover (Dorycnium rectum)	83	54	6	143	Butanoi-HCi	PD	-	Terrill et al., 1992		
Chicory (Chicorium intybus)	1.4	2.6	0.2	4.2	Butanol-HCI	PD	-	Jackson et al., 1996		
Red clover (Trifolium pratense)	0.4	0.6	0.7	1.7	Butanoi-HCI	PC	- Jackson et al., 1996; Sivakumaran et			
Sainfoin (Onobrychis viciifolia)	ND1	ND1	ND <sup>1</sup>	38.1	Butanol-HCI	PD	2040-3060 Bermingham et al., 2001; Min and Hart			
Dock (Rumex obtusifolius)	ND1	ND1	$ND^1$	25.9	Vanillin-HCI	PC		Waghorn and Jones 1989		

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Table 1.4. Concentration, predominate monomer type and mean molecular weight of condensed tannins in the leaves of forage plants.

<sup>1</sup> Not determined <sup>2</sup> PC = Procyanidin, PD = Prodelphinidin <sup>3</sup> DMACA-HCI = 4-timethytaminocinnamatdetxte: hydrochloric acid

#### 59

#### 1.7.1.1. Prevalence of condensed tannins in plants

Much research has been undertaken to characterise CT in temperate forages and the effect of CT on protein digestion in the ruminant. The concentration of CT in some forage plants that have been used in studies with ruminants is summarised in *Table 1.4*. Condensed tannins are present in only trace amounts in perennial ryegrass and white clover foliage (Barry 1989; *Table 1.4*).

## 1.7.1.2. The condensed tannin monomers

The constitutive flavan-3-ol monomers in CT have an A, B and C ring structure (*see Figure 1.10*). Ring A is derived from acetate via malonate and the B and C rings are derived from cinnamate (Haslam 1989).

From the hydroxylation pattern of the flavan-3-ol unit the CT unit can be classified as a procyanidin, which has a catechol hydroxylation pattern with hydroxyl groups at the 4' and 5' carbons of the B ring or a prodelphinidin that has a gallocatechol hydroxylation pattern with hydroxyl groups at the 3', 4' and 5' carbons on the B ring (Haslam 1989).

## 1.7.1.3. Stereochemistry of monomers

The stereochemistry of the C-ring in the flavanol monomers can be either 2,3-*trans* (catechin, and gallocatechin) or 2,3-*cis* (epicatechin, and epigallocatechin). Of these monomers, catechin and epicatechin are the most widely distributed in plants (Haslam 1989).

## 1.7.1.4. Interflavonoid linkage

Within the CT polymer, the monomeric units are linked together with interflavan bonds predominately of the C8 in the A ring to C4 in the C ring. Less common are C4 to C6 linkages that give rise to polymer branching (Haslam 1989).



Figure 1.10. The condensed tannin monomer. When R1 is H, the condensed tannin is an (epi)catechin polymer (procyanidin) and when R1 is OH, the condensed tannin is an (epi)gallocatechin polymer (prodelphinidin) (from Barry and McNabb 1999).

#### 1.7.1.5. Procyanidin to prodelphinidin ratio

Pure prodelphinidin polymers (gallocatechin and epigallocatechin) are rare compared to procyanidin polymers (catechin and epicatechin). Mixed procyanidin/prodelphinidin polymers commonly occur in plants, where both types of flavan-3-ols are randomly distributed in the structure of the proanthocyanidin polymers (Haslam 1989). *Lotus*  *pedunculatus* CT is a mixed polymer made up of predominately prodelphinidin subunits while the CT in *Lotus corniculatus* is predominately procyanidin (Foo *et al.*, 1996, 1997; Meagher *et al.*, 2004).

# 1.7.2. Chemical properties and protein binding activity of condensed tannins

The reactivity of CT has been defined as the ability of the CT to precipitate protein per unit weight (Bate-Smith 1973). Condensed tannins are known to complex with a range of molecules but derive their main biochemical properties from an ability to precipitate protein at a neutral pH (Tanner *et al.*, 2000). There is evidence that stereochemistry and monomeric composition as well as molecular weight can have a significant effect on CT protein binding activity (Jones *et al.*, 1976; Beart *et al.*, 1985; Ayres *et al.*, 1997).

## 1.7.2.1. Molecular weight

Molecular weight of CT polymers varies widely and is dependent on the number of units that make up the polymer (Haslam 1989). Condensed tannins in temperate forages have a mean molecular mass in the range of 2000-4000 (Foo *et al.*, 1982) however, recently it has been demonstrated that the CT of *Lotus pedunculatus* include polymers of very high molecular weight (Meagher *et al.*, 2004).

Molecular weight is an important property for the precipitation ability of CT (Waterman 2000). Tannins produced by different species of plant vary in their capability to precipitate protein and reactivity tends to increase with increased molecular weight (Horigome *et al.*, 1988).

#### 1.7.2.2. Binding of condensed tannin to protein to form complexes

The effectiveness of CT as complexing agents derives from the numerous phenolic groups and aryl rings on the periphery of the molecule, which provides a multiplicity of potential binding sites with favourable steric opportunities for hydrogen bonding (McLeod 1974). The affinity of CT for proteins has been attributed to the strong hydrogen bonding affinity of the carbonyl oxygen of the peptide group of the protein (McLeod 1977; Haslam 1989).

The CT and protein association is largely a surface phenomenon and is thought to take place in two distinct phases. Firstly, the CT molecule seeks out preferred sites and regions on the protein where its numerous aromatic rings are most readily accommodated. This in turn brings aromatic and hydrocarbon groups into close proximity creating a hydrophobic environment. Secondly, the association is reinforced with hydrogen bonds between the phenol residues and polar groups (Haslam 1989). The bonding is dynamic with individual linkages being continually broken and reformed in a random manner (Barry and Manley 1986).

Condensed tannins exhibit considerable variation in their affinity for different proteins, which suggests that CT interact with protein in a specific and selective manner. Those proteins that bind strongly with CT have a high molecular weight, open and flexible tertiary structure and high contents of proline and other hydrophobic amino acids (Hagerman and Butler 1981; Asquith and Butler 1986; Spencer *et al.*, 1988; Waterman 2000). Condensed tannins of higher prodelphinidin content are said to have greater protein binding activity (Jones *et al.*, 1976).

#### 1.7.2.3. pH dependent activity

The CT-protein interaction will be strongest when the pH is near the isoelectric point of the protein, as this minimises the protein-protein electrostatic repulsion (Hagerman and Butler 1981; Mangan 1988). The binding between CT and forage protein is pH dependent and stable complexes will be formed at a pH between 3.5 and 7.5 while the CT and protein will dissociate at a pH below 3.5 (Jones and Mangan 1977). Therefore, CT will form a stable complex with protein in the rumen, which would then dissociate in the acidic conditions of the abomasum (Waghorn *et al.*, 1990).

## 1.7.2.4. Effect of polyethylene glycol

The bond between polyethylene glycol (PEG) and CT is very strong (Barry and Manley 1986). Polyethylene glycol preferentially binds with CT and thus, inhibits the activity of the CT with other substrates, preventing the CT from binding with plant proteins in the rumen (Jones and Mangan 1977; Min *et al.*, 2003). The nutritional effects of CT have generally been assessed by administrating PEG to one group of experimental animals while another group receives a placebo such as water. The effects of CT are determined by comparing data from the CT-active (placebo) and CT-inactive (PEG-dosed) groups (Min *et al.*, 2003).

#### 1.7.3. Condensed tannins and the ruminant

Moderate concentrations of CT in forages can exert beneficial effects on protein metabolism in ruminants and thus improve production (Aerts *et al.*, 1999; Min *et al.*, 2003). Condensed tannins have also been shown to be inhibitory to some species of rumen bacteria (Min *et al.*, 2002).

## 1.7.3.1. Reduced protein degradation in the rumen

In ruminants fed fresh forages there is a substantial degradation (75%) of feed protein to amino acids, most of which is deaminated to ammonia in the rumen. Some of the ammonia will be incorporated into microbial protein. However, the rate at which ammonia is produced exceeds the rate at which it can be incorporated into microbial protein. Consequently, 20-35% of the nitrogen in fresh forage diets is lost as ammonia that is absorbed across the rumen wall and converted to urea in the liver (MacRae and Ulyatt 1974).

Low concentrations of CT in the diet are beneficial in ruminant nutrition due to their effect in reducing rumen degradation of forage protein (Min *et al.*, 2003). Reducing rumen degradation of protein decreases protein lost as ammonia and increases the amount of undegraded plant protein passing into the intestines which is available to the animal (Waghorn *et al.*, 1990; Waghorn and Shelton 1992).

The CT in *Lotus pedunculatus* was found to reduce the disappearance of the predominate plant protein, ribulose-1,5-bisphosphate carboxylase/oxygenase, in the rumen by reducing the rates of both solubilisation and degradation (McNabb *et al.*, 1996; Min *et al.*, 2000). The effect of CT reducing the degradation rate of protein in the rumen was evident from reduced ammonia concentration in the rumen when ruminants were fed *Lotus* sp. that contain CT (Waghorn and Shelton 1995, 1997).

#### 1.7.3.2. Anti-microbial effects

Condensed tannins from *Lotus corniculatus* not only reduce the microbial degradation of protein in the rumen but also inhibit the growth of some species of proteolytic bacteria (Min *et al.*, 2002). It has been suggested that the mechanism by which tannins are antimicrobial is either through enzyme inhibition, disrupting the integrity of cell membranes or through the deprivation of metal ions (Scalbert 1991). There was less growth of ruminal bacteria with the addition of CT from *Desmodium ovalifolium* in biological assays (Nelson *et al.*, 1997). Jones *et al.* (1994) also showed that CT from sainfoin inhibited the growth of some ruminal bacteria and that this was likely to be due to an interaction of the CT with the bacterial cell wall.

## 1.7.3.3. Increased amino acid absorption in intestines

Post ruminal infusion of protein and amino acids identified that wool production and growth in ruminants fed fresh forages is limited by the absorption of essential amino acids from the small intestine (Barry 1981). With *Lotus* species, duodenal non-ammonia nitrogen flow increases linearly with increasing CT concentration and equals nitrogen intake at a CT content of approximately 40g kg<sup>-1</sup> DM (Barry and Manley 1984). The CT in *Lotus corniculatus*, fed to sheep increased the amount of essential amino acids apparently absorbed from the small intestine by 62% compared to those infused with PEG (Waghorn *et al.*, 1987). Sheep fed *Lotus pedunculatus* had a greater flux of sulphur amino acids to the small intestine and increased absorption of methionine compared to sheep infused with PEG (McNabb *et al.*, 1993).

## 1.7.3.4. Digestion and absorption of condensed tannins by the ruminant

Condensed tannins are not broken down in the digestive tract and are unlikely to pass through the gut wall (McLeod 1974). Using  $[^{14}C]$ -labelled condensed tannin, Terrill *et al.* (1994) showed no CT from *Lotus pedunculatus* was digested and absorbed in the small intestine.

## 1.7.3.5. Detrimental effects of condensed tannin in ruminant nutrition

It is thought that CT are produced by the plant as a defence mechanism against herbivory whereby salivary proteins are precipitated making the plant unpalatable (Beart *et al.*, 1985). Furthermore, extracted CT from legume forages has been shown to inhibit cellulose digestion which was correlated to the inhibition of rumen bacterial endoglucanase activity (McAllister *et al.*, 2005).

Condensed tannins in the plant at a concentration greater than 60 g kg<sup>-1</sup> DM have been shown to depress feed intake and reduce the degradation of fibre and protein in the rumen which resulted in an overall decrease in the growth rate of ruminant livestock (Leinmuller *et al.*, 1991). It is possible that these anti-nutritive effects of CT are due to their action on salivary proteins and cellulose digestion.

## 1.7.4. Effects of condensed tannin on rumen skatole and indole formation

Condensed tannins have the ability to reduce protein solubilisation and slow protein degradation in the rumen (Min *et al.*, 2000) therefore, it is likely that this would reduce the amount of free tryptophan available in the rumen for conversion to skatole and indole. This

in turn could reduce the concentration of indole and skatole in the meat and improve the flavour. Currently, there is minimal published research that has looked at the use of dietary CT to reduce skatole and indole formation in the rumen and the effect this could have on meat flavour. However, some work has been reported on the effect of sulla (*Hedysarum coronarium*; a legume forage that contains CT) and the appearance of indoles in milk (Roy *et al.*, 2002; *Table 1.5*).

## 1.7.4.1. Condensed tannin forages and indoles in milk and fat

Priolo *et al.* (2005) did not observe a difference in the concentration of indole or skatole in the fat from lambs fed sulla with and without polyethylene glycol (PEG) supplementation (PEG inactivates the CT). However, a study investigating the concentration of skatole and indole in the milk of ewes fed sulla, demonstrated that CT decreased skatole and indole concentration and their yield in the milk after 14 days on the treatment diet compared to ewes fed sulla that were drenched with PEG (*Table 1.5*). This was associated with lower skatole and indole concentration in the mesenteric artery and mammary vein of the ewes fed sulla (Roy *et al.*, 2002; *Table 1.5*). The sulla fed in the study of Roy *et al.*, (2002) contained a higher concentration of CT (40 g kg<sup>-1</sup> DM) compared to that used in the study of Priolo *et al.* (2005) (18 g kg<sup>-1</sup> DM). Thus, there is reason to believe that the action of forage CT may reduce the amount of indole and skatole deposited in the meat of grazing ruminants. This hypothesis needs to be investigated.

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Table 1.5. Effect of sulla (a condensed tannin-containing forage) with and without polyethylene glycol (PEG) supplementation, on milk skatole/indole concentration and yield and plasma skatole/indole concentration in lactating ewes after 14 days on treatment diet (from Roy *et al.*, 2002).

	Tre	atments	50	D velue	
-	Sulla Sulla+PEG		50	r value	
Milk					
Skatole (ng g <sup>-1</sup> , Day 14)	75.3	134.4	29.9	0.01	
Indole (ng g <sup>-1</sup> , Day 14)	21.2	36.1	4.1	<0.001	
Skatole yield (ug day <sup>-1</sup> , Day 14)	71.9	130.9	30.2	0.02	
Indole yield (ug day <sup>-1</sup> , Day 14)	21.0	37.1	9.9	0.03	
Plasma (ng mL <sup>-1</sup> )					
Skatole - Mesenteric artery	2.2	6.5	2.9	0.08	
Skatole - Mammary vein	2.2	6.5	2.6	0.06	
Indole - Mesenteric artery	0.8	2.8	0.7	0.02	
Indole - Mammary vein	0.7	2.7	0.5	0.005	

# **1.8. CONCLUSIONS AND HYPOTHESES**

- The meat industry is a major export industry for New Zealand with approximately 70% of the meat produced being exported. New Zealand exported 354,099 tonnes of lamb and mutton (equivalent to an export value of \$2.2 billion) and 370,824 tonnes of beef (equivalent to an export value of \$1.7 billion) in the 2002/2003 season. The European Union and USA were the major destinations for sheep meat and beef, respectively (Meat New Zealand Annual Report). Exports of New Zealand meat to Asia are low and represent a significant growth opportunity. Low consumption of New Zealand meat in Asian countries and in some American markets has been partly attributed to poor acceptability of the meat due to the presence of "pastoral" flavours and odours that originate from the grazing of pasture in New Zealand animal production systems.
- Flavour is a sensation that results primarily from the perception of volatile chemicals by receptors in the olfactory epithelium at the back of the nose, with some interplay of taste chemicals perceived by receptors on the tongue. Flavour compounds have threshold concentrations at which they are detected or recognised. Some highly volatile compounds only need to be present at a very low concentration to have a significant flavour effect. Over 1000 volatile compounds have been associated with meat flavour. The fat component of meat provides the volatile compounds that give meat its characteristic flavours.
- Influences on the animal, carcass and meat cuts through out the production process can all affect meat flavour. Of all the animal and environmental factors that can influence

meat flavour, the animal's diet appears to have the most impact. Flavour differences between the meat of animals raised on pasture compared to grain have been well documented. The rejection of meat from animals raised on pasture is generally a consequence of the consumer being more accustomed to the flavour of meat from animals raised on concentrate- or total mixed ration-based diets.

- Flavour descriptors such as "barnyard", "animal", "sheepy", "milky" "faecal" and "grassy" are more intense with pasture-finished lamb. Such descriptors are collectively called "pastoral" flavours because of the production system from which they originate. Undesirable meat flavours that arise because of grazing pasture appear to be intensified by the pasture's clover or legume component.
- Studies comparing chemical and sensory analysis of meat from pasture-raised lambs have found that pastoral flavour is associated with the presence of skatole (3methylindole), and the related compound indole. Indole and skatole are heterocyclic amines that are soluble in fat and are formed in the rumen from the microbial fermentation of tryptophan, which is released during protein degradation. Research with cattle has shown that skatole is formed from tryptophan in a two-step process involving the initial formation of indole acetic acid, with subsequent decarboxylation to skatole. Indole is formed in a one-step process from tryptophan.
- Indoles that are formed in the rumen are absorbed from the digestive tract into the portal blood stream and are then metabolised by the liver. Research with pigs has

indicated that the cytochrome P450 system in the liver is involved in the metabolism of skatole. Cytochrome P450 refers to a group of hemoproteins responsible for oxidative metabolism of small molecular weight, lipophilic compounds. From studies with goats, the major skatole metabolic pathway in the ruminant liver appears to be the formation of methyloxyindoles with subsequent conjugation to glucuronic acid and sulphate, followed by excretion in the urine. Some indole and skatole will bypass liver metabolism and enter the general circulation to be available for deposition in the body fat.

- Feeding grain or concentrate diets up to slaughter will result in a flavour that is preferred by some international markets that object to pastoral flavours specifically, the Asian markets. However, is not practical or economical to implement grain feeding into current New Zealand grazing systems. Other options for reducing the amount of skatole and indole that accumulates in the fat involve reducing their formation in the rumen with the use of ionophore antibiotics or defaunation to reduce the concentration of free tryptophan in the rumen and decrease the conversion of tryptophan to skatole and indole. In pigs, selection for fast growth rate has also resulted in low levels of skatole in the back fat and this is correlated to high levels of cytochrome P450 in the liver. Thus, another alternative to reduce skatole and indole in the fat of ruminants is to improve liver metabolism of skatole.
- Condensed tannins bind to plant protein and slow proteolysis in the rumen. Since

skatole and indole formation is linked to rapid protein degradation in the rumen, CT could potentially be used to reduce skatole and indole formation in the rumen and to improve meat flavour. Condensed tannins are polymerised phenolic compounds that are naturally produced by some plants. The use of forages containing CT in grazing systems may offer a practical solution to ameliorate pastoral flavours in meat destined for overseas markets.

- Feeding CT-containing forages has been shown to reduce indole and skatole concentration in the milk however, no sensory (flavour and odour) qualities were established for this milk. Furthermore, there is no data currently published on the effect of CT-containing forages on the concentration of indolic compounds in meat and the associated sensory characteristics. If dietary CT can reduce the formation of indole and skatole in the rumen and consequently, reduce the concentration of these indolic compounds in the meat, it needs to be determined that there is a detectable sensory effect on pastoral flavours. Therefore, the hypothesis of this thesis is to determine whether dietary CT can reduce the formation of indole and skatole in the rumen to give a significant reduction in skatole and indole concentration in the meat and fat so that pastoral flavours are reduced.
- It has been found that clover or legume diets intensify undesirable flavours in meat.
   White clover usually comprises up to 30% of the botanical composition of pastures in New Zealand animal production systems, with the remaining 70% being predominately perennial ryegrass. White clover is known to be highly degradable in the rumen and

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have a greater soluble protein content, which is likely to lead to a high free amino acid concentration in the rumen. This means that clover and legume diets are more likely to result in a high availability of tryptophan in the rumen for conversion to indole and skatole. Therefore, a second hypothesis of this thesis is to determine whether a legume diet such as white clover gives a significant increase in the formation of indole and skatole in the rumen compared to perennial ryegrass and if this has an effect on pastoral flavour in meat.

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# **CHAPTER 2**

# **Materials and Methods**

# **2.1. INTRODUCTION**

Indole and skatole (3-methylindole) are associated with pastoral flavours in the meat from ruminants and are formed in the rumen and absorbed across the rumen wall into the portal blood. Indole and skatole are metabolised by the liver. However, some indole and skatole will by-pass liver metabolism and enter the systemic blood circulation. Due to the lipophilic nature of the indole and skatole molecules they are absorbed into the adipose tissue of the animal. The indole and skatole present in the meat fat impart undesirable pastoral odour and flavour to those consumers who are sensitive to such odours and flavours.

Indole and skatole are formed in the rumen as a consequence of feeding forages, as minimal indole and skatole are formed when feeding grain or concentrates (Young *et al.*, 2003). The objective of this thesis is to identify if feeding different forages or supplying dietary condensed tannins to ruminants is able to minimise the formation of indole and skatole and ameliorate pastoral odour and flavour in the meat. The effect of different forages and condensed tannins on the formation of indole and skatole and skatole and skatole by measuring the concentration of indole and skatole formed in the rumen and circulating in the blood and consequently, deposited in the fat in a range of experiments. The methods outlined in this section describe procedures for the measurement of indole and skatole in the rumen fluid, blood plasma and fatty tissue.

To determine if treatments had an impact on the odour and flavour of the meat, sensory

assessment of meat and fat samples was undertaken with odour and flavour panels. The panel is atypical to that of the everyday New Zealand consumer of ruminant meat in that the panel members are sensitive to pastoral flavour and odour. Therefore, the panel mimics the sensitivity to pastoral flavours of specific consumers in target markets. The procedures undertaken for sensory assessment using the odour and flavour panel are also outlined in this materials and methods section.

Due to the high resource input required to assess the formation of indole and skatole *in vivo*, an *in vitro* rumen fermentation method is useful to enable indole and skatole formation to be measured under several different treatment conditions. This section also outlines an *in vitro* method for the fermentation of fresh forages with rumen liquor to assess the formation of indole and skatole with different treatments.

# 2.2. MEASUREMENT OF INDOLES IN RUMEN FLUID BY SOLID PHASE EXTRACTION AND HIGH PERFORMANCE LIQUID CHROMATOGRAPHY

Indole and skatole concentration in the rumen fluid was determined using a solid phase extraction (SPE) method followed by high performance liquid chromatography (HPLC). This method is not an exhaustive extraction technique, therefore the indole and skatole concentrations are representative quantitative estimates rather than absolute determinations. The advantage of this method over other absolute extraction methods, such as simultaneous distillation-extraction, is that it allows for a higher sample throughput.

#### 2.2.1. Solid phase extraction

The method for the measurement of indole and skatole in rumen fluid was adapted from the method of Mattivi *et al.* (1999), who determined the concentration of tryptophan-derived indolic compounds in wine. Solid phase extraction involves the following steps (see *Figure 2.1*):

- 1. Sample pre-treatment
- 2. SPE column conditioning
- 3. SPE column equilibration
- 4. Sample loading
- 5. Interference elution
- 6. Analyte elution



Figure 2.1. Steps involved in the solid phase extraction (SPE) method: 1. sample pretreatment, 2/3. Column conditioning and equilibration, 4. Sample loading, 5. Interference elution, 6. Analyte elution.

# 2.2.1.1. ISOLUTE<sup>®</sup> ENV+ resin

The method involved loading a sample of rumen fluid into a 1 mL column containing 50 mg of ISOLUTE<sup>®</sup> ENV+ resin (International Sorbent Technologies, Mid Glamorgan, England). ISOLUTE<sup>®</sup> ENV+ resin is a hyper cross-linked hydroxylated polystyrenedivinylbenzene co-polymer (*Figure 2.2*). The irregular shaped particles have an average diameter of 90  $\mu$ m and a surface area of approximately 1000 m<sup>2</sup> per gram. With this resin, small relatively hydrophobic molecules such as indole and skatole are retained on the resin by hydrophobic interactions allowing the indole and skatole molecules to be extracted out of an aqueous solution. During the elution step, the organic solvent interrupts the hydrophobic interactions allowing the collection of indole and skatole (*Figure 2.3*). Choice of appropriate solvents allows for the separation of indole and skatole from other interfering compounds in the sample.



Figure 2.2. Structure of ISOLUTE® ENV+ resin (Source: International Sorbent Technology)

#### 2.2.1.2. Equilibration buffer

The equilibration buffer solution (BS) used in this SPE method was prepared by combining potassium dihydrogen orthophosphate (2.36 mg mL<sup>-1</sup>) and disodium hydrogen phosphate (3.86 mg mL<sup>-1</sup>) in distilled water. The BS had a pH of 7.0 (at  $20^{\circ}$ C).

# 2.2.1.3. Conditioning and equilibration of the column

To ensure that the surface area of the ISOLUTE<sup>®</sup> ENV+ resin was optimised for interaction with the analytes of interest, the column was conditioned by rinsing with 1 mL 100% methanol (MeOH). Without this step the aqueous sample matrix would not be able to penetrate the pores and wet the surface of the resin. To displace excess MeOH and equilibrate the column for optimal retention of the analyte, the column was rinsed with 1mL of the BS.

## 2.2.1.4. Pre-treatment of the sample

Into a pre-weighed Eppendorf tube, 0.5 mL of rumen fluid was added and the weight recorded. To the rumen fluid, 0.5 mL of 100% MeOH was added and the sample was vortexed until mixed thoroughly. This ensured that indole and skatole were solubilised from particulate material and enhanced the analyte retention on the ISOLUTE<sup>®</sup> ENV+ resin. To remove particulate matter the samples were centrifuged (2000 g for 5 minutes) and the supernatant removed. The pellet was then resuspended in the BS, mixed thoroughly using a vortex and then centrifuged (2000 g for 5 minutes) and this step repeated. The supernatant after each centrifugation was combined.

#### 2.2.1.5. Sample loading

The combined supernatants from the pre-treatment of the rumen fluid sample were loaded onto the ISOLUTE<sup>®</sup> ENV+ column using a disposable glass pipette. All solutions were allowed to elute through the column under gravitational flow.

#### 2.2.1.6. Removing interferences

To eliminate as many components as possible that might interfere with the analysis, the column was sequentially eluted with 1 mL 20% MeOH in BS and 1 mL 55% MeOH in BS after the sample had been loaded. The column was rinsed with 20% and 55% MeOH in BS to remove lipophilic interferences that had weaker hydrophobic interaction to the ISOLUTE<sup>®</sup> ENV+ resin but with insufficient MeOH to interrupt the strong hydrophobic interaction of indole and skatole with the ISOLUTE<sup>®</sup> ENV+ resin.

## 2.2.1.7. Elution of the analyte

Elution of the analytes of interest (indole and skatole) was carried out by eluting the column with 2 mL MeOH and this fraction collected into a test tube (see *Figure 2.3*). To the MeOH elutate 50  $\mu$ L of internal standard (2-methylindole; 2MI; 0.05  $\mu$ g  $\mu$ L<sup>-1</sup>) was added and the solution was then transferred into a vial ready for HPLC analysis.



Figure 2.3. Retention and elution interactions between ISOLUTE® ENV+ resin and the analyte (Source: International Sorbent Technology)

#### 2.2.2. High Performance Liquid Chromatography (HPLC)

The HPLC system consisted of a Shimadzu pump (LC10ADvp), auto-injector (SIL-10ADvp) and detector (RF-10Ax1; Shimadzu, Kyoto, Japan). The chromatography was performed with an isocratic mobile phase consisting of 70% acetic acid solution (1.2 mg mL<sup>-1</sup>) and 30% isopropanol (Hypersolv, BDH Laboratory Supplies, Poole, United Kingdom) at 1 mL min<sup>-1</sup>. The sample injection volume was 5  $\mu$ L and chromatographic separation was carried out on a reverse-phase C18 column (Platinum, 150 x 4.6 mm; Alltech, Deerfield, Illinois, USA). The fluorescence excitation was set to 285 nm and the emission to 350 nm for the detection of indole, skatole, and 2MI (internal standard). An external standard containing indole (0.025  $\mu$ g  $\mu$ L<sup>-1</sup>), 2MI (0.05  $\mu$ g  $\mu$ L<sup>-1</sup>) and skatole (3methylindole; 0.1  $\mu$ g  $\mu$ L<sup>-1</sup>) was also analysed by HPLC to provide a reference for the calculation of indole and skatole concentration in the samples. Data acquisition and peak processing were performed using Shimadzu, Class-VP software (version 5.032, Shimadzu, Kyoto, Japan).

# 2.2.2.1. Data processing

Once peak area data had been obtained using the Shimadzu Class-VP software, the peak areas for indole and skatole were then normalised to the peak area of the internal standard (2MI). The concentration of indole and skatole was calculated based on the peak areas obtained with the external standard and the concentration then adjusted for the weight of the rumen fluid sample.

#### 2.2.3. Quality criteria of determinations of indoles in rumen fluid

For the determination of the accuracy (recovery of added amounts) and precision (repeatability), indole and skatole were spiked into rumen fluid sub-samples. Indole was added to give resulting concentrations of 1.2, 2.4, 3.6, and 6.8  $\mu$ g g<sup>-1</sup> in rumen fluid. Skatole was added to give concentrations of 5.1, 10.5, 15.9 and 31.5  $\mu$ g g<sup>-1</sup> in rumen fluid (*Table 2.1*). After analysis of the spiked samples by SPE and HPLC, the measured concentration was adjusted for endogenous indole and skatole and compared to the concentration of indole and skatole spiked into the rumen fluid samples. The data were used to determine the coefficients of the intra- and inter-assay variation from repeated measurements of the samples on the same day (*n*=4; *Table 2.1*) and from assays completed a week apart (*n*=4; *Table 2.1*).

Volume of standard	Weight of	(µg g <sup>-1</sup> rumen fluid)		Recovery	Coefficient of variation (%)	
added	sample (g)	Expected	Measured	(%)	Intra-assay	Inter-assay
Indole (0.025 µg µL <sup>-1</sup> ):						
25 µL	0.54	1.2	1.4 ± 0.19	117.5	3.0	27.4
50 µL	0.52	2.4	2.2 ± 0.14	91.4	3.2	12.7
75 μL	0.52	3.6	3.3 ± 0.04	90.2	1.0	2.4
150 µL	0.52	7.2	6.8 ± 0.15	94.9	4.1	4.5
Skatole (0.11 µg µL <sup>-1</sup> ):						
25 µL	0.54	5.1	4.8 ± 0.33	94.3	2.0	13.8
50 μL	0.52	10.5	9.4 ±0.31	89.8	4.1	6.6
75 μL	0.52	15.9	14.2 ± 0.31	89.6	2.9	4.4
150 μL	0.52	31.5	28.6 ± 0 <i>.</i> 47	90.8	2.2	3.3

Table 2.1. Accuracy and precision of indole and skatole determination in rumen fluid

# 2.3. MEASUREMENT OF INDOLE AND SKATOLE IN PLASMA BY ETHER EXTRACTION AND HIGH PERFORMANCE LIQUID CHROMATOGRAPHY

The method used to determine indole and skatole in the plasma is adapted from the method of Claus *et al.* (1993), who determined indole and skatole concentration in the blood plasma of pigs.

#### 2.3.1. Calibration

In order to be able to calculate the concentration of indole and skatole in the plasma samples, an external calibration curve was established for both indole and skatole. The calibration curve was generated by running calibration standards of different concentration through the HPLC system to determine the response factor for each analyte (concentration per unit of peak area). The response factor was used to calculate the unknown concentration of indole and skatole in the plasma samples from peak areas measured by HPLC. The response factor is the slope of the linear (calibration) curve generated from the peak area ratio of the analyte (indole or skatole) to 2MI in comparison to the concentration ratio of analyte (indole or skatole) to 2MI.

### 2.3.1.1. Calibration standards

The external calibration standards used for this method contained indole, skatole and the internal standard, 2MI. By the addition of 2MI as an internal standard to both the biological samples and the external calibration standards, the procedure automatically compensated for any procedural losses. The standards were prepared in the following manner:

Standard A (84 ng 2MI  $\mu$ L<sup>-1</sup>): 2.1 mg 2-methylindole dissolved in 25 mL acetonitrile/water (75:25, v/v) Standard B (3.4 ng 2MI  $\mu$ L<sup>-1</sup>): 1 mL of standard A into 25 mL acetonitrile/water (75:25, v/v) Standard C (internal standard; 0.07 ng 2MI  $\mu$ L<sup>-1</sup>): 1 mL of standard B into 50 mL acetonitrile/water (75:25, v/v) Standard D (84 ng indole and skatole  $\mu$ L<sup>-1</sup>): 2.1 mg indole and 2.1 mg skatole in 25 mL acetonitrile/water (75:25, v/v) Standard E (84 ng indole and skatole  $\mu$ L<sup>-1</sup>): 1 mL of standard D into 25 mL acetonitrile/water (75:25, v/v) Standard F (0.13 ng indole and skatole  $\mu$ L<sup>-1</sup>): 1 mL of standard E into 25 mL acetonitrile/water (75:25, v/v)

The external calibration standards that were run to establish the calibration curve were prepared in accordance to the volumes given in *Table 2.2*. The corresponding concentration of indole, skatole and 2MI in each of the calibration standards is also given in *Table 2.2*.

	Standard number					
	1	2	3	4	5	6
Volume (μL)						
L <sup>-1</sup> ) Standard C (2-methylindole; 0.07 ng	250	250	250	250	250	250
Standard F (indole and skatole; 0.13 ng $\mu$ L <sup>-1</sup> )	0	25	75	175	375	775
1 MTrisbuffer (pH 8.3)	4750	4725	4675	4575	4375	3975
Concentration (ng mL <sup>-1</sup> )						
2-methylindole	3.36	3.36	3.36	3.36	3.36	3.36
Indole	0	0.67	2.02	4.70	10.08	20.83
Skatole	0	0.67	2.02	4.70	10.08	20.83

Table 2.2. Volumes of standards used to create calibration standards and the concentration of 2-methylindole, indole and skatole in the calibration standards

#### 2.3.1.2. Response factors and goodness of fit

The response factors obtained from the calibration curves are given in *Table 2.3* along with the  $r^2$  value as an indicator of the linear fit for the data points in the calibration curve.

Table	2.3.	Response	factors	and	r-squared	values	for	the	calibration	curves	ob ta ined	for
indole	and	skatole.										

	Response Factor (Slope)	r <sup>2</sup>
Indole	1.76521	0.992
Skatole	1.09622	0.994

#### 2.3.2. Extraction procedure

The extraction method involved taking 0.5 mL portions of blood plasma and extracting with 2 mL diethylether after the addition of the internal standard (5 ng 2MI in 75  $\mu$ L acetonitrile/water (75:25, v/v)) by vortexing for 30 seconds. After centrifugation (1200 *g* for 15 minutes), the samples were placed in a -20°C freezer until the aqueous phase was frozen. This allowed the ether phase to be easily decanted into tubes containing 1 mL of 0.02 M acetic acid/isopropanol (60:40, v/v; the HPLC mobile phase). Evaporation of the ether was carried out in a water bath in a fume hood at 47 °C after mixing with the acetic acid/isopropanol mobile phase to avoid losses of the volatile indoles. The remaining acetic acid/isopropanol phase was filtered with a 2  $\mu$ m filter (Upchurch Scientific Inc., Oak Harbour, Washington State, USA) and transferred into a 1.5 mL auto-sampler vial.

#### 2.3.3. High Performance Liquid Chromatography (HPLC)

The HPLC system used to chromatically determine indole and skatole concentration in the plasma was the same as that used in the rumen fluid method (see above).

## 2.3.4. Quality criteria of determinations of indoles in plasma

For determining the accuracy (recovery of added amounts) and precision (repeatability), indole and skatole were spiked into blood plasma sub-samples to give concentrations of 5.4, 20.3 and 40.5 ng mL<sup>-1</sup> of both indole and skatole in the plasma (*Table 2.4*). After analysis of the spiked samples by extraction and HPLC, the concentrations were adjusted for endogenous indole and skatole and compared to the concentration of indole and skatole spiked into the blood plasma samples. The data were used to determine recoveries and the coefficient of the intra-assay variation from triplicate measurements (*Table 2.4*)

Volume of standard added	(ng mL <sup>·</sup>	<sup>1</sup> plasma)	Recovery	Intra-assay coefficient	
	Expected	Measured	(%)	of variation (%)	
Indole (0.13 ng µL <sup>-1</sup> ):					
20 µL	5.4	5.5 ± 0.29	101.8	9.2	
75 µL	20.3	21.5 ± 0.71	106.3	5.7	
150 µL	40.5	41.0 ± 1.31	101.1	5.5	
Skatole (0.13 ng µL <sup>-1</sup> ):					
20 µL	5.4	5.1 ± 0.32	93.2	11.0	
75 µL	20.3	18.8 ± 0.82	91.7	7.5	
150 µL	40.5	37.4 ± 1.57	91.1	7.3	

Table 2.4. Precision and accuracy of indole and skatole determination in plasma

# 2.4. MEASUREMENT OF INDOLE AND SKATOLE IN FAT BY SIMULTANEOUS DISTILLATION EXTRACTION AND GAS CHROMATOGRAPHY/MASS SPECTROMETRY

Tail-stub and inter-muscular fat were analysed to determine the concentration of indole and skatole by simultaneous distillation-extraction (SDE) and gas chromatography/mass spectrometry (GC/MS) following a procedure adapted from Ha and Lindsay (1991) by Karl Fraser, AgResearch Ltd, Palmerston North, New Zealand (personal communication).

#### 2.4.1. Simultaneous distillation extraction

A sample of fat (5-10 g; weight recorded) was added to a 250 mL round bottom flask containing 40 mL of saturated sodium chloride and internal standard (50 ng g<sup>-1</sup> d<sub>3</sub>-skatole, donated by D. Rowan, HortResearch Ltd, Palmerston North, New Zealand). The mixture underwent SDE, using Likens-Nickerson apparatus, for 90 minutes with 30 mL of *t*-butyl methyl ether.

#### 2.4.2. Gas chromatography/ mass spectrometry

Analyses were carried out on a Shimadzu (Kyoto, Japan) GC/MS instrument. Samples were loaded into an AOC-20s auto-sampler, and 30 µL aliquots were injected with an AOC-20i auto-injector into an OCI-17 programmable temperature vaporiser/large volume injector (PTV/LVI) fitted to a GC-17A gas chromatograph coupled to a QP-5050A quadrupole mass spectrometer. The glass insert in the injector was packed with Tenax TA 60/80 adsorbent (Alltech, Deerfield, Illinois, USA). After injection of the sample, the injector was held at 50 °C for 0.1 minutes, then heated at 250 °C min<sup>-1</sup> to 60 °C and held for a further 1.8 minutes for solvent evaporation via helium purge then heated at 250°C min<sup>-1</sup> to 250°C and

maintained at this temperature for the remainder of the chromatographic run.

Separation was carried out on a fused-silica capillary column coated with 100% polyethylene glycol (ZB-WAX, film thickness 0.25  $\mu$ m, 30 m × 0.25 mm ID, Phenomenex, California, USA). The carrier gas was helium (>99.995% pure) with a column flow rate of 1.7 mL min<sup>-1</sup>. The split ratio was programmed at 50:1 for 2 minutes to eliminate excess solvent, splitless for 3 minutes during the sample loading step, and then split at 10:1 for the remainder of the run. The column oven was initially held at 50 °C for 4 minutes, and then heated at 4°C min<sup>-1</sup> to 200°C and held for a further 12 minutes. The interface temperature was 250°C. Selected ions monitored for each compound were (primary ion/secondary ion); d<sub>3</sub>-skatole, *m/z* 132/134; indole, *m/z* 117/90; skatole, *m/z* 130/131. Data were recorded and integrated using the Shimadzu integrated software package CLASS-5000.

#### 2.4.3. Quality criteria of determinations of indoles in fat

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The recovery and reproducibility of indole and skatole from animal fat samples by SDE with GC/MS, has been evaluated in the laboratory of Karl Fraser and Geoff Lane, AgResearch Ltd, Palmerston North, New Zealand. The mean recoveries of indole and skatole calculated from standard addition were approximately 117% and 106%, respectively (n=9). The mean intra-assay co-efficient of variation was 6% for indole and 7% for skatole (n=2). The mean inter-assay co-efficient of variation (n=5) was approximately 14% for indole and skatole (Karl Fraser, personal communication).

# 2.5. FLAVOUR AND ODOUR PANELS

The following method for the sensory panels was developed by Tracey Cummings of the Meat Quality and Safety Section (formerly of the Meat Industry Research Institute of New Zealand) at the Ruakura Research Centre, AgResearch Limited. The method has been utilised to assess the sensory aspects of meat in previous research into pastoral flavour (Young *et al.*, 2003).

#### 2.5.1. Preparation of fat for odour assessment

Fat was diced and melted in a glass beaker in a 600 W microwave for 60 seconds. A warmed stainless steel piston was used to manually press the liquid fat to separate out collagenous material and then the liquid given a light centrifuge to remove particulate material. The liquid fat was then transferred to a vial, vacuum packed and stored at -35°C until required for sensory analysis. Fat samples were prepared for sensory evaluation by melting at 100°C and placing 1.5 g of the melted fat into a wide-neck 25 mL round-bottom flask with a stopper. Samples were kept warm in a water bath (80°C) while awaiting assessment.

### 2.5.2. Preparation of meat samples for flavour assessment

After thawing, lean and fat in the muscle groups caudal to the femur were separated and then minced together twice through a 3 mm plate to yield mince containing approximately 15% fat. Samples of the mince were vacuum packed and refrozen (-35°C) ready for thawing, cooking and presentation to a sensory panel within 4 days of preparation. Thawed minces were cooked in stainless steel pots over boiling water to a 75°C endpoint. Samples

were served hot to each of the ten panellists

#### 2.5.3. Group sessions

Prior to the assessment of odours and flavours from the samples the panellists met as a group and were presented with either fat spiked with indole and skatole or appropriate minced meat samples (meat spiked with indole or skatole, or meat pooled from treatments). From these group sessions, a consensus was obtained on the descriptors to be used by the sensory panel and an opportunity provided for the panellists to familiarise themselves with the flavours that were to be detected.

#### 2.5.4. Individual assessment for odour or flavour scoring

The 10 panellists appeared at 10-minute intervals and were presented the session samples in a randomised order. The panellists sat in individual booths under a red light with outwards pressure. A computerised system was used to record panellist responses using Compusense<sup>®</sup> software. Panellists used a touch screen to rate the intensity of each descriptor along a line scale. A maximum of six samples was assessed at any one session.

### 2.5.4.1. Odour

The panellists removed the stopper and smelled the headspace. Panellists assessed the samples for sweet, sheepy, earthy, camphor, faecal, musty and barnyard odours on a 0-9 intensity scale. A list of definitions that is used to help clarify the perceived odours by the panellists is given in *Table 2.5*.

## 2.5.4.2. Flavour

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Panellists assessed the samples first by smelling then by eating, and scored them on a 0-9 scale for the overall odour intensity and the flavour intensities of the seven flavour attributes: sweet, sheepy, earthy, camphor, faecal, musty and barnyard. A list of definitions that is used to help clarify the perceived flavours by the panellists is given in *Table 2.5*.

Table 2.5. Descriptors/definitions associated with the categorical odour/flavour attributes used in sensory analysis.

Odour/Flavour	Definitions
Sweet	sweet, fresh, oily, fatty, beefy
Sheepy	sheepy, muttony, lamb fatty, roast lamb
Earthy	earthy, muddy, mushroomy, fresh rain on hot ground
Camphor	mothballs, "old grannies", sharp, acidic/biting and nose reaction
Faecal	urine, poo, manure, cat piss, cowshit
Musty	damp, stale (stored/old fabric), old/rancid, musky, dirty socks, sweaty
Bamyard	animal, silage, old hay, wet dirty animal, cowshed, milking shed

# 2.6. IN VITRO FORAGE FERMENTATION INCUBATIONS

#### 2.6.1. Forage preparation

Vegetative, leafy plant matter was harvested using scissors. Immediately after harvesting, all plant material was laid out in plastic bags and frozen at -20°C. Triplicate samples (20 g each) of the frozen forages in all the experiments were taken to determine dry matter (DM) by drying in a forced-air oven at 90°C for 24hrs. The fresh, frozen forages were minced by passing 2-3 cm lengths through a meat mincer (Kreft Compact R70, Germany). Minced forage equivalent to 0.5 g of DM was then weighed into 50 mL Schott bottles. The bottles containing the fresh, frozen minced forage were then placed back into the freezer (-20°C) until required.

### 2.6.2. Preparation of incubation bottles

To the bottles containing the frozen minced forage the following was added; 10 mg cellobiose (Sigma Chemical Co. St Louis, Missouri, USA), 12 mL CO<sub>2</sub>-saturated McDougall's buffer (pH 6.8; McDougall 1948) and 0.5 mL cysteine sulphide reducing agent. The bottles were then fitted with lids that had one-way valves to release internal pressure and were left to warm in the incubator (39°C, 90 oscillations per minute) while rumen fluid was collected. Rumen fluid (3 mL) was then added to the bottles under a flow of CO<sub>2</sub> and the bottles returned to the incubator. For the incubations containing polyethylene glycol (PEG) the volume of the McDougall's buffer added was reduced to 10 mL and 80 mg of PEG dissolved in 2 mL McDougall's buffer was added to the incubations.

#### 2.6.2.1. McDougall's Buffer

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To 1 litre of MilliQ water was added:

- 9.80 g sodium bicarbonate (9.8 mg mL<sup>-1</sup>)
- 3.97 g di-sodium hydrogen orthophosphate (anhydrous; 3.97 mg mL<sup>-1</sup>)
- 0.47 g sodium chloride  $(0.47 \text{ mg mL}^{-1})$
- $0.57 \text{ g potassium chloride } (0.57 \text{ mg mL}^{-1})$
- $0.06 \text{ g magnesium chloride } (0.06 \text{ mg mL}^{-1})$
- 0.04 g calcium chloride ( $0.04 \text{ mg mL}^{-1}$ )

The buffer solution was mixed well and saturated with CO<sub>2</sub> until the pH reached 6.8 before including in the *in vitro* incubations.

## 2.6.2.2. Cysteine sulphide reducing agent

315 mg cysteine hydrochloride (6.3 mg mL<sup>-1</sup>)
315 mg sodium sulphide (6.3 mg mL<sup>-1</sup>)
48 mL distilled water
2 mL 1 M sodium hydroxide (1.6 mg mL<sup>-1</sup>)

#### 2.6.3. Rumen fluid inoculate

Four Romney wethers with rumen fistulae were used as a source of rumen fluid to inoculate the incubation bottles. The sheep were grazed outdoors on perennial ryegrass-dominant pasture. Pasture was allocated to the fistulated sheep in daily breaks by using front and back portable fences. This ensured that intakes from day-to-day were similar. The sheep were removed from the pasture and fasted for 2 hours prior to collecting the rumen contents. Rumen contents were squeezed through a double layer of cheesecloth and the fluid collected into a pre-warmed vacuum flask. Equal volumes of rumen fluid from each of the four sheep were pooled and immediately used to inoculate each of the bottles used in the incubations.

### 2.6.4. Sampling protocol and timing

In each experiment, five incubation bottles were set up for each treatment. Samples of the incubation media were taken at 0, 1, 2, 3, 4, 5, 6, 7, 8 and 10 hours after the incubation had commenced. Samples of the media were taken from sequential bottles over the 10 time-points so that each bottle was sampled twice. All treatments were run in one day and repeated on three successive days, so that one replicate of each treatment was run on each of the four days (*Figure 2.4*).



Figure 2.4. Example of the arrangement of bottles and sampling protocol for *in vitro* rumen incubations. The times above the bottles indicate the hour of the *in vitro* at which sampling took place from that bottle. This system was repeated over four days to get 4 replicates of each time point in each treatment.
#### CHAPTER 2: Materials and Methods

# 2.7. CHEMICALS AND COMPOUNDS

The following table (Table 2.5) lists the chemicals and compounds mentioned in this Material and Methods section. Included in Table

2.5 is the molecular weight and supplier/manufacturer.

Table 2.6. Chemicals and compounds used in methods outlined in this Materials and Methods section	n

Chemical/compound name	mical/compound name Formula Me		Supplier/ Manufacturer		
Acetic acid	CH3COOH	60.05	BDH Laboratory Supplies, Poole, United Kingdom		
Acetonitrile	CH₃CN	41.05	BDH Laboratory Supplies, Poole, United Kingdom		
Calcium chloride	CaCl <sub>2</sub>	110.99	Sigma Chemical Co. St Louis, MO, USA		
Cellobiose	C12H22O11	342.30	Sigma Chemical Co. St Louis, MO, USA		
Cysteine hydrochloride	C <sub>3</sub> H <sub>7</sub> NO <sub>2</sub> S.HCl	157.62	Sigma Chemical Co. St Louis, MO, USA		
Diethyl ether	(C2H5)2O	74.12	BDH Laboratory Supplies, Poole, United Kingdom		
Disodium hydrogen orthophosphate	Na₂HPO₄	141.96	Sigma Chemical Co. St Louis, MO, USA		
Indole	C <sub>6</sub> H <sub>7</sub> N	131.17	Aldrich (now Sigma-Aldrich), Auckland, New Zealand		
sopropanol (HiPerSolv)	(CH <sub>3</sub> )₂CHOH	60.09	BDH Laboratory Supplies, Poole, United Kingdom		
Magnesium chloride	MgCl <sub>2</sub>	95.21	Sigma Chemical Co. St Louis, MO, USA		
Methanol	CH₃OH	32.04	BDH Laboratory Supplies, Poole, United Kingdom		
2-methylindole	C₃H₃N	131.17	Aldrich (now Sigma-Aldrich), Auckland, New Zealand		
Polyethylene glycol (PEG)	(CH <sub>2</sub> CH <sub>2</sub> O) <sub>n</sub>	3500	Aldrich (now Sigma-Aldrich), Auckland, New Zealand		
Potassium chloride	KCI	74.55	Sigma Chemical Co. St Louis, MO, USA		
Potassium dihydrogen orthophosphate	KH₂PO₄	136.09	Sigma Chemical Co. St Louis, MO, USA		
Skatole (3-methylindole)	C <sub>9</sub> H <sub>9</sub> N	131.17	Aldrich (now Sigma-Aldrich), Auckland, New Zealand		
Sodium bicarbonate	NaHCO <sub>3</sub>	84.00	Sigma Chemical Co. St Louis, MO, USA		
Sodium chloride	NaCl	58.44	Sigma Chemical Co. St Louis, MO, USA		
Sodium hydroxide	NaOH	40.00	Sigma Chemical Co. St Louis, MO, USA		
Sodium sulphide	Na <sub>2</sub> S	78.04	Sigma Chemical Co. St Louis, MO, USA		
t-butyl methyl ether	C <sub>6</sub> H <sub>14</sub> O	102.18	BDH Laboratory Supplies, Poole, United Kingdom		

#### 115

# 2.8. DISCUSSION

This section has outlined the methods used in the studies of this thesis to measure the concentration of indole and skatole in rumen fluid, blood plasma and the fat of sheep. The measurement methods implemented for indole and skatole in the rumen fluid and blood plasma involved a relatively rapid extraction and analysis technique. This made possible a high throughput of samples and allowed for an adequate number of treatments and sample measurements within each experiment. The SDE method for the measurement of indole and skatole in the fat requires 90 minutes for extraction, which limited the throughput of samples. However, the recovery of indole and skatole using this technique is high. The measurement of indole and skatole in the rumen fluid using SPE and HPLC provided a representative quantitative estimate rather than an absolute concentration. This allowed comparison of indole and skatole concentration between treatments in an experiment and between experiments that have used the same method. However, comparison of indole and skatole concentration between the same nethods may not be valid.

All the methods gave high recoveries from standard additions, although the recovery of skatole from the rumen fluid and blood plasma was observed to be lower than that of indole. The log P value (log P is a measure of lipophilicity) of skatole is higher than that of indole (2.06 vs. 1.94; <u>http://pubchem.ncbi.nlm.nih.gov/</u>). The greater lipophilic nature of skatole suggests that it may have a stronger association with components in the samples making it more difficult to extract from the sample matrix, therefore contributing to the lower recoveries that were observed.

Although the recovery of skatole from rumen fluid and blood plasma was not complete (89-93%), the intra-assay coefficient of variation was reasonably low (1-11%) for both indole and skatole in the rumen fluid and blood plasma, suggesting that the comparison of samples between treatments is applicable. The inter-assay co-efficient of variation for the indole and skatole measured in rumen fluid tended to be higher (2-27%) than that of the intra-assay, indicating a preference for batch processing of samples to reduce the variability in the results. The extraction method of indole and skatole from a fat matrix using SDE has been shown to result high recoveries and a reasonable co-efficient of variation (6-7% intra-assay and 14% inter-assay) however, the higher inter-assay once again indicates a preference for batch processing. When comparing treatments in biological experiments, it is likely that the within-treatment biological variation of indole and skatole will not be exceeded by the analytical variation. Therefore, any observed treatment effects can be considered as genuine and not the result of large analytical variation.

The sensory panel is experienced in the detection of the pastoral odours and flavours present in meat and fat samples. In this manner, the sensory panel provides a measurement of pastoral odour and flavour intensity. Therefore, the sensory panel is a valuable tool for determining whether the differences in indole and skatole concentration observed in the rumen fluid, blood plasma and fat of animals on different treatments is having a detectable sensory impact. It should be noted that the use of a sensory panel is a subjective measurement of odour and flavour. The perception of odour and flavour will be variable from person-to-person and as such the variation in the scores obtained will to a large extent, reflect the variation amongst panellists. To ensure that panellist variation is accounted for appropriately, the effect of panellist is included as a random block effect in statistical analyses of odour and flavour score in the studies of this thesis.

The measurement of fat concentration of indole and skatole on the other hand, is an objective measurement and the difference in indole and skatole concentration in the fat between two treatments needs to be larger than the panellist variation in order for a concentration difference to be detected as a difference in the odour and flavour by the panellists. Another point to consider is that the complexity of pastoral odour and flavour is related to more than just indole and skatole concentration in the fat. Thus, an objective difference in indole and skatole concentration may not be sufficient to indicate a change in pastoral odour and flavour.

The *in vitro* technique was designed to mimic the *in vivo* rumen. The method utilises the fermentation of fresh forages in a rumen fluid media, which is ideal for the study of indole, and skatole that are formed in the rumen of forage fed animals. The *in vitro* technique allowed for the comparison of many more treatments than could be feasibly undertaken *in vivo*. Although the *in vitro* rumen fermentation method is based on providing conditions similar to that of the *in vivo* rumen, it is none-the-less a closed system and not the dynamic environment of the *in vivo* rumen. Therefore, the results from *in vitro* rumen fermentations can not be used to generalise about what would occur in the *in vivo* rumen; rather, it provides an indication of possible differences in the formation of indole and skatole between treatments. Furthermore, the closed system of the *in vitro* rumen fermentation method means that there is a build-up of end-products in the media. This can sometimes, result in the inhibition of rumen microbes through feed-back mechanisms as well as alter

the media to contain a high concentration of end-product acids (in particular volatile fatty acids) which can dramatically shift the pH outside the range for viable rumen microbial function.

Overall, the extraction and analysis methods for the measurement of indole and skatole in rumen fluid, blood plasma and fat have been shown to be robust and reliable. The *in vitro* rumen fermentation and sensory assessment methods are valuable tools that allow greater insight into the formation of indole and skatole and their detection as pastoral flavours.

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# **CHAPTER 3**

Effect of forage condensed tannin concentration, forage maturity and nitrogen fertiliser application on the formation of indole and skatole in *in vitro* rumen fermentations.

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The material presented in this chapter has been submitted as a paper to: Journal of the Science of Food and Agriculture.

# **3.1. ABSTRACT**

The objectives of this work were to establish the effect of varied condensed tannin (CT) concentration in seven different forages, and of plant maturity and nitrogen fertiliser application on the ruminal formation of skatole and indole using an in vitro method designed to mimic rumen fermentation conditions. After 10 hours of incubation, the concentration of indole and skatole was highest when incubating white clover (P < 0.05). Polyethylene glycol addition, to inhibit CT, showed that CT reduced the formation of indole and skatole when incubating Lotus corniculatus, sulla, Dorynium rectum and Lotus *pedunculatus* (P<0.01). Mature forage growth resulted in a lower concentration of indole and skatole being formed in vitro compared to the incubation of new spring growth (P<0.001). A higher application of nitrogen fertiliser to perennial ryegrass-based pasture resulted in a higher crude protein concentration in the plant and a higher concentration of skatole formed in vitro (P<0.001). Forages containing CT reduced indole and skatole formation and plants containing a higher CT concentration tended to be more effective, but compositional differences of CT between forages may also have had an influence. New forage growth or swards that had a high application of nitrogen fertiliser had a higher plant protein concentration and this promoted the formation of indole and skatole.

# **3.2. INTRODUCTION**

Pastoral flavour reduces the acceptance of meat in some markets that are accustomed to meat from grain-based production systems (Rubino *et al.*, 1999; Young *et al.*, 2003). Compared to feeding grain, a higher concentration of indole and skatole was found in the meat of lambs that had been grazing pasture and the skatole and indole concentration was correlated to pastoral flavours using a gas chromatography-olfactory method (Young *et al.*, 2003).

In the rumen, protein from fresh forage diets is rapidly degraded, while the structural carbohydrate is relatively slowly degraded (Dove 1996). The consequence can be a lack of energy for microbial protein synthesis that utilises the peptides, amino acids and ammonia released from the degradation of plant protein. Amino acids not incorporated into microbial protein may be deaminated by rumen microbes and in the case of the amino acid tryptophan, it will under go further decarboxylation reactions to form skatole and indole (Deslandes *et al.*, 2001).

As a plant matures its chemical composition changes (Chaves *et al.*, 2002) and the concentration and digestibility of the protein in the plant, decreases (Waghorn and Barry 1987). It is hypothesised that skatole and indole formation in the rumen may be reduced as the forage eaten by the ruminant matures. Furthermore, the concentration of free amino acids in the rumen is influenced by the initial concentration of protein in the diet (Volden *et al.*, 2001). This suggests that a plant with higher crude protein concentration will release more tryptophan into the rumen and hence, more skatole and indole may be formed.

Previous studies have shown that feeding white clover (*Trifolium repens*) to sheep results in a greater concentration of skatole and indole in the rumen compared to perennial ryegrass (*Lolium perenne*) and *Lotus corniculatus* (Schreurs *et al.*, 2004). The higher concentration of indole and skatole in the rumen when feeding white clover was attributed to the greater protein concentration, greater protein solubility and rapid protein degradation, resulting in a greater availability of tryptophan for conversion to skatole and indole.

Condensed tannins (CT) are polyphenolic compounds that reduce plant protein degradation in the rumen (McNabb *et al.*, 1996). They occur at different concentrations and chemical compositions in a range of plant species (Terrill *et al.*, 1992; Jackson *et al.*, 1996). For example, the CT in *Lotus corniculatus* consists of predominately procyanidin subunits while *Lotus pedunculatus* CT consists of mainly prodelphinidin subunits (Foo *et al.*, 1996, 1997). These differences maybe responsible for the variation in the ability of these forages to slow the degradation of protein in the rumen (McNabb *et al.*, 1998; Aerts *et al.*, 1999). Thus, it is hypothesised that CT-containing forages will alleviate some of the inefficiencies associated with microbial utilisation of amino acids in the rumen, with some variation in the reduction and availability of tryptophan for indole and skatole formation as a consequence of differences between sources of CT.

The objectives of the present work were to establish the effect of incubating seven forages containing different concentrations of CT on the *in vitro* formation of skatole and indole. Polyethylene glycol (PEG) was used to establish the effect that CT had on skatole and indole formation. Further *in vitro* studies were carried out to determine the effect of plant maturity and plant crude protein concentration on *in vitro* formation of indole and skatole.

# **3.3. MATERIALS AND METHODS**

## 3.3.1. Experimental design

Three *in vitro* rumen fermentation experiments were undertaken to determine the effects of three differing forage-based factors on the formation of skatole and indole using minced frozen forages. The first experiment looked at the effect of seven forages with varying CT concentration and chemical composition. The seven forages were white clover (*Trifolium repens*, Grasslands Huia; WC), perennial ryegrass (*Lolium perenne*, Grasslands Nui, wild-type endophyte; PRG), *Lotus corniculatus* (LC), *Lotus pedunculatus* (LP), sulla (*Hedysarum coronarium*; SL), broadleaf dock (*Rumex obtusifolius*; BD) and *Dorycnium rectum* (DR). To determine the effect that CT had on skatole and indole formation, the forages were incubated in the presence and absence of polyethylene glycol (PEG; MW 3350). Polyethylene glycol preferentially binds with CT and inhibits the activity of CT (Jones and Mangan 1977). The PEG is not likely to affect other aspects of *in vitro* fermentation, as digestion was not affected when PEG was supplemented to sheep fed lucerne (a non-CT forage; Wang *et al.*, 1994).

The second experiment investigated the effect of plant maturity on skatole and indole formation by comparing fresh spring growth to that of mature growth for WC, PRG and LC. The third experiment used PRG that had been grown with high nitrogen (High N) fertiliser application compared to PRG that had received no nitrogen fertiliser (Low N), to determine the effect of varied nitrogen content in the plant on skatole and indole formation.

In each experiment, five incubation bottles were set up for each treatment. Samples of the incubation media were taken at 0, 1, 2, 3, 4, 5, 6, 7, 8 and 10 hours after the incubation had

commenced. Samples of the media were taken from sequential bottles over the 10 timepoints, so that each bottle was sampled twice. All treatments were run in one day and repeated on three successive, days so that one replicate of each treatment was run on each of the four days.

# 3.3.2. Forage preparation

## 3.3.2.1. Experiment 1: Varied forage condensed tannin concentration

Fresh spring growth of WC, PRG, LC, SL, and DR were obtained from plots at Aorangi Research Farm, AgResearch Ltd, Manawatu, New Zealand. Leaves from new spring growth of BD were collected from the paddocks at Grassland Research Centre, Palmerston North, New Zealand. Trimmings of spring growth from LP were obtained from outdoor demonstration plots grown at Grassland Research Centre.

## 3.3.2.2. Experiment 2: Forage maturity

Fresh spring growth of WC, PRG and LC was harvested 12 days after grazing. This plant material was defined as being young, new vegetative growth. Exclusion cages were then placed over the harvested areas to allow the plant mass to regrow. The use of exclusion cages ensured that the site of sampling could be easily identified and allowed normal farm operations to continue as animals could then graze the rest of the plot without interfering with regrowth. A second harvest of the plant growth in the exclusion cages occurred 42 days later. This plant material was defined as being mature growth, as it was harvested in the early summer when the WC and LC were just starting to flower and PRG was showing its first seed heads.

## 3.3.2.3. Experiment 3: Nitrogen fertiliser application

After light grazing, two 4m<sup>2</sup> plots were marked out in separate parts of a PRG paddock at Grasslands Research Centre, Palmerston North. Urea was applied to one plot (300 kg N ha<sup>-1</sup>, High N) while the other area received no nitrogen fertiliser (Low N). Exclusion cages covering 0.5 m<sup>2</sup> were placed in the middle of each of the plots to ensure that the sample plant matter was not grazed. The plots were set up in mid-winter, and a second application of urea (300 kg N ha<sup>-1</sup>) was made to the High N plot after 33 days, as there had been substantial rain, which may have caused significant leaching of the first urea application. Furthermore, the second application of urea would maximise N uptake by the plant during the following spring growth. The PRG from the exclusion cages in each plot was harvested 71 days after the initial set up. Although the period over which the plot was set up was quite long, there was significant growth only in the two weeks prior to harvesting. The plant material harvested was defined as being new vegetative growth, as it was harvested in the early spring when the sward was lush with no seed heads present. Any dead matter was removed from the harvested forage samples.

## 3.3.2.4. Post-harvest storage and forage analyses

Immediately after harvesting, all plant material was placed in plastic bags and frozen at - 20°C. Triplicate samples (20 g each) of the frozen forages in all the experiments were taken to determine dry matter (DM) by drying in a forced-air oven at 90°C for 24hrs. The forages were minced by passing 2-3 cm lengths of the frozen forage through a meat mincer (Kreft Compact R70, Germany). Minced forage equivalent to 0.5 g of DM was then weighed into 50 mL Schott bottles. The bottles containing the fresh, frozen minced forage were then placed back in the freezer (-20°C) until required. Representative samples of the minced

forage were freeze-dried and ground (to particle size <1mm), to determine chemical composition by Near Infra-red Reflectance Spectrometry (NIRS; Feedtech, AgResearch Limited, Palmerston North, New Zealand). The CT concentration in the freeze-dried and ground forage samples was determined using the butanol-HCl method (Terrill *et al.*, 1992).

## 3.3.3. Rumen fluid inoculate

Four Romney wethers with rumen fistulae were used as a source of rumen fluid to inoculate the incubation bottles. The sheep were grazed outdoors on perennial ryegrass-dominant pasture. Pasture was allocated to the fistulated sheep in daily breaks by using front and back portable fences. This ensured that intakes from day-to-day were similar. The sheep were removed from the pasture and fasted for 2 hours prior to collecting the rumen contents. Rumen contents were squeezed through a double layer of cheesecloth and the fluid collected into a pre-warmed vacuum flask. Equal volumes of rumen fluid from each of the four sheep was pooled and immediately used to inoculate each of the bottles used in the incubations.

# 3.3.4. In vitro rumen incubation method

The following was added to bottles containing frozen minced forage; 10 mg cellobiose (Sigma Chemical Co. St Louis, Missouri, USA), 12 mL CO<sub>2</sub> saturated McDougall's buffer (pH 6.8; McDougall 1948) and 0.5 mL cysteine sulphide reducing agent. The bottles were then fitted with lids that had one-way valves to vent fermentation gases and were left to warm in the incubator (39°C, 90 oscillations per minute) while rumen fluid was collected. Rumen fluid (3 mL) was then added to the bottles under a flow of CO<sub>2</sub> and the bottles returned to the incubator. For those incubations containing PEG the volume of the

McDougalls buffer added was reduced to 10 mL and 80 mg of PEG dissolved in 2 mL McDougalls buffer was added to the incubations prior to adding rumen fluid. For the forage with the highest CT concentration the ratio of PEG:CT was 1.3:1.

# 3.3.5. Sampling and metabolite analysis

At each time point, a total of 3 mL of the media was taken from the appropriate bottles for skatole and indole, ammonia and volatile fatty acid (VFA) analysis. For skatole and indole analysis, 1.2 mL of the media was frozen in liquid nitrogen and these samples transferred to a -20°C freezer for storage. Skatole and indole in the media samples were measured using solid-phase extraction (Mattivi *et al.*, 1999) followed by high performance liquid chromatography (HPLC). The HPLC system consisted of a Shimadzu pump (LC10ADvp), auto-injector (SIL-10ADvp) and detector (RF-10Axl; Shimadzu, Kyoto, Japan). The chromatography was performed with a mobile phase consisting of 70% acetic acid solution (1.2 mg mL<sup>-1</sup>) and 30% isopropanol (Hypersolv, BDH Laboratory Supplies, England) isocratic at 1 mL min<sup>-1</sup>. Injection volume was 5  $\mu$ L with chromatographic separation carried out on a reverse-phase platinum C18 column (150 x 4.6 mm; Alltech, Deerfield, Illinois, USA). The fluorescence excitation was set to 285 nm and the emission to 350 nm for the detection of skatole, indole and 2-methylindole (internal standard). Data acquisition and peak processing were performed using Shimadzu, Class-VP software (version 5.032, Shimadzu, Kyoto, Japan).

Another 1.2 mL of the media was centrifuged (16000 g for 15 minutes) and the supernatant used for VFA analysis by liquid-gas chromatography (Attwood *et al.*, 1998). A further 0.6 mL of incubation media was added to 10  $\mu$ L of concentrated hydrochloric acid and

centrifuged (16000 g for 15 minutes). The supernatant was analysed for ammonia by reductive amination of 2-oxoglutarate giving a decrease in absorbance at 340 nm due to the oxidation of NADPH proportional to the ammonia concentration (Neeley and Phillipson 1988).

# 3.3.6. Statistical analysis

For all experiments, statistical analysis was carried out using PROC MIXED of SAS (2003). In Experiment 1 the skatole, indole, ammonia and volatile fatty acid concentration was analysed using a block design with a model that included the fixed effects of forage, PEG and the interaction of forage and PEG. Replication was included as a random effect. A block design was also used for Experiment 2 with fixed effects of forage type, forage maturity and the interaction of forage type and maturity. In Experiment 3, a linear model was used which included the fixed effect of nitrogen status. Replication was also included as a random effect in both Experiment 2 and 3.

# 3.4. RESULTS

# 3.4.1. Experiment 1: Varied forage condensed tannin concentration

# 3.4.1.1. Nutrient composition of the forages

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Neutral detergent fibre was considerably higher and the crude protein (CP) concentration much lower in PRG compared to the other forages (*Table 3.1*). The CP concentration was high in WC, LC, SL and BD. Organic matter digestibility was high for all the forages, except for PRG and LP at 0.83 and 0.81, respectively. Soluble sugar and starch concentration was approximately twice as high in SL compared to the other forages at 202 g kg<sup>-1</sup> DM. The metabolisable energy content of the forages was highest with SL and BD (13.2-13.4 MJ kg<sup>-1</sup> DM) and lowest with PRG and LP (11.4-11.5 MJ kg<sup>-1</sup> DM). The WC, LC and DR had intermediate metabolisable energy contents (12.2-12.6 MJ kg<sup>-1</sup> DM). The condensed tannin (CT) concentration was the highest in LP and DR at 99 and 122 g kg<sup>-1</sup> DM, respectively. A moderate concentration (20-40 g kg<sup>-1</sup> DM) of CT was found in LC, SL and BD, while WC and PRG contained only trace concentrations of CT (*Table 3.1*).

CHAPTER 3: Forage factors on indole/skatole formation	131
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	White clover	Perennial ryegrass	Lotus comiculatus	Sulla	Dorycnium rectum	Lotus pedunculatus	Broadleaf dock
Nutrient Composition:	·						_
Dry matter (g kg <sup>·1</sup> )	189	222	177	151	211	166	139
Organic matter digestibility	>0.87	0.83	0.86	>0.87	>0.87	0.81	>0.87
MetabolisableEnergy(MJ kg <sup>-1</sup> DM)	12.6	11.5	12.2	13.4	12.6	11.4	13.2
Neutral detergent fibre (g kg <sup>-1</sup> DM)	210	411	185	138	<b>2</b> 59	255	158
Crude Protein (g kg <sup>-1</sup> DM)	276	122	266	254	208	194	289
Soluble sugars and starch (g kg <sup>-1</sup> DM)	100	128	107	202	91	116	119
Total Condensed tannin (gkg <sup>-1</sup> DM)	1.3	0.5	35.4	37.7	121.8	98.5	21.2

Table 3.1. Experiment 1. Nutrient composition of the minced forages used in the *in vitro* incubations.

## 3.4.1.2. Metabolite formation

The indole and skatole concentration (expressed per gram of CP added) at the end of the *in vitro* incubation period (10 hours) was highest for WC (*Table 3.2*). Compared to WC, a lower concentration of indole occurred with SL (P<0.05) but, indole concentration was the lowest with PRG, LP and BD (P<0.05). Skatole concentration was also the lowest when incubating LP and BD (P<0.05) and was intermediate with PRG, LC, SL and DR compared to WC (P<0.05; *Table 3.2*).

Ammonia concentration was the highest when incubating WC and LC and was the lowest with PRG (P<0.05; *Table 3.2*). Sulla gave the highest total volatile fatty acid (VFA) concentration, with intermediate values for WC, PRG, LC and BD (P<0.05) and the lowest values for DR and LP (P<0.05; *Table 3.2*). The molar proportion of acetate:propionate:butyrate formed was 58:29:10 when incubating SL and this was consistent with the molar proportions formed with the incubation of WC. A higher molar proportion of acetate (and corresponding lower proportions of propionate and butyrate) were found when incubating PRG and LC (P<0.05), and was the highest when incubating DR, LP and BD (P<0.05; *Table 3.2*). Branched-chain VFA concentration per unit of CP added was highest when incubating SL, followed by WC (P<0.05) with lowest values observed with DR, LP and BD (P<0.05; *Table 3.2*).

### CHAPTER 3: Forage factors on indole/skatole formation

Table 3.2. Experiment 1. Concentration of indole and skatole, ammonia and volatile fatty acids (VFA) in the media after 10 hours of incubating seven forages in the absence of polyethylene glycol.

133

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	White clover	Perennial ryegrass	Lotus corniculatus	Sulla	Dorycnium rectum	Lotus pedunculatus	Broad leaf dock	S.E.M.
Indole <sup>†</sup> (µg g <sup>-1</sup> CP)	292ª	45 <sup>c</sup>	200 <sup>a b</sup>	171 <sup>b</sup>	200 <sup>b</sup>	38°	 17°	42.5
Skatole <sup>†</sup> (µg g <sup>-1</sup> CP)	575°	151°	356 <sup>b</sup>	302 <sup>b</sup>	122 <sup>c ¢</sup>	43 <sup>c.d</sup>	28 <sup>d</sup>	41.5
Ammonia <sup>†</sup> (mmol g <sup>-'</sup> CP)	2.02ª	0.38 <sup>c</sup>	1.84ª	0.96 <sup>°</sup>	0.81 <sup>b</sup>	0.77 <sup>b</sup>	0.74 <sup>b</sup>	0.09
Total VFA (mmol L <sup>*</sup> )	142.6 <sup>b</sup>	128.0 <sup>b.c</sup>	129.7 <sup>b.c</sup>	175.1ª	83.7 <sup>d</sup>	86.3 <sup>d</sup>	105.5°°	9.4
- Acetate (molar %)	57.9 <sup>e</sup>	59.8 <sup>d</sup>	59.7 <sup>d</sup>	57.5 <sup>e</sup>	63.1 <sup>b</sup>	65.3ª	61.1°	0.47
- Propionate (molar %)	28.3ª	26.4 <sup>b</sup>	28.1ª	28.9 <sup>a</sup>	26.2 <sup>b</sup>	23.6°	25.8 <sup>b</sup>	0.37
- Butyrate (molar %)	9.5°	11.0ª	8.7 <sup>d</sup>	10.3 <sup>b</sup>	7.9 <sup>e</sup>	8.9 <sup>d</sup>	10.5 <sup>a,b</sup>	0.30
- Branched chain VFA <sup>†</sup> (mmol g <sup>'</sup> CP)	0.50*	0.20 <sup>c.d</sup>	0.32 <sup>c</sup>	0.80*	0.18 <sup>d</sup>	0.18 <sup>d</sup>	0.17 <sup>4</sup>	0.05

Indole, skatole, ammonia and branched chain VFA concentration has been adjusted for differences in the crude protein (CP) added to the incubations. ab.c.de Means in the same row with different superscripts are significantly different (P<0.05)</p>



Figure 3.1. Experiment 1. Indole formation *in vitro* when incubating seven different forages in the presence ( $\Box$ ) and absence ( $\bullet$ ) of polyethylene glycol (PEG). PEG inhibits the activity of condensed tannins. Results are the least square means of four replicates and the error bars are the standard error of the mean. \*, P<0.05; \*\*, P<0.01; \*\*\*, P<0.001.



Figure 3.2. Experiment 1. Skatole formation *in vitro* when incubating seven different forages in the presence ( $\Box$ ) and absence ( $\bullet$ ) of polyethylene glycol (PEG). PEG inhibits the activity of condensed tannins. Results are the least square means of four replicates and the error bars are the standard error of the mean. \*, P<0.05; \*\*, P<0.01; \*\*\*, P<0.001.

### 3.4.1.3. Addition of polyethylene glycol to incubations

Adding polyethylene glycol (PEG) to the incubations caused a significant increase in indole concentration with PRG and LC (P<0.05) and for SL, DR and LP (P<0.001). The effect of PEG was most pronounced with DR and LP, with significant differences occurring as early as 2 and 3 hours of incubation (*Figure 3.1*). Polyethylene glycol addition caused a significant increase in skatole concentration when incubating LC, SL, DR and LP (P<0.01). The effect of PEG on skatole formation was evident after 6-8 hours of incubation (*Figure 3.2*).

# 3.4.2. Experiment 2: Forage maturity

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# 3.4.2.1. Nutrient composition of the forages

The mature forages all had a higher neutral detergent fibre and acid detergent fibre concentration compared to the new spring growth (young forages; *Table 3.3*). Crude protein concentration, metabolisable energy content and organic matter digestibility was lower in the mature forage growth compared to the young forages. Mature PRG had a higher soluble sugar and starch concentration compared to that in the new spring growth of PRG however, the opposite was found with the soluble sugar and starch concentration in WC and LC (*Table 3.3*).

The WC and LC had similar CP and neutral detergent fibre concentration, while in comparison the PRG had a lower CP concentration and much higher neutral detergent fibre concentration (*Table 3.3*). Metabolisable energy content was highest in WC and lowest in PRG with intermediate metabolisable energy content in the LC. *Lotus corniculatus* was the

only forage to contain a significant amount of CT at 26-31 g kg<sup>-1</sup> DM. The CT concentration was higher in the mature LC (*Table 3.3*).

# 3.4.2.2. Metabolite formation

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There was a significant effect of plant maturity on depressing indole and skatole formation (P<0.001; *Table 3.4*). Plant maturity did not have an effect on ammonia, total VFA or BCVFA concentration. Maturity had an effect on the molar proportions of acetate, propionate and butyrate when incubating white clover (P<0.01). When incubating mature white clover, compared to the young WC, the molar proportion of acetate increased (58.3% vs. 61.6%), and correspondingly, the molar proportions of propionate and butyrate decreased (27.9% vs 25.9% and 9.7% vs. 8.7%, respectively; *Table 3.4*)

Indole, skatole and ammonia formation was the highest when incubating WC, intermediate when incubating LC and lowest when incubating PRG (P<0.05; *Table 3.4*). Total VFA formation was similar between the forages. The molar proportion of acetate was highest with LC (P<0.05) while incubating perennial ryegrass produced a higher molar proportion of butyrate (P<0.05). Molar proportion of propionate was not affected by forage type (*Table 3.4*). The incubation of PRG gave a higher concentration of BCVFA compared to WC and LC (P<0.05; *Table 3.4*).

### CHAPTER 3: Foruge factors on indole/skatole formation

Table 3.3. Experiment 2. Nutrient composition of foliage from young and mature white clover, perennial ryegrass, and *Lotus corniculatus*. The foliage was minced and used in the *in vitro* incubations.

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	White clover		Perennia	l ryegrass	Lotus co	rniculatus
	Young	Mature	Young	Mature	Young	Mature
Nutrient Composition:				,		
Dry matter (g kg <sup>-'</sup> )	181	296	222	283	177	159
Organic matter digestibility	>0.87	0.82	0.76	0.72	0.85	0.79
Metabolisable Energy (MJ kg <sup>-1</sup> DM)	12.7	11.4	10.7	10.3	11.9	11.2
Neutral detergent fibre (g kg <sup>-1</sup> DM)	211	294	471	499	213	275
Acid detergent fibre (g kg ' DM)	156	243	255	278	165	212
Crude protein (g kg DM)	271	214	109	91	250	228
Soluble sugars and starch (g $kg^{1}DM$ )	169	119	128	144	159	127
Total Condensed tannin (g kg DM)	2.5	2.9	2.3	2.5	25.6	31.1

<u>138</u>

#### CHAPTER 3: Forage factors on indole/skatole formation

Table 3.4. Experiment 2. Mean concentration of indole and skatole, ammonia and volatile fatty acids (VFA) in the media after 10 hours of incubating new spring growth (young) and mature foliage growth from white clover, perennial ryegrass and Lotus corniculatus. Results are the least square means of four replicates.

	White clover		Perennia	Perennial ryegrass		Lotus comiculatus	
	Young	Mature	Young	Mature	Young	Mature	3.L.IW.
Indole <sup>†</sup> (µg g <sup>-1</sup> CP)	374 <sup>a</sup>	298 <sup>b</sup>	22 <sup>ª</sup>	24 °	129°	78 <sup>d</sup>	18.5
Skatole <sup>†</sup> (µg g <sup>-1</sup> CP)	895°	512°	259 <sup>d</sup>	140 <sup>e</sup>	678 <sup>b</sup>	511°	30.0
Ammonia <sup>†</sup> (mmolg <sup>-1</sup> CP)	1.23 <sup>b</sup>	1.41 <sup>a</sup>	0.26 <sup>c</sup>	0.15°	1.26 <sup>a.b</sup>	1.20 <sup>b</sup>	0.09
Total VFA (mmol L <sup>-1</sup> )	149.0 <sup>a.b</sup>	122.2 <sup>b</sup>	163.7ª	136.1 <sup>ª.b</sup>	133.8 <sup>a,b</sup>	137.3 <sup>a,b</sup>	13.0
- Acetate (molar %)	58.3 <sup>b</sup>	61.6 <sup>ª</sup>	58.5 <sup>b</sup>	58.7 <sup>b</sup>	61.2 <sup>ª</sup>	61.6 <sup>ª</sup>	0.52
- Propionate (molar %)	27.9 <sup>ª</sup>	25.9 <sup>b</sup>	27.3 <sup>a</sup>	27.3 <sup>a</sup>	27.1 <sup>e</sup>	27.0 <sup>®</sup>	0.39
- Butyrate (molar %)	9.7 <sup>b</sup>	8.7°	11.5 <sup>ª</sup>	11.1 <sup>a</sup>	8.5 <sup>c</sup>	8.6°	0.29
- Branched chain VFA <sup>†</sup> (mol g <sup>-1</sup> CP)	0.37 <sup>b</sup>	0.38 <sup>b</sup>	0.64ª	0.76 <sup>a</sup>	0.28 <sup>b</sup>	0.27 <sup>b</sup>	0.07

<sup>†</sup> Indole, skatole, ammonia and branched chain VFA concentration has been adjusted for differences in the crude protein (CP) added to the incubations. a.b.c.d.e Means within the same row with different superscripts are significantly different (P<0.05).

139

# 3.4.3. Experiment 3: Nitrogen fertiliser application

### 3.4.3.1. Nutrient composition of the forages

The PRG that had been given no application of nitrogen (Low N) had a higher dry matter content than that of the High N PRG, while the neutral detergent fibre concentration, metabolisable energy content and organic matter digestibility were similar for the two forages (*Table 3.5*). The High N PRG had a much higher CP concentration and a lower soluble sugars and starch concentration than that of the Low N PRG.

Table 3.5. Experiment 3. Nutrient composition of the minced foliage from perennial ryegrass given low (Low N) and high (High N) applications of nitrogen fertiliser and then used in the *in vitro* incubations.

	Perennia	l ryegrass
	Low N	High N
Nutrient Composition:		
Dry matter (g kg <sup>-1</sup> )	239	181
Organic matter digestibility	0.85	0.85
Metabolisable Energy (MJ kg <sup>-1</sup> DM)	11.8	11.9
Neutral detergent fibre (g kg <sup>-1</sup> DM)	445	434
Crude Protein (g kg <sup>-1</sup> DM)	193	292
Soluble sugars and starch (g kg <sup>-1</sup> DM)	118	95
Total Condensed tannin (g kg <sup>-1</sup> DM)	ND	2.3

ND = not detected

## 3.4.3.2. Metabolite formation

Incubation of the High N PRG for 10 hours resulted in a higher concentration of skatole and ammonia compared to the incubation of the Low N PRG (*P*<0.001; *Table 3.6*). No differences between the forages occurred in the 10-hour concentration of indole and total VFA or the molar proportion of acetate, propionate or butyrate. Although the unadjusted branched-chain VFA concentration was similar for the two treatments (data not shown) upon adjustment for CP concentration the concentration of branched-chain VFA were lower for the High N PRG compared to the Low N PRG (*P*<0.001; *Table 3.6*).

Table 3.6. Experiment 3. Mean concentration of indole and skatole, ammonia and volatile fatty acids (VFA) in the media after 10 hours of incubating the minced foliage from perennial ryegrass given low (Low N) and high (High N) applications of nitrogen fertiliser. Results are the least square means of four replicates.

	Perennial	ryegrass	SEM	P
	Low N	High N	- U.L.M.	•
Indole <sup>†</sup> (µg g <sup>-1</sup> CP)	226.8	200.6	46.4	NS
Skatole <sup>†</sup> (µg g <sup>-1</sup> CP)	114.5	290.8	29.7	***
Ammonia <sup>†</sup> (mmol g <sup>-1</sup> CP)	0.86	1.97	0.08	***
Total VFA (mmol L <sup>-1</sup> )	129.8	118.1	5.7	NS
- Acetate (molar %)	56.9	57.4	0.84	NS
- Propionate (molar %)	26.5	27.1	0.55	NS
- Butyrate (molar %)	12.9	11.9	0.31	NS
- Branched chain VFA <sup>†</sup> (mmol g <sup>-1</sup> CP)	0.39	0.27	0.02	***

<sup>T</sup> Indole, skatole, ammonia and branched chain VFA concentration has been adjusted for differences in the crude protein (CP) added to the incubations.

NS: not significant (i.e., P>0.05) \*\*\* P<0.001

# 3.4.4. Indole, skatole, ammonia and branched-chain VFA correlations

For the data from Experiments 1, 2 and 3 there was a positive correlation between ammonia concentration per unit of CP added and indole and skatole concentration per unit of CP added. The correlation of indole and ammonia using the data from Experiment 1 in the absence of PEG gave an *r*-value of 0.50 (P<0.01) and this relationship was similar when including the data from Experiments 2 and 3 (r = 0.52; *Table 3.7*). The strongest correlation to ammonia was found with skatole when comparing the forages from Experiment 1 in the absence of PEG (r = 0.64; *Table 3.7*). This relationship of ammonia and skatole was significantly different from zero (P<0.001). Including the forages from Experiment 2 and 3 weakened the correlation of ammonia and skatole reducing the *r*-value to 0.42 however, this correlation was still

significantly different from zero (P<0.001). Indole concentration was correlated to skatole concentration in all of the comparisons made (r = 0.55-0.66; *Table 3.7*).

Table 3.7. Correlation of 10-hour in vitro incubation concentrations of indole, skatole, ammonia and branched-chain volatile fatty acids (BCVFA) per gram of crude protein added for forages from Experiment 1, 2 and 3.

	r	Р
Experiment 1: Forages incubated without PEG (n=28)		
- Ammonia and Indole	0.50	**
- Ammonia and Skatole	0.64	***
- Ammonia and BCVFA	0.40	٠
- BCVFA and Indole	0.21	NS
- BCVFA and Skatole	0.39	*
- Indole and Skatole	0.66	***
Experiment 1, 2 and 3: Forages incubated without PEG (n=60)		
- Ammonia and Indole	0.52	***
- Ammonia and Skatole	0.42	***
- Ammonia and BCVFA	-0.15	NS
- BCVFA and Indole	-0.02	NS
- BCVFA and Skatole	0.04	NS
- Indole and Skatole	0.55	***

NS: not significant (i.e., P>0.05), \* P<0.05, \*\* P<0.01, \*\*\* P<0.001

# 3.5. DISCUSSION

This study has identified non-CT-containing legumes, early vegetative growth and application of N fertiliser as being factors favouring the formation of indole and skatole in *in vitro* rumen incubations of fresh plant material and this affect appeared to be mediated through plant protein concentration. The high concentration of indole and skatole found in the *in vitro* fermentation of white clover complements the results of *in* vivo work (Schreurs et al., 2004). Forages with a higher CT concentration, such as LP and DR (99-122 g kg<sup>-1</sup> DM), were better at reducing the formation of skatole and indole than those forages with a lower CT concentration (LC and SL). Inclusion of PEG in the incubations confirmed that CT was having a highly significant effect on indole and skatole formation from LC, SL, DR and LP. Perennial ryegrass and BD also gave low concentrations of indole and skatole. However, incubations with PEG suggest that CT were not involved. A higher concentration of indole and skatole was formed with the incubation of young spring foliage compared to mature foliage of a lower CP content. The application of nitrogen fertiliser to perennial ryegrass pasture increased the CP concentration in the plant and increased the concentration of skatole formed during fermentation.

High concentrations of indole and skatole formed when incubating WC in Experiment 1 and Experiment 2, are probably due to the higher solubility and degradability of the protein in this forage (Min *et al.*, 2000). The high protein solubility and degradability were also responsible for the higher ammonia and branched-chain VFA concentration when incubating WC (Schreurs *et al.*, 2004). Plants containing a higher CT concentration (LP and DR) were more effective at lowering the formation of indole and skatole than plants with a lower CT concentration (LC and SL). Increasing the CT concentration in incubations containing plant protein from WC has given an incremental decrease in the degradation of the protein by rumen microbes (Aerts *et al.*, 1999). Thus, it is likely that the lower indole, skatole and ammonia concentration from the incubation of forages with a higher CT concentration is due to a greater reduction in the degradation of plant protein. However, the 2.8 fold difference in CT concentration with an 8.3 fold difference in skatole concentration between LC and LP suggests that factors other than CT concentration need to be considered.

Variation in responses to CT from different forages may also depend on the reactivity of the CT (McNabb *et al.*, 1998). Condensed tannins may exert their effect on plant protein degradation in the rumen by interacting directly with plant protein and/or with proteolytic enzymes of microbial origin. Aerts *et al.* (1999) showed that a CT extract from LP was more effective at reducing the *in vitro* degradation of plant protein than an extract from LC, while Min *et al.* (2002) showed that CT from LC reduced rumen proteolytic enzyme activity. Differences in the reactivity of the CT from different CT-containing forages may explain why some forages are more effective at reducing skatole and indole formation. Differences in CT reactivity may be responsible for the unproportional decrease in skatole and indole when comparing LC and LP, and for the lower indole and skatole when incubating LP compared to DR even though DR contained a higher CT concentration.

There is considerable variation in the composition of CT in forage plants (Foo *et al.*, 1982; Jackson *et al.*, 1996), and the reactivity of CT has been reported to increase with increasing prodelphinidin content (Jones *et al.*, 1976) and molecular weight (Horigome *et al.*, 1988). Recent studies suggest that polymers of very high molecular weight may

be less active (Sivakumaran *et al.*, 2004). The CT of LC is composed of predominately procyanidin units with an average molecular weight of approximately 1900 (Foo *et al.*, 1996). The CT of LP is composed of predominately prodelphinidin units (Foo *et al.*, 1997), comprising both a low molecular weight component (average MW 2200) and a higher molecular weight component (average MW approximately 13000; Meagher *et al.*, 2004). The CT in DR is also composed primarily of prodelphinidin units, and can be separated into fractions of low (MW 3300), medium (MW 12200), and high (MW 35600) molecular weight (Sivakumaran *et al.*, 2004). The high molecular weight fraction was the major component (73%) of the DR CT and showed lower activity against several rumen microbes than the medium and low molecular weight fractions (Sivakumaran *et al.*, 2004). Thus, the lower concentration of indole and skatole observed with the *in vitro* fermentation of LP compared to LC and DR maybe due to a combination of CT content, polymer composition and size dispersion.

Using CT extracts from various forages McNabb *et al.* (1998) showed that 25-50  $\mu$ g of a CT extract from LC, LP and SL was required to precipitate the plant protein included in incubations while only 5  $\mu$ g of a CT extract from BD was required. The lower concentration of CT from BD required to precipitate plant protein may explain why a lower concentration of indole, skatole and ammonia was found when incubating BD in this study. However, the lack of response to the inclusion of PEG in the incubation with BD suggests that it is not a CT effect. In a study by Waghorn and Jones (1989), PEG addition did not increase rumen ammonia concentration when including BD (18-41 g CT kg<sup>-1</sup> DM in the leaves) at 10% in a luceme and grass-based diet although it was expected that rumen ammonia concentration would increase as a result of protein being released from CT-protein complexes. No explanation was given for this lack of PEG effect, even though it was found that the CT from BD was efficient at precipitating plant protein and reducing the concentration of soluble nitrogen in the rumen. A similar lack of effect of PEG was found on indole and skatole concentration when incubating BD in this study. It is possible that PEG may not bind effectively with the CT from BD or a much higher ratio of PEG is required to bind with the CT from BD and effectively release protein. The polyphenols in BD have galloyl derivatives attached (Meagher *et al.*, 2005) which may interfere with the binding of the PEG.

The higher fibre concentration in the PRG indicates that the plant cell walls were likely to be more lignified (Chaves *et al.*, 2002). This in turn, will slow the release of protein from plant cells in the incubations and limit the availability of tryptophan and amino acids for the formation of indole, skatole and ammonia. The higher molar proportion of acetate when incubating LP and DR is due to the higher fibre content in these forages, while the higher concentration of VFA when incubating SL is likely to be a consequence of a higher soluble sugars and starch content.

As a plant matures the chemical composition of the plant changes. Specifically, the structural carbohydrate and lignin increases and CP decreases (Chaves *et al.*, 2001) and this effect was evident in Experiment 2. Increased lignification associated with increasing plant maturity decreases rumen solubility, degradability and degradation rate of CP (Chaves *et al.*, 2002), which will slow the release of amino acids in the rumen. Therefore, the lower CP concentration along with reduced CP degradability may have been responsible for the lower indole, skatole and ammonia formation with the mature forage compared to the young spring forage growth in Experiment 2.

Keane and Allen (1999) found that a nitrogen fertiliser application of 204-227 kg ha<sup>-1</sup> to a sward that contained 44% PRG, 47% other perennial grasses and 5% *Trifolium* sp. significantly increased the CP concentration in the sward compared to a lower nitrogen fertiliser application of 57 kg ha<sup>-1</sup>. In the present *in vitro* study, a higher nitrogen fertiliser application increased the CP concentration in PRG pasture and increased skatole and ammonia formed in the *in vitro* media. The metabolisable energy content of the High N and Low N PRG was the same, so the higher CP in the High N PRG may have resulted in a lack of energy for microbial protein synthesis to utilise the higher concentration of peptides, amino acids and ammonia released from the degradation of plant protein. This suggests that protein degradation by rumen microbes to peptides, amino acids and ammonia was greater than that being converted into microbial protein when incubating the High N PRG, therefore increasing the availability of tryptophan to be converted to skatole.

The significant and positive correlation between ammonia and indole and skatole formed suggests that ammonia formation can be used as an appropriate indicator of the extent of indole and skatole formation from any particular forage. A higher concentration of ammonia was formed in *in vitro* rumen incubations when forages contained a high concentration of CP and low soluble carbohydrate and fibre concentration (Burke *et al.*, 2000) thus, such characteristics of forages are also likely to lead to a higher concentration of indole and skatole being formed. Furthermore, a faster rate of protein degradation is likely to lead to a higher rumen ammonia concentration (Dove 1996; Wallace *et al.*, 1997). From the *r*-values in *Table 3.7*, it can be calculated that between 18-41% of the variation between treatments in indole and skatole production can be explained by variation in ammonia production (an indicator of

deamination). Whilst this is statistically significant, it is not large and indicates that other factors must also be influencing indole and skatole production, in addition to deamination. Some of these factors have been studied in detail in the present experiments. Indole and skatole concentration were correlated in all comparisons (*Table 3.7*) and this is likely to be a consequence of the formation of indole and skatole from tryptophan sharing common steps in the formation pathway.

New Zealand pastures can contain up to 30% of the botanical composition as WC. Grazing animals for meat production on pasture swards high in WC will increase the rumen production of indole and skatole, increasing the risk of undesirable pastoral flavours being present in the meat products. This study has shown that forages containing CT can reduce the production of indole and skatole and those forages containing a higher CT concentration, such as LP and DR, were more effective. However, the compositional differences in the CT of different forages are also likely to have an influence on reducing indole and skatole formation in the rumen. Pure PRG produced a low indole and skatole concentration and thus, PRG may not contribute to pastoral flavour problems in NZ meat to the same extent as legumes. Some caution is needed, as high applications of nitrogen fertiliser to PRG will promote a greater formation of skatole. Grazing young forage growth is likely to further increase the risk of pastoral flavour being present in the meat, as the protein in the plant will be more soluble and degradable and promote a greater production of indole and skatole.

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# **CHAPTER 4**

Concentration of indoles and other rumen metabolites in sheep after a meal of fresh white clover, perennial ryegrass or *Lotus corniculatus* and the appearance of indoles in the blood.

The material presented in this chapter has been submitted as a paper to: Journal of the Science of Food and Agriculture.

# 4.1. ABSTRACT

The objective of this study was to determine the effect of feeding three fresh forage diets, white clover (WC), perennial ryegrass (PRG) or Lotus corniculatus (LC), on the formation of skatole and indole in the rumen. The formation of skatole and indole in the rumen and their appearance in the blood was also compared. Peak rumen skatole and indole concentration per kg crude protein intake (CPI) were higher when feeding WC compared to PRG and LC (P < 0.05) and this was associated with a higher rumen concentration per kg CPI of ammonia, branched chain volatile fatty acids, total nitrogen and soluble nitrogen (P < 0.05). Greater indole and skatole concentration when feeding WC can be attributed to high solubility and rapid degradation of the forage protein. Lotus corniculatus had a similar nutrient composition to WC but the condensed tannins in LC slowed protein degradation and reduced indole and skatole formation. Indole and skatole concentration peaked in the plasma 1-2 hours after the end of feeding, indicating that skatole and indole are rapidly absorbed from the rumen into the blood. High indole and skatole formation with low intakes of WC indicate that the WC component of traditional New Zealand pastures may be the primary cause of undesirable pastoral flavours that result from the presence of indoles in the meat. To ameliorate undesirable flavours, producers reliant on pastoral systems will need to consider using alternative forages such as LC to reduce protein solubility and degradation rate.

### **4.2. INTRODUCTION**

In the European Union, Asia and North America, grain and other concentrates constitute the basis of diets used for ruminant animal production. Consumers accustomed to meat from these production systems will often consider meat from pasture-based animal production systems to have an undesirable flavour which is commonly described as, "barnyard", "animal", "sheepy", "milky" "faecal" and "grassy" (Rousset-Akrim *et al.*, 1997; Priolo *et al.*, 2001; Young *et al.*, 2003). Such flavours are collectively called pastoral flavours. Consequently, consumption of New Zealand (NZ) sheep meat is low in Asia, partially due to pastoral flavours (Prescott *et al.*, 2001).

In a study by Young *et al.* (2003) pastoral flavour was associated with the meat from lambs that had grazed on pasture but not with lamb from animals fed a concentrate diet. The pastoral flavour in the pasture-grazed lamb was correlated to the skatole and indole concentration in the meat using a gas chromatography-olfactory technique. Indole and skatole are formed in the rumen from the microbial deamination and decarboxylation of tryptophan (Deslandes *et al.*, 2001). Formation of indolic compounds in the rumen has been found to be dependent on diet (Carlson *et al.*, 1983) and to be influenced more by rumen fermentation conditions than tryptophan concentration (Carlson and Breeze 1984). High skatole concentration has been associated with a higher concentration of ammonia and volatile fatty acids in the rumen (Carlson *et al.*, 1983; Hammond *et al.*, 1984a).

Condensed tannins (CT) are polyphenolic compounds that naturally occur in some plant species, such as *Lotus corniculatus*. Condensed tannins slow protein degradation in the rumen (McNabb *et al.*, 1996) and thus, it is hypothesised that CT-containing forages

will allow for a more efficient use of amino acids by rumen microbes and reduce the availability of tryptophan for indole and skatole formation. Feeding lactating ewes with the CT-containing forage, sulla, reduced the concentration of skatole in the blood and milk compared to supplementing sulla-fed sheep with polyethylene glycol (Roy et al., 2002). This suggests that the CT in sulla reduced skatole formation. The objective of this study was to determine the effect of three fresh forage diets namely, white clover, perennial ryegrass or *Lotus corniculatus*, on the formation of indole and skatole in the rumen to provide the first direct measurement of the effect of a CT-containing forage and a direct comparison of white clover and perennial ryegrass on indole and skatole formation. Indole and skatole concentration were compared to volatile fatty acid (VFA), nitrogen and ammonia concentration in the rumen, to determine the effect of the forages on rumen fermentation and to gain a better understanding of the rumen fermentation conditions that affect the formation of indolic compounds. A second objective was to compare the formation of skatole and indole in the rumen with their appearance in the blood to establish the dynamics of skatole formation in the rumen and transfer to blood in forage fed sheep.

### **4.3. MATERIALS AND METHODS**

### 4.3.1. Experimental design

Experiment 1 investigated the formation of skatole and indole and other metabolites in the rumen when feeding fresh forages. White Clover (*Trifolium repens*, cv. Grasslands Huia, WC), Perennial Ryegrass (*Lolium perenne*, wild-type endophyte, cv. Grasslands Nui, PRG) and *Lotus corniculatus* (LC) were each fed to six rumen fistulated sheep over sequential periods of three weeks. The first week was allowed for adjustment of the animal and its rumen microbial population to the new forage. In the second week, the rumen of each animal was evacuated to determine rumen pool size. In the third week, rumen samples were obtained for profiling the formation of skatole and indole and other rumen metabolites of interest.

In Experiment 2 the appearance of skatole and indole in the blood was compared with rumen formation of these two compounds when feeding fresh perennial ryegrass. Two rumen fistulated sheep with jugular catheters were fed perennial ryegrass twice daily for 16 days. The first 9 days were allowed for adaptation followed by two sampling days a week apart. Both rumen and blood samples were taken over a 15-hour period on each sample collection day.

### 4.3.2. Animal housing and care

For Experiment 1, six Romney wethers (mean initial liveweight 43.2 kg; aged between 14-15 months) with rumen cannulae (85mm ID flexible rumen cannula) were housed indoors in metabolism crates. In Experiment 2, after a 3-month period outdoors on pasture two Romney wethers from Experiment 1 were again housed indoors in metabolism crates.

For two-weeks prior to surgery to insert fistula, the sheep were held on a feed pad and fed fresh perennial ryegrass twice a day. After fistulation, the animals were held in recovery cages and provided with lucerne chaff twice daily. After a 10-day period of post-surgery recovery, the animals were transferred to metabolism crates for the commencement of the experiment.

In order to maintain the health of the animals, prior to the commencement of both experiments, the animals used were given a dose of minerals (Nutrimol Classic, Maxicorp Limited, Palmerston North, New Zealand), a prophylactic treatment for both internal parasites (Ivomec, Merck, Sharp and Dohme, Auckland, New Zealand) and external parasites (Wipeout, Schering-Plough, Upper Hutt, New Zealand), and a slow release zinc capsule to prevent facial eczema (Time Capsule, AgResearch, New Zealand). The sheep in both experiments had access to water at all times.

#### 4.3.3. Experiment 1: Profile of rumen metabolites when feeding fresh forages

### 4.3.3.1. Forages and Feeding

Pure swards of vegetative forage were harvested daily at 09:30h using a sickle-bar mower and then stored at 4°C until feeding. Animals received meals of forage twice a day at 08:00h and 16:00h and were allowed a two-hour feeding period at each meal. Each sheep was offered 4-5kg of fresh forage at each meal and the weight of feed remaining after 2 hours was recorded for each sheep and then removed.

At each meal, representative samples of the forage offered and refused from each of the sheep were pooled and triplicate samples of 200g were taken to determine dry matter (DM) content by placing in a forced-air oven at 90°C for 24 hours. Samples of the feed offered were pooled over weekly intervals then freeze-dried and ground to pass through

a 1mm screen for composition analysis by near infra-red reflectance spectrometry (NIRS; FeedTech, AgResearch Ltd, Palmerston North, New Zealand). The freeze-dried and ground forage samples were also used to determine CT concentration using the butanol-HCl method (Terrill *et al.*, 1992).

### 4.3.3.2. Rumen Pool Size

After the sheep had been given a 7-9 day adaptation period to each forage, the rumen of each sheep was evacuated after the 2-hour morning feed period had ended. Two evacuations were undertaken on separate days for each sheep.

The procedure for evacuation was as follows. The entire rumen content of each sheep was removed through the rumen fistula by hand and the weight of rumen contents recorded. Duplicate samples of the rumen contents for each sheep were placed in a forced-air oven set at 90°C and dried to determine the dry matter proportion of the rumen contents. Before the rumen contents were placed back in the animal, samples were taken for the determination of indole, skatole, soluble nitrogen and ammonia in the rumen fluid and for total nitrogen in whole rumen contents using the analytical methods described below.

### 4.3.3.3. Rumen fluid sampling and analysis

After bailing, the sheep were given a three-day interlude before sampling the rumen contents to obtain concentration profiles of ammonia, soluble nitrogen, volatile fatty acids (VFA), indole and skatole in rumen fluid and total nitrogen in whole rumen contents following a 2-hour meal. Rumen samples were taken prior to feeding and then every 30 minutes throughout feeding and at 30 (x7), 45 (x2) and 60 minute intervals for a further 6 hours after feeding. Thus, the times of sampling relative to the start of

feeding were -15, 30, 60, 90, 120, 150, 180, 210, 240, 270, 300, 330, 375, 420, and 480 minutes.

At each time point, sub-samples of the whole rumen contents were collected and placed in a vial and frozen at -20°C then freeze-dried and ground in preparation for analysis by the Dumas method (NA1500 Series 2 Nitrogen Analyser, Carlo Erba Instruments, Milan, Italy) to determine the total nitrogen concentration in the rumen samples.

Rumen contents were squeezed through a double layer of cheesecloth to obtain rumen fluid. The pH of the rumen fluid was measured at each time point (PHM210 standard pH meter, Radiometer, France). An 8 mL sub-sample of the rumen fluid was frozen in liquid nitrogen and stored at -20°C to await analysis for skatole and indole by solid phase extraction using the method of Mattivi *et al.* (1999) followed by high performance liquid chromatography (HPLC).

The HPLC system consisted of a Shimadzu pump (LC10ADvp), auto-injector (SIL-10ADvp) and detector (RF-10Axl; Shimadzu, Kyoto, Japan). The chromatography was performed with a mobile phase consisting of 70% acetic acid solution (1.2 mg mL<sup>-1</sup>, BDH Laboratory Supplies, Poole, United Kingdom) and 30% isopropanol (Hypersolv, BDH Laboratory Supplies, Poole, United Kingdom) isocratic at 1 mL min<sup>-1</sup>. Injection volume was 5  $\mu$ L with chromatographic separation carried out on a reverse-phase C18 column (Platinum, 150 x 4.6 mm; Alltech, Decrfield, Illinois, USA). The fluorescence excitation was set to 285 nm and the emission to 350 nm for the detection of skatole, indole and 2-methylindole (internal standard). Data acquisition and peak processing were performed using Shimadzu, Class-VP software (version 5.032, Shimadzu, Kyoto, Japan). This method is not an exhaustive extraction technique therefore, the indole and skatole concentrations are representative estimates rather than quantitative determinations.

A further 1.5 mL of the rumen fluid was added to 25  $\mu$ L concentrated hydrochloric acid (BDH Laboratory Supplies, Poole, United Kingdom), centrifuged (11000 g for 15 minutes) and the supernatant stored at -20°C for ammonia analysis. Ammonia concentration in the rumen fluid was determined using a commercial assay (Sigma Diagnostics Ltd, St Louis, Missouri, USA) based on the principle of reductive amination of 2-oxoglutarate to give a decrease in absorbance at 340 nm due to oxidation of NADPH proportional to the ammonia concentration (Neeley and Phillipson 1988).

Sub-samples of rumen fluid were centrifuged at 20000 g for 15 minutes to obtain a supernatant. A 10 mL sample of the supernatant was stored at -20°C for soluble nitrogen analysis. A further 3 mL of the supernatant was frozen (-20°C) for VFA analysis by gas-liquid chromatography (Attwood *et al.*, 1998).

# 4.3.4. Experiment 2: Comparison of blood plasma and rumen fluid indole and skatole

### 4.3.4.1. Forages and Feeding

Perennial ryegrass was harvested daily and stored as described in Experiment 1. In Experiment 2, the two animals were fed fresh perennial ryegrass for two 2-hour meal periods each day at 09:00 and 16:00 hours. The animals were offered 3-4 kg of fresh forage at each meal with refusals weighed at the end of the 2-hour meal period. For two days prior to sampling and on the sampling day, animals were fed in the morning (09:00) only and then placed back on twice daily feeding once sampling had concluded.

Forage dry matter and chemical composition was determined by the same method used in Experiment 1.

### 4.3.4.2. Rumen fluid sampling and analysis

The two sheep were sampled for rumen fluid on the same day, with samples being taken prior to the start of the morning feed (time 0) and then at hourly intervals from the start of feeding. The last sample was taken 15 hours after the start of feeding. Whole rumen contents were obtained via the rumen fistula and squeezed through a double layer of cheesecloth. A 4 mL sub-sample of the rumen fluid was then frozen in liquid nitrogen and stored at -20°C until subsequent analysis for indole and skatole using the same HPLC method as described in Experiment 1. A second sampling day occurred one week later.

### 4.3.4.3. Blood sampling and plasma analysis

Blood samples were taken via a jugular catheter. Temporary jugular catheters were inserted a week after the animals had been placed in metabolism crates. Samples of blood were taken at the same time intervals as rumen fluid, that being prior to feeding (time 0), and then at hourly intervals from the start of feeding for 15 hours.

Blood was collected by flushing the catheter with approximately 2 mL of 50 iu heparin in saline then withdrawing 4-5 mL of blood which was discarded. The blood sample was then aspired from the catheter into a 7.5 mL LH S-Monovette tube (Lithium Heparin 15 iu mL<sup>-1</sup> of blood, Sarstedt, Germany) using a S-Monovette adapter. Blood samples were immediately placed on ice. After sampling, the catheter was flushed with 5-8 mL of 50 iu heparin in saline. Blood was then centrifuged (3270 g for 15 minutes) and the plasma removed and frozen at -85°C for the analysis of indoles at a later stage. Plasma samples were analysed for indole and skatole by high performance liquid chromatography following the method of Claus *et al.* (1993).

### 4.3.5. Statistical analyses

For the data from Experiment 1 and 2, splines were fitted for each sheep using the statistical package ASRcml (Gilmour et al., 2002). A spline is a polynomial curve fitted between points so that variation is represented smoothly. From the predicted values obtained from the splines the values for maximum concentration and time to maximum were obtained for each sheep on each of the treatment diets. Maximum concentration and time to maximum were then statistically analysed using PROC MIXED of SAS (Version 9.1, 2003) with a linear model that included the fixed effect of forage and had sheep as a random block effect. Due to differences in the variance (heterogeneous variance) for each forage treatment, the error structure was defined as a block of the residual for each forage treatment. The data was adjusted for crude protein intake or dry matter intake, as there was a strong positive correlation between intake and the concentration of metabolites investigated. Rumen pool data was also adjusted for intakes and analysed using PROC MIXED of SAS with a linear model that included the fixed effects of forage. In Experiment 2 the time to maximum for the concentration of metabolites in the rumen fluid and blood plasma were compared using PROC MIXED of SAS with a model that included the fixed effects of fluid type (rumen or plasma) and the fixed effect of sampling day (day 1 or 2) and the interaction of fluid type and sampling day. The effect of sheep was included as a random block effect.

# 4.4. RESULTS

### 4.4.1. Composition of diets and intakes

The chemical composition of the white clover (WC), perennial ryegrass (PRG), and *Lotus corniculatus* (LC) fed in Experiment 1 and of the perennial ryegrass fed in Experiment 2 is presented in *Table 4.1*. Crude protein concentration was similar in WC and LC while the concentration in PRG was much lower. Fibre concentration (NDF) in PRG was approximately twice as high as that in WC or LC. *Lotus corniculatus* was the only forage to contain a significant concentration of condensed tannin at 14 g kg<sup>-1</sup> DM. The perennial ryegrass fed in Experiment 2 was of similar composition to that used in Experiment 1 apart from having a higher crude protein concentration.

In Experiment 1 the dry matter intake (DMI) was lowest when feeding WC and highest when feeding PRG. The corresponding crude protein intake (CPI) was lowest when feeding white clover and twice as high when feeding PRG and LC (*Table 4.1*). Dry matter intake of perennial ryegrass for the 2-hour morning meals in Experiment 2 was approximately 520 g and the corresponding CPI was 117 g.

### 4.4.2. Rumen pools

The rumen dry matter and liquid volumes were lower when feeding WC compared to the other forages (P<0.05), reflecting in part the much lower DMI (P<0.05). The rumen total nitrogen, ammonia, indole and skatole pool relative to CPI was the highest when feeding WC and lowest when feeding LC (P<0.05; *Table 4.2*).

CHAPTER	4:	Postorand	lial ind	lole/s	katol	e formation
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	Experiment 1				Experiment 2
	White clover	Perennial ryegrass	Lotus corniculatus	S.E.M	Perennial ryegrass
Chemical Composition:					
Dry matter (g kg <sup>-1</sup> )	122	233	148		195
Neutral detergent fibre (g kg <sup>-1</sup> DM)	242	455	227	-	45 <b>7</b>
Crude Protein (g kg <sup>·1</sup> DM)	265	172	248	-	225
Organic matter digestibility	0.83	0.82	0.79	-	0.75
Soluble sugars and starch (g kg <sup>-1</sup> DM)	104	107	128	-	69
Total Condensed tannin (g kg <sup>·1</sup> DM)	3.6	0.2	14.2	-	ND
Intakes:					
Dry matter intake (g per 2-hour meal)	114 <sup>a</sup>	351 <sup>b</sup>	278 <sup>c</sup>	18.3	520
Crude protein intake (g per 2-hour meal)	30ª	60 <sup>b</sup>	69 <sup>b</sup>	3.4	117

Table 4.1. Nutrient composition of the diets offered and average intakes of six sheep on the days of sampling in Experiment 1 when feeding fresh perennial ryegrass, white clover and *Lotus corniculatus* and in Experiment 2 when feeding perennial ryegrass.

165

ND = not determined ab.c. Means in the same row with different superscripts are significantly different (P<0.05).

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	White clover	Perennial ryegrass	Lotus corniculatus	Pooled SEM
Rumen dry matter concentration (g kg <sup>-1</sup> )	94	110	105	7.4
Intakes:				
- Dry matter (g per 2-hour meal)	132ª	297 <sup>b</sup>	222 <sup>b</sup>	49.7
- Crude Protein (g per 2-hour meal)	38 <sup>ª</sup>	64 <sup>b</sup>	56 <sup>ab</sup>	12.6
Rumen volumes (after 2 hour meal):				
- Rumen dry matter (g)	358°	589 <sup>b</sup>	498 <sup>⊳</sup>	70.7
- Rumen liquid (L)	3.3 <sup>a</sup>	4.7 <sup>b</sup>	4.2 <sup>b</sup>	0.39
Rumen pools:1				
- Total nitrogen (g per kg CPI)	852ª	466 <sup>b</sup>	376 <sup>b</sup>	189.8
- Ammonia (g per kg CPI)	59 <sup>ª</sup>	37 <sup>ab</sup>	23 <sup>b</sup>	18.7
- Indole (mg per kg CPI)	308 <sup>a</sup>	205 <sup>ab</sup>	104 <sup>b</sup>	79.9
- Skatole (mg per kg CPI)	590 <sup>a</sup>	314 <sup>b</sup>	256 <sup>b</sup>	174.2

Table 4.2. Experiment 1. Least square means for rumen pools of total nitrogen, ammonia, indole and skatole of six sheep after a two-hour feeding period on white clover, perennial ryegrass and Lotus corniculatus. Included in the table is the intake of dry matter and crude protein, rumen dry matter concentration and rumen volumes during the period of pool size estimation.

 $^1$  Total nitrogen, ammonia, indole and skatole pool data are adjusted for crude protein intake (CPI),  $^{\rm ab}$  Means in the same row with different superscripts are significantly different (P<0.05).



#### 4.4.3. Skatole and indole production in the rumen

The concentration of indole per kg CPI in the rumen fluid increased after the start of feeding and reached maximal concentration 2-2.9 hours after the start of feeding (*Figure 4.1*). Maximum indole concentration per kg CPI in the rumen fluid was higher when feeding WC compared to PRG and LC (P<0.05; *Table 4.3*). A shorter time was required to reach maximum indole concentration when feeding WC compared to LC (*Table 4.4*). Skatole concentration per kg CPI in the rumen fluid also increased over the feeding period (*Figure 4.2*) and maximum concentration was higher when feeding WC (P<0.05; *Table 4.3*). Skatole concentration reached its maximum at 2.6 hours when feeding WC which was sooner than when feeding PRG (P<0.05; *Table 4.4*). The rumen fluid skatole and indole concentration per kg CPI had declined to pre-feeding concentration at approximately 6 hours after feeding.

### 4.4.4. Total nitrogen, soluble nitrogen and ammonia

Total rumen nitrogen and soluble nitrogen concentration per kg CPI did not vary significantly over the 8 hours after the start of feeding (*Figure 4.3* and *Figure 4.4*) however, the concentration of these nitrogen fractions were higher when feeding WC compared to PRG and LC (P<0.001). Ammonia concentration in the rumen per kg CPI was higher when feeding WC compared to PRG and LC (P<0.05; *Table 4.3*). The time taken to reach maximum ammonia concentration in the rumen was approximately 2-2.3 hours and was not different when feeding the different forages (*Table 4.3*).



Figure 4.1. Experiment 1. Mean concentration of indole adjusted for crude protein intake (CPI) in the rumen of sheep (n=6) fed white clover  $(\triangle)$ , perennial ryegrass  $(\bullet)$  or *Lotus corniculatus*  $(\Box)$ . Error bars shown are the SEM and the double-ended arrow indicates the time over which feeding took place.



Figure 4.2. Experiment 1. Mean concentration of skatole adjusted for crude protein intake (CPI) in the rumen of sheep (n=6) fed white clover  $(\triangle)$ , perennial ryegrass  $(\bullet)$  or *Lotus corniculatus*  $(\Box)$ . Error bars shown are the SEM and double-ended arrow indicates the time over which feeding took place.

CHAPTER 4 P	ostorandial indol	e/skatole forma	tion

Table 4.3. Experiment 1. Maximum rumen concentration of indolic compounds, ammonia and volatile fatty acids (VFA) adjusted for intake of crude protein (CPI) or dry matter (DMI) when feeding white clover, perennial ryegrass or Lotus corniculatus. Also included for comparison is minimum rumen pH.

	White clover	Perennial ryegrass	Lotus corniculatus
Maximum rumen concentration <sup>1</sup> ;			
- Indole (μg g <sup>·1</sup> per kg CPI)	96 ± 31.1ª	37 ± 9.3 <sup>b</sup>	23 ± 3.8 <sup>b</sup>
- Skatole (µg g <sup>-1</sup> per kg CPI)	164 ± 36.2 <sup>ª</sup>	67 ± 24.3 <sup>b</sup>	48 ± 10.0 <sup>b</sup>
- Ammonia (mmol L <sup>-1</sup> per kg CPI)	1062 ± 310.8ª	341 ± 152.1 <sup>b</sup>	294 ± 53.1 <sup>b</sup>
- Total VFA (mmol L <sup>-1</sup> per kg DMI)	872 ± 287.8	436 ± 175.1	384 ± 60.0
- Acetate (molar %)	64.1 ± 2.4 <sup>ab</sup>	61.8 ± 2.3 <sup>b</sup>	69.1 ± 1.1 <sup>*</sup>
- Propionate (molar %)	17.1 ± 2.3	20.9±0.9	20.6 ± 1.1
- Butyrate (molar %)	6.8 ± 1.2 <sup>ª</sup>	10.7 ± 0.9 <sup>b</sup>	$7.9 \pm 0.3^{a}$
- Total Branched Chain VFA (mmol L <sup>-1</sup> per kg CPI)	239 ± 96.4ª	81 ± 36.0 <sup>b</sup>	67 ± 10.6 <sup>b</sup>
Minimum pH	6.9 ± 0.10 <sup>a</sup>	6.2 ± 0.15 <sup>b</sup>	6.6 ± 0.07 <sup>b</sup>

Values obtained from spline curves (polynomial regressions) fitted for individual sheep so that the variance is represented smoothly ab Means in the same row with different superscripts are significantly different (P<0.05).

#### 169

CHAPTER 4: Postprandial indole/skatole formation

	White clover	Perennial ryegrass	Lotus corniculatus
Time to maximum rumen concentration (hours after the start of feeding) <sup>1</sup> :			
- Indole	$2.0 \pm 0.38^{b}$	2.4 ± 0.25 <sup>ab</sup>	2.9 ± 0.33 <sup>*</sup>
- Skatole	2.6 ± 0.27 <sup>b</sup>	$3.4 \pm 0.29^{\circ}$	3.1 ± 0.13 <sup>ab</sup>
- Ammonia	2.2 ± 0.16	$2.3 \pm 0.30$	2.1 ± 0.10
- Total VFA	2.6 ± 0.14	3.0±0.25	2.8 ± 0.15
- Acetate	2.9 ± 0.08	3.2 ± 0.48	2.9±0.14
- Propionate	2.4 ± 0.08	2.6 ± 0.09	2.5 ± 0.08
- Butyrate	2.7 ± 0.11	3.0 ± 0.12	2.8 ± 0.20
- Total Branched Chain VFA	1.7 ± 0.24	1.4 ± 0.14	NA
Time to minimum pH	$2.5 \pm 0.02$	$2.5 \pm 0.02$	2.5 ± 0.02

Table 4.4. Experiment 1. Time taken after the start of feeding to reach maximum rumen concentration of indolic compounds, ammonia, volatile fatty acids (VFA) when feeding white clover, perennial ryegrass or Lotus corniculatus. Also included for

Values obtained from spline curves (polynomial regressions) fitted for individual sheep so that the variance is represented smoothly

NA: Not applicable as concentration declined over time. a.<sup>b</sup> Means in the same row with different superscripts are significantly different (P<0.05).

comparison is time taken to reach minimum rumen pH.

170



Figure 4.3. Experiment 1. Least squares means of total nitrogen concentration per kg crude protein intake (CPI) in the rumen of sheep (n=4) fed white clover ( $\triangle$ ), perennial ryegrass ( $\bullet$ ) or *Lotus corniculatus* ( $\Box$ ). SEM = 1.47 g g<sup>-1</sup> per kg CPI. Double-ended arrow indicates the time over which feeding took place.



Figure 4.4. Experiment 1. Least squares means of soluble nitrogen concentration per kg crude protein intake (CPI) in the rumen of sheep (n=4) fed white clover ( $\triangle$ ), perennial ryegrass ( $\bullet$ ) or *Lotus corniculatus* ( $\Box$ ). SEM = 50.6 mg g<sup>-1</sup> per kg CPI. Double-ended arrow indicates the time over which feeding took place.

### 4.4.5. Volatile fatty acids

The maximum concentration of total volatile fatty acids (VFA) in the rumen per kg DMI was highest when feeding WC and lowest when feeding LC. The large variability in rumen total VFA concentration between sheep resulted in no statistical differences being observed between forages for the rumen total VFA concentration (*Table 4.3*). The maximum molar percentages of rumen acetate:propionate:butyrate were 64:17:7 for WC, 62:21:11 for PRG and 69:21:8 for LC. The molar percentage of propionate was not different for the three forages. The molar proportion of acetate was higher when feeding LC compared to PRG (P<0.05) and butyrate was higher when feeding PRG compared to WC and LC (P<0.05). Rumen BCVFA concentration per kg CPI, was higher when feeding WC (*Table 4.3*).

### 4.4.6. pH

Rumen pH declined over the feeding period to reach a minimum at approximately 2.5 hours after the start of feeding. The pH minimum was significantly lower when feeding PRG and LC compared to WC (*Table 4.3*).

### 4.4.7. Plasma and rumen comparison

*Figure 4.5* shows the concentration of indole in the rumen and plasma over a 15 hour period after the start of feeding perennial ryegrass in the morning for two hours in Experiment 2. Maximum indole concentration in the rumen was 49  $\mu$ g g<sup>-1</sup> per kg CPI and in the plasma was 23 ng mL<sup>-1</sup> (*Figure 4.5*). Indole concentration peaked in the rumen 3.0 hours after the start of feeding and declined to minimum (0.5  $\mu$ g g<sup>-1</sup> per kg CPI) at 10 hours. The peak concentration of indole in the plasma occurred 45 minutes after the rumen peak however, these peak times were not statistically different (*P*=0.07; *Figure 4.5*).

Maximum skatole concentration in the rumen for Experiment 2 was 66  $\mu$ g g<sup>-1</sup> per kg CPI and in the plasma was 31 ng mL<sup>-1</sup> (*Figure 4.6*). The minimum rumen concentration of skatole (31  $\mu$ g g<sup>-1</sup> per kg CPI) occurred at 3.8 hours after the start of feeding while the maximum rumen concentration of skatole did not occur until 10.5 hours after the start of feeding. Peak plasma concentration of skatole occurred at 3.8 hours after the start of feeding, earlier than the maximum skatole concentration in the rumen (*P*<0.05) but at the same time as the minimum skatole concentration in the rumen (*Figure 4.6*).



Figure 4.5. Experiment 2. Mean concentration indole in the rumen fluid ( $\bullet$ ) and blood plasma ( $\Box$ ) of sheep fed perennial ryegrass. Rumen concentration has been adjusted for crude protein intake (CPI). Error bars indicate the range and double-ended arrow indicates the period over which feeding took place.



Figure 4.6. Experiment 2. Mean concentration of skatole in the rumen fluid ( $\bullet$ ) and blood plasma ( $\Box$ ) of sheep fed perennial ryegrass. Rumen concentration has been adjusted for crude protein intake (CPI). Error bars indicate the range and double-ended arrow indicates the period over which feeding took place.

### 4.5. DISCUSSION

This study has shown that feeding WC resulted in a higher rumen concentration and pool size of skatole and indole per unit of crude protein intake compared to PRG and LC. The higher indole and skatole formation when feeding white clover was associated with a higher rumen concentration of ammonia, branched chain volatile fatty acids, total nitrogen and soluble nitrogen per unit of CP intake. Although *Lotus corniculatus* is a legume like white clover and has a similar nutrient composition, it did not result in a high concentration of indole and skatole in the rumen.

The high rumen concentration of soluble nitrogen and ammonia found when feeding white clover is indicative of highly soluble protein and rapid degradation of plant protein in white clover. In the rumen, plant protein is degraded by rumen microbes to form peptides, amino acids and ammonia (Mackie and White 1990; Cotta and Russell 1996). Highly soluble and degradable plant protein results in the rapid formation of peptides, amino acids and ammonia relative to the energy available in the form of adenosine triphosphate (ATP) to convert them into microbial protein (Dove 1996). Skatole and indole are likely to be formed when protein solubility and degradability is high, since there will be more free amino acids released into the rumen and degraded to form reduction products (e.g., indole and skatole from tryptophan) providing an electron sink for reducing equivalents, allowing the generation of ATP for rumen microbes (Carlson and Breeze 1984). Therefore, the highly soluble protein and rapid degradation of plant protein in white clover is likely to be

total nitrogen, ammonia, indole and skatole in the rumen relative to crude protein intake when feeding WC (*Table 4.2*).

Hammond *et al.*, (1984a) found that a higher pH (in the range 7.0-7.5) promoted a greater conversion of tryptophan to skatole in an *in vitro* rumen system, compared to a lower pH (in the range 4.5-6.5). Feeding white clover in this study gave an overall higher pH in the rumen (*Table 4.3*) and this was likely to be a consequence of the higher ammonia concentration and was likely to promote indole and skatole formation.

Protein is less soluble and degradable in legumes that contain condensed tannin (CT) (Min *et al.*, 2000). The CT present in LC slows protein degradation in the rumen (Min *et al.*, 2000), resulting in more plant protein by-passing rumen fermentation and less protein being broken down to amino acids and ammonia. Consequently, a low concentration of ammonia in the rumen has often been observed when feeding LC. In this study, feeding LC was associated with a lower concentration of ammonia, soluble nitrogen, indole and skatole in the rumen per unit of CP consumed. The lower protein degradability that results from feeding a CT-containing forage like LC was likely to have reduced the availability of tryptophan for conversion to skatole and indole in the rumen.

The protein content of the perennial ryegrass diet was much lower and the fibre content much higher than that of WC or LC. It is likely that the combination of these two nutrient components resulted in the low concentration of indole and skatole in the rumen when feeding PRG. The high fibre content means that the forage is less rapidly degraded and the low crude protein limits the availability of tryptophan for conversion to indole and skatole.

Straight-chain VFAs are the products of carbohydrate digestion in the rumen. Burke *et al.*, (2002) showed that feeding white clover produced greater VFA concentrations compared to other forages. In agreement with this, a higher rumen total VFA concentration per kg of dry matter intake was observed when feeding WC compared to PRG and LC in this study. The higher concentration of indole and skatole observed when feeding WC was also associated with a higher total VFA concentration that compliments the observations of Hammond *et al.*, (1984b) who found that a lower VFA concentration in the rumen was associated with a lower skatole concentration. Although feeding PRG gave a slightly lower molar proportion of acetate and corresponding higher molar proportion of butyrate, the difference in the molar proportions of straight-chain VFA between forages was small in comparison to the differences in rumen indole and skatole concentration between forages. This suggests that skatole and indole formation is not directly associated with carbohydrate digestion.

Branched-chain VFAs (BCVFA) are formed in the rumen from branched-chain amino acids by deamination (Allison et al., 1974), in a similar manner to which indole and skatole are formed from tryptophan. Maximal BCVFA concentrations were higher when WC was fed. This is consistent with a tendency for BCVFA to be formed in the rumen when there is a high concentration of free amino acids above the capacity for microbial protein synthesis because of high protein solubility and rapid protein degradation.

The difference in the time to maximum for the different metabolites is evidence of differences in the biosynthetic pathways and the way in which the metabolites are utilised. The longer time to reach maximum concentration of skatole in the rumen in comparison to indole is likely to be due to the additional decarboxylation reaction slowing the conversion

of tryptophan to skatole relative to indole. Branched-chain VFA can be utilised as an energy source and a precursor for protein synthesis by rumen microbes. Branched-chain VFA are also absorbed from the rumen and utilised by the ruminant. The shorter time to reach maximum concentration for BCVFA is likely to be due to microbial utilisation or rumen absorption of BCVFA exceeding the formation of BCVFA at the end of the feeding period. It is likely that the BCVFA are utilised by rumen microbes to fill any deficits for energy or microbial protein precursors induced by feeding (Allison 1962).

Of the rumen metabolites investigated, ammonia concentration peaked second after BCVFA. This indicates that the ammonia formation exceeded microbial requirements for ammonia at an earlier stage than that of other metabolites. This earlier peak in ammonia suggests that protein degradation was rapid giving a rapid formation of ammonia soon after feeding. This indicates that after feeding the utilisation of the ammonia by microbes is limited by energy availability for protein synthesis as indicated by the much later peak in VFA concentration.

Indole and skatole are absorbed directly from the rumen into the portal blood (Hammond *et al.*, 1983; Roy *et al.*, 2004). Skatole and indole are metabolised by the liver and any indole or skatole that escapes liver metabolism enters the systemic circulation. In Experiment 2, a rise in rumen indole concentration, approximately 1 hour after the end of feeding, was associated with a corresponding rise in the indole concentration in the plasma (*Figure 4.5*). This agrees with the results of Hammond *et al.*, (1984b) who reported that increased rumen concentration of indole was associated with an increase in plasma concentration of indole in blood collected from the jugular vein of steers.

Plasma skatole concentration also peaked approximately 1 hour after the end of feeding (*Figure 4.6*) confirming the findings of previous research with steers, which found that skatole and indole are rapidly absorbed from the rumen into the blood (Yokoyama *et al.* 1975; Hammond *et al.*, 1983). However, the skatole concentration in the rumen remained high throughout the sampling period (comparable to the maximum seen in Experiment 1), reaching a minimum as the skatole concentration in the plasma reached its peak. The skatole concentration in the rumen reached a maximum 8 hours after feeding. Plasma volumes remain relatively constant, while the rumen volume will vary because of feeding and passage of digesta from the rumen. The blood plasma concentration of skatole clearly indicates that there was a net increase in the skatole being absorbed from the rumen during feeding in Experiment 2. The decline in rumen concentration during feeding may have been due to an increased rumen volume as a consequence of feeding. The DMI in Experiment 2 was approximately twice that of Experiment 1 so a dilution effect was likely to be more evident. However the rumen indole data did not show this effect so the discrepancy remains largely unexplained.

High skatole and indole concentration in the fat of sheep raised on pasture has been associated with undesirable faecal odours and pastoral flavours (Young *et al.*, 2003). The high rumen concentration of indole and skatole per kg CP intake for sheep fed white clover observed in this study indicates that traditional New Zealand pastures that incorporate WC are not likely to promote desirable meat flavours for discerning international markets. Feeding pure WC may have resulted in an unbalanced nutrient intake or bloat-like effects which resulted in a negative feedback mechanism on feed intake and contributed to a low intake of WC in this study. Adjustment for CP intake was necessary so that comparisons could be made between forages (as this was the objective) on the formation of indole and skatole. However, this adjustment has indicated that when sheep graze conventional pastures (comprising of up to 30% WC) the WC component of the dietary intake is likely to be small but can result in an unproportionally higher formation of indole and skatole in the rumen compared to diets not containing WC.

For sheep fed a maize-based concentrate diet, there was a lower skatole concentration in the fat, and sensory analysis resulted in no pastoral odour or flavours being observed (Young *et al.*, 2003). Feeding concentrates is impractical and uneconomically for most New Zealand production systems that rely on the grazing of pasture. Thus, the solution to improving meat flavour will need to be forage-based so it can be incorporated into current grazing systems. It would appear that higher dietary protein concentration in plants along with a high solubility and degradability in the rumen is important for promoting skatole and indole synthesis as indicated by higher ammonia, total nitrogen and soluble nitrogen concentration in the rumen when feeding white clover. This study showed that indole and skatole production in the rumen was lowered by feeding the CT-containing forage LC, as forage protein was protected from degradation in the rumen. Further research is required to determine the effect of different CT-containing forages on skatole and indole formation in the rumen and the effect that a CT-containing forage diet has on meat flavour.

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# **CHAPTER 5**

Skatole and indole concentration and the odour of fat from lambs that had grazed perennial ryegrass/white clover pasture or Lotus corniculatus.

The material presented in this chapter has been submitted as a paper to: Animal Feed Science and Technology.

An experiment was conducted for 111 days in the summer of 2002/2003 to compare the effect of grazing lambs on condensed tannin-containing Lotus corniculatus L. (LC; cv. Grasslands Goldie; n=12) and perennial ryegrass/white clover pasture (PRG/WC; Lolium perenne/Trifolium repens; n=12) on the concentration of indole and skatole in rumen fluid, blood plasma and body fat and upon the odour of the fat. Rumen fluid and blood samples were obtained on days 0, 22, 58, 87 and 111 of the experiment. Fat from inter-muscular and tail-stub depots was obtained at slaughter. In the rumen fluid prior to slaughter, the mean skatole concentration was 0.65  $\mu$ g g<sup>-1</sup> when grazing LC compared to 1.45  $\mu$ g g<sup>-1</sup> when grazing PRG/WC (P<0.001), with no treatment differences in indole concentration (0.48 versus 0.45  $\mu$ g g<sup>-1</sup>). In the plasma prior to slaughter there was no significant difference in skatole concentration but indole concentration was 1.85 ng mL<sup>-1</sup> and 7.22 ng mL<sup>-1</sup> when grazing LC and PRG/WC, respectively (P<0.001). The concentration of skatole in the tailstub fat was lower and less variable (P < 0.05) in the lambs that had grazed LC. No differences were observed in the odour of the inter-muscular fat from lambs that had grazed LC compared to PRG/WC, suggesting that the reductions in indole and skatole formation from grazing LC were not large enough to produce a detectable difference in fat odour. The experiment was complicated by the on-set of drought conditions during the second half. Had the lambs been slaughtered at the mid-point of the experiment when the difference in the plasma indole and skatole concentration for lambs grazing LC in comparison to PRG/WC pasture was the largest then, there may have been a larger difference in fat concentration of indole and skatole, and a detectable effect on fat odour. The difference in metabolite concentration in rumen fluid obtained via rumen fistula or stomach lavage was
investigated in an initial experiment with four rumen fistulated wethers. The indole, skatole, ammonia and total volatile fatty acid (VFA) concentration did not differ significantly in the rumen fluid obtained by the two methods but the molar proportions of VFA in the rumen fluid differed between the two methods (P<0.01).

### **5.2. INTRODUCTION**

When lambs are grazed on perennial ryegrass (*Lolium perenne*)/white clover (*Trifolium repens*)-dominant pasture the meat from these animals has been described as having a pastoral flavour (Young *et al.*, 2003). In markets where the consumer is accustomed to meat from animals raised on grain or concentrates, pastoral flavour is considered an undesirable attribute of meat. Pastoral flavour has been correlated to elevated levels of indole and skatole in lamb meat (Young *et al.*, 2003). Skatole and indole are formed in the rumen from the microbial deamination and decarboxylation of tryptophan (Deslandes *et al.*, 2001).

Research from an indoor feeding trial has indicated that the white clover (WC) component of the pasture is likely to be responsible for pastoral flavour, as indole and skatole concentration in the rumen per unit of crude protein intake was much higher when feeding pure WC compared to pure perennial ryegrass (PRG; Schreurs *et al.*, 2006a; Chapter 4). In contrast, feeding the legume *Lotus corniculatus* L. (LC; cv. Grasslands Goldie) which contains condensed tannins (CT), reduced the concentration of indole and skatole in the rumen compared to feeding white clover (Schreurs *et al.*, 2006a; Chapter 4). This effect was probably due to CT in LC slowing protein degradation in the rumen and limiting the amount of tryptophan available to rumen microbes for conversion to indole and skatole. It has not yet been established if grazing CT-containing forages like LC can sufficiently reduce the formation of indole and skatole in the rumen to give a consequent reduction in blood plasma and body fat concentration compared to grazing PRG/WC-dominant pasture. Differences in the flavour of meat have been observed when animals are fed different forages. Meat from animals on legume diets has been attributed as having a more intense and less desirable meat flavour than that of animals fed grass diets (Cramer *et al.*, 1967; Shorland *et al.*, 1970). Evidence is required as to whether feeding animals CT-containing forages alters the sensory attributes of meat flavour and in particular, ameliorates pastoral flavour problems compared to feeding conventional PRG/WC pastures. The objective of this study was to determine if rumen and plasma skatole and indole concentration are reduced in lambs grazing LC and to establish if there is a difference in the odour of the fat compared to similar lambs grazing New Zealand PRG/WC-dominant pastures.

Previous rumen indole and skatole measurements from *in vivo* experiments have been obtained from rumen fluid obtained from rumen fistulated animals. Due to the surgery required to insert cannulae and the intensive management required to maintain fistulated animals, rumen fistulation can not feasibly be carried out on a large number of animals required for grazing studies. An alternative to obtain rumen fluid is to use a stomach lavage with non-fistulated animals. It is possible that the two techniques of obtaining rumen fluid could give differences in indole and skatole concentration and in the course of this study we have compared the concentration of indole and skatole, and other rumen metabolites obtained by these two techniques.

# **5.3. MATERIALS AND METHODS**

#### 5.3.1. Experimental design

#### 5.3.1.1. Experiment 1: Grab vs. stomach lavage

Rumen fluid samples were obtained by both a grab sample via the rumen fistulae and by stomach lavage from the same four rumen fistulated wethers in order to evaluate if there is a difference in the concentration of indole and skatole using the two techniques. The four wethers grazed together on perennial ryegrass-dominate pasture and were sampled four times with a 48-hour period between each sampling. Samples were analysed for indole, skatole, ammonia and volatile fatty acids (VFA).

#### 5.3.1.2. Experiment 2: Grazing of pasture and Lotus corniculatus on fat odour

The animals used in this experiment were twenty-four cryptorchid (Suffolk × Romney) lambs grazing swards of LC (n=12) or PRG/WC pasture (n=12). The lambs were randomly selected from a larger group of sixty of lambs involved in a rotational grazing experiment at Riverside farm, Massey University in the Wairarapa, New Zealand (Ramírez-Restrepo *et al.*, 2005). The experiment was carried out from the 18 November 2002 and lasted for a total of 111 days. The twenty-four randomly selected lambs were used to measure the concentration of indoles in the rumen fluid, blood plasma and body fat. Rumen fluid and blood samples were obtained on days 0, 22, 58, 87 and 111 of the experiment. Nine non-experimental weaned lambs (pre-treatment) that had been on PRG/WC pasture since birth, were slaughtered at a commercial abattoir the start of the trial. Fat from the pre-treatment lambs and those slaughtered after experimental grazing of PRG/WC pasture or LC was assessed for indole and skatole concentration and odour.

#### 5.3.2. Forages and grazing management

Although *in vitro* experiments indicated that *Lotus pedunculatus* was likely to be the optimal CT-containing legume to reduce the formation of indole and skatole (Schreurs *et al.*, 2006b; Chapter 3) it was decided to use *Lotus corniculatus* in this study. *Lotus corniculatus* was chosen because *Lotus pedunculatus* proved to be difficult to grow which was further complicated by the lack of enough viable seed. Attempts to establish a plot of *Lotus pedunculatus* using old seed proved to be unsuccessful while large plots of *Lotus corniculatus* were readily available at the time.

Management and grazing of the lambs in the Riverside Farm trial are described in detail by Ramírez-Restrepo *et al.* (2005). Briefly, pure vegetative swards of perennial ryegrass/ white clover pasture (*Lolium perenne/Trifolium repens*; PRG/WC) and *Lotus corniculatus* (cv. Grasslands Goldie, LC) were rotationally grazed in weekly breaks with free access to water at all times. Over the experimental period the pre- and post-grazing green (non-dead matter) dry matter (DM) masses for PRG/WC pasture were 2360 kg ha<sup>-1</sup> and 1420 kg ha<sup>-1</sup> respectively and for LC, 3200 kg ha<sup>-1</sup> and 2070 kg ha<sup>-1</sup> respectively. Feed allowances were set at 6 kg green DM lamb<sup>-1</sup> day<sup>-1</sup> for the initial 56 days of grazing and then increased to 8 kg green DM lamb<sup>-1</sup> day<sup>-1</sup> until the end of the trial. At the end of the grazing break, hand plucked samples to represent what lambs were eating were obtained from exclusion cages (1.4 m x 0.9 m) placed in the breaks prior to grazing. Hand-plucked samples were pooled and stored at -20°C for nutritive analysis. Botanical composition was determined from a pooled sample of weekly samples collected from each grazing treatment over the course of the experiment (Ramírez-Restrepo *et al.*, 2005).

#### 5.3.3. Sampling

The rumen fluid and blood samples were taken two days after animals had been moved to a new grazing break. The lambs were taken off the diets at approximately 09:00 hours and held in portable yards for a two-hour period while sampling of rumen fluid and blood took place. Rumen fluid (20-50 mL) was obtained by stomach lavage and 4 mL of the fluid snap frozen in liquid nitrogen and stored at -20°C to await analysis for indoles. Another 4 mL of the rumen fluid was centrifuged (16000 g for 15 minutes) and the supernatant stored at - 20°C for VFA analysis. A further 1 mL of rumen fluid was added to 15  $\mu$ L of concentrated hydrochloric acid and centrifuged (16000 g for 15 minutes). The supernatant was stored at - 20°C for ammonia analysis.

Blood was obtained by jugular venipunture into evacuated 10 mL Vacutainers<sup>®</sup> with ethylenediaminetetraacetic acid anticoagulant. Blood was then centrifuged (3270 g for 15 minutes) and the plasma removed and stored at -85°C for the analysis of indoles.

Experimental lambs were slaughtered at a commercial abattoir after 111 days of grazing the treatment diets. Fat was collected from inter-muscular depots in the hind legs and from the tail stub of each carcass.

#### 5.3.4. Laboratory analyses

#### 5.3.4.1. Forage analyses

Forage analysis is fully explained by Ramírez-Restrepo *et al.* (2005). Briefly, freeze-dried forage samples were ground to pass through a 1 mm diameter mesh sieve. Total nitrogen was determined in the ground forage samples using the Dumas principle. Metabolisable

Energy (ME; MJ kg<sup>-1</sup> DM) was estimated as 16.3 x digestible organic matter in the dry matter (Ulyatt *et al.*, 1980). The CT in the forage samples was determined using the butanol-HCl method of Terrill *et al.* (1992).

#### 5.3.4.2. Rumen fluid analysis for indoles, volatile fatty acids and ammonia

Skatole and indole in the rumen fluid samples was measured using solid-phase extraction (Mattivi *et al.*, 1999) followed by high performance liquid chromatography (HPLC). The HPLC system consisted of a Shimadzu pump (LC10ADvp), auto-injector (SIL-10ADvp) and detector (RF-10Axl; Shimadzu, Kyoto, Japan). The chromatography was performed with a mobile phase consisting of 0.02 M acetic acid/2-propanol (70:30 v/v; Hypersolv, BDH Laboratory Supplies, Poole, UK) at a flow rate of 1 mL min<sup>-1</sup>. The injection volume was 5  $\mu$ L and chromatographic separation was carried out on a reverse-phase C18 column (Platinum; 150 x 4.6 mm; Alltech, Deerfield, Illinois, USA) operated at 50°C. The fluorescence excitation was set to 285 nm and the emission to 350 nm for the detection of skatole, indole and 2-methylindole (internal standard). Data acquisition and peak processing were performed using Shimadzu, Class-VP software (version 5.032, Shimadzu, Kyoto, Japan).

Volatile fatty acids in the rumen fluid were analysed by gas-liquid chromatography following the method of Attwood *et al.* (1998). Ammonia concentration in the rumen fluid was analysed by reductive amination of 2-oxoglutarate giving a decrease in absorbance at 340 nm due to the oxidation of NADPH proportional to the ammonia concentration (Neeley and Phillipson 1988).

#### 5.3.4.3. Plasma analysis for indoles

Plasma samples were analysed for indole and skatole by HPLC following the method of Claus *et al.* (1993). The method involved taking 0.5 mL portions of blood plasma and extracting with 2 mL diethylether after the addition of the internal standard (6.3 ng 2-methylindole in 75  $\mu$ L acetonitrile/water (75:25, v/v)) by vortexing for 30 seconds. After centrifugation (1200 *g* for 15 minutes) the samples were frozen (-20°C). The ether phase was decanted into tubes containing 1 mL of 0.02 M acetic acid/2-propanol (60:40, v/v; the HPLC mobile phase), to avoid losses of the volatile indoles during the subsequent evaporation of the ether phase. Evaporation of the ether was carried out in a water bath at 47 °C. The remaining acetic acid/2-propanol phase was filtered with a 2  $\mu$ m filter (Upchurch Scientific Inc., Oak Harbour, Washington State, USA) and transferred into a 1.5 mL autosampler vial.

#### 5.3.4.4. Fat analysis for indoles

Tail stub fat was analysed to determine the concentration of indole and skatole. A sample of fat (5-10 g; weight recorded) was added to a 250 mL round bottom flask containing 40 mL of saturated sodium chloride and internal standard (50 ng g<sup>-1</sup> d<sub>3</sub>-skatole; donated by D. Rowan, HortResearch Limited, Palmerston North, New Zealand). The mixture was then extracted by steam distillation using Likens-Nickerson apparatus for 90 minutes with 30 mL of *t*-butyl methyl ether.

Analyses were carried out on a Shimadzu GC-MS 5050A (Kyoto, Japan) instrument. Samples were loaded into an AOC-20s auto-sampler, and 30 µL aliquots were injected with an AOC-20i auto-injector into an OCI-17 programmable temperature vaporiser/large volume injector fitted to a GC-17A gas chromatograph coupled to a QP-5050A quadrupole mass spectrometer. The glass insert in the injector was packed with Tenax TA 60/80 adsorbent (Alltech, Deerfield, Illinois, USA). After injection of the sample, the injector was held at 50°C for 0.1 minutes, then heated at 250°C min<sup>-1</sup> to 60 °C and held for a further 1.8 minutes for solvent evaporation, then heated at 250°C min<sup>-1</sup> to 250°C and maintained at this temperature for the remainder of the chromatographic run.

Separation was carried out on a fused-silica capillary column coated with 100% polyethylene glycol (ZB-WAX, film thickness 0.25  $\mu$ m, 30 m × 0.25 mm ID, Phenomenex, California, USA). The carrier gas was helium (>99.995% pure) with a column flow rate of 1.7 mL min<sup>-1</sup>. The split ratio was programmed at 50:1 for 2 minutes to eliminate excess solvent, splitless for 3 minutes during the sample loading step, and then split at 10:1 for the remainder of the run. The column oven was initially held at 50 °C for 4 minutes, and then heated at 4°C min<sup>-1</sup> to 200°C and held for a further 12 minutes. The interface temperature was 250°C. Selected ions monitored for each compound were (primary ion/secondary ion); d<sub>3</sub>-skatole, *m/z* 132/134; indole, *m/z* 117/90; skatole, *m/z* 130/131. Data were recorded and integrated using Shimadzu software (CLASS-5000).

#### 5.3.5. Odour assessment of fat by a sensory panel

Inter-muscular fat from 9 lambs slaughtered prior to the beginning of the trial (pretreatment), 9 LC grazed lambs and 9 PRG/WC grazed lambs (27 samples in total) was sampled for assessment of odour by a sensory panel. The fat was diced and melted in a glass beaker in a 600 W microwave for 60 seconds. A warmed stainless steel piston was used to manually press the liquid fat to separate out collagenous material and then the liquid given a light centrifuge to remove particulate material. The liquid fat was then transferred to a vial, vacuum packed and stored at -35°C until required for sensory analysis.

Fat samples were prepared for sensory evaluation by melting at 100°C and placing 1.5 g of the melted fat into a wide-neck 25 mL round-bottom flask with a stopper. Samples were kept warm in a water bath (80°C) while awaiting odour assessment. The panellists appeared at 10-minute intervals and were presented with the session samples in a randomised order. The panellists removed the stopper and smelled the headspace. Panellists assessed the samples for sweet, sheepy, earthy, camphor, faecal, musty and barnyard odours on a 0-9 intensity scale.

Ten panellists assessed each of the 27 samples. Six samples (2 pre-treatment, 2 PRG/WC, 2 LC) were presented at each session, thus requiring a total of 5 sessions to assess all the fat samples. Three randomly allocated samples were repeated in the 5<sup>th</sup> session to balance the design.

The members of the analytical odour panel were experienced at identifying odour notes and intensities of sheep meat and had initially been selected through a screening process. They have been used for the assessment of sheep meat flavours and odours for at least 8 years. Prior to the assessment of odours from the samples, the panellists met as a group and were presented with fat samples spiked with indole and skatole. From these group sessions, a consensus was obtained on the descriptors to be used in the sensory panel.

#### 5.3.6. Calculations and statistical analyses

Concentrations of indole and skatole in the rumen fluid, plasma and fat were determined from the peak area ratios and response factors relative to the internal standard. Log<sub>e</sub> transformations were under taken for the purpose of providing a normal distribution for the statistical analysis of rumen and plasma metabolites and odour scores with the least square means obtained by back-transformation. As standard errors cannot be calculated by backtransformation, the data are presented with 95% confidence intervals.

Data from the Riverside farm experiment and grab versus stomach lavage experiment were analysed using PROC MIXED of SAS (2003). The models for both experiments included the fixed effects of treatment (PRG/WC or LC; grab or stomach lavage) and day of sampling and the interaction of forage and sample day. Incorporated into the model was the random effect of animal. Residual errors were modelled using a compound symmetry structure. The concentration of indole and skatole in the fat was also analysed using PROC MIXED (SAS 2003). The data had a heterogeneous variance and so the model corrected for this by using log-transformed data. To test if the variances in the rumen fluid, blood plasma and fat concentration of indole and skatole between diet treatments were different, the Levine's test was used (PROC GLM; SAS 2003).

Odour scores from the trained panellists were examined using PROC GENMOD of SAS (2003). The model included the fixed effect of treatment and the random effect of treatment (pre-treatment, PRG/WC pasture or LC) and the random block effect of panellist. The model accounted for the many zero values in the odour score data by assuming a Poisson distribution. To test if there was a relationship between indole and skatole concentration in

the fat and the odour scores detected by each panellist, the interaction between indole or skatole concentration in the fat and panellist was assessed. For those interactions that were significant at the 5% level a Spearman Rank-order correlation (PROC CORR, SAS 2003) was carried out to assess the nature of the relationship between odour scores and fat concentration of indole and skatole for each panellist.

# 5.4. RESULTS

# 5.4.1. Experiment 1: Comparison of rumen fluid obtained from stomach lavage and fistula

No significant differences were observed in the concentration of indole, skatole, ammonia or total VFA in rumen fluid obtained from a grab sample of rumen contents via a rumen fistula or from stomach lavage (P>0.05; *Table 5.1*). There were however, significant differences in the proportion of VFA between fistula and stomach lavage samples. The proportion of acetate was higher and the proportion of propionate and butyrate lower in rumen fluid obtained by stomach lavage. Branched-chain VFA also tended to be lower in stomach lavage samples of rumen fluid (*Table 5.1*)

#### 5.4.2. Experiment 2: Grazing of pasture and Lotus corniculatus on fat odour

#### 5.4.2.1. Botanical and chemical composition of the forages

Details on the botanical and chemical composition of the PRG/WC and LC grazed in this study are given by Ramírez-Restrepo *et al.* (2005). The lambs grazing PRG/WC pasture selected 17% of their diet as WC while 80% of the diet was made up of PRG (leaves and stems). The diet selected by the lambs grazing LC consisted predominately of the LC leaf,

with only small amounts of WC consumed. Drought conditions came into effect during the second half of the experiment. Consequently, the quality of the PRG/WC pasture declined over the second half of the trial as indicated by an increased proportion of dead matter in

the sward, lower digestibility and ME values (Ramírez-Restrepo et al., 2005; Figure 5.1.A).

The crude protein concentration in the LC was higher than that in the PRG/WC pasture at the start of the experiment. However, as drought conditions came into effect there was a decline in the crude protein concentration in both the LC and PRG/WC pasture. The extent to which the crude protein declined in the second half of the experiment was greater in the LC compared to the PRG/WC pasture, so that in the last weeks of the experiment the LC had a lower crude protein concentration compared to the PRG/WC pasture (*Figure 5.1.B*). The CP:ME (g MJ<sup>-1</sup>) was similar for the two forages at the start of the experiment. The CP:ME decreased in the LC over the period of the experiment from a value of 2.0 to 1.2. This was largely a consequence of the decreasing CP in the LC over this time. The CP:ME value for the PRG/WC was relatively stable over the time of the experiment at about 1.8.

The CT concentration in the LC diet selected was approximately 40 g kg<sup>-1</sup>DM, while the PRG/WC diet selected by the lambs contained only trace amounts of CT at 1.7 g kg<sup>-1</sup>DM (Ramírez-Restrepo *et al.*, 2005).

#### CHAPTER 5: Pasture vs. L. corniculatus and fat indole/skatole

Table 5.1. Experiment 1. Least squares (LS) mean concentration of metabolites and the proportion of volatile fatty acids (VFA) in rumen fluid of four sheep. Rumen fluid was obtained via grab sample through a rumen fistula or by stomach lavage.

	Rumen fistula				
	LS Mean <sup>†</sup>	95% Confidence Interval	LS Mean <sup>†</sup>	95% Confidence Interval	۴
Indole (µg g <sup>-1</sup> )	1.9	1.6-2.4	1.5	1.1-1.8	0.1182
Skatole (µg g <sup>-1</sup> )	3.5	2.9-4.3	2.7	2.2-3.3	0.1325
Ammonia (mmol L <sup>-1</sup> )	18.3	16.1-20.7	20.0	17.6-22.6	0.2709
Total VFA (mmol L <sup>-1</sup> )	90.0	75.7-107.1	86.6	72.8-103.0	0.6757
Acetate (%)	55.6	53.6-57.6	62.0	59.8-64.2	0.0193
Propionate (%)	23.4	22.2-24.6	21.2	20.1-22.3	0.0365
Butyrate (%)	12.4	11.5-13.2	10.4	9.8-11.2	0.0501
Total Branched Chain VFA (%)	5.3	4.8-5.8	4.6	4.1-5.0	0.0887

LS Means were obtained from back transformation of data that had a log, transformation for the purpose of statistical analysis.

200



Figure 5.1. Experiment 2. Mean values of (A) metabolisable energy concentration (ME, MJ kg<sup>-1</sup> DM) and (B) crude protein (g kg<sup>-1</sup> DM) in the diet selected by lambs grazing ( $\Box$ ) *Lotus corniculatus* L. (cv. Grasslands Goldie) or ( $\bullet$ ) perennial ryegrass/white clover (*Lolium perenne/Trifolium repens*) pasture. Adapted from Ramírez-Restrepo *et al.* (2005).



Figure 5.2. Experiment 2. Indole (A) and skatole (B) concentration in rumen fluid obtained from lambs ( $\bullet$ ; n=12) grazing perennial ryegrass/white clover (*Lolium perenne/Trifolium repens*) pasture or ( $\Box$ ; n=12) Lotus corniculatus L. (cv. Grasslands Goldie). Vertical bars represent the 95% confidence interval.

#### 5.4.2.2. Rumen indole and skatole

Indole and skatole concentration varied between sampling dates (P<0.001). Indole concentration in rumen fluid when grazing LC was lower at day 58 of grazing compared to rumen indole concentration when grazing PRG/WC (P<0.001; *Figure 5.2.A*). Differences in rumen indole concentration between the LC and PRG/WC treatments were not significant at any of the other sampling dates. Skatole concentration in rumen fluid was lower when grazing LC compared to PRG/WC at day 87 and prior to slaughter at day 111 (P<0.001; *Figure 5.2.B* and *Table 5.2*).

At day 87 the rumen fluid concentration of indole and skatole decreased in comparison to previous sampling time-points. This corresponded to a time when the forage crude protein decreased because of drought conditions.

#### 5.4.2.3. Rumen metabolites prior to slaughter

In rumen fluid samples obtained prior to slaughter, no difference in rumen ammonia concentration was observed. The rumen concentration of VFA prior to slaughter was approximately 8% lower when grazing LC than PRG/WC (P<0.001; *Table 5.2*). The proportion of acetate was lower (P<0.001) and the proportion of propionate, butyrate and branched-chain VFA was higher when feeding LC compared to PRG/WC (P<0.01; *Table 5.2*). Prior to slaughter, the variation in the rumen indole and skatole concentration was similar for the lambs that had grazed PRG/WC pasture and LC (*Table 5.3*).

#### CHAPTER 5: Pasture vs. L. corniculatus and fat indole/skatole

Table 5.2. Experiment 2. Mean concentration of metabolites and the proportion of volatile fatty acids from the rumen fluid of lambs after 111 days of grazing perennial ryegrass/white clover pasture (PRG/WC; *Lolium perenne/Trifolium repens*; n=12) or *Lotus corniculatus* L.( cv. Grasslands Goldie; n=12) in the summer of 2002/2003.

	PRG/WC Pasture		La	•	
	LS Mean <sup>†</sup>	95% Confidence Interval	LS Mean <sup>†</sup>	95% Confidence Interval	F
Indole (µg g <sup>.1</sup> )	0.45	0.32-0.59	0.48	0.35-0.62	0.779
Skatole (µg g <sup>-1</sup> )	1.45	1.24-1.68	0.65	0.51-0.80	<0.001
Ammonia (mmoi L <sup>-1</sup> )	7.79	6.93-8.75	8.47	7.54-9.49	0.333
TotalVFA (mmolL <sup>-1</sup> )	61.4	54.0-69.9	42.4	37.2-48.3	<0.001
Acetate (%)	65.2	63.8-66.6	58.8	57.6-60.1	<0.001
Propionate (%)	18.7	17.7-19.8	21.3	20.2-22.5	<0.01
Butyrate (%)	11.5	10.9-12.2	13.1	12.4-13.9	<0.01
Total Branched Chain VFA (%)	3.2	2.9-3.6	5.0	4.5-5.5	<0.001

LS Means were obtained from back transformation of data that had a log, transformation for the purpose of statistical analysis.

204

#### 5.4.2.4. Plasma indole and skatole

Plasma indole concentration was lower at day 58, 87 and 111 in those animals that were grazing LC compared to PRG/WC (P<0.001; *Figure 5.3.A*). Plasma concentration of indole when grazing LC remained constantly low throughout the experiment, while indole concentration in the plasma when grazing PRG/WC tended to increase over time (*Figure 5.3.A*).

Skatole concentration in the plasma was lower at day 22 and 58 when feeding LC compared to PRG/WC (P<0.05, *Figure 5.3.B*). A decrease (P<0.01) in plasma skatole concentration at day 87 in lambs grazing PRG/WG resulted in no difference in plasma skatole concentration between the treatments prior to slaughter (*Figure 5.3.B*).

Prior to slaughter the plasma indole concentration was much less varied in those lambs that had been grazing LC compared to PRG/WC pasture (P=0.001; *Table 5.3.*). The *z*-values also indicate that the variation of plasma skatole concentration prior to slaughter tended to be less for those lambs hat had been grazing LC compared to PRG/WC pasture however, statistical analysis using the Levine's test indicated that the variance in plasma skatole was not significantly different for the two grazing treatments.



Figure 5.3. Experiment 2. Indole (A) and skatole (B) concentration in plasma obtained from jugular blood of lambs grazing ( $\bullet$ ; n=12) perennial ryegrass/white clover (*Lolium perenne/Trifolium repens*) pasture or ( $\Box$ ; n=12) *Lotus corniculatus* L. (cv. Grasslands Goldie). Vertical error bars represent the 95% confidence interval.

#### 5.4.2.5. Skatole and indole concentration in tail stub fat

The difference between mean indole concentration in the fat of lambs in the different grazing treatments was not significant at the 5% level however, there was a tendency for the indole concentration to be higher in the pre-treatment animals (P<0.13; *Figure 5.4.A*). Mean concentration of skatole in the tail-stub fat was lower when grazing LC compared to PRG/WC (P=0.06; *Figure 5.4.B*). Comparison of pre-treatment fat skatole concentration to the concentration after the grazing of treatment diets indicated a tendency for skatole concentration to decrease in those lambs that grazed LC (*Figure 5.4.B*).

Indole concentration in the tail-stub fat ranged from 6-25 and 5-21 ng g<sup>-1</sup> for the lambs slaughtered after grazing PRG/WC pasture or LC respectively. Skatole concentration in the tail-stub ranged from 41-154 and 31-86 ng g<sup>-1</sup> for the PRG/WC pasture and LC grazing treatments, respectively. The absolute value of residuals, computed in the Levine's test as a measure of the variance for indole concentration in the fat, was similar for the fat from lambs grazed on LC or PRG/WC pasture (*Table 5.3*). The absolute value of the residuals for the skatole concentration in the fat was lowest in the fat from lambs that had grazed LC (*P*=0.01; *Table 5.3*).



Figure 5.4. Experiment 2. Concentration of indole (A) and skatole (B) in the tail-stub fat of lambs (O) slaughtered prior to allocation to treatments (pre-treatment) or after 111 days grazing perennial ryegrass/white clover (PRG/WC; *Lolium perenne/Trifolium repens*) pasture or *Lotus corniculatus* L. (cv. Grasslands Goldie). • indicates the mean concentration in each treatment.

#### CHAPTER 5: Pasture vs. L. corniculatus and fat indole/skatole

Table 5.3. Experiment 2. Mean, range and absolute value of residuals (z; a measure of variance) of indole and skatole concentration observed in the rumen and plasma prior to slaughter (after 111 days grazing) and in the tail-stub fat of lambs that grazed perennial ryegrass/white clover pasture (PRG/WC; Lolium perenne/Trifolium repens) or Lotus corniculatus L. (cv. Grasslands Goldie).

	PRG/WC Pasture		Lotus corniculatus			Levine's test <sup>†</sup>		
	Mean	Range	z	Mean	Range	z	Р	
Indole								
Rumen (µg g <sup>-1</sup> )	0.4	0.2-1.0	0.2	0.5	0.02-0.8	0.2	0.75	
Plasma (ng mL'')	7.2	2.4-23.6	5.7	1.9	0.6-6.9	1.4	0.001	
Tail-stub fat (ng g <sup>-1</sup> )	13.3	6.2-25.0	3.5	12.6	4.7-21.2	3.9	0.89	
Skatole								
Rumen (µg g <sup>·1</sup> )	1.5	0.8-2.4	0.2	0.7	0.4-1.3	0.3	0.27	
Plasma (ng mL°)	11.1	4.2-25.2	4.1	8.6	4.4-14.3	2.7	0.27	
Tail-stub fat (ng g <sup>-1</sup> )	88.1	40.7-154.1	36.6	56.4	30.7-86.4	15.4	0.01	

Levine's test used to test if variances are equal

#### 209

#### 5.4.2.6. Odour assessment of inter-muscular fat

The mean score for sweet odour was lower for those lambs that were slaughtered pretreatment compared to those lambs that had grazed PRG/WC pasture or LC (P<0.01; *Table* 5.4). The barnyard odour was higher in the fat of those lambs that been slaughtered pretreatment compared to those that had grazed PRG/WC pasture (P<0.05; *Table* 5.4). The strongest odours observed were sheepy, barnyard and sweet odours (*Table* 5.4). There was a significant effect of panellist on the scores for all odours (P<0.001). There was a significant interaction between the indole and skatole concentration and the sheepy and camphor odour scores that were observed by the different panellists (P<0.01). Sheepy odour scores were negatively correlated to indole concentration for 5 of the 10 panellists (significant for 3 panellists; P<0.05) and to skatole concentration for 6 panellists (significant for 2 panellists; P<0.05). Camphor odour scores were positively correlated with indole concentration for 7 panellists (none significant) and were positively correlated with skatole concentration for 8 panellists (significant for 3 panellists; P<0.05). CHAPTER 5: Pasture vs. L. corniculatus and fat indole/skatole

Table 5.4. Experiment 2. Least squares (LS) mean odour scores of inter-muscular fat from lambs slaughtered prior to being allocated to treatments (n=9) or after 111 days of grazing perennial ryegrass/white clover pasture (PRG/WC; Lolium perenne/Trifolium repens; n=9) or Lotus corniculatus L. (cv. Grasslands Goldie; n=9) in the summer of 2002/2003.

	Pre-treatment		F	PRG/WC Pasture	Lotus comiculatus		
	LS Mean <sup>†</sup>	95% Confidence Interval	LS Mean <sup>†</sup>	95% Confidence Interval	LS Mean <sup>†</sup>	95% Confidence Interval	
Sweet	2.2	1.4-3.3	2.5	1.7-3.7	2.9	2.2-3.8	
Sheepy	2.9	2.1.4.2	2.9	1.8-4.7	3.3	2.2-4.8	
Earthy	1.9	1.1-3.1	2.0	12-3.2	2.0	1.2-3.3	
Camphor	1.2	0.7-2.1	1.2	0.7-2.1	1.0	0.5-2.0	
Faecal	1.9	1.0-3.6	1.6	0.9-2.8	1.6	0.8-3.3	
Musty	2.0	1.2-3.5	1.8	1.1-3.0	1.9	1.1-3.5	
Barnyard	3.0	2.0-4.4	2.4	1.6-3.6	2.6	1.7-3.9	

<sup>T</sup>LS Means were obtained from back transformation of data that had a log<sub>a</sub> transformation for the purpose of statistical analysis.

211

# 5.5. DISCUSSION

Grazing LC resulted in a lower mean skatole concentration and fewer high outlying concentration values of skatole (>100 ng g<sup>-1</sup>) in the tail stub fat of lambs compared to grazing PRG/WC pasture. Overall, the panel did not find a significant difference in the odour of the inter-muscular fat from the lambs that had grazed LC compared to PRG/WC. However, some panellists were sensitive to higher levels of skatole in the samples, associating higher levels positively with "camphor" odour, and negatively with "sheepy" odour. Grazing LC also reduced the concentration of indole and skatole in rumen fluid and plasma during some stages of the experimental period. It appears that the differences in mean rumen, plasma and fat concentration of indole and skatole and the reduction in high outliers were not substantial enough to give a difference in the overall mean odour of the fat.

Flavour is a sensory perception that is the result of the three distinct sensations of taste, odour and "mouth feel". The five taste sensations (sour, sweet, bitter, salt and umami (savoury)) cannot account for all the different flavours. Odour is the other main contributor to flavour (Fisher and Scott 1997). Fat odour was assessed in this study to determine the effects of the grazing treatments on odours that could be associated with pastoral flavour in lamb meat. No difference was observed for the odour of intermuscular fat from lambs slaughtered after grazing PRG/WC or LC.

The concentration of skatole in the tail-stub fat was lower in those lambs that had been grazing LC compared to pre-treatment lambs (raised on PRG/WC pasture since birth) and to lambs that had grazed PRG/WC pastures during Experiment 2. This was a consequence of less variability in the fat concentration of skatole in those lambs that

had grazed LC, with no animals having an outlying concentration of skatole (>100 ng  $g^{-1}$ ) in the fat. Although a difference was observed in the concentration of skatole in the tail-stub fat for the lambs on each of the grazing treatments, this was not large enough to give a detectable difference in the fat odour. Young *et al.* (2003) observed a difference in pastoral odour for fat samples obtained from lambs that had grazed pasture compared to fat from lambs that had consumed a concentrate diet. The fat concentration of indole and skatole of the lambs that had grazed both LC and PRG/WC in this current study were much higher than those observed from concentrate-fed lambs (with no pastoral odours observed) but comparable to the concentration observed for pasture-fed lambs in the experiment of Young *et al.* (2003).

The white clover component of the PRG/WC pasture in this study was approximately 115 g kg<sup>-1</sup> DM (Ramírez-Restrepo *et al.*, 2005). This low content of WC in the PRG/WC pasture was likely to attribute to the lack of difference observed in the odour between the PRG/WC and LC treatments. The WC component of pasture is thought to be responsible for pastoral flavours in meat, as *in vivo* studies have shown that the concentration of indole and skatole in the rumen is much greater when feeding WC compared to PRG and LC (Schreurs *et al.*, 2006a; Chapter 4). If the white clover component had been greater then it is possible that the difference in the indole and skatole formed and deposited in the fat may have been larger between the LC and PRG/WC treatments and resulted in a detectable difference in the odour of the fat.

The significant effect of panellist on the odour scores suggests that the individual panellists have different sensitivities to the odours present in the fat samples. Skatole has an odour sometimes described as "mothball" or "camphor" (Annor-Frempong *et al.*,

1997). The significant interaction of skatole concentration and panellist, along with the positive correlation (for some panellists) of skatole concentration with camphor odour scores indicates that some panellists are more sensitive to a higher skatole concentration in the fat observed as a camphor odour. Sheepy odours are generally associated with branched-chain fatty acids (Wong *et al.*, 1975). Hence the negative correlation observed between indole and skatole concentration in the fat and sheepy odour for some panellists is likely to be a consequence of indole and skatole interfering with the detection of branched-chain fatty acids.

The detection of undesirable pastoral flavours by consumers may be primarily a consequence of the consumption of meat from animals that have a exceptionally high concentration of indole and/or skatole in the meat. The between-animal variation in indole and skatole concentration observed in the fat of the lambs in the different treatments was also observed in the blood plasma but not in the rumen fluid. This suggests that some lambs grazing PRG/WC pasture were less effective than others at clearing from the body the indole and skatole formed in the rumen, with the result being a higher circulating concentration of indole and skatole in the blood and larger deposits of indole and skatole in the fat. The results from the panel assessment of odour and the variation in indole and skatole concentration in the fat indicate that consumers will have a varied response to high indole and skatole in the meat with some consumers being particularly sensitive. The ability of grazing LC to reduce the variability in the concentration of skatole in the fat has the potential to provide a more consistent flavour in meat cuts exported to discerning international markets. This would reduce the probability of a consumer that is sensitive to pastoral flavours obtaining a meat product with a high concentration of indole or skatole.

Differences in the rumen concentration of indole and skatole during the grazing are likely to be a consequence of different levels of crude protein in the forage and the degradation and utilisation of plant protein in the rumen. In the case of LC, this was likely to be affected by the CT content. The lower rumen indole concentration at day 58 and the lower rumen skatole concentration at days 87 and 111 when grazing LC may be due to the LC having a lower crude protein concentration at this stage of the experiment, rather than any specific effect of CT. The lower ratio of CP:ME in the LC compared to the PRG/WC at the end of the experiment (when drought conditions had been imposed on the forages) also suggests that there would have been better utilisation of forage nitrogen by rumen microbes when feeding LC at this stage of the experiment. The abundant supply of energy relative to forage nitrogen would have allowed forage nitrogen to be incorporated into microbial protein more efficiently, thereby minimising the availability of tryptophan in the rumen for the biosynthesis of indole and skatole.

Ammonia concentration in the rumen reflects the extent of protein degradation (Dove 1996). The similar ammonia concentration in the rumen of the lambs in both treatments prior to slaughter is in contrast to earlier *in vivo* findings (Schreurs *et al.*, 2006a; Chapter 4) and suggests that at this stage of the trial there was no difference in the efficiency of forage protein utilisation. The similar indole concentration in the rumen of the lambs in both treatments is in keeping with this. On the other hand, the lower skatole concentration in the rumen of lambs that grazed LC may have been due to the CT in LC having a specific inhibitory effect on the microbes involved in the final stages of skatole biosynthesis (Tavendale *et al.*, 2005) in combination with the overall lower CP concentration and lower CP:ME ratio in the LC diet at the end of the experiment. However at day 45, when the CP:ME ratio was similar for both the treatment diets, the

difference in the plasma indole and skatole concentration between lambs grazing PRG/WC pasture and LC (*Figure 5.3*) appears to be near its maximum. This indicates that the CT was possibly reducing indole and skatole biosynthesis in the rumen and reducing the concentration of indole and skatole circulating in the blood. Had slaughter occurred at this time (prior to the onset of drought conditions), then the difference in the concentration of indole and skatole in the fat from the lambs on the two grazing treatments may have been much greater and possibly detectable by the sensory panel.

Comparison of the rumen fluid obtained by a grab sample via the fistula or from stomach lavage from the same animals used in Experiment 1 showed no difference in the concentration of indole, skatole, ammonia, and total VFA. This suggests that both methods can be used to get samples to measure rumen metabolites and the results from each method will be comparable. There was however, a discrepancy in the proportions of VFA obtained by the two methods. The proportion of acetate was higher and the proportion of propionate and butyrate lower in stomach lavage samples of rumen fluid compared to grab samples from the fistula. The contents of the rumen are distributed into two layers. The fluid component of the contents contains the liquid and fine particulate material. Floating on top of this is a raft of fibrous material. The difference in the proportion of VFA in the rumen fluid obtained by the two methods was likely to be a consequence of sampling from different strata of the rumen contents.

This study indicated that at some time-points during the trial, grazing lambs on LC lowered the concentration of indole and skatole formed in the rumen, transported in the blood plasma, and lowered the skatole deposited in the fat, compared to lambs that grazed PRG/WC pasture. Due to changes in crude protein concentration throughout the

experiment it is difficult to know whether the lower indole and skatole concentration in the rumen fluid and plasma of the lambs grazing LC was a consequence of CT slowing protein degradation or simply due to a lower crude protein intake or a combination of these factors. There are indications of a specific effect of CT on skatole concentration, however further experiments will need to consider the use of polyethylene glycol to establish the CT effect. Despite the lower concentration of skatole in the fat of lambs that had grazed LC, no difference in the fat odour was detected. It is possible that the CT in LC was not sufficiently effective at reducing indole and skatole formation to lower the fat concentration to a level that was detectably different from those lambs that grazed on PRG/WC pasture.

Considering the flavour of meat from individual animals is important for the provision of consistently desirable, high value meat products as the frequency of occurrence of high outliers may be more significant than effects on population means. A proportion of the meat eating population will be sensitive to adverse pastoral flavours from the presence of indole and skatole in the meat. The interaction of these sensitive consumers with sheep meat from individual animals containing high indole or skatole concentration (>100 ng g) will result in consumers with a negative image of sheep meat quality which reduces the overall marketability. The ability of the LC to reduce the variability of skatole concentration in the fat and minimise the number of animals with an outlying high concentration of skatole in the meat suggests that CT forages hold some potential to provide consistently flavoured meat products from grazing systems. Future grazing experiments of this type with LC should examine the effects on meat flavour as well as fat odour, as Schreurs *et al.*, (2006c; Chapter 7) found that administering a grape seed extract containing CT to lambs fed forage diets, significantly

reduced a range of pastoral flavours in cooked meat but minimal effects on fat odour were detected.

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# **CHAPTER 6**

# Controlling the formation of indole and skatole in *in vitro* rumen fermentations using condensed tannin

The material presented in this chapter has been submitted as a paper to: Journal of the Science of Food and Agriculture.

# 6.1. ABSTRACT

Indole and skatole formed from the rumen fermentation of tryptophan have been correlated to the presence of undesirable pastoral flavours in meat from grazing ruminants. A series of four *in vitro* rumen fermentation experiments were carried out to determine the effectiveness of condensed tannin (CT) to reduce the formation of indole and skatole. Experiment 1 used ratios of fresh white clover (WC) with CT-containing Lotus pedunculatus (LP; 97 gCT kg<sup>-1</sup> DM), while Experiments 2 and 3 used extracts of CT from LP and grape seed. In a fourth experiment the mechanisms behind the action of CT where elucidated by delaying the addition of tryptophan and polyethylene glycol (PEG) to incubations of LP. Increasing the ratio of LP to WC decreased the formation of indole and skatole. Grape seed and LP CT extract included in incubations at 40 and 80 g kg<sup>-1</sup> DM with WC and PRG were more effective at reducing indole and skatole formation than lower concentrations of CT extract (P < 0.05). Including fresh LP in ratios with WC gave a linear decrease in indole and skatole concentration (indicating dilution) while including a CT extract in the incubations gave an exponential decrease in indole and skatole concentration (suggesting binding). Rumen microbes that were exposed to LP CT in planta for up to six hours and then provided with tryptophan were still able to convert tryptophan to indole and skatole. Adding PEG to incubations of LP after six hours was able to inhibit the activity of plant CT and increased the availability of substrate for indole and skatole formation. These studies have shown that a higher concentration of CT is more efficient for reducing indole and skatole formation and that CT contained within plants acts differently in rumen microbial fermentations than additions of extracted CT. Under the conditions of these experiments, there was no evidence that CT contained in LP affected the protein present in WC in a mixed fermentation.
## **6.2. INTRODUCTION**

Pastoral flavour reduces the acceptance of meat in some markets that are accustomed to meat from grain-based production systems (Rubino *et al.*, 1999; Young *et al.*, 2003). Compared to feeding grain, a higher concentration of indole and skatole was found in the meat of lambs that had been grazing pasture and this skatole and indole was correlated to pastoral flavours using a gas chromatography-olfactory method (Young *et al.*, 2003).

In the rumen, protein from fresh forage diets is rapidly degraded to peptides, amino acids and ammonia. The amino acids released into the rumen can be utilised by rumen microorganisms as a nitrogen and energy source, forming ammonia in the process (Hino and Russell 1985). Amino acids not incorporated into microbial protein may be deaminated by rumen microbes and in the case of the amino acid tryptophan it undergoes further decarboxylation reactions to form indole and skatole (Deslandes *et al.*, 2001).

Previous *in vitro* studies have shown that the fermentation of white clover in rumen fluid results in a high concentration of indole and skatole being formed, while with the forage legume *Lotus pedunculatus* less indole and skatole was formed (Schreurs *et al.*, 2006; Chapter 3). The higher concentration of skatole and indole with the rumen fermentation of white clover is likely to be a consequence of greater protein solubility and more rapid protein degradation, resulting in a greater availability of tryptophan for conversion to indole and skatole. The condensed tannin (CT) in *Lotus pedunculatus* slows the degradation of forage protein (McNabb *et al.*, 1996), which would limit the availability of tryptophan to be converted to indole and skatole and there is also evidence that CT inhibits the rumen microorganisms involved in the formation of indole and skatole (Tavendale *et al.*, 2005).

The objectives of this work were to determine the efficacy of CT in the forage or added as an extract to reduce the *in vitro* rumen formation of skatole and indole. Polyethylene glycol (PEG), which preferentially binds with CT and inhibits the activity of CT (Jones and Mangan 1977), was used to establish the effect that CT had on skatole and indole formation. The PEG is not likely to affect other aspects of *in vitro* fermentation, as digestion was not affected when PEG was supplemented to sheep fed lucerne (a non-CT forage; Wang *et al.* 1994). A further *in vitro* rumen fermentation study was carried out to establish if CT were inhibiting protein degradation and/or the microbes involved in indole and skatole formation by delaying the addition of tryptophan and PEG to an *in vitro* rumen incubation of a CT-containing forage.

## 6.3. MATERIALS AND METHODS

### 6.3.1. Experimental design

Four *in vitro* rumen fermentation experiments were undertaken to determine the effect of CT on the formation of indole and skatole using fresh minced forages. The first experiment utilised fresh minced white clover (*Trifolium repens*; WC), which is known to produce a high concentration of indole and skatole *in vitro*, incubated at different ratios with *Lotus pedunculatus* (LP), which contains CT. The ratios of WC:LP used were 100:0, 75:25, 50:50, 25:75 and 0:100 on a dry matter basis. To determine the effect that CT had on skatole and indole formation, all ratios of the forages were incubated in the presence and absence of polyethylene glycol (PEG; MW 3350).

The second experiment investigated the effect of adding a CT extract from LP on indole and skatole formation during the incubation of WC. The CT extract was added at 0, 10, 20, 40, and 80 g per kg of dry matter (DM) used in the incubations. In Experiment 3 a commercially available CT extract from grape (*Vitis vinifera L*.) seed, was incubated with both WC and perennial ryegrass (PRG).

A fourth experiment aimed to determine the mechanism by which CT was inhibiting indole and skatole formation. This experiment involved the *in vitro* incubation of LP with the addition of tryptophan alone or PEG alone or both tryptophan and PEG added at 0, 1, 2, 4 or 6 hours after the incubation had commenced.

In each experiment, five incubation bottles were set up for each treatment. Samples of the incubation media were taken at 0, 1, 2, 3, 4, 5, 6, 7, 8 and 10 hours after the incubation had commenced. Samples of the media were taken from sequential bottles

over the 10 time-points, so that each bottle was sampled twice. All treatments were run in one day and repeated on three successive days, so that one replicate of each treatment was run on each of the four days.

## 6.3.2. Forage preparation

Fresh spring growth of WC (for Experiments 1, 2 and 3) and PRG (for Experiment 3), were obtained from plots at Aorangi Research Farm, AgResearch Ltd, Manawatu, New Zealand. Trimmings of spring growth from LP (for Experiments 1 and 4) were obtained from outdoor demonstration plots grown at the Grassland Research Centre. Immediately after harvesting, all plant material was placed in plastic bags and frozen at -20°C. Triplicate samples (20 g each) of the frozen forages in all the experiments were taken to determine DM by drying in a forced-air oven at 90°C for 24 hrs. The forages were minced by passing 2-3 cm lengths of the frozen forage through a meat mincer (Kreft Compact R70, Germany). Minced forage equivalent to 0.5 g of DM was then weighed into 50 mL Schott bottles fitted with one-way valves in the lids. The bottles containing the fresh, frozen minced forage were then placed back in a freezer (-20°C) until required. Representative samples of the minced forage were freeze-dried and ground (to particle size <1 mm) to determine chemical composition by Near Infra-red Reflectance Spectrometry (NIRS; Feedtech, AgResearch Limited, Palmerston North, New Zealand). The CT concentration in the freeze-dried and ground forage samples was determined using the butanol-HCl method (Terrill et al., 1992).

## 6.3.3. Extraction of CT from Lotus pedunculatus.

The CT extract from *Lotus pedunculatus* was prepared following the method of Sivakumaran *et al.* (2004a). Fresh forage was extracted with acetone/H<sub>2</sub>O (70:30 v/v; BDH, New Zealand Ltd) containing ascorbic acid (1 g L<sup>-1</sup>; BDH Laboratory Supplies,

Poole, UK) in a blender (Varning VCM62, AB Hallde Maskiner, Sweden). Acetone was removed *in vacuo* (40°C) and the resulting aqueous extract washed three times with dichloromethane (BDH Laboratory Supplies, Poole, UK) to remove lipids and chlorophyll. The aqueous extract was concentrated under reduced pressure and freezedried. The freeze-dried extract was reconstituted in aqueous methanol/H<sub>2</sub>O (1:1 v/v; BDH Laboratory Supplies, Poole, UK) and filtered through a Buchner funnel using Whatman no. 40 filter paper and centrifuged (1000 g for 15 minutes). The extract solution was then eluted onto a column containing Sephadex LH20 (Pharmacia). The CT-containing fraction was eluted from the column using aqueous acetone/H<sub>2</sub>O (70:30 v/v), concentrated *in vacuo* (40°C) and freeze-dried.

### 6.3.4. Rumen fluid inoculate

Four Romney wethers with rumen fistulae were used as a source of rumen fluid to inoculate the incubation bottles. The sheep were grazed outdoors on perennial ryegrass dominant pasture. Pasture was allocated to the fistulated sheep in daily breaks by using front and back portable fences. This ensured that intakes from day-to-day were similar. The sheep were removed from the pasture and fasted for 2 hours prior to collecting the rumen contents. Rumen contents were squeezed through a double layer of cheesecloth and the fluid collected into a pre-warmed vacuum flask. Equal volumes of rumen fluid from each of the four sheep were pooled and immediately used to inoculate each of the bottles used in the incubations.

## 6.3.5. In vitro rumen incubation method

To the bottles containing the frozen minced forage the following was added; 10 mg cellobiose (Sigma Chemical Co. St Louis, Missouri, USA), 12 mL CO<sub>2</sub> saturated McDougall's buffer (pH 6.8; McDougall 1948) and 0.5 mL cysteine sulphide reducing

agent. The bottles were then fitted with lids that had one-way valves and were left to warm in the incubator (39°C, 90 oscillations per minute) while rumen fluid was collected. Rumen fluid (3 mL) was then added to the bottles under a flow of CO<sub>2</sub> and the bottles returned to the incubator. For those incubations containing PEG, the volume of the McDougall's buffer added was reduced to 10 mL and 80 mg of PEG dissolved in 2 mL McDougall's buffer was added to the incubations. When tryptophan (Sigma, St Louis, Missouri, USA) was added to the incubations in Experiment 4 the volume of McDougall's buffer initially added to the incubations was reduced by 1 mL and 121 μg of tryptophan was dissolved in 1 mL of McDougall's buffer and then included in the incubations. The CT extracts from *Lotus pedunculatus* (Experiment 2) and grape seed (Experiment 3; Grape Seed P.E. 120:1, C.Z. Medipro Botanical Laboratories, China; 99.1% proanthocyanidins) were added as dry powders after the minced forages had been weighed into the incubation bottles.

### 6.3.6. Sampling and metabolite analysis

At each time point, a total of 3 mL of the media was taken from the appropriate bottles for skatole and indole, ammonia and volatile fatty acid (VFA) analysis. For skatole and indole analysis, 1.2 mL of the media was frozen in liquid nitrogen and these samples transferred to a -20°C freezer for storage. Skatole and indole in the media samples were measured using solid-phase extraction (Mattivi *et al.*, 1999) followed by high performance liquid chromatography (HPLC). The HPLC system consisted of a Shimadzu pump (LC10ADvp), auto-injector (SIL-10ADvp) and detector (RF-10Axl; Shimadzu, Kyoto, Japan). The chromatography was performed with a mobile phase consisting of 70% acetic acid solution (1.2 mg mL<sup>-1</sup>) and 30% isopropanol (Hypersolv, BDH Laboratory Supplies, Poole, UK) isocratic at 1 mL min<sup>-1</sup>. The injection volume was 5 µL and chromatographic separation was carried out on a reverse-phase C18 column (Platinum, 150 x 4.6 mm; Alltech, Deerfield, Illinois, USA). The fluorescence excitation was set to 285 nm and the emission to 350 nm for the detection of skatole, indole and 2-methylindole (internal standard). Data acquisition and peak processing were performed using Shimadzu, Class-VP software (version 5.032, Shimadzu).

Another 1.2 mL of the media was centrifuged (16000 g for 15 minutes) and the supernatant used for VFA analysis by liquid-gas chromatography (Attwood *et al.*, 1998). A further 0.6 mL of incubation media was added to 10  $\mu$ L of concentrated hydrochloric acid and centrifuged (16000 g for 15 minutes). The supernatant was analysed for ammonia by reductive amination of 2-oxoglutarate giving a decrease in absorbance at 340 nm due to the oxidation of NADPH proportional to the ammonia concentration (Neeley and Phillipson 1988).

## 6.3.7. Statistical analysis

For all experiments, statistical analysis of skatole, indole, ammonia and volatile fatty acid concentration was carried out using PROC MIXED of SAS (2003). Replication was included as a random effect in all analyses.

*Experiment 1:* A block design was used with a model that included the fixed effects of forage ratio, PEG and the interaction of forage ratio and PEG. *Experiment 2:* A linear model was used with the fixed effect of CT concentration added (0, 10, 20, 40 or 80 g kg<sup>-1</sup> DM). *Experiment 3:* A block design was used which included the fixed effects of CT concentration, forage (WC or PRG) and the interaction of CT concentration and forage. *Experiment 4:* A block design was used with the fixed effects of treatment (plus tryptophan, plus PEG or plus tryptophan and PEG), addition time (0, 1, 2, 4 or 6 hours) and the interaction of treatment and addition time.

For Experiments 2 and 3 the indole and skatole concentrations were regressed (PROC REG, SAS 2003) against the  $\log_e+1$  transformed concentration of the CT included in the incubations. The log transformation of the concentration of CT included in the incubation converted the exponential fit of the data to a linear fit enabling linear regression analysis to evaluate the relationship between the CT added to the incubations and indole and skatole formation. For Experiments 1-3 linear, quadratic and cubic contrasts were carried out using the contrasts command in SAS (2003).

# 6.4. RESULTS

## 6.4.1. Nutrient composition of the forages

Neutral detergent fibre (NDF) concentration was considerably higher and the crude protein concentration lower in the PRG used in Experiment 3 compared to the WC used in Experiments 1, 2 and 3 and to the LP used in Experiment 4 (*Table 6.1*). The crude protein (CP) concentration was similar in the WC and LP used in the experiments. Organic matter digestibility (OMD) was high for all the forages. Soluble sugar and starch (SSS) concentration was similar for all forages but tended to be slightly higher in the WC and PRG used in Experiment 3. Condensed tannin (CT) concentration was the highest in LP at 97 and 98 g kg DM<sup>-1</sup> for Experiments 1 and 4 respectively (*Table 6.1*).

# 6.4.2. Experiment 1: Metabolite formation when incubating ratios of white clover and *Lotus pedunculatus*

The indole and skatole concentration per gram of crude protein (CP) at the end of the *in vitro* incubation period (10 hours) linearly decreased as the proportion of WC included in the incubations decreased and the proportion of LP increased (P<0.001). Consequently, indole and skatole concentration was highest when incubating 100WC:0LP at 425 and 582 µg g<sup>-1</sup> CP respectively, and lowest when incubating 0WC:100LP at 114 and 41 µg g<sup>-1</sup> CP, respectively (P<0.05; *Table 6.2*).

Ammonia concentration per gram of CP added and total volatile fatty acid (VFA) concentration followed a similar linear trend (P<0.001), with an increased proportion of LP giving a decreased ammonia and total VFA concentration in the incubations (*Table 6.2*). The molar proportion of acetate linearly increased (P<0.001) while the molar proportion of propionate linearly decreased (P<0.001) with an increased proportion of LP in the incubations (*Table 6.2*). There was no clear trend in the molar proportion of

butyrate across the different ratios of WC:LP incubated. The concentration of branched chain VFA was higher when there was a higher proportion of WC included in the incubations and linearly decreased with increasing proportions of LP (P<0.001; *Table 6.2*).

# 6.4.3. Addition of polyethylene glycol to incubations with ratios of white clover and *Lotus pedunculatus*

Adding polyethylene glycol (PEG) to the incubations caused a significant increase in indole and skatole concentration with the ratios of WC:LP of 50:50, 25:75 and 0:100 (P<0.001; *Figure 6.1 and 6.2*), with the effect becoming apparent after approximately 6 hours of incubation. The difference in the indole and skatole concentration in incubations with and without PEG was the greatest when incubating 0WC:100LP.

# 6.4.4. Experiment 2: Metabolite formation when incubating white clover with a CT extract from *Lotus pedunculatus*

Increasing the amount of CT extract from LP added to incubations with WC progressively decreased the concentration of indole, skatole and ammonia formed in a non-linear manner (P<0.05; *Table 6.3*). Therefore, the concentration of indole, skatole and ammonia was highest when incubating WC without the addition of the CT extract from LP (0 g kg<sup>-1</sup> DM) and lowest when including the LP CT extract at 80 g kg<sup>-1</sup> DM to incubations with WC. Further regression analyses indicated that there was a significant negative relationship between indole and skatole concentration and the log transformed LP CT concentration included in incubations with WC (P<0.001; *Figure 6.3*), indicating an exponential decline. The log of the concentration of CT included in the incubations explained 91 and 68% of the variability in indole and skatole concentration respectively ( $r^2$  values).

### CHAPTER 6: CT to control indole/skatole formation

#### Table 6.1. Experiment 1. Nutrient composition of the minced forages used in the in vitro incubations of Experiments 1-4

	Ex	periment 1	Experiment 2	Exp	periment 3	Experiment 4
	White clover	Lotus pedunculatus	White clover	White clover	Perennial ryegrass	Lotus pedunculatus
Chemical Composition:						
Dry matter (g kg <sup>-1</sup> )	184	145	182	155	174	219
Neutral detergent fibre (g kg <sup>-1</sup> DM)	207	245	215	210	507	267
Crude Protein (g kg <sup>-1</sup> DM)	279	256	269	282	112	295
Organic matter digestibility	>0.87	>0.87	>0.87	>0.87	>0.87	0.84
Soluble sugars and starch (g kg $^{-1}$ DM)	158	154	156	178	184	158
Total Condensed tannin (g kg <sup>-1</sup> DM)	ND	97.3	ND	ND	0.4	97.9

#### 233

CHAPTER 6: CT to control indole/skatole formation

			75WC:25LP 50WC:50LP 2			SEM	Contrasts <sup>1</sup>		
	100WC:0LP	/5WC:25LP		25WC:75LP	0WC:100LP		Linear	Quadratic	Cubic
Indole <sup>†</sup> (µg g <sup>-1</sup> CP added)	425 <sup>ª</sup>	352 <sup>ab</sup>	326 <sup>b</sup>	175 <sup>c</sup>	114 <sup>c</sup>	31.3		NS	NS
Skatole <sup>†</sup> (µg g <sup>-1</sup> CP added)	582ª	427 <sup>b</sup>	314 <sup>c</sup>	195 <sup>d</sup>	41 <sup>e</sup>	44.6		NS	NS
Ammonia <sup>†</sup> (mmol g <sup>.1</sup> CP added)	1.17 <sup>a</sup>	0.91 <sup>b</sup>	0.92 <sup>b</sup>	0.70 <sup>c</sup>	0.65 <sup>c</sup>	0.07		NS	NS
Total VFA (mmol L <sup>-1</sup> )	135.0 <sup>ª</sup>	121.2 <sup>b</sup>	110.3b <sup>c</sup>	103.9 <sup>c</sup>	98.3°	4.5		NS	NS
- Acetate (molar %)	52.5°	52.8°	55.4 <sup>b</sup>	56.8 <sup>b</sup>	60.2 <sup>ª</sup>	0.91		NS	NS
- Propionate (molar %)	31.5"	32.6 <sup>ª</sup>	28.8 <sup>b</sup>	29.3 <sup>b</sup>	26.0 <sup>c</sup>	0.90	•••	NS	NS
- Butyrate (molar %)	12.2ª	11.1 <sup>b</sup>	12.4 <sup>ª</sup>	11.1 <sup>b</sup>	11.3 <sup>b</sup>	0.41	NS	NS	NS
- Branched chain VFA <sup>†</sup> (mmolg <sup>-1</sup> CP added)	0.31ª	0.26 <sup>ab</sup>	0.23 <sup>bc</sup>	0.19 <sup>c</sup>	0.17 <sup>c</sup>	0.02		NS	NS

Table 6.2. Experiment 1. Concentration of indole and skatole, ammonia and volatile fatty acids (VFA) in the media after 10 hours of incubating ratios of white clover (WC) and Lotus pedunculatus (LP) in the absence of polyethylene glycol.

T Indole, skatole, ammonia and branched chain VFA concentration has been adjusted for differences in the crude protein (CP) added to the incubations.

Indole, Skatole, animonia and prance chain VFR contermation has been adjusted to an TNS (noi significant),  $^{\circ}$  (P<0.05),  $^{\circ}$  (P<0.01),  $^{\circ}$  (P<0.001) abc.d.e Means in the same row with different superscripts are significantly different (P<0.05)

#### 234



Figure 6.1. Experiment1. Indole formation *in vitro* when incubating ratios of white clover (WC) and *Lotus pedunculatus* (LP) in the presence ( $\Box$ ) and absence ( $\bullet$ ) of polyethylene glycol which inhibits the activity of condensed tannins. Results are the least square means of four replicates and the error bars are the standard error of the mean. \*, P<0.05; \*\*, P<0.01; \*\*\*, P<0.001.



Figure 6.2. Experiment 1. Skatole formation in vitro when incubating ratios of white clover (WC) and Lotus pedunculatus in the presence ( $\Box$ ) and absence ( $\bullet$ ) of polyethylene glycol which inhibits the activity of condensed tannins. Results are the least square means of four replicates and the error bars are the standard error of the mean. \*, P<0.05; \*\*, P<0.01; \*\*\*, P<0.001.

Total VFA concentration linearly decreased with increased addition of CT from LP to the incubations of WC (P<0.001), while the molar proportion of acetate linearly increased (P<0.001; *Table 6.3*). The molar proportions of propionate and butyrate were similar across the different percentages of CT added to the incubations. The concentration of branched chain VFA per gram of CP added to the incubations decreased in a quadratic manner as the quantity of CT added to the incubations increased (P<0.001; *Table 6.3*).

# 6.4.5. Experiment 3: Metabolite formation when incubating white clover and perennial ryegrass with a CT extract from grape seed

Increasing the amount of CT extract from grape seed added to incubations with WC and PRG decreased the formation of indole and skatole per gram of CP added in a nonlinear manner (P<0.01; *Table 6.4*) and decreased ammonia formation linearly. Indole and ammonia concentration per gram CP were higher when incubating PRG compared to WC, while skatole concentration per gram of CP added was higher when incubating WC (P<0.001; *Table 6.4*). Further regression analysis indicated that there was a significant negative relationship between indole and skatole concentration and the log transformed CT concentration included in incubations with both WC and PRG (P<0.001; *Figure 6.4*) indicating an exponential decline. The concentration of CT included in the incubations with WC explained 54 and 62% of the variability in indole and skatole concentration respectively ( $r^2$  values) and explained 76% of the variability in indole and 85% of the variability in skatole when incubating PRG. CHAPTER 6: CT to control indole/skatole formation

Table 6.3. Experiment 2. Mean concentration of indole and skatole, ammonia and volatile fatty acids (VFA) in the media after 10 hours of incubating white clover with condensed tannins (CT) extracted from Lotus pedunculatus added at 0-80 g kg<sup>-1</sup> of the dry matter content of the forages added to incubations. Results are the least square means of four replicates.

	Amount of CT added (g kg <sup>-1</sup> DM)					Contrasts <sup>‡</sup>			
	0	10	20	40	80	5.E.M	Linear	Quadratic	Cubic
Indole <sup>†</sup> (µg g <sup>-1</sup> CP added)	687 <sup>ª</sup>	539 <sup>b</sup>	361°	248 <sup>d</sup>	55°	25.9	•••		NS
Skatole <sup>†</sup> (µg g <sup>-1</sup> CP added)	1463 <sup>ª</sup>	1327ª	788 <sup>b</sup>	651 <sup>b</sup>	242°	73.9			NS
Ammonia <sup>†</sup> (mmol g <sup>-1</sup> CP added)	2.79 <sup>ª</sup>	2.56 <sup>b</sup>	2.15 <sup>c</sup>	1.79 <sup>d</sup>	1.22 <sup>€</sup>	0.08	***	•	NS
Total VFA (mmol L <sup>-1</sup> )	136.8 <sup>ª</sup>	133.8 <sup>ab</sup>	121.6 <sup>c</sup>	126.5 <sup>bc</sup>	118.5°	3.1	***	NS	NS
- Acetate (molar %)	51.3°	52.0 <sup>bc</sup>	52.0 <sup>bc</sup>	53.1 <sup>b</sup>	55.7ª	0.58		NS	NS
- Propionate (molar %)	30.6 <sup>ab</sup>	30.6 <sup>ab</sup>	31.2 <sup>ª</sup>	30.8 <sup>ab</sup>	29.7 <sup>b</sup>	0.46	NS	NS	NS
-Butyrate (molar %)	12.6 <sup>ª</sup>	12.4ª	12.1 <sup>ª</sup>	12.1ª	11.5 <sup>b</sup>	0.31	••	NS	NS
- Branched chain VFA <sup>†</sup> (mmol g <sup>-1</sup> CP added)	0.50 <sup>a</sup>	0.44 <sup>b</sup>	0.36 <sup>c</sup>	0.31 <sup>d</sup>	0.22 <sup>e</sup>	0.02		•••	NS

Indole, skatole, ammonia and branched chain VFA concentration has been adjusted for differences in the crude protein (CP) added to the incubations. \* NS (not significant), \* (P<0.05), \*\* (P<0.01), \*\*\* (P<0.001) a.b.c.d.® Means within the same row with different superscripts are significantly different (P<0.05).</p>

238



Figure 6.3. Experiment 2. The concentration of indole ( $\bullet$ ) and skatole ( $\triangle$ ) plotted against the log<sub>e</sub>+1 scale of the *Lotus pedunculatus* condensed tannin (CT) included at 0, 10, 20, 40 and 80 g kg<sup>-1</sup> DM (equivalent to 0, 1, 2, 4 and 8 % of the DM) in *in vitro* incubations with fresh minced white clover.

The concentration of total VFA and branched chain VFA per unit of CP decreased as the amount of CT extract from grape seed increased in incubations with both WC and PRG (*Table 6.4*), with the decrease being linear for WC (P<0.001) and quadratic for PRG (P<0.05). Incubation of WC resulted in a higher concentration of total VFA compared to PRG (P<0.01). The molar proportion of acetate tended to increase with increased amount of grape seed CT added to the incubations, with the response being linear for WC (P<0.001) and quadratic for PRG (P<0.01). The molar proportion of propionate did not show a consistent trend across the different amounts of CT added when incubating WC; however, the molar percentage of propionate quadratically decreased with increased grape seed CT when incubating PRG (P<0.01; *Table 6.4*). The molar proportion of butyrate was similar at all percentages of grape seed CT added to incubations with PRG however, the molar proportion of butyrate linearly decreased with increased grape CT when incubating WC (P<0.001; *Table 6.4*).

# 6.4.6. Experiment 4: Skatole and indole formation when adding tryptophan and PEG to incubations with *Lotus pedunculatus*

The addition of tryptophan only, or PEG only, to incubations with LP resulted in an increase in the concentration of indole (*Figure 6.5*) or skatole (*Figure 6.6*) after the time at which the substance was added. The increased formation in indole was immediate while for skatole there was a 2-hour time lag before any increase was seen. When adding tryptophan and PEG at the same time there was also an increase in the concentration of indole or skatole formed (*Figure 6.5* and *Figure 6.6*) with the increase being approximately equal to the sum of concentrations obtained when adding tryptophan and PEG separately.

CHAPTER 6: CT to control indole/skatole formation

	CT added to incubations (g kg <sup>-1</sup> DM)						Contrasts <sup>1</sup>		
	0	10	20	40	80	S.E.M.	Linear	Quadratic	Cubic
White clover									
Indole <sup>†</sup> (µg g <sup>-1</sup> CP added)	197ª	131 <sup>b</sup>	125 <sup>b</sup>	45°	28°	17.0	***		NS
Skatole <sup>†</sup> (µg g <sup>-1</sup> CP added)	830 <sup>ª</sup>	715 <sup>b</sup>	466 <sup>c</sup>	267 <sup>d</sup>	164 <sup>d</sup>	53.1	***	**	NS
Ammonia <sup>†</sup> (mmol g <sup>-1</sup> CP added)	1.6ª	1.4 <sup>b</sup>	1.4 <sup>bc</sup>	1.2 <sup>c</sup>	0.9 <sup>d</sup>	0.10	***	NS	NS
Total VFA (mmol L <sup>-1</sup> )	120ª	118ª	112 <sup>ab</sup>	103 <sup>bc</sup>	94 <sup>c</sup>	4.0	***	NS	NS
Acetate (molar %)	52.2 <sup>bc</sup>	51.7°	52.0°	54.1 <sup>b</sup>	57.0 <sup>ª</sup>	0.74	***	NS	NS
Propionate (molar %)	29.6	29.9	30.2	30.1	29.6	0.56	NS	NS	NS
Butyrate (molar %)	13.6 <sup>ab</sup>	14.0 <sup>ª</sup>	13.8 <sup>ab</sup>	12,5 <sup>b</sup>	10.8 <sup>c</sup>	0.62	***	NS	NS
Branched chain VFA <sup>†</sup> (mmol g <sup>-1</sup> CP added)	0.32 <sup>ª</sup>	0.30 <sup>ab</sup>	0.26 <sup>b</sup>	0.19 <sup>c</sup>	0.13 <sup>c</sup>	0.02	***	NS	NS
Perennial ryegrass									
Indole <sup>†</sup> (µg g <sup>-1</sup> CP added)	527ª	212 <sup>b</sup>	194 <sup>b</sup>	8°	11 <sup>c</sup>	39.1	***	***	NS
Skatole <sup>†</sup> (µg g <sup>-1</sup> CP added)	669a	462 <sup>b</sup>	320°	129 <sup>d</sup>	104 <sup>d</sup>	30.8	***	***	NS
Ammonia <sup>†</sup> (mmol g <sup>-1</sup> CP added)	1.9 <sup>a</sup>	1.5 <sup>bc</sup>	1.5 <sup>⊳</sup>	1.2 <sup>cd</sup>	0.9 <sup>d</sup>	0.17	***	NS	NS
Total VFA (mmol L <sup>-1</sup> )	120	101 <sup>b</sup>	101 <sup>b</sup>	98 <sup>b</sup>	89 <sup>c</sup>	4.0	***		**
Acetate (molar %)	53.2°	55.9 <sup>b</sup>	56.3 <sup>b</sup>	58.5ª	59.2ª	0.54	***	**	NS
Propionate (molar %)	29.2ª	28.2 <sup>b</sup>	27.2 <sup>b</sup>	26.1°	26.1 <sup>c</sup>	0.57	***	••	NS
Butyrate (molar %)	12.9 <sup>a</sup>	12.2 <sup>ab</sup>	12.4 <sup>ab</sup>	12.2 <sup>ab</sup>	12.0 <sup>b</sup>	0.40	NS	NS	NS
Branched chain VFA <sup>†</sup> (mmol g <sup>-1</sup> CP added)	0.36 <sup>a</sup>	0.24 <sup>bc</sup>	0.27 <sup>b</sup>	0.20 <sup>c</sup>	0.14 <sup>d</sup>	0.02	***		NS

Table 6.4. Experiment 3. Mean concentration of indole and skatole, ammonia and volatile fatty acids (VFA) in the media after 10 hours of incubating white clover or perennial ryegrass with condensed tannins (CT) extracted from grape seed added at 0-80 g kg<sup>-1</sup> of the dry matter content of the forage added to incubations. Results are the least square means of four replicates.

<sup>1</sup> Indole, skatole, ammonia and branched chain VFA concentration has been adjusted for differences in the crude protein (CP) added to the incubations. <sup>1</sup> NS (not significant), \* (P<0.05), \*\* (P<0.01), \*\*\* (P<0.001) <sup>a,b,c,d</sup> Means within the same row with different superscripts are significantly different (P<0.05).



Figure 6.4. Experiment 3. The concentration of indole (•) and skatole ( $\triangle$ ) plotted against the log<sub>e</sub>+1 scale of the grape seed condensed tannin (CT) included at 0, 10, 20, 40 and 80 g kg<sup>-1</sup> DM (equivalent to 0, 1, 2, 4 and 8 % of the DM) in *in vitro* incubations with fresh minced white clover (A) and perennial ryegrass (B).



(B) Addition of PEG



(C) Addition of tryptophan and PEG



Figure 6.5. Experiment 4. *In vitro* formation of indole when incubating *Lotus pedunculatus* with the addition of tryptophan (A), polyethylene glycol (PEG; B) or both tryptophan and PEG (C) at 0 ( $\Box$ ), 1 ( $\blacktriangle$ ), 2 ( $\diamond$ ), 4 ( $\bullet$ ) or 6 ( $\circ$ ) hours after the start of the incubation period.

### (A) Addition of tryptophan



(B) Addition of PEG



(C) Addition of tryptophan and PEG



Figure 6.6. Experiment 4. In vitro formation of skatole when incubating *Lotus* pedunculatus with the addition of tryptophan (A), polyethylene glycol (PEG; B) or both tryptophan and PEG (C) at 0 ( $\Box$ ), 1 ( $\blacktriangle$ ), 2 ( $\diamond$ ), 4 ( $\bullet$ ) or 6 ( $\circ$ ) hours after the start of the incubation period.

## 6.5. DISCUSSION

Increasing the concentration of CT decreased the concentration of indole and skatole formed *in vitro* from rumen fermentation of fresh forages. This was shown with the use of CT contained within forage material included at increasing ratios with non-CT forage and with the addition of a CT extract at increasing concentration with non-CT forage. Previous *in vitro* work showed that plants with a higher CT concentration formed less indole and skatole in rumen incubations (Schreurs *et al.*, 2006; Chapter 3) however, the work was confounded by the effect of different CT polymer composition that occurs in different plants. This study has shown that when the effect of CT composition is removed, there is a strong effect of CT concentration on the formation of indole and skatole.

Digestion of fresh-forage protein in the rumen is the result of the combined processes of solubilisation and degradation. Protein solubilisation in the rumen is an important determinant of the susceptibility of that protein to degradation (Min *et al.*, 2000). Condensed tannins bind with plant protein to form insoluble CT-protein complexes. By the formation of these complexes, the CT slows the degradation of forage protein in the rumen (McNabb *et al.*, 1996). Experiment 1 showed that when there was more than 50% LP in the fresh forage included in the incubations, the indole concentration was significantly lower than with WC alone. Skatole concentration was significantly lower than with only WC when 25% LP was included in the incubations. The reduction in indole and skatole formation was approximately proportional to the amount of LP included in the incubations as shown by the significant linear negative contrasts, indicating a dilution effect as the proportion of LP was

reduced. This suggests that the CT in LP was protecting the protein in LP from degradation, with no cross over of the CT to protect the protein from WC.

For Experiments 2 and 3, the indole and skatole concentration showed a good fit to a negative linear relationship with the log of the CT concentration included in the incubations. Such a near-exponential trend is typical of substrate binding interactions (Bistrovic *et al.*, 1984; Pope *et al.*, 1998) and suggests the added CT extract from LP and grape seed was able to bind with the protein from WC and protect it from microbial degradation therefore, resulting in a dose-dependent decrease in the formation of indole and skatole. This conclusively demonstrates that CT present within a plant behaves differently to extracted CT when both are added to *in vitro* rumen microbial fermentations.

Preferential binding of CT to protein when added as a forage component explains the differences in the relationship between CT concentration and indole and skatole concentration in Experiment 1 compared to Experiments 2 and 3. In Experiment 1 a linear relationship was observed between the portion of LP added to the incubations and indole and skatole concentration. In Experiment 1 the CT in LP (97 g kg<sup>-1</sup> DM) preferentially bound with the protein from LP (83 g kg<sup>-1</sup> DM), resulting in a low concentration of free CT (14 g kg<sup>-1</sup> DM) available to bind with the protein from WC or rumen microbes (Barry and Manley 1986). The exponential relationship in Experiment 2 and 3 (*Figure 6.3 and 6.4*) indicates that the CT from LP bound with the protein in the incubation (either plant or microbial), with less effect seen at increments of higher concentration due to the binding capacity being approached.

Condensed tannins in the rumen may exert their effects by interacting with plant protein but may also influence proteolytic enzymes of microbial origin Haslam 1996) or inhibit the growth and function of rumen microbes (Sivakumaran *et al.*, 2004b). Feeding LP has been associated with lower rumen ammonia concentration as a consequence of CT slowing the degradation of forage protein and improving the utilisation of rumen nitrogen (Waghorn *et al.*, 1994). Increasing the proportion of LP forage, LP CT extract and grape seed CT extract included in the incubations all decreased the concentration of ammonia formed. The reduced ammonia concentration with the addition of CT to *in vitro* incubations in this present study concur with the reduced rumen ammonia concentration found when feeding CT-containing forages *in vivo* compared to non-CT forages. This indicates that the degradation of forage protein had been slowed by including CT in the incubations.

Burke *et al.* (2002) showed that WC gave a higher rumen concentration of VFA compared to other forages. *In vitro* incubations of WC in this study also gave a higher total VFA concentration. Forages with a higher fibre content tend to result in a higher molar ratio of acetate:propionate being formed in the rumen, which is likely to be the reason for the increasing acetate:propionate ratio as the proportion of WC in the incubations of Experiment 1 decreased. However, when a CT extract from LP or grape seed was included in incubations with WC there was also an increase in the acetate:propionate ratio with increased CT addition. The CT may have had an effect on the fermentation of plant carbohydrates mediated through direct microbial inhibition or inhibition of microbial enzymes or maybe direct association with carbohydrate (Terrill *et al.*, 1992). Branched chain VFA are formed in the rumen from branched chain amino acids (Allison 1978) thus, it appears that the reduced BCVFA with decreased WC:LP

ratio (Experiment 1) and increased CT added (Experiments 2 and 3) is a consequence of the CT limiting the availability of amino acids that can be converted to BCVFA.

Increased indole and skatole production from adding tryptophan to incubations with fresh LP (Experiment 4) indicates that microbes involved in the formation of indole and skatole are not affected by the CT within LP. Adding tryptophan at all time points resulted in an increase in the formation of indole and skatole when incubating LP. Thus, it appears that lack of substrate was inhibiting the formation of indole and skatole when incubating LP. This is further indicated by the increase in the formation of indole and skatole when incubating LP. This is further indicated by the increase in the formation of indole and skatole after the addition of PEG to the incubations. Our interpretation is that the CT in LP was slowing the degradation of plant protein and inhibiting the availability of tryptophan for indole and skatole formation. Adding PEG is likely to have released the plant protein from CT-protein complexes allowing the protein to undergo microbial degradation, increasing the availability of tryptophan for conversion to indole and skatole. The combined addition of tryptophan and PEG resulted in an additive effect on the formation of indole and skatole indicating that the microbes were able to utilise both the tryptophan released from the degradation of plant protein and that added to the incubation, to form indole and skatole.

Tavendale *et al.* (2005) found that the CT from *Dorycnium rectum* inhibited the rumen microbes that formed skatole from indole acetic acid, an intermediate in the pathway to skatole from tryptophan. In Experiment 4 the CT was added to the incubations as a component of the fresh LP while in the experiment of Tavendale *et al.* (2005) the CT from *Dorycnium rectum* was added as an extract to the incubations. This current study has shown that adding CT as an extract to incubations provides a higher concentration

of free CT in the incubation media available to bind and inhibit rumen microbes while providing the CT *in planta* results in preferential binding of the CT to the forage protein, leaving less free CT to inhibit rumen microbes (Barry and Manley 1986).

This study has confirmed the findings of previous studies that indole and skatole formation is minimised when forages are fermented in the presence of a higher CT concentration. The rumen microbes do not appear to be affected when CT is provided as a component of fresh LP, as rumen microbes that were exposed to CT *in planta* for a period of up to six hours and then provided with tryptophan, were still able to convert the tryptophan to indole and skatole. Thus, it appears that the CT in LP preferentially limits the availability of substrate rather than inhibiting the rumen microbes to reduce indole and skatole formation. Further research is required to determine the effect of different CT composition on indole and skatole formation.

In order to minimise pastoral flavours in meat products from grazing ruminants, forage with a higher CT concentration (approximately 80 g kg<sup>-1</sup> DM) will be most effective at slowing plant protein degradation and minimising the tryptophan available to be converted to indole and skatole. In a mixed sward comprising of both CT-containing forages and non-CT forages, the CT-containing plant would need to have a CT concentration of approximately 187 g kg<sup>-1</sup> DM in order to provide 80 g kg<sup>-1</sup> DM of free CT (Barry and Manley 1986). This concentration of CT in a forage plant is rare and would decrease the palatability of the forage resulting in preferential selection of the non-CT forage by the grazing ruminant. Therefore, grazing of pure CT-containing forage swards is preferential to reduce indole and skatole formation as the grazing of mixed swards is unfeasible.

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# **CHAPTER 7**

The effect of supplementation of a white clover or perennial ryegrass diet with grape seed extract on indole and skatole metabolism and the sensory characteristics of lamb

The material presented in this chapter has been submitted as a paper to: Journal of the Science of Food and Agriculture.

# 7.1. ABSTRACT

Condensed tannin in the form of a grape seed extract (GSE) was dosed twice daily to weaned wether lambs fed white clover (WC) or perennial ryegrass (PRG) over a 9-week period to determine if the "pastoral" flavour and odour of meat could be altered. The concentration of the pastoral flavour compounds, indole and skatole, were determined in rumen fluid, blood plasma and inter-muscular fat. The odour and flavour of fat and meat from the slaughtered lambs was assessed by a trained panel. The rumen fluid and blood plasma concentration of indole and skatole was higher in those lambs fed WC compared to PRG (P < 0.05) and the overall meat flavour intensity was greater when feeding WC (P < 0.01). The observed concentration of indole and skatole in the fat between WC and PRG feeding treatments was not statistically different. Power analysis indicated that with the 20 lambs per feeding treatment the variance around the observed means was too great to result in a significant difference in fat concentration of indole and skatole. Increasing the number of lambs per feeding treatment group to 65 would result in a smaller variance and the higher fat skatole concentration observed in lambs fed WC compared to PRG would be significant (P < 0.05). Dosing with GSE gave a small reduction in skatole concentration in the rumen fluid and reduced plasma concentration of indole and skatole (P < 0.001). Odour and flavour scores of the fat and meat samples were not particularly high however, dosing with GSE lowered the overall and sweet odour and the sheepy, camphor, faecal and barnyard flavour (P < 0.05). Although the plasma concentration of indole and skatole suggests that GSE reduced indole and skatole formation, the twice daily dosing of the GSE to the rumen environment was not sufficient to reduce their concentration in the fat. Hence, the small difference in the scores for pastoral odour and flavour attributes associated with GSE treatment may arise from other factors involving protein degradation. From a primary investigation, there

was no difference in the concentration of indole and skatole in fat samples collected from carcasses before and after chilling. Further investigations into meat pastoral flavour are warranted through feeding condensed tannin-containing forages.

# **7.2.** INTRODUCTION

The type of feed consumed by a ruminant will affect the flavour of its meat. Feeding pasture comprised of perennial ryegrass and white clover to sheep was associated with characteristic pastoral flavours in the meat compared to meat from sheep fed concentrate diets (Young *et al.*, 2003). Furthermore, animals on legume diets have a less desirable meat flavour than animals fed grass diets (Cramer *et al.*, 1967, Shorland *et al.*, 1970). Pastoral flavours are particularly undesirable attributes of meat for those consumers accustomed to meat from animals produced on grain- or concentrate-based diets. In such cases, the pastoral flavour is described as "off", "animal-like", "grassy" or "faecal-like" (Young *et al.*, 2003 and references cited therein).

Pastoral flavours in sheep meat have been linked to the presence of indole and skatole in the meat using coupled sensory and chemical analyses in the form of gas chromatography olfactory (Young *et al.*, 2003). Skatole and indole are formed in the rumen from the degradation of tryptophan (Deslande *et al.*, 2001). A higher indole and skatole concentration in the meat of ruminants grazing pasture has been attributed to the high concentration of protein in plant material and the rapid degradation of that protein, resulting in a greater degradation of tryptophan to indole and skatole (Lane and Fraser 1999).

Condensed tannins are polyphenols that have the ability to slow the degradation of protein in the rumen. *In vitro* and *in vivo* studies have shown that CT-containing forages are able to reduce the formation of indole and skatole (Schreurs *et al.*, 2006a,b,c; Chapter 3, 4 and 5). Furthermore, rumen concentration of indole and skatole were higher in sheep that were fed white clover compared to perennial ryegrass and *Lotus* 

*corniculatus* (Schreurs *et al.*, 2006b; Chapter 4). This suggests that non-CT legumes in the pasture sward may be primarily responsible for pastoral flavours associated with indole and skatole. The objective of this study was to determine the differences in the rumen fluid, blood plasma and fat concentration of indole and skatole and the sensory attributes of meat from lambs fed white clover in comparison to perennial ryegrass, and the effects of supplementing these diets with condensed tannin in the form of a grape seed extract. It had not been established if chilling of the carcass results in a substantial loss of indole and skatole from fat depots. A second objective was to compare the concentration of indole and skatole in the fat from pre- and post-chilled carcasses to establish the best procedure for the sampling of fat for experimental purposes.

## 7.3. MATERIALS AND METHODS

## 7.3.1. Experimental design

**7.3.1.1.** Experiment 1: Indole and skatole in the fat of pre- and post-chilled carcasses An initial experiment was undertaken to determine if the chilling of carcasses had an effect on indole and skatole concentration in the fat. Fat was collected from the brisket, tail-stub and inter-muscular depots of four sheep carcasses randomly selected from the slaughter chain (Ashhurst Stock Processing, Ashhurst, New Zealand). The left side of the carcass was used to obtain fat prior to chilling and post-chilling fat samples were obtained from the right side of the carcass.

# 7.3.1.2. Experiment 2: Sensory aspects of meat from lambs fed white clover or perennial ryegrass and supplemented with a grape seed extract

The main experiment (Experiment 2) was conducted with lambs that were housed indoors at Grasslands Research Centre, AgResearch Limited, Palmerston North from the  $3^{rd}$  of March to  $3^{rd}$  May 2004 (9 weeks). Late season wether lambs were obtained from Tuapaka Farm, Massey University, Manawatu. Lambs were allocated to WC (n=20) or PRG (n=22) feeding groups. Half the lambs fed WC (n=10) were dosed twice daily with a grape seed extract (GSE) while remainder (n=10) were not supplemented with GSE. Similarly, twelve lambs fed PRG were dosed with the GSE while the remaining lambs fed PRG (n=10) were not dosed. The four treatment groups were balanced for initial rumen skatole concentration and initial live weight. The concentration of indole, skatole and other metabolites was determined in the rumen fluid and plasma of the lambs from samples obtained at weeks 0, 4 and 9 of the experiment. After 9 weeks on the treatment diets the lambs were slaughtered and fat and meat samples obtained for chemical and sensory analysis.
#### 7.3.2. Animal housing and management

Prior to being allocated to the treatment diets, the lambs were grazed outdoors on a perennial ryegrass dominant pasture, supplemented with hay. The lambs were penned indoors in groups of 5-6 (treatment groups halved) which allowed for easier handling of the forages and reduced the competition amongst animals at the feed bins during feeding time. Animals within treatments were randomly allocated to pen groups.

All lambs were treated for internal parasites (Leviben<sup>®</sup>, Young's Animal Health Limited, New Zealand) prior to the start of the experimental period and then every 4 weeks during the experimental period. Every animal received a zinc bolus (Time Capsule<sup>™</sup>, AgResearch Limited, New Zealand) prior to the commencement of the trial for the prevention of facial eczema.

#### 7.3.3. Feeding and dosing the grape seed extract

Forages were grown as pure swards at Aorangi Research Farm, Manawatu, New Zealand. Vegetative forage growth was harvested daily at 09:30h using a 4 disc bar mower, modified with a trailing collection chute, behind a tractor. Forages were transported to the Grasslands Research Centre in large hessian sacks. Upon arrival, the forage was immediately weighed into portions for each pen group. The portion of forage weighed for each group was based on offering 1.5 kg dry matter (DM) per lamb per day in the first 5 weeks of the trial and 2 kg DM per lamb per day in the last 4 weeks of the trial. The forage was then stored at 4°C until feeding. Refusals were collected and weighed for each group prior to offering new forage at 08:00h and 16:00h each day and in this manner, the lambs had access to forage over the full 24-hour period.

At each feeding time (08:00h and 16:00h) representative samples of the forage offered and refused from each group were pooled into treatment groups and triplicate samples of 200 g were taken to determine DM content by placing the samples in a forced-air oven for 24 hours. Samples of the feed offered were pooled over weekly intervals then freeze-dried and ground to pass through a 1 mm screen for composition analysis by near infra-red reflectance spectrometry (NIRS; Feedtech, AgResearch Limited, Palmerston North, New Zealand).

Once the lambs were offered their new feed, they were given 30 minutes to eat before commencing with the dosing of the grape (*Vitis vinifera*) seed extract (GSE). The GSE powder (Grape Seed P.E. 120:1, C.Z. Medipro Botanical Laboratories, China; 99.1% proanthocyanidins) was made into a solution (0.33 g mL<sup>-1</sup>) with fresh tap water. Each lamb in the GSE treatment groups was dosed with 50 mL of the GSE solution at each meal (33 g of GSE per day), while the animals in the non-GSE treatment groups received 50 mL of tap water as a placebo dose.

#### 7.3.4. Animal measurements

Live weight was measured every Monday, Wednesday and Friday of the trial using electronic scales (Trutest, Auckland, New Zealand). Initial carcass weight of the 42 experimental lambs was estimated through the slaughter of 9 non-experimental lambs at the start of the experiment to determine the ratio of carcass weight to live weight and that was used to calculate the initial carcass weight from the initial live weight of the experimental lambs. At the end of the 9-week period, the experimental lambs were slaughtered and carcass weight and carcass fatness (GR, measured as the subcutaneous fat depth over the 12<sup>th</sup> rib at a point 11 cm from the midline; Kirton 1989) was recorded for each lamb.

#### 7.3.5. Sampling

Rumen fluid (20-50 mL) was obtained by stomach lavage approximately 3-4 hours after offering feed in the morning. A 4 mL sub-sample of the rumen fluid was snap frozen in liquid nitrogen and stored at -20°C to await analysis for indoles. Another 4 mL of the rumen fluid was centrifuged (16000 g for 15 minutes) and the supernatant stored at - 20°C for volatile fatty acid (VFA) analysis. A further 1 mL of the rumen fluid was added to 15  $\mu$ L concentrated hydrochloric acid and then centrifuged (16000 g for 15 minutes). The supernatant was stored at -20°C for ammonia analysis.

For each lamb, blood was obtained by jugular venipunture into two evacuated 10 mL Vacutainers<sup>®</sup>, one with ethylenediaminetetraacetic acid (EDTA) anticoagulant for indole and skatole analysis, and the other with lithium heparin anticoagulant for urea and ammonia analysis. The blood was obtained approximately 3-4 hours after feed was offered in the morning. Blood from both tubes was then centrifuged (3270 g for 15 minutes) and 4 mL of the plasma from the EDTA Vacutainer<sup>®</sup> snap-frozen in liquid nitrogen and stored at -85°C for the analysis of indoles.

For the analysis of ammonia concentration in the plasma, 1.5 mL of plasma from the Vacutainer<sup>®</sup> containing lithium heparin anticoagulant was added to 0.5 mL of 30% trichloroacetic acid (w/w) and then centrifuged (3270 g for 15 minutes). The supernatant was then filtered (45  $\mu$ m) into a vial and stored at -85°C for ammonia analysis by flow injection analysis (FIA). The remaining plasma from the lithium heparin Vacutainer<sup>®</sup> was transferred to a vial and stored at -85°C for urea analysis.

Experimental lambs were slaughtered at a commercial abattoir (Ruakura Abattoir, Hamilton, New Zealand) after 9 weeks on the experimental diets. Prior to slaughter the lambs were fasted for a 24-hour period. Lambs were slaughtered by exsanguination after captive bolt stunning. The carcasses received no electrical stimulation. After the carcasses were chilled for 24 hours to reduce the carcass temperature to 7°C, fat was collected from inter-muscular depots in the hind limbs and the GR measurement was made. Fat was stored at -85°C to await chemical analysis for indole and skatole and also for odour assessment by the sensory panel. Following collection of the fat the left hind limb was removed, vacuum packed and stored at -85°C. Muscle and fat from the hind limb were minced to provide samples for the assessment of flavour.

#### 7.3.6. Laboratory methods

#### 7.3.6.1. Rumen fluid analysis for indoles, volatile fatty acids and ammonia

Skatole and indole in the rumen fluid samples was measured using solid-phase extraction (Mattivi *et al.*, 1999) followed by high performance liquid chromatography (HPLC) as described by Schreurs *et al.* (2006c; Chapter 5). An internal standard (2-methylindole) was added to the sample extract to allow indole and skatole concentration to be calculated from the peak areas generated by HPLC.

Volatile fatty acids in the rumen fluid were analysed by gas-liquid chromatography following the method of Attwood *et al.*, (1998). Ammonia concentration in the rumen fluid was analysed using a commercial assay (Sigma Diagnostics Ltd, St Louis, Missouri, USA) which utilised glutamate dehydrogenase for the reductive amination of 2-oxoglutarate to give a decrease in absorbance at 340 nm due to the oxidation of NADPH proportional to the ammonia concentration (Neeley and Phillipson 1988).

#### 7.3.6.2. Plasma analysis for indoles, ammonia and urea

Blood plasma was analysed for indole and skatole using the ether extraction method of Claus *et al.* (1993). An internal standard (2-methylindole) was added to the samples and indole and skatole concentration determined using the same HPLC system that was used for rumen fluid (Schreurs *et al.*, 2006c; Chapter 5)

Blood plasma ammonia concentration was determined by FIA (Tecator, FIAstar Flow Injection 5010 Analyser, 590nm). Urea concentration in the plasma was determined using a commercial assay (Roche Diagnostics Ltd, Basel, Switzerland) which utilises urease and glutamate dehydrogenase and detects the production of NADH at 340nm. The assay converts urea to ammonia therefore, a second assay (Sigma diagnostics Ltd, St Louis, Missouri, USA) was carried out to measure the endogenous ammonia so that the true urea concentration could be calculated. The urea and ammonia assays were performed on a Cobas Fara II Analyser (Hoffman la Roche, Basel, Switzerland).

#### 7.3.6.3. Fat analysis for indoles

Fat obtained from pre-chilled and post chilled carcasses and the inter-muscular fat that had been prepared for odour assessment (see below) was analysed to determine the concentration of indole and skatole following the method of Schreurs *et al.* (2006c; Chapter 5) utilising steam distillation extraction and gas chromatography-mass spectrometry (GC-MS). An internal standard (d<sub>3</sub>-skatole) was added to the sample to allow indole and skatole concentration to be calculated from the peak areas generated by GC-MS.

#### 7.3.7. Odour assessment of fat by sensory panel

Inter-muscular fat was assessed for odour by a trained sensory panel. The fat was diced and melted in a glass beaker in a 600 W microwave for 60 seconds. A warmed stainless steel piston was used to manually press the liquid fat to separate out collagenous material and then the liquid given a light centrifuge (to remove particulate material. The liquid fat was then transferred to a vial, vacuum packed and stored at -35°C until required for sensory analysis. Ten panellists assessed each of the 42 samples. Six randomly picked samples were presented at each session, thus requiring a total of 7 sessions to assess all the fat samples.

Fat samples were prepared for sensory evaluation by melting them at 100°C and placing 1.5 g of the melted fat into a wide-neck 25 mL round-bottom flask with a stopper. Samples were kept warm in a water bath (80°C) while awaiting odour assessment. The panellists appeared at 10-minute intervals and were presented the session samples in a randomised order. The panellists removed the stopper and smelled the headspace. Panellists assessed the samples for sheepy, barnyard, musty, camphor, sweet and faecal odours on a 0-9 intensity scale.

The members of the analytical odour panels were highly trained and very sensitive to the odours of interest. The panellists were selected through a screening process and had been used for the assessment of sheep meat flavours and odours for 8 years. Prior to odour assessment of the samples, the panellists met as a group and were presented with fat samples spiked with indole and skatole. From these group sessions, a consensus was obtained on the descriptors to be used by the sensory panel.

#### 7.3.8. Flavour assessment of minced meat by sensory panel

After thawing, lean meat and fat from the muscle groups caudal to the femur were separated and then minced together twice through a 3 mm plate to yield minces containing approximately 15% fat. Samples of the mince were vacuum packed and refrozen (-35°C) ready for thawing, cooking and presentation to a sensory panel within 4 days of preparation.

Thawed minces were cooked in stainless steel pots over boiling water to a 75°C endpoint. Samples were served hot to each of the ten panellists in seven sessions with the meat of six animals presented per session. Samples were presented to each panellist in a randomised order. Panellists assessed the samples first by smelling, then by eating, and scored them on a 0-9 scale for the overall odour intensity and the intensities of the seven flavour attributes: sweet, sheepy, earthy, faecal, camphor, musty and barnyard.

Prior to running the sensory flavour panel a group session was held where the panellists were presented with samples of mince, pooled from animals in each of the treatments groups. This allowed the panellists to concur on the flavour descriptors to be used and provided an opportunity for them to familiarise themselves with the flavours that were to be detected.

#### 7.3.9. Statistical analysis

Concentrations of indole and skatole in the rumen fluid, plasma and fat were determined from the peak area ratios and response factors relative to the internal standard. The internal standard for rumen fluid and blood plasma analyses was 2-methylindole and for fat analyses, d<sub>3</sub>-skatole. The pre-chill and post-chill concentration of indole and skatole in the fat was statistically analysed using the two sample paired t-test for means in SAS Analyst (SAS 2003).

PROC MIXED in SAS (SAS 2003) was used to assess the fixed effects of forage (WC or PRG), GSE supplementation (+GSE or --GSE) and the interaction of forage and GSE supplementation. Pen grouping was used as a random block effect when statistically analysing DMI, CPI, live weight, carcass weight, GR and the rumen and plasma metabolites. Initial live weight, initial carcass weight and final carcass weight were used as a covariate when statistically analysing final live weight, final carcass weight and GR respectively. Residual errors were modelled using a compound symmetry structure. Statistical analyses were undertaken with log transformations on the data for rumen and plasma metabolites and fat concentration of indole and skatole (log+1), and the least square means obtained by back-transformation. Log transformations adjusted the data to a normal distribution for statistical analysis. Differences in the variance of the indole and skatole concentration in the fat were evaluated using the Levine's test (SAS 2003).

Odour and flavour scores from the trained sensory panel were examined using PROC GENMOD of SAS (SAS 2003), which accounts for the many zero values in this data by assuming a Poisson distribution. The model included the fixed effects of forage (WC or PRG), and GSE supplementation (+GSE or –GSE) and the interaction of forage and GSE supplementation. The effect of panellist was included in the model as a random block effect. The odour and flavour score data were log transformed in order to provide a normal distribution for statistical analysis, and least square means were obtained by back-transformation.

Power analysis was performed (PROC GLMPOWER, SAS 2003) using the mean and variance measures of the fat skatole concentration data. A power of 80% and a significance level of P<0.05 was assumed.

## 7.4. RESULTS

# 7.4.1. Experiment 1: Indole and skatole in the fat of the pre- and post-chilled carcass

No difference was observed in the indole and skatole concentration in the fat from pre-

chilled sheep carcasses compared to the fat from chilled carcasses (Table 7.1). The

intermuscular fat tended to show some decline in indole and skatole concentration from

sheep carcasses before and after chilling compared to the tail stub and brisket fat (Table

7.1).

Table 7.1. Experiment 1. Comparison of indole and skatole concentration in the fat of sheep (n=4) obtained from carcasses prior to chilling (pre-chill) and after chilling for 24 hours (post-chill). The values presented for each treatment group are the mean  $\pm$  SEM.

	Pre-chill	Post-chill	Р
Indole (ng g <sup>-1</sup> )			
- Brisket	15.6 ± 3.3	14.5 ± 2.8	0.809
- Tail stub	17.2 ± 5.3	16.6 ± 5.2	0.934
- Inter-muscular	8.1 ± 1.7	5.7 ± 0.8	0.275
Skatole (ng g <sup>-1</sup> )			
- Brisket	44.3 ± 13.5	42.1 ± 13.2	0.909
- Tail stub	30.1 ± 11.8	27.6 ± 12.0	0.886
- Inter-muscular	21.9 ± 8.9	15.2 ± 5.7	0.556

# 7.4.2. Experiment 2: Sensory aspects of meat from lambs fed white clover or perennial ryegrass and supplemented with a grape seed extract

#### 7.4.2.1. Composition of diets and intakes

The metabolisable energy content, crude protein concentration and the organic matter digestibility of the WC were higher than that of the PRG. The neutral detergent fibre

and soluble sugar and starch content were lower in the WC compared to the PRG during the experiment (*Table 7.2*).

 Table 7.2. Experiment 2. Composition of white clover and perennial ryegrass offered to lambs. Values presented are the mean and SEM for the 9 week period of the experiment.

	White clover	Perennial ryegrass
Metabolisable energy (MJME kg <sup>-1</sup> DM)	12.0 ± 0.05	10.4 ± 0.14
Neutral detergent fibre (g kg <sup>-1</sup> DM)	330 ± 5.2	536 ± 13.1
Organic matter digestibility	0.85 ± 0.004	0.73 ± 0.01
Crude protein (g kg <sup>-1</sup> DM)	297 ± 4.0	184 ± 7.8
Soluble sugar and starch (g kg $^{-1}$ DM)	100 ± 7.2	126 ± 7.5

In week 1 of the experiment there was a difference (P < 0.001) in the dry matter intake (DMI) between those animals dosed with the grape seed extract (GSE) and those that received the placebo when fed WC. This difference was not observed during the remaining 8 weeks of the experiment (*Figure 7.1.A*). Those animals fed WC had a higher (P < 0.05) DMI than those fed PRG during weeks 1-8 of the experiment. In week 9, the DMI was similar for all treatment groups. Differences in DMI between WC and PRG were particularly large during weeks 5-8 of the experiment (*Figure 7.1.A*). During this time, the PRG offered to the lambs was from a more mature sward and the feed offered was observed to contain a greater proportion of dead material.

Crude protein intake (CPI) was much greater when feeding WC compared to feeding PRG during the whole experiment (P<0.001; *Figure 7.1.B*). No difference was observed for the CPI of animals dosed with GSE compared to those that received the placebo dose of water, except in week 1 when CPI was lower for those animals fed WC and dosed with GSE compared to those that were fed WC with the placebo (P<0.05; *Figure 7.1.B*).



Figure 7.1. Experiment 2. (A) Dry matter intake (DMI) and (B) crude protein intake (CPI) for lambs fed white clover with ( $\blacksquare$ ) and without ( $\Box$ ) a supplement of grape seed extract or perennial ryegrass with ( $\bullet$ ) and without ( $\circ$ ) a grape seed extract supplement. Vertical bars represent the pooled standard error of the mean.

#### 7.4.2.2. Ammonia and VFA in rumen fluid prior to slaughter

Feeding WC resulted in a higher concentration of ammonia and total VFA in the rumen fluid sampled prior to slaughter compared to feeding PRG (P<0.001). The molar proportion of acetate was lower (P<0.05) and consequently the proportion of propionate and butyrate higher (P<0.001) when feeding WC compared to PRG (*Table 7.3*). The rumen fluid concentration of branched-chain VFA was higher when feeding WC compared to PRG (P<0.001). Supplementing lambs with GSE resulted in a lower concentration of total VFA and molar proportion of propionate (P<0.05; *Table 7.3*)

#### 7.4.2.3. Rumen indole and skatole

Indole and skatole concentration in rumen fluid samples was similar for all treatment groups at the start of Experiment 2. Feeding WC resulted in a significant increase in the rumen concentration of indole and skatole at weeks 4 and 9 compared to week 0 (P<0.001). Consequently, the lambs fed WC had a higher indole and skatole concentration in the rumen fluid at weeks 4 and 9 compared to those lambs fed PRG (P<0.001; *Figure 7.2*). Feeding PRG resulted in a significant increase in rumen indole concentration at week 9 (P<0.001) but not at week 4 compared to week 0. Compared to the concentration at week 0, the skatole concentration in the rumen skatole skatole concentration in the rumen fluid at week 4 compared to week 0. Compared to the concentration at week 0, the skatole concentration in the rumen was lower at weeks 4 and 9 when feeding PRG (P<0.01; *Figure 7.2*). Supplementing lambs with GSE resulted in a lower concentration of skatole in the rumen fluid at week 9, prior to slaughter (P<0.05; *Table 7.3*).

CHAPTER 7: Grape seed CT when feeding forages

Table 7.3. Experiment 2. Mean concentration of metabolites and the proportion of volatile fatty acids from the rumen fluid of lambs prior to slaughter. Lambs were fed white clover or perennial ryegrass with (+) or without (-) supplementation with a grape seed extract (GSE). The LS Means were obtained from back transformation of log+1 transformed data.

271

	White	White clover		Perennial ryegrass		cance of	95% Confidence Interval		
	-GSE (n=10)	+GSE (n=10)	-GSE (n=10)	+GSE (n=12)	Forage	GSE	Forage x GSE	Minus	Plus
Indole (µg g <sup>-1</sup> )	3.7	3.6	1.1	1.1		NS	NS	0.18	0.19
Skatole (µg g <sup>-1</sup> )	8.5	6.8	1.7	1.6		•	NS	0.44	0.48
Ammonia (mmol L <sup>-1</sup> )	34.8	32.5	21.1	17.2	•••	NS	NS	1.78	1.91
Total VFA (mmol L <sup>-1</sup> )	92.6	88.6	61.5	54.1			NS	4.64	4.95
Acetate (molar %)	56.4	57.9	63.7	63.3		NS	NS	0.66	0.67
Propionate (molar%)	24.2	22.7	19.2	18.5			NS	0.43	0.44
Butyrate (molar %)	12.7	12.1	11.7	12.3	•	NS	NS	0.38	0.40
Total Branched Chain VFA (mmol L-1)	4.5	4.7	2.5	2.4	•••	NS	NS	0.16	0.17

NS (not significant), \* (P<0.05), \*\* (P<0.01), \*\*\* (P<0.001)



Figure 7.2. Experiment 2. Indole (A) and skatole (B) concentration in the rumen fluid over a 9 week treatment period for lambs fed white clover with ( $\blacksquare$ ) and without ( $\Box$ ) a supplement of grape seed extract or perennial ryegrass with ( $\bullet$ ) and without ( $\circ$ ) a grape seed extract supplement. Vertical bars represent the 95% confidence interval.

#### 7.4.2.4. Plasma indole and skatole

Blood plasma indole and skatole concentration was similar for all groups at the start of the experiment. At week 4 feeding WC resulted in a higher concentration of indole and skatole compared to feeding PRG (P<0.001) and supplementing the lambs with GSE also lowered indole and skatole concentration in the blood plasma (P<0.001; *Figure 7.3*).

Indole and skatole concentration in the blood plasma samples from those lambs fed WC declined from week 4 to week 9 while in the animals fed PRG without GSE, the indole concentration increased. As a result of concentration changes from week 4 to 9 there was less difference between treatments for the concentration of indole and skatole in the plasma at week 9 (*Figure 7.3*). However prior to slaughter (week 9), plasma skatole concentration remained higher in those lambs that were fed WC compared to PRG (P<0.01) and it was still evident that supplementing with the GSE resulted in a lower indole and skatole concentration in the plasma (P<0.001; *Table 7.4*).

#### 7.4.2.5. Plasma ammonia and urea

Supplementing with GSE decreased the concentration of ammonia in the plasma (P<0.01; *Table 7.4*). Plasma urea concentration was higher when feeding WC compared to PRG (P<0.001; *Table 7.4*). There was a significant interaction of the forage and GSE (P<0.05) on the plasma urea concentration indicating that GSE supplementation was only effective at reducing plasma urea when feeding WC (*Table 7.4*).



Figure 7.3. Experiment 2. Plasma indole (A) and skatole (B) concentration over a 9 week treatment period for lambs fed white clover with ( $\blacksquare$ ) and without ( $\Box$ ) a supplement of grape seed extract or perennial ryegrass with ( $\bullet$ ) and without ( $\circ$ ) a grape seed extract supplement. Vertical bars represent the 95% confidence interval.

CHAPTER 7: Grape seed CT when feeding forages

Table 7.4. Experiment 2. Mean concentration of metabolites prior to slaughter in the blood plasma of lambs fed white clover or perennial ryegrass with (+) or without (-) supplementation with a grape seed extract (GSE). The LS Means were obtained from back transformation of log+1 transformed data.

	White	clover	over Perennial		Signifi	Significance of effects <sup>†</sup>			95% Confidence interval	
	-GSE (n=10)	+GSE (n=10)	-GSE (n=10)	+GSE (n=12)	Forage	GSE	Forage x GSE	Minus	Plus	
Indole (ng mL <sup>-1</sup> )	9.0	5.2	16.1	4.1	NS	***	NS	1.76	2.21	
Skatole (ng mL <sup>-1</sup> )	75.8	44.0	48.5	25.4			NS	10.2	13.0	
Ammonia (µg mL <sup>-1</sup> )	0.88	0.76	1.06	0.75	NS	••	NS	0.07	0.07	
Urea (µmol mL <sup>-1</sup> )	11.6	10.2	7.1	7.2		NS	·	0.34	0.35	

<sup>T</sup>NS (not significant), •(P<0.05), \*\* (P<0.01), \*\*\* (P<0.001)

275

#### 7.4.2.6. Live weight, live weight gain, carcass weight and fatness values

There were no differences in the mean initial live weight for the four treatment groups. The lambs fed WC had a higher final live weight and carcass weight than the lambs fed PRG (P<0.05; *Table 7.5*). The live weight change and carcass weight gain were higher when feeding WC compared to PRG (P<0.05). Supplementing GSE to lambs fed WC resulted in a lower final live weight and carcass weight compared to feeding WC alone (*Table 7.5*). There was a tendency for GSE supplementation to reduce live weight gain (P=0.087) and carcass weight gain (P=0.13) when feeding WC (*Table 7.5*). The GR measurements indicate that those lambs that were fed WC without GSE had a greater carcass fatness compared to lambs in the other treatment groups (*Table 7.5*).

Table 7.5. Experiment 2. Effect of feeding white clover or perennial ryegrass fed to lambs with (+) or without (-) supplementation with a grape seed extract (GSE) on live weight, carcass weight and carcass fatness. The values presented for each treatment group are the mean  $\pm$  SEM.

	White	clover	Perennial	ryegrass
	-GSE	+GSE	-GSE	+GSE
n	10	10	10	12
Initial live weight (kg)	27.6 ± 1.1	30.1 ± 2.1	28.7 ± 1.4	31.6 ± 1.8
Final live weight (kg) <sup>1</sup>	45.1 ± 1.0 <sup>a</sup>	41.3 ± 1.4 <sup>b</sup>	$34.8 \pm 0.9^{\circ}$	$34.5 \pm 0.7^{\circ}$
Live weight change (g day <sup>-1</sup> )	241 ± 15°	$190 \pm 24^{a}$	$80 \pm 9^{b}$	$90 \pm 9^{b}$
Carcass weight (kg) <sup>2</sup>	$20.5 \pm 0.6^{a}$	18.4 ± 0.7 <sup>b</sup>	$13.1 \pm 0.6^{c}$	$13.0 \pm 0.5^{\circ}$
Carcass weight gain (g day <sup>-1</sup> )	127 ± 9 <sup>a</sup>	103 ± 13 <sup>a</sup>	$13 \pm 6^{b}$	23 ± 8 <sup>b</sup>
Carcass fatness (GR; mm) <sup>3</sup>	10.9 ± 1.2 <sup>ª</sup>	8.1 ± 0.6 <sup>b</sup>	$7.8 \pm 0.5^{b}$	$8.0 \pm 0.4^{b}$

a,b,c Means in the same row with different superscripts are significantly different (P<0.05)

<sup>1</sup> data analysed with initial live weight as a covariate

<sup>2</sup> data analysed with initial carcass weight as a covariate

<sup>3</sup> data analysed with carcass weight as a covariate

#### 7.4.2.7. Indole and skatole concentration in the fat

The concentration of indole and skatole was higher in the inter-muscular fat from lambs fed WC compared to those fed PRG but the difference was not statistically significant. Indole concentration in the intermuscular fat was lower when feeding PRG supplemented with the GSE compared to the other treatments (P<0.05; *Figure 7.4*). The variance of indole and skatole concentration in the fat was not observed to be different between the treatments.



Figure 7.4. Experiment 2. Concentration of indole ( $\blacksquare$ ) and skatole ( $\Box$ ) in the intermuscular fat of the hind legs of lambs that were fed fresh white clover (WC) or perennial ryegrass (PRG) with (+) or without (-) supplementation with a grape seed extract (GSE). Error bars are the 95% confidence interval.

#### 7.7.2.8. Odour of fat and flavour of meat

The trained sensory panel found that the overall odour of the inter-muscular fat from lambs supplemented with GSE was less intense than that of the fat from lambs that did not receive GSE (P<0.05; *Table 7.6*). Supplementation with GSE decreased the intensity of sweet odours (P<0.05; *Table 7.6*). No differences were found for the other odour attributes.

The flavour of minced meat samples was also assessed by the sensory panel and it was found that the overall flavour was affected by the forage fed, with WC lambs having a higher overall flavour intensity (P<0.01; *Table 7.7*). The GSE supplementation lowered overall flavour intensity for lambs fed WC. Sheepy, camphor, faecal and barnyard flavours were less intense from meat samples obtained from lambs supplemented with GSE (P<0.01; *Table 7.7*).

CHAPTER 7: Grape seed CT when feeding forages

	White	clover	Perennia	lryegrass	Signif	icance of	effects <sup>†</sup>	95% Confide	nce interva
	-GSE (n=10)	+GSE (n=10)	-GSE (n=10)	+GSE (n=12)	Forage	GSE	Forage x GSE	Minus	Plus
Overall	4.1	3.6	3.9	3.8	NS	•		0.36	0.40
Sweet	2.5	1.9	2.1	2.1	NS		NS	0.34	0.41
Sheepy	2.4	2.1	2.3	2.0	NS	NS	NS	0.33	0.39
Earthy	1.1	0.8	1.2	1.1	NS	NS	NS	0.23	0.30
Camphor	0.8	0.7	0.7	0.6	NS	NS	NS	0.19	0.26
Faecal	0.6	0.5	0.7	0.5	NS	NS	NS	0.19	0.28
Musty	0.7	0.7	0.8	0.7	NS	NS	NS	0.22	0.31
Bamyard	1.2	1.2	1.3	1.1	NS	NS	NS	0.29	0.39

Table 7.6. Experiment 2. Mean odour scores of inter-muscular fat from lambs fed white clover or perennial ryegrass with (+) or without (-) supplementation with a grape seed extract (GSE). The 95% confidence interval was calculated from log transformed pooled SEM.

<sup>†</sup> NS (not significant), \* (P<0.05), \*\* (P<0.01), \*\*\* (P<0.001)

279

CHAPTER 7: Grape seed CT when feeding forages

Table 7.7: Experiment 2. Mean flavour scores of mince composed of lean and fat (15%) from the hind limb of lambs fed white clover or perennial ryegrass with (+) or without (-) supplementation with a grape seed extract (GSE). The 95% confidence interval was calculated from log transformed pooled SEM.

280

	White	White clover Perennial ryegrass			Signif	icance of e	effects <sup>†</sup>	95% Confide	ence interval
	-GSE (n=10)	+GSE (n=10)	-GSE (n=10)	+GSE (n=12)	Forage	GSE	Forage x GSE	Minus	Plus
Overall	5.1	4.9	4.6	4.7		NS	•	0.32	0.34
Sweet	3.0	3.0	2.9	3.2	NS	NS	NS	0.36	0.41
Sheepy	4.0	3.7	3.4	3.3	NS		NS	0.36	0.40
Earthy	1.2	1.2	1.3	1.1	NS	NS	NS	0.30	0.40
Camphor	0.6	0.5	0.6	0.4	NS	•••	NS	0.16	0.23
Faecal	0.3	0.3	0.7	0.3	NS	•••	NS	0.13	0.20
Musty	0.7	0.9	1.0	1.0	NS	NS	NS	0.24	0.34
Barnyard	2.4	1.9	2.2	1.8	NS		NS	0.31	0.37

<sup>†</sup>NS (not significant), \* (P<0.05), \*\* (P<0.01), \*\*\* (P<0.001)

### 7.5. DISCUSSION

This study has shown that although feeding WC gave a higher concentration of indole and skatole in the rumen and plasma compared to lambs fed PRG, the concentration of indole and skatole in the fat between WC and PRG feeding treatments was not statistically different. However, the overall meat flavour intensity was higher for lambs fed WC than PRG. Rumen fluid skatole and blood plasma indole and skatole concentration were lowered by GSE supplementation, suggesting that there was some effect of the GSE on reducing protein degradation in the rumen and consequently reducing the formation of indole and skatole.

When taking fat samples from the carcass of an animal there is the option of obtaining fat prior to chilling or after chilling. No substantial loss of indole or skatole was observed in the fat from pre-chilled compared to post-chilled carcasses indicating that for experimental purposes, there is no preference for obtaining fat samples before or after the carcass has been chilled.

Feeding WC resulted in a higher DMI than feeding PRG, particularly in weeks 5-8 of the trial. The higher concentration of crude protein in WC resulted in the CPI being much greater when feeding WC compared to PRG throughout the experiment. The higher digestibility and lower neutral detergent fibre content of the WC compared to the PRG would have resulted in a more rapid degradation of the plant material in the rumen and a faster outflow of digesta from the rumen when feeding WC (Ulyatt 1981). This was likely to be why higher intakes were observed when feeding WC compared to PRG.

The higher indole and skatole concentration in the rumen when feeding WC is a predictable consequence of the higher CPI. However, the CPI was 1.5 times greater while the skatole concentration in the rumen was 5 times greater when feeding WC compared to PRG at week 9. Therefore, the higher indole and skatole concentration in the rumen of lambs fed WC cannot be explained by differences in CPI alone. The higher ammonia concentration in the rumen of lambs fed WC cannot be lambs fed WC compared to those fed PRG indicates that the protein in WC is likely to have been degraded more rapidly in the rumen compared to the protein in PRG. Thus, a greater proportion of tryptophan would have been available for indole and skatole formation, relative to CPI, when feeding WC compared to PRG.

Branched chain VFA are formed from the microbial fermentation of amino acids in the rumen (Allison 1978) therefore, the higher total branched chain VFA concentration when feeding WC further indicates that feeding WC increased the availability of free amino acids in the rumen. Volatile fatty acids formed in the rumen are used as an energy source for metabolic processes, such as growth, in ruminants. The deposition of fat is one mechanism by which an animal stores excess energy. Feeding WC resulted in a higher rumen VFA concentration and a higher ratio of propionate:acetate compared to PRG. This shows that the WC had a higher degradability and a higher nutritive value compared to PRG. Consequently, the live weight and carcass weight were higher and there was greater carcass fat deposited by the lambs that were fed WC. Supplementing the lambs fed WC with GSE reduced the formation of VFA, in particular the molar proportion of propionate, and this would have contributed to the lower live weight, carcass weight and carcass fat observed in these lambs. These findings on rumen

metabolites are in keeping with previous studies of rumen-fistulated sheep (Schreurs *et al.*, 2006b; Chapter 4).

Prior to slaughter the GSE supplementation had only slightly but significantly, reduced the skatole concentration in rumen fluid samples. These effects were much smaller than the differentials in rumen skatole observed in rumen-fistulated sheep fed WC or the CT-containing forage *Lotus* corniculatus (Schreurs *et al.*, 2006b; Chapter 4). The effect of GSE supplementation on indole and skatole formation was more evident in the blood plasma samples, with a lower indole and skatole concentration measured at week 4 and prior to slaughter in the lambs dosed with GSE. Furthermore, the lower ammonia and urea concentration in the plasma when dosing with GSE and feeding WC provided further evidence of reduced protein degradation in the rumen limiting the production and absorption of ammonia. Similar effects on plasma urea were observed by Speijer *et al.* (2004) when feeding the CT-containing forage *Lotus corniculatus*.

Considering the differences between the rumen fluid, blood plasma and inter-muscular fat, the pulsatile nature of GSE dosing and continuous availability of forage to the lambs, suggests that the GSE was only having a short-term effect on reducing indole and skatole formation in the rumen. At the time of sampling, the effects of GSE were small or non-significant in the rumen fluid although evident in the blood plasma. Previous studies have shown that indole and skatole concentration in the rumen of sheep peaks 2-3 hours after the commencement of feeding, while plasma concentration of indole and skatole peaks 3-4 hours after the start of feeding (Schreurs *et al.*, 2006b; Chapter 4). The sampling of rumen and plasma occurred at a mid point between the two dosing times (approximately 3-4 hours after offering feed and dosing with GSE). This

sampling time appeared to be appropriate for observing the effects of GSE in the plasma but may have needed to be carlier to observe the effects of the GSE in the rumen.

*In vitro* research has indicated that it is preferential to supply CT within the forage to optimise substrate protein binding and reduce indole and skatole formation in the rumen but exogenous CT will still bind to the forage protein of WC and PRG (Schreurs *et al.*, 2006d; Chapter 6). However, dosing the GSE may have resulted in the CT binding with rumen microbes and extra-cellular microbial enzymes, as the majority of plant protein may still have remained within plant cells (Barry and Manley 1986). Thus, the small reduction in rumen and plasma indole and skatole observed with the supplementation of GSE in this study may be a consequence of microbial inhibition (Tavendale *et al.*, 2005) rather than plant protein binding and reduction in tryptophan supply.

The plasma data at week 4 and to a lesser extent at week 9 suggests that feeding WC results in a higher circulating concentration of indole and skatole compared to PRG. This reasonably, could have been expected to increase the corresponding concentration in the fat (Schreurs *et al.*, 2006c; Chapter 5). However, the observed concentration of indole and skatole in the inter-muscular fat of lambs that had been fed WC compared to PRG was not statistically different. Power analysis (assuming a power of 80%) indicated that 65 lambs per treatment group (instead of 20) would have detected a significantly greater fat skatole concentration (P<0.05) in lambs fed WC compared to PRG It is possible that the concentration of indole and skatole in the fat the concentration of indole and skatole in the fat main for 24-hours prior to slaughter. Fat depots may have been mobilised during this fasting period and at the same time altered the concentration of indole and skatole in the fat.

Compared to week 4, differences in rumen indole and skatole concentration between lambs fed WC and PRG remained large prior to slaughter (week 9) while the difference in the blood plasma concentration of indole and skatole between lambs fed WC and PRG was smaller. The higher indole and skatole concentration circulating in the blood of lambs fed WC may have resulted in an up-regulation of indole and skatole metabolism in the liver during the course of the experimental period. The improved metabolism may have minimised the observable differences in indole and skatole concentration in the fat of lambs that had been fed WC compared to PRG. The discrepancy between the large difference in skatole and indole concentration in the rumen of lambs fed WC compared to PRG, and the small difference in the fat is unexpected and further research is warranted in order to elucidate the role of liver metabolism on the deposition of indole and skatole in the adipose tissue.

Although there were minimal differences in the concentration of indole and skatole in the fat, there were some minor but significant odour and flavour effects observed as a consequence of feeding WC and PRG and of supplementing with GSE. It should be noted that although the forage and GSE treatments resulted in significant effects being observed in the odour and flavour intensities, the intensity scores for most of the odour and flavour attributes were low (<3) and the changes due to the effect of the treatments were small. The overall odour was less intense in the fat from lambs dosed with GSE and the overall flavour was less intense when feeding PRG compared to WC. The way in which overall odour and flavour have been influenced by the treatments is probably a consequence of odour being assessed with fat while lean muscle was combined with fat to produce the minced samples for flavour assessment. The treatments were likely to

affect the components of the two matrices differently. The effect of forage type interacting with GSE supplementation to affect the overall flavour is therefore, likely to be due to the interaction of the lean and fat.

Camphor, faecal and barnyard are among the descriptors used to characterise pastoral flavours that are associated with the presence of indole and skatole in meat (Young *et al.*, 2003; Annor-Frempong *et al.*, 1997). As the observed difference in the concentration of indole and skatole in the fat was not statistically different between the treatments in this study, the small but significant effect of GSE at lowering camphor, faecal and barnyard flavour intensities suggest that factors other than indole and skatole, which influence the pastoral flavour in the meat, may have been affected. It is possible that GSE supplementation was having a greater influence on the fat concentration of other compounds that have been associated with pastoral flavours but were not measured in this study. Phenols and methylphenols have been implicated as contributing towards pastoral flavour in sheep meat (Young *et al.*, 1997). Phenol and 4-methylphenol formation in the sheep rumen have been linked to forage protein intake but feed effects on the rumen formation of these phenols differed from that of indole and skatole (Fraser *et al.*, 2003).

In conclusion, feeding sheep WC compared to PRG resulted in a higher indole and skatole concentration in the rumen fluid and blood plasma and a more intense overall meat flavour but, a causal relationship has not been established. Dosing condensed tannin in the form of GSE lowered the concentration of indole and skatole in the blood plasma and resulted in less intense sheepy, camphor, faecal and barnyard flavours in the meat, however these flavour effects could not related to differences in indole or skatole

concentration in the fat. It is likely that the intermittent supply of GSE to the rumen environment was not substantial enough to keep the production of indole and skatole minimised to lower the fat concentration of indole and skatole. Furthermore, in order for CT to reduce indole and skatole formation it is necessary for the CT to be supplied in a manner that allows close contact of plant protein with the free CT. Previous studies (Schreurs *et al.*, 2006c,d; Chapter 5,6) have indicated that providing CT in forages is likely to be the best mechanism for this. Further studies are needed to test if feeding a CT-containing forage, where the intake of CT would be continuous, is able to sufficiently reduce indole and skatole in the fat to ameliorate pastoral flavour in lamb.

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## **CHAPTER 8**

## **General Discussion**

## **8.1. INTRODUCTION**

A plethora of research has indicated that feeding pasture to ruminants results in a difference in the flavour of the meat compared to feeding grain or concentrate diets (see Chapter 1; *Table 1.2*). The characteristic flavour of the meat from animals raised on pasture is euphemistically called "pastoral" flavour. Pastoral flavour is considered an undesirable attribute of meat for consumers accustomed to meat from animals raised on grain or concentrate diets. Pastoral flavours are described as "off", "animal-like", "grassy" "barnyard" or "faecal-like" (Young *et al.*, 2003 and references cited therein).

The sensory detection of flavour is a consequence of specific volatile flavour compounds binding to olfactory receptors during eating. Many compounds have been linked to pastoral flavours (Melton 1990; Ha & Lindsay 1991; Lane and Fraser 1999; Young et al., 1997, 2003; Priolo et al., 2004). However, a gas chromatographyolfactory method directly linked skatole to pastoral odours (Young et al., 2003). Indole and skatole are formed in the rumen from the microbial fermentation of tryptophan (Deslandes et al., 2001). Fresh pasture diets containing readily degradable protein, provide the potential for an elevated concentration of tryptophan in the rumen and studies looking at skatole-induced pulmonary emphysema and oedema indicated that lush pasture was causative of high formation of skatole in the rumen (Carlson and Bray 1983). Furthermore, due to the highly soluble and degradable nature of the protein in white clover, it was hypothesised that this forage may result in a greater formation of indole and skatole in the rumen. Feeding legumes, such as white clover, have been noted as imparting a less desirable meat flavour than grasses (Cramer et al., 1967; Shorland et al., 1970; Duckett and Kuber 2001; Table 8.1), although the mechanism of these flavour differences has not been determined. It is possible that such flavour

differences were due to a higher indole and skatole concentration in the meat from animals fed legumes.

In the rumen condensed tannins (CT) slow the rate of plant protein degradation to peptides, amino acids and ammonia and allow a greater proportion of forage protein to pass undegraded into the intestines (McNabb *et al.*, 1996). A lower concentration of free amino acids in the rumen from the action of CT could be expected to reduce the tryptophan available to be converted to indole and skatole. Indole and skatole concentration in the blood and milk of ewes fed the CT forage sulla was lower than that of ewes that were fed sulla with polyethylene glycol to inhibit CT action (Roy *et al.* 2002, 2004). There have been no studies to investigate the possible links between the formation of indole and skatole in the rumen with the indole and skatole concentration in the blood and the consequent effect this has on pastoral flavour when animals are fed different forage diets or when CT is included in the diet.

It was hypothesised at the start of this study that due to high protein solubility and degradability in the rumen, the white clover component of conventional grazed pasture may largely be responsible for the high concentration of indole and skatole in the fat of animals. Furthermore, forages containing CT maybe able to slow the degradation of protein in the rumen and lower the formation of indole and skatole, ultimately ameloriating the intensity of pastoral flavours in the meat. In order to test these hypotheses; indole and skatole formation was compared with different forages (including white clover and perennial ryegrass) *in vitro* (Chapter 3) and *in vivo* (Chapter 4 and 5). Likewise, the effect of CT on the formation of indole and skatole was investigated *in vitro* (Chapter 6) and *in vivo* (Chapter 7). To establish if treatments that

resulted in a lower rumen indole and skatole concentration resulted in a lower concentration in the fat, indole and skatole concentration was measured in the blood plasma and body fat of sheep fed different forage diets and lambs supplemented with dietary CT (Chapters 5 and 7). Sensory panels were used to establish if the different forage diets and dietary CT treatments resulted in a difference in the fat or meat odour (Chapters 5 and 7) or flavour (Chapter 7).

This General Discussion outlines methodological considerations and limitations of the experimental designs reported in this thesis. Secondly, the major findings of the experimental chapters will be summarised. This discussion will end by profiling the implications of this study and consider the need for further research.

Reference	Forage diets considered <sup>T</sup>	Meat flavour/odour results			
Cramer et al., (1967)	White clover Perennial ryegrass	Feeding clover gave a stronger flavour compared to feeding ryegrass			
Shorland <i>et al.</i> , (1970)	White clover Perennial ryegrass	Feeding clover gave a stronger flavour compared to feeding ryegrass			
Czochanska <i>et al</i> ., (1970)	White clover Perennial ryegrass	Feeding clover gave a stronger flavour compared to feeding ryegrass			
Nicol & Jagusch (1971)	Lucerne Ryegrass pasture	Lucerne gave more intense odour			
Park <i>et al.</i> , (1972)	Lucerne Rape Vetch	Lucerne and vetch considered to give more intense flavour and the rape gave a sickly flavour			
Park <i>et al</i> ., (1975)	Lucerne Grass pasture	Lucerne gave a more intense flavour			
Nixon (1981)	<i>Lotus pedunculatus</i> Red clover White clover Lucerne Perennial ryegrass	Off-flavours more predominant when feeding ryegrass			
Bailey <i>et al</i> ., (1994), Trial 1	<i>Lotus corniculatus</i> Lucerne Perennial ryegrass White clover	Flavour intensity was highest from lambs fed ryegrass and clover and lowest from lambs fed Lotus			
Bailey <i>et al</i> ., (1994), Trial 2	<i>Lotus corniculatus</i> Lucerne Perennial ryegrass White clover	No difference in flavour intensity between forages fed			
Bailey et al., (1994), Trial 3	Cowpea Lucerne	No difference in grassy or lamby flavours			
Bailey et al., (1994), Trial 4	Cowpea Sudan grass	No difference in grassy or lamby flavours			
Young <i>et al</i> ., (1994)	Perennial ryegrass Tall fescue Cocksfoot <i>Phalaris</i> Lucerne Chicory Prairie grass	Feeding <i>Phalaris</i> gave a stronger foreign flavour. No other flavour effects of forages observed.			
Fraser et al., 2004	Red clover Lucerne Perennial ryegrass	No difference in flavour between the forages fed.			
Chapter 5 (Experiment 2)	Perennial ryegrass/white clover Lotus corniculatus	No odour differences observed			
Chapter 7 (Experiment 2)	White clover Perennial ryegrass	Overall flavour intensity was higher from lambs fed white clover			

Table 8.1. Influence of different forages on the odour and flavour of lamb meat

<sup>†</sup> White clover (*Trifolium repens*), Perennial ryegrass (*Lolium perenne*), Lucerne (*Medicago sativa*), Rape (*Brassica rapa*), Vetch (*Vicia sativa*), Red clover (*Trifolium pratense*), Cowpea (*Vigna unguiculata*), Sudan grass (*Sorghum vulgara sudanensis*), Tall fescue (*Festuca arundinacea*), Cocksfoot (*Dactylis glomerata*), *Phalaris (Phalaris aquatics*), Chicory (*Chicorium intybis*), Prairie grass (*Bromus wildenowii*).
# 8.2. METHODOLOGICAL CONSIDERATIONS

This section covers some methodological considerations for general aspects of the experiments and specifically the experimental design used to test the hypotheses. Some of the methodological considerations relating to specific analytical methods have already been outlined in Chapter 2.

## 8.2.1. Experimental design

The first hypothesis was tested by comparing the formation of indole and skatole with CT and non-CT forages both in vitro and in vivo. Traditionally, the effects of CT in forages are compared in experiments by comparing polyethylene glycol (PEG)-dosed sheep with sheep not receiving PEG. Polyethylene glycol preferentially binds with CT thus inactivating the CT (Jones and Mangan 1977). Although PEG was incorporated in the in vitro rumen fermentations to establish the effect of CT, PEG dosing was not utilised in the *in vivo* experiments. In Chapter 5 (Experiment 2) the extensive grazing system meant PEG-dosing was not feasible, while in Chapter 7 (Experiment 2) the controlled dosing of the exogenous grape seed extract (CT source) to sheep fed non-CT forages made the need for dosing with PEG redundant. In Chapter 4 (Experiment 1), PEG dosing could have been implemented to further elucidate the effects of the CT in the Lotus corniculatus, however, the aim of this experiment was to simply compare rumen formation of indole and skatole between forages rather than to elucidate the specific effects of CT. Future experiments to specifically determine the effects of CT in CT-containing forages on indole and skatole formation in the rumen will need to consider further work using PEG as in the work of Roy et al. (2002).

In the *in vivo* experiment of Chapter 4 (Experiment 1) the three forages; white clover, perennial ryegrass and *Lotus corniculatus*, were fed in a sequential order. As such, carry-over effects from the forage previously fed can not be ruled out. A cross over design would have been preferential (*Table 8.2*) but greater animal numbers would have been required to generate appropriate replication for each forage sequence, imposing a greater ethical cost. Furthermore, the cross over design would also require all three forages to be available at the same time. At the time of the experiment in Chapter 4 (Experiment 1) the simultaneous availability of the three forages was not possible, due to agronomic and environmental constraints on their growth. Another alternative design would have been to utilise more animals and have separate groups of animals fed each forage, but this also would have required forages to be available at the same time. To over-come any possible carry-over effects a 7-10 day adaptation was used after the sheep were introduced to each new forage.

	Sheep							
	1	2	3	4	5	6		
Period 1	WC	PRG	LC	WC	PRG	LC		
Period 2	PRG	LC	WC	LC	WC	PRG		
Period 3	LC	WC	PRG	PRG	LC	WC		

Table 8.2. Crossover design applicable to Experiment 1 of Chapter 4 when feeding white clover (WC), perennial ryegrass (PRG) and *Lotus corniculatus* (LC).

## 8.2.2. In vitro rumen fermentations

Previous *in vitro* rumen fermentations have reported the use of a lucerne standard to compare between treatments run on separate days and had replicate bottles within the

same incubation to generate replication (Burke *et al.*, 2000). The *in vitro* method utilised in these studies did not incorporate a lucerne standard, but instead incubated all treatments on one day and repeated all the treatments on the successive three days to generate replicates. Due to the size of the incubator (holds approximately 95 incubation bottles) this method is limited to running a maximum of 18 treatments (5 bottles per treatment) however, repeating treatments across days (as done in this present study) is a better form of replication than repeated samples within an incubation.

#### 8.2.3. Animal numbers

In Chapter 7 (Experiment 2) differences in the rumen and plasma concentration of indole and skatole between nutritional treatments were not reflected as a difference in the fat, while in Chapter 5 (Experiment 2) the observed differences in rumen and plasma indole and skatole between nutritional treatments only just reached significance in the fat. Power analysis has indicated that greater animal numbers in each treatment would result in some of the differences in fat indole and skatole between nutritional treatments being detected as significant at P<0.05. The use of greater animal numbers, maybe in extensive grazing experiments, needs to be considered for future experiments where indole and skatole concentration in the body fat is to be determined.

# 8.2.4. Single time-point sampling of dynamic biological systems

The animal is a dynamic biological system and the concentration of indole and skatole in the rumen, plasma and fat will be a constantly changing entity. As Chapter 4 (Experiment 2) showed, the concentration of indole and skatole in the rumen fluid and blood plasma changes in relation to the time of feeding. This can make the detection of treatment responses difficult when using single time-point sampling after the application of a treatment, as indicated in Chapter 7 (Experiment 2). To ensure that treatment effects are observed, multiple sampling time points should be considered for rumen fluid and blood plasma samples in future experiments. Alternatively, treatments that give a prolonged effect (e.g., continuous feeding or infusion of CT rather than a single-dose) should be utilised.

## 8.2.5. Accuracy and precision of analyses

These have been outlined in detail in the materials and methods chapter (Chapter 2). In general, the methods used to analyse indole and skatole in the rumen fluid, blood plasma and fat had a suitable level of accuracy and precision. Analytical variation was smaller than that of within-treatment biological variation, making it possible to detect genuine treatment differences. Inter-assay variation tended to be greater than that of intra-assay variation indicating a preference for batch processing of samples.

## 8.2.6. Sensory panels

Sensory evaluation may result in greater variability in the results between panellists than between treatment replicates (samples from individual sheep). This means that subtle differences in indole and skatole concentration in the fat may not be detected as a difference in the mean sensory scores. In order to aid the detection of treatment differences it may have been more effective to pool the treatment replicates and carry out repeated sensory assessment on treatment aggregates. However, such a design would not allow for valid statistical analysis as all replication of the treatments would be lost and as such, prevents sensory analysis of individual animals that may have a particularly high or conversely, low concentration of indole and skatole in the fat (such as that undertaken in Chapter 5).

In order to characterise differences in meat flavour between treatments an option is to use sensory difference tests such as the triangle test or duo-trio test. In the triangle test the panellist is presented with three samples for sensory evaluation, two from the same treatment (or a control) and one from an alternative treatment. The panellist is then asked to pick out the odd sample and rate its sensory characteristics. The duo-trio test asks the panellist to determine if samples are different to a control. Such sensory tests could then be linked with intensity rating of specific odour and flavour attributes to further define the degree and the nature of the difference being observed. This method provides a way to directly compare the sensory characteristic of treatments and may have been able to better establish if flavour or odour differences existed between nutritional treatments in Chapter 5 (Experiment 2) and Chapter 7 (Experiment 2). However, difference tests may be too general and not identify the direction (more or less intense) or magnitude of pastoral flavours. Therefore, intensity rating of pastoral flavour attributes (as used in this study) is more specific for determining the difference in pastoral flavour attributes between treatments.

# 8.3. THE EFFECT OF DIFFERENT FORAGES ON INDOLE AND SKATOLE FORMATION IN THE RUMEN

### 8.3.1. Differences in the formation of indole and skatole between forages

White clover (WC) resulted in a much higher formation of indole and skatole in both *in vitro* and *in vivo* studies (Chapter 3, 4, 6 and 7) compared to perennial ryegrass. In comparison, the formation of indole and skatole with perennial ryegrass (PRG) is generally only 6-41% of that formed with WC (*Table 8.3*). For this reason, it was concluded that it is likely to be the WC component of conventional pastures that is largely responsible for pastoral flavours.

Forages containing condensed tannin lowered the formation of indole and skatole both *in vitro* (Chapter 3 and 6) and *in vivo* (Chapter 4 and 5), although the CT-containing forage legumes and non-CT forage legumes had a similar crude protein concentration.

#### 8.3.2. Forage-based factors that influence indole and skatole formation

Forages vary in their propensity to form indole and skatole and this can be related to crude protein concentration in the plant and to the degradability of that protein. The higher crude protein concentration in WC compared to PRG partly contributes to the difference in indole and skatole formation in the rumen with these two forages. However, the higher concentration of indole and skatole formed with WC has been linked to the higher solubility and degradability of the protein, as indicated by a correlated higher concentration of rumen ammonia both *in vitro* (Chapter 3 and 6) and *in vivo* (Chapter 4 and 7).

Increased forage maturity lowers indole and skatole formation and this is due to reduced crude protein concentration and reduced degradability of the forage with age, as indicated by lower ammonia concentration formed with mature forages (Chapter 3). In addition, a higher application of nitrogen fertiliser increased the crude protein concentration in perennial ryegrass and resulted in a greater formation of skatole (Chapter 3). The higher fibre content of PRG is thought to slow the microbial degradation of the forage and consequently reduce the formation of indole and skatole.

Comparison of forages fermented *in vitro* (Chapter 3) showed that with forages of a higher CT concentration such as *Lotus pedunculatus* (98 gCT kg<sup>-1</sup> DM) and *Dorycnium rectum* (122 gCT kg<sup>-1</sup> DM), the indole and skatole formed was only 7-21% of that formed with WC (*Table 8.3*). With forages of an intermediate CT concentration such as sulla (*Hedysarum coronarium*; 38 gCT kg<sup>-1</sup> DM) and *Lotus corniculatus* (35 gCT kg<sup>-1</sup> DM), the indole and skatole concentration formed was 53-68% of that of WC (*Table 8.3*). More details on the effect of concentration on indole and skatole formation are given in section 8.4 of this General Discussion.

Some exceptions were encountered. With broadleaf dock the CT concentration was low (21 gCT kg<sup>-1</sup> DM) and the concentration of indole and skatole formed were also low. In addition, the *Dorycnium rectum* had a higher CT concentration than *Lotus pedunculatus* but the indole and skatole concentration formed with *Dorynium rectum* was higher than that with *Lotus pedunculatus* (Chapter 3). It is possible that chemical composition of the CT in different forages also has an effect but this needs further investigation.

	Treatment <sup>1,2</sup>	Concentration relative to control (%) <sup>1</sup>				
	reatment	Indole	Skatole			
IN VITRO						
Hammond & Carlson 1980	Tryptophan (c)	100	100			
	Tryptophan +Monensin	13	8			
Tavendale et al., 2005	Tryptophan (c)	100	100			
	Tryptophan +CT (from DR)	65	30			
Chapter 3 (Experiment 1)	WC(c)	100	100			
	PRG	15	26			
	LC	68	62			
	SL	59	53			
	LP	13	7			
	DR	68	21			
	BD	6	3			
Chapter 3 (Experiment 2)	Young WC (c)	100	100			
	Mature WC	80	57			
	Young PRG	6	29			
	Mature PRG	6	16			
	Young LC	34	76			
	Mature LC	21	57			
Chapter 6 (Experiment 1)	100WC:0LC (c)	100	100			
	75WC:25LC	83	73			
	50WC:50LC	77	54			
	25WC:75LC	41	34			
	OWC:100LC	27	7			
Chapter 6 (Experiment 2)	WC (c)	100	100			
	WC+CT (from LP)	8	17			
Chapter 6 (Experiment 3)	WC (c)	100	100			
	WC +GSE (CT)	14	20			
	PRG	130	81			
	PRG +GSE (CT)	6	12			
IN VIVO						
Carlson et al., 1983	Pasture (c)	100	100			
	Pasture +Monensin	50	53			
Potchoiba et al., 1984 (Expt1)	Pasture (c)	100	100			
	Pasture +Monensin	200	64			
Potchoiba et al., 1984 (Expt2)	Pasture (c)	100	100			
	Pasture +Monensin	50	84			
Potchoiba et al 1992	Pasture (c)	100	100			
	Pasture +Monensin	127	56			
Chapter 4 (Experiment 1)	WC (c)	100	100			
	PRG	39	41			
	LC	24	29			
Chapter 5 (Experiment 2)	PRGMIC Pacture (c)	100	100			
Chapter 5 (Experiment 2)		107	100			
		107	40			
Chapter / (Experiment 2)		100	100			
		97	08			
		30	20			
	FRG TOSE (C1)	30	19			

Table 8.3. Influence of different forages and condensed tannin (CT) on the formation of indole and skatole in the rumen relative to control values.

<sup>1</sup> Control treatment indicated with (c). Treatment effects are relative to the control (100%) <sup>2</sup> Abbreviations: DR, *Dorycnium* rectum; WC, White clover; PRG, Perennial ryegrass; LC, *Lotus corniculatus*; SL, Sulla; LP, Lotus pedunculatus; BD, Broadleaf dock; GSE, Grapeseed extract

# 8.3.3. White clover vs. perennial ryegrass effects on meat flavour

Feeding white clover in comparison to perennial ryegrass did not result in a substantial difference in the concentration of indole and skatole in the fat, even though differences were observed in the concentration of indole and skatole in the rumen and blood. The overall flavour tended to be more intense for the meat obtained from WC fed lambs however, the intensity scores for each treatment were low and hence the treatment differences were small.

# 8.4. WHAT ROLE DO CONDENSED TANNINS HAVE IN LOWERING INDOLE AND SKATOLE FORMATION AND AMELIORATING PASTORAL FLAVOUR?

Condensed tannin-containing forages are just as effective if not more effective at lowering indole and skatole concentration as monensin (*Table 8.3*). Monensin (Hammond and Carlson 1980; Carlson *et al.*, 1983; Potchoiba *et al.*, 1984, 1992) and the use of CT from *Dorycnium rectum* in incubations with tryptophan (Tavendale *et al.* 2005) have indicated that microbial inhibition can reduce the formation of indole and skatole but, this study has shown that CT effects are greater when acting on both the forage protein and the microbes (*Table 8.3*).

## 8.4.1. Concentration of condensed tannin (CT)

*In vitro* fermentation of forages with a range of CT concentrations indicated that the formation of indole and skatole was affected by CT concentration (Chapter 3, Experiment 1). However, these results are confounded by the different chemical compositions of the CT polymer in each plant. In Chapter 6 the effect of chemical composition differences between CT from forages was removed by the incubation of only *Lotus pedunculatus* at different ratios with white clover or CT extracts added to the incubations at different concentrations with fresh white clover or perennial ryegrass.

Chapter 6 showed that increasing the CT concentration as a component of *Lotus pedunculatus* with white clover only resulted in a reduction in the formation of indole and skatole proportional to the amount of *Lotus pedunculatus* added to the incubations (linear response). It was concluded that the CT was only slowing the degradation of protein in the LP with no cross-over to the protein in white clover.

In contrast, increasing the concentration of CT extract added to rumen fermentations resulted in an exponential increase in protein (plant and microbial) binding, due to the binding capacity being reached at a higher CT concentration (Chapter 6, Experiments 2 and 3). It was concluded that the concentration of CT available in the rumen will influence the amount of CT available to interact with forage protein and rumen microbes. Therefore, a forage containing a higher CT concentration is preferential for reducing indole and skatole formation. It should be noted however, that a high CT concentration in the plant may have anti-nutritional effects on rumen digestion and on feed intake by the animal.

#### 8.4.2. Endogenous vs. exogenous plant CT

Condensed tannins in the rumen exert their effects by binding to plant protein to form insoluble complexes that slow the degradation of forage protein but may also inhibit the function of rumen microbes (Haslam 1996).

The *in vitro* studies of Chapter 6 indicated that an exogenous CT added to rumen fermentations was able to bind with the protein from minced white clover and perennial ryegrass to lower the formation of indole and skatole. Adding CT as an exogenous extract to *in vitro* rumen fermentations provides a higher concentration of free CT to bind with all protein sources whether it be forage or microbial. Further incubations with tryptophan and PEG added to rumen fermentations with *Lotus pedunculatus* confirmed that CT in the plant preferentially binds to forage protein while free CT is able to inhibit rumen microbes as well as the degradation of forage protein (Chapter 6).

With endogenous plant CT, the CT is released from plant vacuoles at the same time as protein during mastication and microbial degradation, optimising the interaction of the

CT with forage protein to form insoluble complexes (Barry and McNabb 1999; Min *et al.* 2003). When an exogenous CT in the form of a grape seed extract was dosed to sheep that were continously fed white clover or perennial ryegrass the effect on the rumen concentration of indole and skatole was minimal. In the *in vivo* animal, the dosed CT would pass out of the rumen during the period between doses while forage intake would continue. Therefore, it was likely that any forage protein entering the rumen between dosing times had limited interaction with the exogenous CT which, limited the effect of the CT on forage protein degradaton.

Therefore, the ultimate scenario for reducing the formation of indole and skatole in the rumen is to feed a forage that has a high concentration of CT, to optimise forage protein binding. Grazing mixed swards would require an unrealistically high CT concentration in the CT-containing forage of the sward in order to get an effective reduction of indole and skatole formation. Therefore, it is preferential to graze a sward of pure CT forage with a high CT concentration (approximately 80 g kg<sup>-1</sup> DM) to finish lambs before slaughter. A prime candidate forage for this would be *Lotus pedunculatus* 

# **8.4.2. Effect of CT on the indole and skatole concentration in the blood and fat and the pastoral odour and flavour**

Prior to the studies of this thesis, sulla (a CT-containing forage; 40 gCT kg<sup>-1</sup> DM) fed to sheep lowered the concentration of indole and skatole in the blood compared to similar animals fed sulla and dosed with PEG (CT inactive; Roy *et al.*, 2002). It was hypothesised that the CT in sulla had the ability to reduce the formation of indole and skatole in the rumen and this was reflected in the blood indole and skatole concentration. The present studies have established, by direct measurements, that feeding *Lotus corniculatus* (14-40 gCT kg<sup>-1</sup> DM) and dosing grape seed extract lowered the concentration of indole and skatole in the rumen compared to non-CT treatments (*Table 8.4*) and that this was paralleled by lower blood plasma concentration of indole and skatole.

Recently, Priolo *et al.* (2005) reported a study implementing the same treatments as Roy *et al.* (2002) however, the sulla used by Priolo *et al.* (2005) had a considerably lower concentration of CT (18 gCT kg<sup>-1</sup> DM) and they did not observe a difference in the concentration of indole and skatole in the fat between sulla and sulla plus PEG treatments (*Table 8.4*). Similarly, dosing a grape seed extract (source of CT) to lambs fed WC or PRG had no observable effect on fat indole and skatole concentration in these studies (Chapter 7, Experiment 2; *Table 8.4*). In contrast, grazing the CT forage *Lotus corniculatus* was able to reduce the concentration of skatole in the fat compared to grazing perennial ryegrass/white clover pasture (Chapter 5, Experiment 2).

Intermittent dosing of lambs with exogenous CT is therefore less effective at reducing rumen indole and skatole formation and consequently can not lower the concentration of indole and skatole in the fat. However, the results from Chapter 5 suggest that a more continuous intake of CT would keep rumen indole and skatole production minimised and can reduce indole and skatole concentration in the fat. Further grazing experiments using greater animal numbers are required to confirm this.

Not only did grazing *Lotus corniculatus* lower the concentration of skatole in the fat, but it was also able to reduce the between animal variability in the fat concentration of skatole. None of the lambs slaughtered after grazing *Lotus corniculatus* had a high outlying concentration (>100 ng g<sup>-1</sup>) of skatole in the fat. This experiment was able to show that some of the panellists were sensitive to outlying, high concentrations of indole and skatole in the fat of some lambs. Therefore, CT forages that reduce between animal variation of fat indole and skatole concentration may have a role in reducing the proportion of meat cuts that have a high outlying concentration of indole and skatole and so minimise the possibility of pastoral flavour detection for those consumers that are sensitive to high skatole in the meat.

Table 8.4	. Effect	of	dietary	condensed	tannins	on	the	indole	and	skatole	concen	tration
(parts per	r billion;	; pp	b) in the	e blood plas	ma and l	bod	y fat					

	Poforonco	Plasm	a (ppb) <sup>1</sup>	Fat (ppb)		
	Reference	Indole	Skatole	Indole	Skatole	
Non-CT treatment						
Sulla+PEG <sup>2</sup>	Roy et al., 2002	2.8	6.5	ND	ND	
Sulla+PEG <sup>2</sup>	Priolo et al., 2005	ND	ND	39	51	
Pasture (PRG/WC) <sup>2</sup>	Chapter 5 (Experiment 2)	7	11	13	88	
White clover	Chapter 7 (Experiment 2)	11	76	8	79	
Perennial ryegrass	Chapter 7 (Experiment 2)	16	49	8	62	
CT treatment						
Sulla	Roy et al., 2002	0.8	2.2	ND	ND	
Sulla	Priolo et al., 2005	ND	ND	36	91	
Lotus corniculatus	Chapter 5 (Experiment 2)	2	9	13	56	
White clover +GSE <sup>2</sup>	Chapter 7 (Experiment 2)	5	44	9	86	
Perennial ryegrass +GSE <sup>2</sup>	Chapter 7 (Experiment 2)	4	25	5	73	

<sup>1</sup> Plasma from the blood of the jugular vein apart from the study by Roy *et al.*, 2002 where mesenteric artery values are reported

<sup>2</sup> Abbreviations: PEG, Polyethylene glycol; GSE, Grape seed extract; PRG/WC, Perennial ryegrass/White clover ND; Not determined

#### 8.4.3. Dietary CT and its effect on pastoral odour and flavour in sheep meat

Grazing *Lotus corniculatus* (Chapter 5) was able to lower the concentration of skatole in the fat compared to lambs grazed on perennial ryegrass/white clover but, the difference in these treatment effects was not large enough to give a difference in the pastoral odour. The CT concentration in the LC may not have been high enough to obtain the difference in the concentration of indole and skatole in the fat required to obtain a sensory difference.

Dosing grape seed extract as a source of CT resulted in less intense pastoral flavours (sheepy, camphor, faecal and barnyard) however, this effect was not related to a lower indole and skatole concentration in the fat. It is possible that the grape seed extract was affecting the formation of other flavour compounds that originate from the degradation of forage protein in the rumen. Microbial degradation of tryptophan results in the formation of indole and skatole in the rumen but other amino acids degraded by rumen microbes may also result in the formation of flavour compounds that have pastoral flavour attributes (*Figure 8.1*). Phenols associated with pastoral flavours (Young *et al.*, 1997, 2003) are produced in the rumen from microbial fermentation of tyrosine (Ha and Linsay 1991) while branched chain fatty acids are associated with sheepy flavour notes in sheep meat (Wong *et al.* 1975; Suzuki and Bailey 1985) and can be derived from branched-chain volatile fatty acids that are formed in the rumen from the fermentation of the amino acids leucine, isoleucine and valine (Allison 1978). Sulphur containing compounds that have pastoral flavour attributes can be derived from methionine and cysteine degradation in the rumen (Keen 1998; *Figure 8.1*).

Forage CT are not absorbed from the ruminant digestive system (Terrill *et al.*, 1994) and are therefore, not nutrients in their own right. They confer nutritional benefits in ruminants through interacting with plant components that are nutrients, notably protein. As shown in *Figure 8.1*, CT are likely to reduce pastoral flavour in meat by reducing the rumen microbial degradation of forage protein and amino acids, with tryptophan being one of these.

Figure 8.1. Suggested rumen microbial metabolism of plant protein to compounds implicated in the cause of pastoral odour and flavour in the meat of sheep grazing fresh forage diets. Sites of condensed tannin (CT) inhibition are indicated. This figure created using data from: Wong *et al.* (1975); Ha and Lindsay (1991); Young *et al.* (1997, 2003); Le *et al.* (2005) and this study.



# **8.5. CONCLUSIONS AND FUTURE RESEARCH**

The use of alternative forages is considered a potential solution to provide a more desirable product for discerning markets that have an objection to pastoral flavours associated with the presence of a high indole and skatole concentration in the meat. This thesis has endeavoured to study different forages and in particular, condensed tannin-containing forages to investigate the feasibility of using alternative forages for improved meat flavour. Finding a practical solution to ameliorate pastoral flavours in meat has the potential to open up new markets for New Zealand sheep meat and increase exports of sheep meat from New Zealand.

## 8.5.1. Major conclusions of this thesis

From the results of this thesis, the following conclusions can be made:

- Feeding white clover will result in a higher concentration of indole and skatole being formed in the rumen and this is due to the higher crude protein concentration and higher protein solubility and degradability in this forage compared to other forages such as perennial ryegrass. Conventional pastures grazed in New Zealand can be comprised of up to 30% white clover. The white clover component of pastures is likely to be more influential on the pastoral flavour of meat than perennial ryegrass (*Figure 8.2*).
- Grazing condensed tannin-containing forages should be considered to ameliorate pastoral flavour in sheep meat (*Figure 8.2*). Grazing forages with a high concentration of condensed tannin (approximately 80 g kg<sup>-1</sup> DM) such as *Lotus pedunculatus* will minimise indole and skatole formation in the rumen due to slowing the degradability of forage protein and inhibiting the rumen microbes involved in the formation of indole and skatole. Having a high concentration of CT

ensures there is sufficient CT to bind protein in the forage and also provides free CT for inhibiting microbial fermentation of tryptophan to indole and skatole.

• As indicated from *in vitro* studies, minimising the use of nitrogen fertiliser and the grazing of new, young forage growth is likely to reduce the indole and skatole formed in the rumen and aid in reducing pastoral flavour (*Figure 8.2*). However, the grazing of young leafy swards is required to maximise animal production in New Zealand and other scenarios for minimising pastoral flavour should firstly be considered.

#### 8.5.2. Future research

- In the one experiment that assessed meat flavour of lambs fed white clover compared to perennial ryegrass, no difference in the fat concentration of indole and skatole was observed. Further research using greater animal numbers per treatment is required to substantiate if white clover in conventional pasture contributes towards the presence of pastoral flavour in sheep meat through changes in the indole and skatole concentration in the body fat.
- As a continuation to the experiments in this study, grazing studies are needed to assess if forages with a high CT concentration (e.g. *Lotus pedunculatus*) are able to ameliorate pastoral flavours in comparison to white clover and/or perennial ryegrass. These studies need to have an emphasis on flavour assessment and therefore, sufficient animal numbers to ensure that differences in the fat concentration of indole and skatole are detected.

- In this study, differences in blood plasma indole and skatole between treatments were not always evident in the fat. To understand the mechanism behind these results, further investigation into the metabolism of indole and skatole by the ruminant and the control of indole and skatole deposition in the fat is warranted.
- This study has identified that the chemical composition (as well as concentration) of the CT polymer may affect the formation of indole and skatole. The affect of the chemical composition of the CT polymer on indole and skatole formation needs to be investigated. The ultimate goal of such an experiment would be to identify the optimal chemical composition of the CT polymer required to reduce indole and skatole formation in the rumen in an effort reduce pastoral flavour in meat. Once the optimal chemical composition of the CT polymer is identified, plant-breeding methods could be implemented to design a forage with optimum CT composition and concentration to amend pastoral flavour problems in meat products from grazing ruminants.
- It will be beneficial to carry out experimental work where a CT-containing forage is fed, with and without PEG dosing and the effects observed from the rumen to the blood and then on to the fat with final sensory evaluation of the meat. This would provide conclusive understanding of the effect that CT are having on pastoral flavour when feeding forages.
- Reducing the degradation of forage protein with the use of CT forages will reduce the indole and skatole formed in the rumen but, will also have an effect on other compounds produced from protein degradation in the rumen and linked to pastoral

flavours (e.g., phenols and branched-chain volatile fatty acids; *Figure 8.1*). Continued research into pastoral flavour mitigation using nutritional treatments such as condensed tannins should consider measuring these other flavour related compounds.

- Incorporation of white clover in conventional pastures is vital in agronomic terms as the *Rhizobium* in the root nodules of legumes fix atmospheric nitrogen. Furthermore, white clover is a highly nutritious animal feed compared to grasses and is required in New Zealand swards to optimize animal growth and production. It may be possible to incorporate a CT forage such as *Lotus pedunculatus* into grazing systems as a finishing feed prior to slaughter. This would enable optimal utilisation of conventional pastures with the added benefits of producing meat with low pastoral flavour intensity. It will be imperative to know the length of time needed prior to slaughter to graze a CT forage to remove pastoral flavours from the meat. Serial slaughter trials maybe required to investigate this.
- The poor agronomic characteristics of CT-containing forages for New Zealand conditions make them difficult to grow and limit the potential for their use in grazing systems as a method to ameliorate pastoral flavour. Future research needs to consider incorporating high CT concentrations into forage legumes well adapted to New Zealand conditions (such as white clover). Modern technologies such as genetic engineering could be considered to produce such plants.



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