METABOLISM OF RUMEN INFUSED SKATOLE AND EFFECTS ON HEPATIC GENE EXPRESSION IN SHEEP

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ABSTRACT

Skatole (3-methylindole) contributes to the unique flavour palate known as pastoral flavour (characterised by 'sheepy or 'grassy' odours) that differentiates meat products of pasture-fed ruminants from those finished under grain-based production systems. Pastoral flavour is undesirable to some consumer groups who are sensitive or unaccustomed to meat from temperate pastoral production systems yet is largely appreciated by traditional consumers. The biosynthesis of skatole in the rumen requires bacterial deamination of the amino acid L-tryptophan, hence its rate of production can be manipulated by reducing the rumen formation of free amino acids or decreasing the activity of skatole forming microbes. Ruminants grazing New Zealand improved pasture species, in particular white clover, ingest sufficient rumen degradable protein to allow post-prandial skatole formation at a rate greater than the first-pass detoxification capacity of the liver. Skatole accumulation in body fat occurs when its absorption from the reticulorumen exceeds the pre and post-hepatic detoxification capacity of the body.

It was hypothesised that under conditions of minimal rumen skatole production a 72 hour administration of exogenous skatole would result in an increase in rumen skatole to a plateau concentration and induce differential expression of detoxification genes in the ovine liver. These hypotheses were tested using a continuous intraruminal infusion of skatole (140 mg/h), measurement of skatole concentration in rumen fluid, peripheral plasma and inter-muscular fat and transcriptional analysis of hepatic tissue using DNA microarrays.

Twelve, ten month old castrate male sheep (*Ovis aries*) from a single sire were exposed to a contrasting level of skatole for a 72 hour period. Rumen skatole production was minimised by feeding a diet with low rumen degradable protein content. Sheep received a constant rumen infusion of propylene glycol carrier with or without skatole (28 mg/mL). Samples of rumen fluid and peripheral blood were collected at 0, 2, 4, 6, 8, 12, 16, 24, 48 and 72 hours for determination of skatole concentration. Sheep were euthanased 72 hours after commencement of infusion and samples of liver and inter-

muscular fat were collected. Total hepatic RNA was isolated, purified and used for microarray hybridisation. cDNA was synthesised and cyanine dye incorporated in preparation for hybridisation to expressed sequence tag bovine microarrays with greater than 97% homology to ovine protein coding sequence. The experiment consisted of an 18 array augmented loop design balanced for dye bias.

Rapid appearance of rumen-infused skatole in the peripheral blood and inter-muscular fat confirmed the high rate of skatole absorption and deposition in sheep. A two compartment model fitted to the rumen and blood concentration of treated sheep enabled estimation of the rate of transfer from rumen to peripheral plasma ($k = 0.23$) and the rate of elimination from peripheral circulation ($k = 2.10$). A negative correlation ($P < 0.05$) between the rate of elimination and level of skatole deposition in inter-muscular fat was also found.

Expressed sequence tags with significant ($FDR < 0.01$) differential expression in either direction represented about 14% of those assessed. Genes encoding enzymes with xenobiotic detoxification activity were induced in ovine hepatic tissue in response to skatole exposure. Of these, only five had a fold change greater than 2.0; three encoded cytosolic phase I oxidoreductase enzymes involved in detoxification; aldehyde dehydrogenase 1 family member A1, NAD(P)H dehydrogenase quinone 1 and leukotriene B4 12-hydroxydehydrogenase. The metabolic oxidoreductase enzyme stearoyl-CoA desaturase was also induced along with phase II detoxification enzyme glutathione S-transferase. Induction of these genes, specifically those with known catalytic activity towards toxic xenobiotics, indicates that the ovine liver is a site of detoxification for skatole or its intermediary metabolites. Further investigation is required to determine the role of these genes in the regulation of skatole detoxification in the ovine liver and the possibility to reduce pastoral flavour in forage grazing ruminants through modulation of the activity of these genes or enzymes.

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LIST OF ABBREVIATIONS

pH	hydrogen potential
BW	bodyweight
CA	California
cDNA	complementary DNA
cm	centimetre(s)
CP	crude protein
d_2	deuterium
DM	dry matter
DNA	deoxyribonucleic acid
°C	degree Celsius
EB	equilibration buffer
EST	expressed sequence tag
FDR	false discovery rate
FID	flame ionization detection
GC-MS	gas chromatography-mass spectrometry
g	gram(s)
g	gravities of centrifugal force
>	greater than
h	hour
HPLC	high performance liquid chromatography
HCl	hydrochloric acid
IL	Illinois
i.m.	intramuscular
i.v.	intravenous
kg	kilogram
<	less than
L	litre
m/z	mass-to-charge ratio
MJ	mega joule
ME	metabolisable energy
m	metre(s)
MeOH	methanol
3MI	3-methylindole
2MI	2-methylindole
µg	microgram(s)
µL	microlitre(s)
mg	milligram(s)
mL	millilitre(s)
mm	millimetre
min	minute(s)
MO	Missouri
M	molarity, moles per litre
ng	nanogram(s)
NJ	New Jersey
NY	New York
NZ	New Zealand
NADPH	nicotine adenine dinucleotide phosphate
n	number of observations
ON	Ontario

OD	optical density
o.d.	outer diameter
%	percent
PMT	photomultiplier tube
PCR	polymerase chain reaction
PD	propane-1,2-diol
®	registered trademark
rpm	revolutions per minute
RDP	rumen degradable protein
RNA	ribonucleic acid
SPE	solid phase extraction
TX	Texas
TM	trade mark
UV	ultraviolet light
USA	United States of America
UK	United Kingdom
VFA	volatile fatty acid
VFI	voluntary feed intake
v	volume
H_2O	water
w	weight
WI	Wisconsin

CHAPTER 1

LITERATURE REVIEW

1.0 INTRODUCTION

New Zealand is the world's largest sheep meat exporter (44%) and is currently suffering a period of reduced prices compounded by a high foreign exchange rate. Expansion of high-value trade in wealthy emerging markets offers one solution to address unsustainably low farm gate revenue ($2.80/kg lamb carcass). Both highly affluent (e.g. Japan) and growth economies (e.g. China) in Asia represent significant marketing opportunities for sheep meat exporters. New Zealand's exports of sheep meat to most countries in Asia are relatively low, total exports to the north Asian region (Japan, Korea, China and Taiwan) comprised just 11% of total exports in the 2006/2007 year.

There is significant evidence (Schreurs *et al.,* 2007a; Young *et al.,* 2003) that the flavour of sheep meats produced from temperate pastoral production systems such as New Zealand's is too strong for Asian consumers. Whilst the same flavours are desirable to the palate of traditional consumers a milder product is required specifically for these potential consumers.

Pastoral flavour is partially caused by the deposition of skatole in inter-muscular fat. Skatole accumulation is multi-factorial and displays considerable variation between animals. One method to increase product acceptance is to eliminate animals from the supply chain that have accumulated skatole at concentrations above the detection threshold for a given market. Ameliorating skatole accumulation in sheep meat currently requires costly dietary additions to the pastoral system to reduce skatole formation in the rumen. Scientific advances in the pork industry suggest that post-absorption metabolism of skatole may be the determining factor of accumulation in pigs.

This review investigates the characteristics of New Zealand's low input pastoral production system that gives rise to pastoral flavour, the biosynthesis of skatole in ruminants and its effects upon the sensory characteristics of sheep meats. It reviews the current knowledge of skatole metabolism in ruminant and monogastric animals and considers the relevance of skatole metabolism to the New Zealand sheep industry.

1.1 RUMEN DIGESTION OF TEMPERATE FORAGE

New Zealand agriculture is based upon the ability of ruminant livestock to graze temperate forages year round. The low input management of highly digestible yet persistent temperate forages has resulted in a reliance on improved cultivars of perennial ryegrass (*Lolium perenne*) and white clover (*Trifolium repens*). Typical improved pastures contain 10 to 30% white clover to increase feeding value (Caradus *et al.*, 1995). Reliance upon these pasture species limits the nutrient intake of grazing ruminants to the seasonally variable nutrient composition and digestibility of these forages. This feeding system is in contrast to ration based systems of ruminant production where diets are formulated to meet the nutritional requirements of the animal.

During grazing sheep bite leaves and stems of pasture forages and chew briefly whilst rolling the material into a saliva coated bolus that is swallowed into the rumen. In grass fed sheep half of the leaf and stem particles entering the rumen are greater than 4 mm in length (Fraser and Barker, 1998) and require further reduction in size through re-chewing (rumination) and microbial fermentation to attain a size < 4 mm where outflow from the rumen becomes increasingly more likely. The rumen is the first compartment of the specialised ruminant digestive system that comprises the fore-stomachs; rumen, reticulum and omasum that precede the abomasum or true stomach. The rumen and reticulum comprise 10 to 20% of the ruminants live weight and support a population of anaerobic micro-organisms (8×10^{10}/mL) (Waghorn and Barry, 1987).

The proportion of ingested plant material that is digested is a major determinant of nutritive value. Temperate forages have a high organic matter digestibility averaging

85% during winter and spring. In both grasses and legumes digestibility declines as the proportion of stem increases with the onset of flowering. At the same time the digestibility of the stem also declines due to the increasing proportion of structural carbohydrate (cellulose and hemicellulose) and lignin, and a decrease in readily fermentable carbohydrates (soluble sugars and pectin) and protein (Ulyatt 1980). Digestibility falls to below 65% during summer senescence before rising rapidly in response to vegetative growth during autumn (Ulyatt 1980). Similarly, the nutrient density of fresh forages changes with seasonal variation in intracellular water content. These physical changes in plant characteristics impact upon skatole formation by rumen bacteria because they influence the quantity and quality of the macronutrients in the grazing animals diet and thus the substrates of rumen fermentation.

Rumen digestion of fresh forages is the result of two competing processes: degradation and passage (Dijkstra *et al.,* 2007). Degradation occurs primarily through the activity of rumen micro-organisms and the action of plant proteases in initial protein degradation (Attwood, 2005). The time that a feed is retained in the rumen for microbial digestion is a major determinant of the efficiency of microbial protein synthesis, as the rumen operates as a partially stirred, continuous-flow fermenter (Dijkstra *et al.,* 2007). Within the rumen separate pools of liquid, escapable particles and retained particles can be distinguished with differing flow characteristics. The most important factors that determine the probability of particles passing out of the rumen are particle size and specific gravity (Dijkstra *et al.,* 2007).

A symbiotic relationship exists between the ruminant animal and rumen microbes that enables the digestion of up to 90% of insoluble structural carbohydrates including cellulose and hemicellulose, major constituents of plant cell walls, which cannot be

efficiently broken down by mammalian digestive enzymes (Waghorn and Barry, 1987). The first step of microbial mediated protein degradation in the rumen is the attachment of bacteria to feed particles, followed by activity of cell-bound microbial proteases. Approximately 70 to 80% of ruminal micro-organisms attach to undigested feed particles in the rumen, and 30 to 50% of those have proteolytic activity. *Butyrivibrio fibrisolvens* and *Prevotella ruminicola* are among the most proteolytic bacteria in the rumen (Stewart *et al.,* 1997). A large number of different microbial species attach to feed particles in an aggregation, acting symbiotically to degrade and ferment nutrients.

Products of carbohydrate fermentation feed the microbial population and provide energy to the ruminant via production of volatile fatty acids; acetate, propionate and butyrate. The volatile fatty acids are absorbed directly from the rumen and are transported via the gastric vein and subsequently the portal blood drainage to the liver.

The ruminant buffers the rumen fermentation to maintain a near neutral pH via secretion of saliva containing sodium bicarbonate. Saliva production is stimulated by increasing intake volume and duration of feeding and rumination (Carter 1990) with daily production between 6 to 16 litres in sheep (Kay 1960). A large proportion of rumen liquid pool also comes from the release of intracellular fluids from ingested plant material that typically comprise 80 to 90% water (Ulyatt and Waghorn, 1993).

The rumen degradation of the protein in fresh pasture, and the efficiency of its assimilation into microbial protein for use by the animal is of fundamental importance to ruminant nutrition. The protein available to ruminants is the sum of rumen un-degraded (bypass) protein and the protein rich bodies of rumen micro-organisms flowing out of the rumen. One goal of pastoral ruminant nutrition is to incorporate

rumen degradable protein (RDP) of temperate forages into microbial protein as efficiently as possible, thus decreasing nitrogen losses to the environment.

1.1.1 Forage Protein Composition

Forage proteins can be divided into three fractions in decreasing order of rumen degradability and nutritional value. Fraction 1, the dominant protein form, is the highly abundant leaf protein, ribulose-1, 5-bisphosphate carboxylase (Rubisco; EC 4.1.1.39). Ribusco, contained within chloroplasts, is the first enzyme in the photosynthetic carbon dioxide fixation in temperate grasses and legumes and constitutes about 40% of leaf protein (Mangan, 1982). Rubisco is soluble, rapidly degraded in the rumen (Nugent and Mangan, 1981) and provides a large proportion of the dietary protein of grazing livestock.

Fraction 2 protein is a complex mixture of leaf enzymes derived from both the chloroplasts and the cytoplasm. It constitutes about 25% of the total leaf protein (Mangan, 1982). Some of these proteins contribute to rumen bypass proteins due to their low rate of ruminal proteolysis (Nugent and Mangan, 1978).

Fraction 3 consists of insoluble chloroplast membrane proteins that constitute about 30% of the total leaf protein. Fraction 3 proteins are of low nutritional value and a high proportion by-pass rumen digestion due to their low solubility (Mangan, 1982).

1.1.2 Ruminant Protein Ingestion and Digestion

The digestion of forage proteins in the reticulorumen can be attributed to the combined processes of solubilisation and degradation (Min *et al.,* 2000). Solubilisation is the release of protein from plant cells into the rumen environment during chewing and is an important prerequisite for degradation (Mangan 1982). Degradation is the catabolism of protein by microbial and plant enzymes that yields peptides, amino acids and ammonia.

When fed to ruminants forage proteins are rapidly solubilised and release between 56 and 65% of their nitrogen content during mastication. Dietary nitrogen, is related to the crude protein content of a forage, it is assumed that each gram of crude protein contains 0.16 grams of nitrogen (Waghorn and Clark 2004). Min *et al.* (2000) reported that approximately 30% of the total nitrogen in white clover forage is instantly solubilised upon ingestion and the rate of rumen solubilisation of the remaining insoluble fraction is 15%/hour. Under these conditions the rumen digestion of carbohydrate is efficient yet 70% of the ingested nitrogen is degraded to ammonia in the reticulorumen, with only 30% outflowing to the lower digestive tract as bypass peptides and proteins or microbial protein (Min *et al.,* 2000; Waghorn and Barry 1987).

Peptides and amino acids arising from the intra and extracellular proteolytic activity in the rumen are transported inside microbial cells where peptides are further degraded to amino acids. Free amino acids can be incorporated into microbial protein or further deaminated to volatile fatty acids, CO_2 and ammonia. The fate of peptides and amino acids absorbed by rumen microbes is dependant upon the availability of energy from carbohydrate fermentation. If energy is available amino acids are transaminated or used directly for microbial protein synthesis (growth). However if energy is limiting the absorbed amino acids are deaminated and their carbon skeleton fermented to yield

volatile fatty acids. Non-protein nitrogen, composed of nitrogen present in DNA, RNA, ammonia, amino acids and small peptides is also used for microbial growth (Bach *et al.*, 2005).

The proteolytic activity of rumen fluid, the numbers of proteolytic bacteria and the predominant proteolytic species present are all influenced by diet (Broderick *et al.*, 2001). Nugent and Mangan (1981) found that changing the diet of cows from a hay and concentrate diet to fresh Lucerne caused a ninefold increase in proteolytic activity associated with a change in the predominant proteolytic bacteria from *Butyrivibrio fibrisolvens* to *Streptococcus bovis*. It has also been reported that reducing the rumen degradability of supplemental protein by replacing casein with ovalbumin results in a 30% decrease in rumen proteolytic activity and an increase in the growth of *Butyrivibrio fibrisolvens* (Wallace *et al.*, 1987).

Until 1996 the accepted dogma of rumen protein degradation was that microbial proteases mediated the hydrolysis of plant proteins once they were released from cells and exposed to the rumen environment. However a new concept suggests an important role of plant proteases in the initial breakdown of proteins in fresh forages (Theodorou *et al.*, 1996). Kingston-Smith and Theodorou (2000) theorise that plant material ingested by grazing ruminants is largely composed of intact plant cells that remain metabolically viable within the rumen. These ingested cells respond to the 39°C, dark and anaerobic conditions of the rumen resulting in a period of proteolysis that releases small peptides and amino acids into the rumen fluid via diffusion. Eventually the combined actions of rumination and microbial cellulotic degradation ruptures the cell wall and exposes the remaining intracellular proteins to microbial proteolytic enzymes

(Figure 1.1). Once exposed to the rumen environment the solubility of plant proteins determines the extent to which it is hydrolysed.

Zhu *et al.* (1999) detected similar rates of Rubisco degradation in both the presence and absence of rumen micro-organisms *in vitro*, and Kingston-Smith *et al.* (2005) reported the disappearance of 95 to 97% of leaf protein from dialysis tubing and polythene bags where rumen microbes were excluded, providing evidence that plant proteases contribute significantly to plant protein breakdown in the rumen. Wallace *et al.* (2001) reported that while the rate of protein breakdown by plant proteases is low (estimated as being 10% of that usually observed in the rumen fluid) their location within plant cells provides plant proteases with a disproportionate contribution to the initial stages of protein degradation in the rumen.

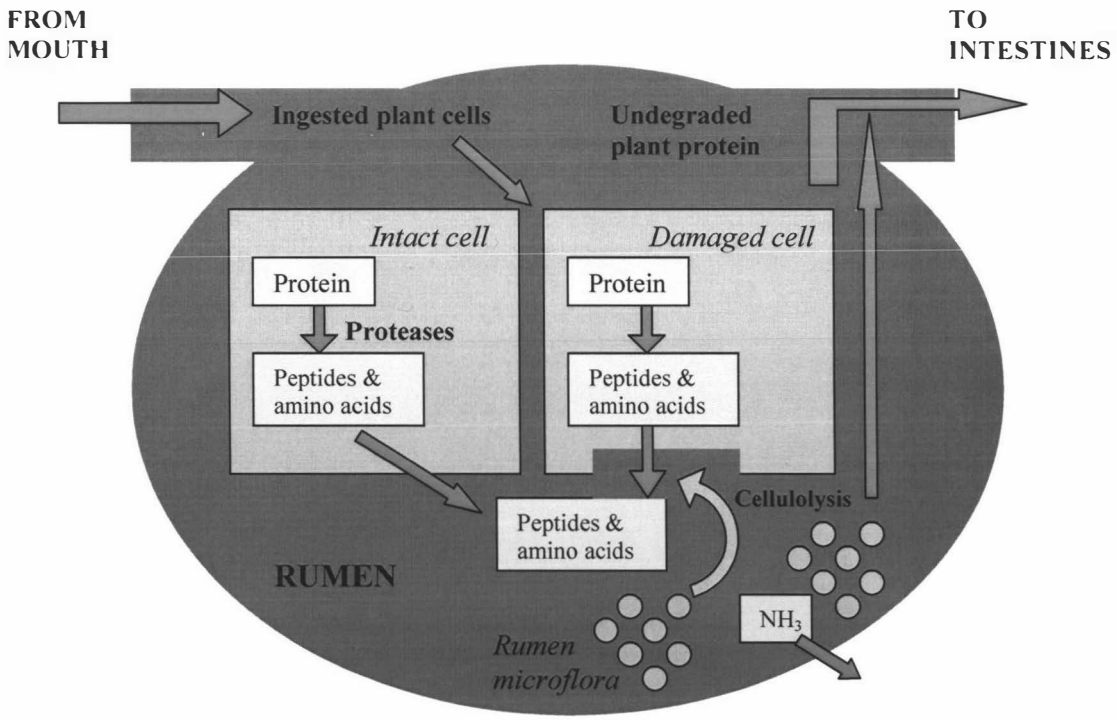

Figure 1.1. Model of forage protein degradation in the rumen: the involvement of plant and microbial processes (Kingston-Smith and Theodorou 2000).

Rumen nitrogen output consists of ammonia nitrogen, undegraded plant protein and microbial protein. Microbial protein synthesis provides the majority of protein supplied to the small intestine accounting for 50 to 80% of total absorbable protein (Storm and Ørskov, 1983). When intake of rumen degradable energy substrates is insufficient to match the energy requirements for microbial growth, protein is used as an energy yielding substrate and degraded to ammonia which is absorbed, metabolised to urea and excreted in the urine. Losses of ammonia nitrogen can be reduced by decreasing protein degradation in the rumen or increasing nitrogen use by rumen micro-organisms (Bach *et al.,* 2005).

Until recently proteolysis has been considered the rate limiting step in ruminal protein degradation. However, Broderick *et al.* (1991) demonstrated that rapidly degraded proteins may result in the accumulation of peptides and amino acids within the first two hours following feeding, suggesting that the rate of deamination may play an important role in the control of protein degradation. Cardozo *et al.* (2004) reported that the concentration of peptides, amino acids and ammonia were within the same range for up to eight hours post feeding, suggesting that amino acid uptake could be a limiting factor of protein degradation in the rumen.

The most important factors affecting microbial protein degradation are the type of protein, nutrient interaction (energy yielding substrates) and the predominant microbial population (dependant upon diet, rumen passage rate and rumen pH) (Bach *et al.,* 2005).

1.1.3 Effect of Forage Species on Nutrient Digestion

The two primary temperate forage plants, perennial ryegrass (*Lolium perenne*) and white clover (*Trifolium repens*) have marked differences in their rumen degradation when fed in the fresh form (Table 1.1).

Table 1.1 Comparison of the composition, and rumen digestion characteristics of the predominant temperate forage species when fed in fresh form to growing cattle (Beever et al., 1986).

	Perennial ryegrass *Lolium perenne*	White clover *Trifolium repens*
Chemical composition		
Organic matter (g/kg DM)	893	903
Water soluble carbohydrate (g/kg)	179	93
Cellulose (g/kg DM)	271	327
Lignin (g/kg DM)	22	30
Total nitrogen (g/kg DM)	23	46
Intakes		
Organic matter (g/kg LWT)	15.5	21.7
Nitrogen (g/kg LWT)	0.37	1.01
Apparent digestibility		
Organic matter	0.823	0.832
Nitrogen	0.662	0.818
Instantly degradable fraction		
Organic matter (g/g)	0.157	0.498
Nitrogen (g/g)	0.117	0.508
Rumen fermentation characteristics		
Volatile fatty acids (mmol/L)	110	124
Ammonia nitrogen (mg/L)	44	278

Of particular relevance to the current review is the marked difference between these forages in the relative efficiency of nitrogen utilisation. For example the mean flow of non-ammonia nitrogen to the small intestine of cattle is almost 30% higher when fed clover diets compared to grass diets (Beever *et al.,* 1986). Total nitrogen consumption on clover diets is almost twice that estimated for grass diets (1.18 vs 0.62 g/kg LWT per day). The increased nitrogen intake on clover diets is a consequence of both the higher nitrogen content of clover (60% higher than ryegrass) and increased (40%) organic

matter intake of animals fed white clover (Beever *et al.*, 1986). However substantial losses of this additional dietary nitrogen supply results in a much smaller post-rumen non-ammonia nitrogen yield than may otherwise be expected. Beever *et al.* (1986) reported that cattle fed early season perennial ryegrass or white clover synthesised microbial nitrogen at a rate of 1.12 or 0.59 g/g of nitrogen intake respectively. A major cause of the reduced rate of synthesis from white clover compared to ryegrass protein substrates is the high solubility of clover nitrogen constituents which are rapidly degraded to ammonia. Ammonia production from highly digestible forages exceeds the capacity of rumen microbes to assimilate the available nitrogen into microbial protein (Beever and Siddons, 1986).

Miller *et al.* (2001) compared two cultivars of perennial ryegrass, differing by 40 g/kg DM in water soluble carbohydrate concentration. A higher proportion of nitrogen intake was excreted in the milk (0.07 g/g) of cows fed the grass higher in water soluble carbohydrate. This can be explained by a higher efficiency of rumen microbial synthesis due to a more synchronous balance of nitrogen to energy-yielding substrates. Kolver *et al.* (1998) reported that a transient improvement in capture of rumen nitrogen can be achieved via synchronisation of the dietary nitrogen to carbohydrate ratio through supplementation resulting in a 33% reduction in peak post-prandial rumen ammonia concentration.

These results demonstrate that efficiency of nitrogen utilisation by the pasture fed ruminant can be improved by balancing the availability of rumen-degradable nitrogen and energy through supplementation with energy supplying concentrates. Steinshamn *et al.* (2006) achieved increases in nitrogen utilisation (11.7 g nitrogen per kg nitrogen intake per kg DM concentrate fed) and decreases in urinary nitrogen excretion (4.0g/kg

DM concentrate fed) by dietary supplementation with 1.8 kg DM of grain based concentrate. This study demonstrates the improvement in total nitrogen utilisation that can be achieved through increasing the supply of rumen fermentable energy substrates for microbial growth.

1.1.4 Asynchrony of Protein and Energy Digestion

A characteristic of the rumen fermentation of highly digestible fresh forages is an asynchrony between the rapid rate of proteolysis relative to energy generation by celluloysis (Kingston-Smith and Theodorou, 2000). These conditions limit growth of the rumen microbial population as rumen microbes use carbohydrates as their primary source of energy (Tas *et al.,* 2006). This process results in pulsatile fermentation in the rumen of animals grazing pasture following each feeding bout.

The carbohydrates of fresh forages are mainly present as soluble sugars, fructosans and cell wall polysaccharides. Upon entering the rumen environment the water soluble carbohydrate fraction is almost instantly and completely degraded (Boudon *et al.,* 2002) whilst the degradability of structural polysaccharides is considerably slower. The difference between the nitrogen and carbohydrate content in temperate pasture plants and the rate of solubilisation and degradation causes an imbalance in the relative supply of rumen-degradable protein and energy (Tas *et al.,* 2006). The resulting loss of nitrogen as ammonia from the rumen represents a loss of dietary protein of up to 0.35 g/g of protein intake (van Vuuren *et al.,* 1992). Nitrogen losses are magnified when the crude protein content of the diet exceeds approximately 210 g/kg of digestible organic matter (Beever *et al.,* 1986; Ulyatt *et al.,* 1988). For this reason the production of high protein ruminant meat products from temperate pastures is relatively inefficient with

only 20 to 30% of ingested nitrogen accounted for by gains in body mass (Dewhurst *et al.*, 1996).

In theory the rumen degradation of protein and carbohydrate from ingested forages must be synchronised to optimise microbial protein synthesis and allow the most efficient use of dietary nitrogen (Min *et al.*, 2000). However it is likely that in the complex ecosystem of mixed ruminal micro-organisms when nutrient supply is synchronised for a specific subpopulation, it may not be synchronised for other populations, therefore average microbial efficiency remains fairly stable (Bach *et al.*, 2005).

The fermentation of ingested plant material in the reticulo-rumen confers an adaptive advantage to the ruminant animal enabling its subsistance on poor quality (low-protein) diets through the microbial synthesis of protein from non-protein nitrogen (MacRae and Ulyatt 1974). Also a high proportion of plant proteins are trapped in a fibre matrix that needs to be degraded before microbial proteases can gain access to proteins for degradation.

A combination of cellulolytic and proteolytic enzymatic activity is required for maximum protein degradation (Bach *et al.*, 2005). Cellulolytic microbes that degrade structural carbohydrates have low maintenance requirements, grow slowly and use ammonia as their main nitrogen source, whereas amylolytic micro-organisms that degrade nonstructural carbohydrates (starch) have higher maintenance requirements, grow rapidly and use ammonia, peptides and amino acids as nitrogen sources (Russell *et al.*, 1992). When high-concentrate rations are fed, starch degrading bacteria predominate and fibre digestion is limited by the reduced number of celluloytic bacteria, thus reducing the degradation of protein. Therefore this nutrient-microbial interaction

may indirectly effect protein degradation by altering both the pH and predominant microbial population.

Reduction in cellulolytic bacteria as a consequence of low pH leads to a reduction in fibre degradation, reducing access of proteolytic bacteria to proteins, indirectly diminishing protein degradation (Bach *et al.,* 2005).

1.1.5 Microbial Protein Synthesis

Rumen microbes require nitrogen and energy for growth. When rumen degradable protein intake is adequate, carbohydrate availability determines microbial protein synthesis in the rumen and efficiency of ammonia utilisation (Firkins *et al.,* 2006). Optimal microbial growth in the rumen occurs at around 25 g nitrogen/kg organic matter truly digested in the rumen (Czerkawski, 1986), and 32 g nitrogen/kg carbohydrate degraded in the rumen (Sinclair *et al.,* 1991). Nitrogen arising at higher levels exceeds the protein assimilation capacity of the microbial population and increases the rumen ammonia pool and loss of non-protein nitrogen from the animal as urea.

The most common assessment of efficiency of microbial protein synthesis is determination of grams of microbial nitrogen per unit of rumen available energy, typically expressed as true organic matter or carbohydrates fermented. In addition to the efficiency of microbial protein synthesis, the efficiency of nitrogen use is a good measurement for describing the efficiency of nitrogen capture by ruminal microbes. Optimal bacterial growth in the rumen occurs when efficiency of microbial protein synthesis is 29 g of bacterial nitrogen/kg of fermented organic matter and efficiency of

nitrogen use is 69%, implying that bacteria would require about 1.3 x rumen-available nitrogen per unit of bacterial nitrogen (Bach *et al.,* 2005).

1.1.6 Involvement of Protozoa in Protein Digestion

Protozoa can account for about 40% of the rumen microbial biomass (Russell and Rychlik, 2001) and play a major role in protein degradation due to their high capacity for proteolytic and deaminase activities (Ushida *et al.,* 1984). Protozoa engulf large molecules; protein, carbohydrate and bacteria and thus also regulate bacterial nitrogen turnover within the rumen.

Reduction in the protozoal population leads to an increase in the bacterial population and the outflow protein from the rumen, due to more ammonia being assimilated into microbial protein when the bacterial population is increased (Newbold and Hillman, 1990).

Protozoa contribute little to the total microbial outflow from the rumen (from 5 to 15% according to Leng (1989) and up 20% according to Ushida *et al.,* (1984). Thus reducing the protozoal biomass in the rumen may lead to increased availability of microbial protein to the host. This is supported by Santra and Karim (2000) who demonstrated an increase in ruminant nitrogen retention as a consequence of protozoal defaunation.

1.1.7 Protein Degradation and End-Products

The proteolytic activity in rumen fluid is primarily the result of bacterial enzymes. The predominant proteolytic bacteria are; *Streptococcus bovis, Prevotella ruminicola, Ruminobacter amylophilus and Butyrivibrio fibrisolvens* (Wallace and Brammall, 1985). The resultant amino acids are either incorporated into microbial proteins,

degraded further via deamination in a process that yields ammonia, or by-pass rumen digestion and leave the rumen intact.

The source of dietary amino acids for the host animal is the outflow of microbial protein, un-degraded dietary protein, peptides and amino acids to the abomasum. Rumen digestion of fresh forages typically results in degradation of 70% of crude protein content with approximately 30% escaping the reticulorumen (Waghorn and Barry, 1987) and entering the abomasum for peptic digestion and absorption in the small intestine.

Forage derived amino acids in excess of requirements for microbial protein synthesis are deaminated to provide energy for growth of the microbial population. The fate of ingested nitrogen is described in Table 1.2, where fresh forage of either high or low protein content was offered to sheep. These results illustrate the inefficiency of nitrogen utilisation by ruminants fed high quality fresh forages.

The deamination of amino acids yields ammonia and other non-protein nitrogenous compounds. Ammonia is toxic and cannot be used by the animal, some is eliminated in the faeces however the majority is rapidly absorbed from the rumen. Some absorbed ammonia is exhaled in the breath but the bulk is converted to urea via an energy consuming process and excreted in the urine (Greaney, 1996).

Table 1.2. Calculated absorption of amino acids from the small intestine and nitrogen balance of sheep fed 1 kg dry matter of fresh forage containing 12.5 and 25% crude protein (Waghorn and Barry, 1987).

	Crude protein (%)	
	12.5	25
Dry matter intake (g)	1000	1000
Metabolisable energy of diet (MJ ME/kg DM)	10	11.5
Nitrogen intake (g/d)	20	40
Nitrogen in faeces (g/d)	4.0	6.4
Nitrogen in urine (g/d)	15.0	30.6
Nitrogen retained (g/d)	1.0	3.0
Non-ammonia nitrogen at duodenum (g)	17.4	28.6
Protein nitrogen absorbed from feed (g)	4.4	7.3
Protein nitrogen absorbed from microbes (g)	6.7	11.0
Total protein nitrogen absorbed (g)	11.1	18.3
(g protein absorbed)	(69)	(114)
Metabolisable energy of protein absorbed as amino acids (KJ/d)	1368	2186
(% of metabolisable energy intake)	(14%)	(19%)
Approximate loss of amino acids between intestinal lumen and portal blood (g nitrogen /d)	7.2	7.2
Amino acid entering portal blood (g nitrogen /d)	3.9	11.1
Amino acid entering portal blood (g/d)	28	81

[1]Net protein absorption. Assumptions: (1) Metabolisable energy of protein = heat of combustion (2740 kJ/mole) – heat of combustion of urinary urea, and (2) 115 g protein yields 133 g amino acid upon hydrolysis.

1.2 Rumen Skatole Biosynthesis

1.2.1 Tryptophan Degradation and Skatole Formation

1.2.1.1 L-tryptophan the precursor to skatole formation

The production of skatole from the amino acid tryptophan was discovered during research into a bovine pulmonary disease, known as acute bovine pulmonary edema and emphysema, a naturally occurring and often fatal affliction of goats, cattle and to a much lesser degree, sheep (Dickinson *et al.,* 1976; Carlson *et al.,* 1968; Bradley *et al.,* 1978).

Acute bovine pulmonary edema and emphysema is observed following an abrupt change in diet when ruminants are introduced to rapidly digestable forages from a diet of poor quality or fibrous roughage (Carlson *et al.,* 1968). Furthermore it has been demonstrated that oral tryptophan administration can cause similar clinical pulmonary toxicity resulting in death. However tryptophan dosed to cattle via either intra-peritoneal or intra-venous routes does not induce pulmonary toxicity (Carlson *et al.,* 1968) illustrating the importance of the rumen metabolism of tryptophan in the development of tryptophan-induced toxicity.

The *in vitro* incubation of L-tryptophan with fresh strained bovine rumen fluid under anaerobic conditions identified skatole as the major fermentation product (Carlson *et al.,* 1972). Administration of skatole by either intrarumen or intravenous routes resulted in the development of cellular tissue damage and clinical symptoms identical to those observed following oral administration of tryptophan, thus identifying skatole as a toxic derivative of tryptophan metabolism in the rumen (Carlson *et al.,* 1972).

To determine if a change in diet alone would result in elevated levels of skatole in cattle, Hammond *et al.* (1979) adapted cattle to a diet of poor quality summer pasture then abruptly shifted them to a pasture of highly digestible vegetative forage. This experiment replicated the conditions responsible for the development of naturally occurring bovine pulmonary disease. Although no quantitative measurement of the relative qualities of these two pasture types was reported, it can be assumed that the rumen degradable protein content of the vegetative pasture was much greater than the dry summer pasture. The change in pasture quality from summer dry to vegetative resulted in an increase in mean rumen skatole concentration from 0.1 μg/mL to a peak of 1.8 μg/mL, causing cattle to develop clinical signs of toxicity in 78 hours (Hammond *et al.,* 1979) demonstrating that forage quality alone has a dramatic effect on the generation of skatole. This study however, did not ascertain if this short-term elevation in rumen skatole formation was due to either a greater skatole forming potential of the predominant rumen microbial population that existed under roughage feeding conditions; or simply an increase in the availability of tryptophan due to the greater rumen degradable protein content of the lush forage.

Tryptophan constitutes 1.2 to 2.3% of the total amino acid in plant leaf protein, whilst the fraction 1 leaf protein Rubisco contains 186 mg tryptophan/g of protein nitrogen (Mangan 1982). Therefore the bacterial production of skatole from tryptophan deamination is proportional to the rumen degradability of forage derived tryptophan.

Honeyfield and Carlson (1990b) have demonstrated that the quantity and nutrient composition of forage fed to cattle for 3 weeks prior to an oral dose of tryptophan alters the rumen production of skatole. The increased capacity of rumen micro-organisms to produce skatole was not known but could be related to changes in relative bacterial

populations (i.e. increased number of skatole producing organisms) or a metabolic change within the skatole producing microbes.

1.2.1.2 Microbial conversion of L-tryptophan to skatole

In vitro studies have shown that the major pathway of ruminal tryptophan degradation involves conversion to indoleacetic acid which is subsequently decarboxylated to skatole (Yokoyama and Carlson, 1974; Carlson and Breeze 1984 Tavendale *et al.*, 2005). These studies demonstrated that skatole, indole and indoleacetic acid are the major metabolites of tryptophan fermentation by rumen micro-organisms and that skatole is the main metabolite produced. The incubation of ^{14}C-tryptophan with rumen fluid confirms that skatole is formed as the major fermentation product in addition to a smaller amount of indoleacetic acid and indole (Carlson *et al.*, 1972). The major route of skatole formation from tryptophan is a two-step process involving the initial formation of indoleacetic acid from tryptophan, and the subsequent decarboxylation of indoleacetic acid to skatole (Figure 1.2) by an enzyme that has not yet been identified (Yokoyama and Carlson, 1974; Deslandes *et al.*, 2001).

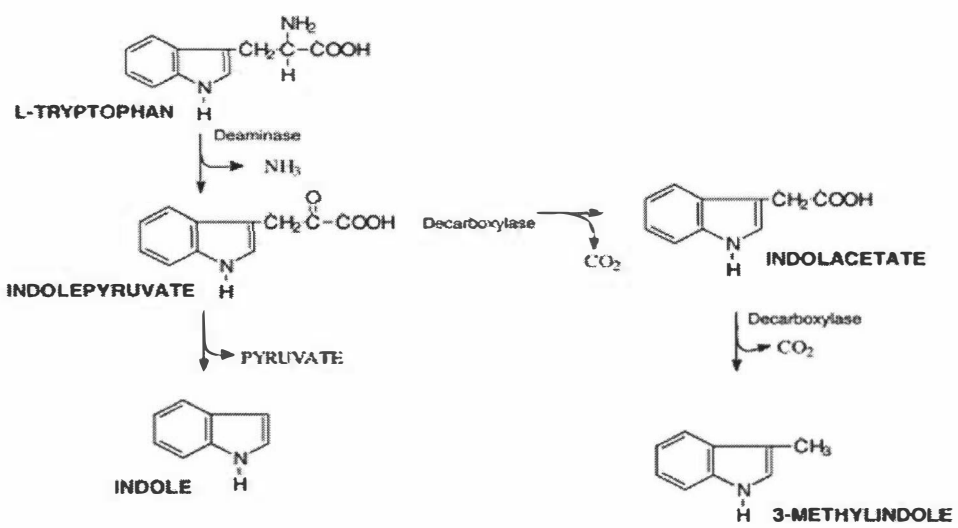

Figure 1.2. Tryptophan fermentation leading to the formation of 3-methylindole (skatole) and indole (Deslandes et al., 2001).

The formation of skatole in the rumen occurs relative to the availability of tryptophan for bacterial deamination and *in vivo* rumen concentrations of up to 9.0 μg/mL have been measured following intrarumen tryptophan administration (Yokoyama *et al.*, 1975).

Recently it has been demonstrated that the formation of skatole from tryptophan is isomer specific. The incubation of both D- and L- isomers of tryptophan with rumen bacteria and protozoa demonstrated that D-tryptophan was not degraded by these rumen micro-organisms and thus no skatole was produced from this isoform. In contrast L-tryptophan was readily degraded by a mixed suspension of rumen bacteria and protozoa that produced skatole at a rate of 6 μmol/g microbial nitrogen/h (Mohammed *et al.*, 2003).

Additions of various indolic compounds to *in vitro* incubations containing rumen inocula has identified the major precursors of skatole formation by rumen microbes. The addition of tryptophan, indole pyruvic acid, indole acetic acid, indole acetamide and indole carbinol resulted in the production of skatole ranging in mean molar yields of 29 to 54% after 8 hours of incubation (Tavendale *et al.*, 2005; Table 1.3).

Table 1.3. Skatole molar yield from various indolic precursors spiked into rumen inocula and incubated for 10 hours (Tavendale et al., 2005).

Compound[A]	Yield of skatole (%)	Control[B] (μM)
Casein	28.7***	0
L-Tryptophan	54.2***	1.2
Indole-3-pyruvic acid	41**	n.d.
Indole-3-acetic acid	29.4***	0.8
Indole-3-acetamide	42.1**	n.d.
Indole-3-carbanol	28.6***	n.d.

Significance of formation relative to control: ** $P < 0.01$; *** $P < 0.001$.
n.d. compound not detected.
[A]Concentration of precursor after spiking: 380 μM.
[B]Concentration of precursor in absence of added substrates.

Initial concentration of L-tryptophan (380 μM) decreased rapidly (67 μM/h) in incubation with rumen inocula and indole acetic acid was formed at a rate of 31 μM/h, reaching a maximum concentration (70 μM) following 2 hours of incubation. Concurrently skatole was formed rapidly (27 μM/h) for 6 hours, consistent with indole acetic acid acting as an intermediate compound in the transformation of tryptophan to skatole. The addition of indole acetic acid to rumen inocula confirmed its role as a precursor of skatole formation. Incubation of L-tryptophan did not result in detectable formation of indole pyruvic acid, indole acetamide or indole carbinol indicating that they are not end products of microbial fermentation but may be short-lived intracellular intermediates. A biochemical pathway from L-tryptophan to skatole through the intermediate indole acetic acid was proposed by Tavendale *et al.* (2005) (Figure 1.3).

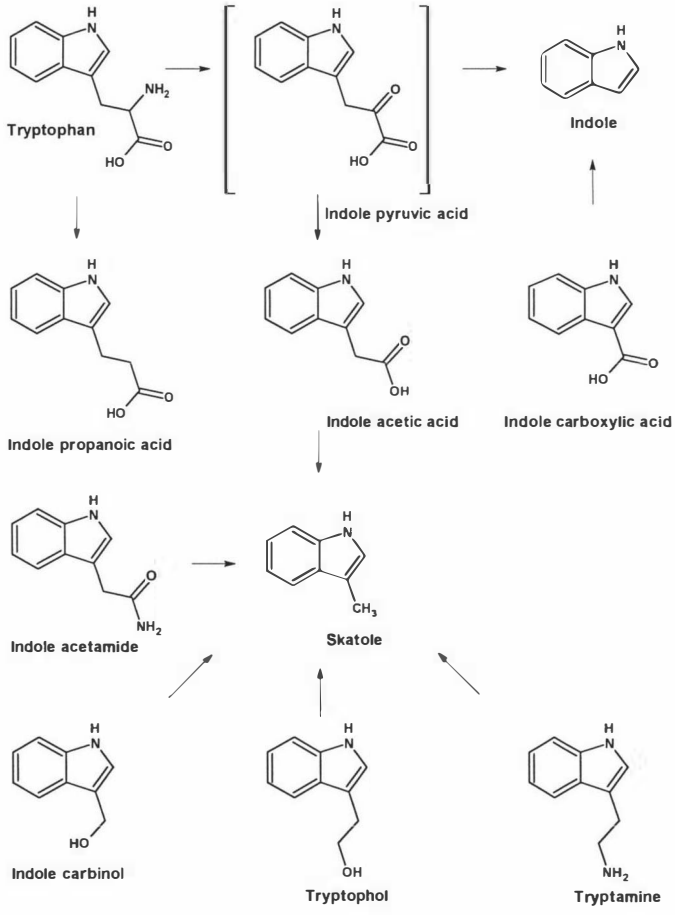

Figure 1.3. Proposed transformation pathway of L-tryptophan to skatole and indole via intermediate indolic compounds (Tavendale et al., 2005).

1.2.2 Microbes and Microbial Interaction in Skatole Formation

1.2.2.1 Bacteria involved in skatole formation

Many bacteria are capable of producing the skatole precursor indoleacetic acid from L-tryptophan, however until recently only six bacterial species capable of producing skatole had been identified. These are; *Lactobacillus* sp. Strain 11201, *Clostridium scatologenes*, *Clostridium nauseum*, *Pseudomonas* sp., *Rhizobium* sp. and *Lactobacillus helveticus* (Deslandes *et al.,* 2001). Of these *Clostridium scatologenes* is the only isolated bacterium known to produce skatole directly from tryptophan (Jensen *et al.,* 1995) however this bacteria has not been found in the rumen population.

The bacteria recognised as responsible for skatole production in ruminants is *Lactobacillus* sp. strain 11201 (Deslandes *et al.,* 2001), it is an obligate anaerobe and cannot form skatole directly from L-tryptophan. Following the catabolism of L-tryptophan to indoleacetic acid *Lactobacillus* sp. 11201 catabolises indoleacetic acid to form skatole. Yokoyama *et al.* (1977) isolated four strains of the *Lactobacillus* species from a tryptophan enrichment of bovine rumen fluid from rumen fistulated steers fed a Lucerne hay diet. The bacteria were gram-positive, non-motile and non-spore-forming rods (Figure 1.4).

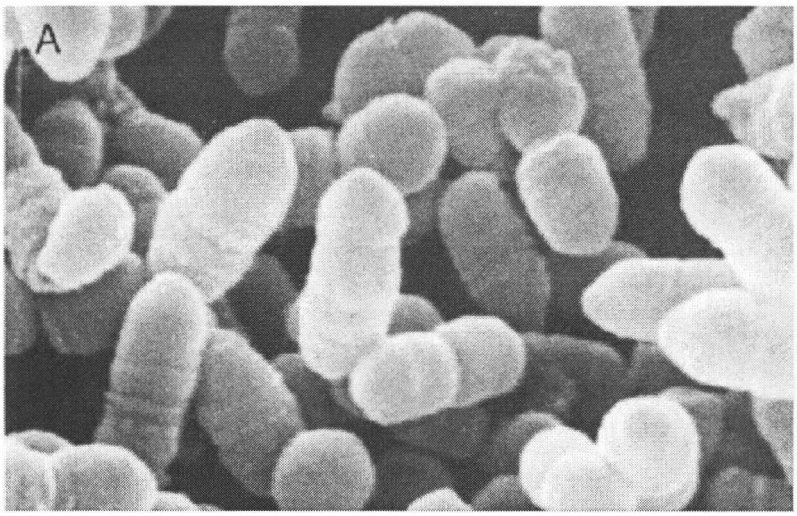

Figure 1.4. A scanning electron micrograph of Lactobacillus *sp. strain 11201 after treatment with sonication. Magnification x24,000 (Honeyfield and Carlson 1990a).*

Honeyfield and Carlson (1990a) demonstrated that skatole formation is not a primary function of *Lactobacillus* sp. 11201 as enzymatic formation of skatole occurred only when the bacteria were incubated with the substrate indoleacetic acid. The maximal enzymatic formation of skatole from indoleacetic acid occurred at a rate of 3.2 nmol/minute when indoleacetic acid was incubated with 50 µg/mL of microbial protein. Under experimental conditions increasing concentration of indoleacetic acid above 1 mmol saturated the enzyme (Figure 1.5).

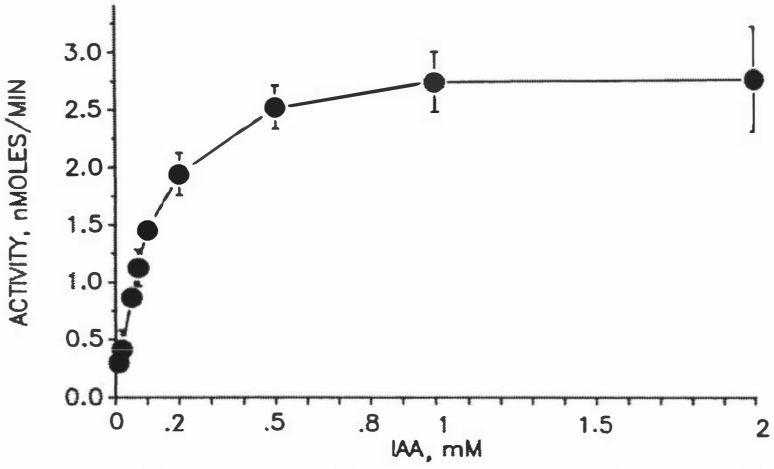

Figure 1.5. Enzymatic formation of skatole with 50 µg/mL of Lactobacillus *sp. strain 11201 protein from the pellet of sonicated cells with increasing concentration of indoleacetic acid (Honeyfield and Carlson 1990a).*

Increasing skatole concentration had a negative feedback effect on both the proliferation of *Lactobacillus* sp. 11201 cells and the ability of the bacterium to produce skatole from indoleacetic acid. This effect was demonstrated through addition of exogenous skatole (Figure 1.6).

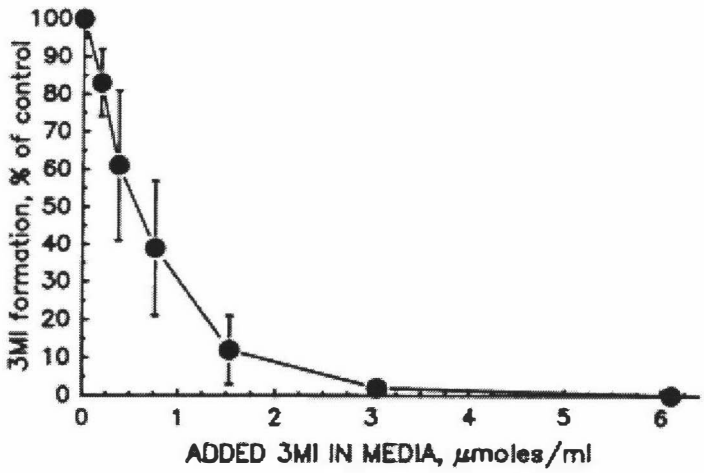

Figure 1.6. Effects of increasing concentration of skatole on the ability of Lactobacillus *sp. strain 11201 to produce skatole from indoleacetic acid (5.7 mM indoleacetic acid; incubated for 24 h) (Honeyfield and Carlson 1990b).*

Attwood *et al.* (2006) screened a culture collection of proteolytic and ammonia hyper-producing rumen bacteria for the production of indolic compounds. Of the bacteria screened none produced skatole, but *Clostridium sticklandii* produced indoleacetic acid from tryptophan degradation. However they were able to identify five skatole producing bacteria from the rumen of sheep and cattle grazing ryegrass-white clover pasture, using tryptophan enrichments of strained rumen fluid. A single skatole forming bacterium was isolated from a sheep rumen. This bacteria designated TrE7262, has a 98% 16s rRNA homology with *Clostridium sporogenes* ATCC 3584. The further four bacterial isolates came from the rumens of dairy cattle. These isolates have been identified based upon the homology (>90%) of their 16s rRNA sequence as; *Prevotella dentalis* DSM 3688, *Clostridium aminophilum* F, *Actinomyces meyeri* CIP 103148 and *Clostridium sporogenes* ATCC 3584. The isolates identified as *Clostridium sporogenes* from both

sheep and cattle share a 99.1% homology in 16s rRNA sequence and both form skatole from indoleacetic acid. Both *Clostridium aminophilum* and *Actinomyces meyeri* are capable of producing trace skatole quantities from tryptophan, however they produce more skatole when indoleacetic acid is provided as substrate. The identification of the role of these bacteria in rumen skatole formation is a significant advance in the understanding of skatole formation in ruminants.

Lactobacillus sp. strain 11201 could not be isolated from the rumen contents of the pasture-fed sheep and cattle studied by Attwood *et al.* (2006). The authors speculate that this may be due to the toxicity of skatole to bacteria of the *Lactobacillus* species or may reflect the presence of different rumen bacterial populations producing indolic compounds in ruminants consuming fresh forages. Skatole acts as a non competitive inhibitor of the enzyme responsible for its formation. Skatole concentrations above 66 μg/mL lyse many bacteria and slow the growth of *Lactobacillus* sp. 11201 however it is not lysed in the presence of 600 to 700 μg skatole/mL (Honeyfield and Carlson 1990b). Resistance to these concentrations of skatole suggests that the failure to isolate *Lactobacillus* sp. from the rumen contents of pasture-grazed ruminants may be due to changes in bacterial populations rather than the negative feedback of skatole specifically upon *Lactobacillus* sp. 11201. Additional research is required to define the contribution these bacteria make to the formation of skatole in ruminants grazing pastures with high rumen degradable protein content and factors influencing the rate of skatole formation.

1.2.2.2 Interaction in mixed-microbial suspensions

Investigation of tryptophan metabolism by mixed rumen protozoa, bacteria and their combination has enabled the identification of the relative contribution of these micro-organisms to the production of skatole. Mohammed *et al.* (2003) and Okuuchi *et al.* (1993) have demonstrated that skatole is formed solely by rumen bacteria and not by protozoa, however the interaction of the two micro-organisms in mixed cultures has relevance to the *in vivo* production of skatole from tryptophan in grazing ruminants. Okuuchi *et al.* (1993) found that rumen protozoa have an excellent ability to transaminate indolepyruvic acid to yield microbial tryptophan thus stimulating the bacterial production of indoleacetic acid and skatole. Mohammed *et al.* (2003) reported that 43% of tryptophan was converted to skatole in mixed bacterial and protozoal cultures after 24 hours of incubation. This is close to the value of 39% conversion reported by Yokoyama and Carlson (1974) during the 24 hour incubation of radio-labelled tryptophan. The combination of protozoa and bacteria clearly stimulates the bacterial conversion of tryptophan to skatole. The rate of skatole formation from tryptophan is twice as high in mixed suspensions of bacteria and protozoa than bacteria alone (6 vs. 3 μmol/g microbial nitrogen/h) following a 12 hour incubation period (Mohammed *et al.*, 2003).

1.2.2.3 Rumen environmental effects on skatole formation

Rumen conditions that alter the bacterial population have an indirect effect upon rumen skatole production. Forage quality alone has been shown to affect skatole production by altering the fermentation conditions of the rumen and the avalibility of skatole precursor, tryptophan (Hammond *et al.*, 1979).

Different skatole producing bacteria have been isolated from the rumen of animals fed fibrous or highly digestible forages (Yokoyama *et al.,* 1977; Attwood *et al.,* 2006). It is unknown why skatole production differs due to changes in diet and how much this is influenced by changes in bacterial population. The direct effects of some rumen conditions on skatole production have been examined. Honeyfield and Carlson (1990b) observed a moderate reduction in growth of *Lactobacillus* sp. 11201 at pH 5.5, compared with 7.5, and found an 18% reduction in skatole forming enzyme activity at pH 5.7 compared to the activity at pH 7.5. Hammond *et al.* (1984) also reported an effect of pH on the *in vitro* skatole production of mixed rumen micro-organisms and measured maximal conversion of tryptophan to skatole at pH levels near to neutral. Yokoyama and Carlson (1974) tested the effect of increasing concentrations of glucose (0 to 110 mM) on skatole formation during *in vitro* incubations with strained rumen fluid. Increasing glucose concentrations resulted in a progressive decrease in tryptophan conversion to skatole of up to 24% and an increase in indoleacetic acid production by up to 7%. These results indicate that increasing glucose concentration reduces the conversion of indoleacetic acid to skatole to a greater extent than the degradation of tryptophan to indoleacetic acid (Yokoyama and Carlson 1974). However these results are confounded by the effect of pH decrease from 6.1 in the control to 4.2 in the flask containing the highest glucose concentration. This may explain why attempts to control ruminal production of skatole *in vivo* by administration of readily fermentable carbohydrates have not proven effective (Hammond *et al.,* 1978). Bray and Emmerson (1994) speculate that the increased capacity for mixed rumen micro-organisms to produce skatole at decreasing levels of glucose concentration may be evidence of selective inhibition within the microbial population that favours skatole-forming organisms. This observation may explain the pronounced short term amplification of

skatole production when ruminants fed poor quality forage are abruptly reintroduced to highly digestible fresh forage.

1.2.3 Effect of Diet on Skatole Formation

Formation of skatole in the rumen and its appearance in the blood is directly related to the solubility and rumen degradability of forage proteins. Schreurs *et al.* (2007c) have demonstrated that peak rumen skatole concentration per unit of crude protein intake is significantly higher when sheep are fed white clover compared to perennial ryegrass or the tannin containing forage *Lotus corniculatis* (164, 67 and 48 μg/kg crude protein intake) due to the differing rate of protein degradation of these forages (Figure 1.7). The formation of skatole from white clover is also more rapid than from perennial ryegrass, attaining its peak concentration 90 minutes earlier. The high rumen skatole formation observed from low intake of white clover (132 g DM per feed) in this study indicates that white clover contributes a large proportion of the rumen degradable protein for skatole formation from New Zealand rye-clover pastures.

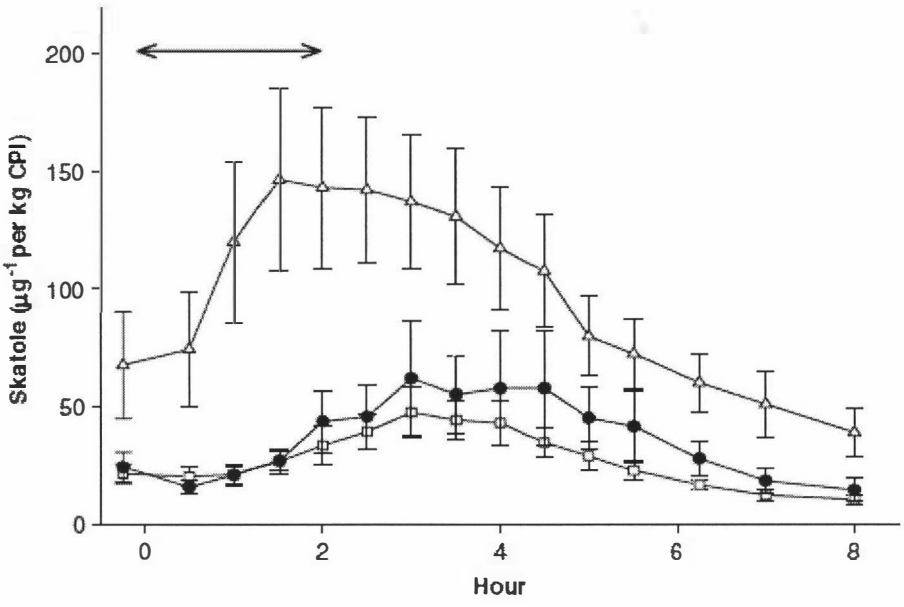

Figure 1.7. Mean concentration of skatole adjusted for crude protein intake in the rumen of sheep (n=6) fed white clover (Δ), *perennial ryegrass* (●) *or* Lotus corniculatus (□). *Error bars are the standard error of the mean; the double-ended arrow indicates the time over which feeding took place (Schreurs* et al., *2007c).*

The condensed tannin (CT) content of *Lotus corniculatus* (14 g/kg DM) slowed protein degradation and reduced both ammonia and skatole formation compared to non-tannin forages perennial ryegrass and white clover (Schreurs *et al.*, 2003).

CTs are polymers of flavan-3-ol (e.g. catechin) or flavan-3,4-diol (proanthocyanidins) linked by carbon-carbon or carbon-oxygen-carbon bonds (Figure 1.8). The polymers are highly variable in their constituent monomers, stereochemistry, size and intermolecular linkages in addition to their distribution within the plant. The CTs of *Lotus corniculatus* consist of relatively homogenous polymers of epicatechin-type procyanidin units (McNabb *et al.*, 1998).

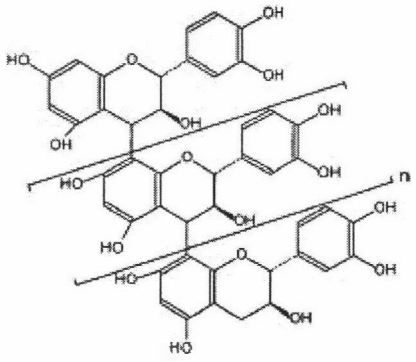

Figure 1.8. Structure of condensed tannin polymer (McMahon et al., 2000).

CTs have a strong affinity for hydrogen bonding with other compounds including amino acids. Bonding affinity varies for individual amino acids and tannins bind to one or more sites of a protein changing its conformation. Such conformational changes protect proteins from rumen microbial degradation by inducing steric interference and preventing enzymatic hydrolysis (McMahon *et al.*, 2000).

Degradation of the dominant soluble protein of fresh forages, Rubisco, is also significantly reduced by CTs from *Lotus corniculatus and L. pedunculatus.* Protection of Rubisco via the formation of tannin-protein complexes inhibits microbial protease activity and results in increased flow of undegraded protein from the rumen (McNabb *et al.*, 1996; Aerts *et al.*, 1999; Min *et al.*, 2000).

Schreurs *et al.* (2007d) used *in vitro* incubations to establish the effect of seven different forages with varied CT content (Table 1.4), plant maturity and nitrogen fertiliser application on the ruminal formation of skatole. The formation of skatole from these fresh forages was greatest in vegetative swards of non-tannin plants grown under a high application of nitrogen fertiliser. Skatole formation was negatively correlated with forage CT concentration ($r = -0.39$; $P < 0.05$) in these tannin containing forages and supports the finding of Aerts *et al.* (1999) that an increasing concentration of free CTs

results in an incremental decrease in white clover protein degradation by rumen microbes.

Table 1.4. Chemical composition and in vitro *skatole yield of fresh spring growth of forages incubated with rumen fluid inoculate from pasture grazed sheep (Schreurs* et al.*, 2007d).*

	White clover	Perennial ryegrass	*Lotus corniculatus*	Sulla	*Dorycnium rectum*	*Lotus pedunculatus*	Broadleaf dock
Dry matter (g/kg)	189	222	177	151	211	166	139
OMD[1]	>0.87	0.83	0.86	>0.87	>0.87	0.81	>0.87
ME[1] (MJ/kg DM)	12.6	11.5	12.2	13.4	12.6	11.4	13.2
NDF[1] (g/kg DM)	210	411	185	138	259	255	158
CP[1] (g/kg DM)	276	122	266	254	208	194	289
SSS[1] (g/kg DM)	100	128	107	202	91	116	119
CT[1] (g/kg DM)	1.3	0.5	35.4	37.7	121.8	98.5	21.2
Skatole (µg/g CP)	575	151	356	302	122	43	28

[1]OMD = organic matter digestibility, ME = metabolisable energy, NDF = neutral detergent fibre, CP = crude protein, SSS = soluble sugars and starch, CT = total condensed tannin.

Addition of CT inhibitor polyethylene glycol to *in vitro* incubations caused a significant increase in the formation of skatole in all tannin-containing forages in Table 1.4, the effect being most pronounced with the highest tannin containing plants. This effect confirmed that CTs are responsible for the significant reduction in skatole formation from fresh forages *Lotus corniculatus*, Sulla, *Dorycnium rectum*, and *Lotus pedunculatus* (Schreurs *et al.*, 2007d).

The ability of CTs to inhibit skatole formation has been demonstrated by increased skatole formation in incubations of rumen microbes, *Lotus pedunculatus* CT and tryptophan with addition of polyethylene glycol, which preferentially binds with CTs and inhibits their ability to bind amino acids (Schreurs *et al.*, 2007a). Addition of CT extracted from *Lotus pedunculatus* or grape seed to *in vitro* incubations of fresh white clover (*Trifolium repens*) or perennial ryegrass (*Lolium perenne*), at a rate of 40 to 80 g

of tannin per kg forage dry matter, gave an exponential decrease in the formation of skatole with increasing CT addition (Schreurs *et al.*, 2007a).

In vitro incubation of fresh *Lotus pedunculatus* together with white clover with rumen microbes decreased skatole formation in proportion to the inclusion of *Lotus pedunculatus*. This indicates a dilution effect and suggests that the protective effect of CTs in one component of a mixed forage diet will not confer significant protection to soluble proteins arising from ingestion of non-tannin forages as observed following the addition of CT extract (Schreurs *et al.*, 2007a).

Schreurs *et al.* (2007b) dosed lambs fed either fresh white clover or perennial ryegrass with free CT extracted from grape seeds. The skatole concentration in intra-muscular fat between feeding treatments was not significantly different, however the administration of free CT reduced skatole concentration in rumen fluid and plasma (Schreurs *et al.*, 2007c). Schreurs *et al.* (2007e) have demonstrated that lambs fed a sole diet of condensed tannin forage *Lotus corniculatus* accumulate less skatole in adipose tissue compared to lambs fed non-tannin forages perennial ryegrass and white clover.

As a plant matures its composition changes due to increasing lignin and a decrease in crude protein content, that results in decreased rumen solubility and rate of crude protein degradation. Schreurs *et al.* (2007d) report that forages harvested following 42 days of spring growth had higher neutral detergent fibre content and lower crude protein, metabolisable energy and organic matter digestibility compared to those harvested following 12 days of growth. The effect of maturity significantly decreased skatole formation per gram of crude protein in white clover, perennial ryegrass and *Lotus corniculatus* by 43%, 46% and 24% respectively (Schreurs *et al.*, 2007d). Of

interest is the reduced impact of plant maturity in the tannin containing forage indicating that the factor limiting relative skatole formation from this forage in its vegetative state is the tannin precipitation of proteins.

Application of urea fertiliser (600 kg nitrogen/ha) to spring grown perennial ryegrass reduced dry matter, neutral detergent fibre and soluble sugar content and increased (34%) crude protein content. *In vitro* incubation of high nitrogen fertilised grass yielded 60% more skatole than low nitrogen fertilised grass (Schreurs et al., 2007d). This result illustrates the potential impact of feeding nitrogen boosted pastures to the skatole production of grazing livestock. CT binding is not limited to proteins of plant origin as they also bind directly to hydrolytic enzymes, rendering them inactive and have a bacteriostatic effect that inhibits proteolytic bacteria by binding to their cell surfaces. *Streptococcus bovis* is susceptible to CTs from several plants and its growth prevented by concentrations as little as 100 μg/mL (McMahon et al., 2000).

Tavendale *et al.* (2005) have demonstrated that CT from the forage legume *Dorycnium rectum* has an inhibitory effect on the conversion of protein to skatole and indole by rumen microbes by reducing the conversion of plant protein to skatole and indole by 75%. At least part of this effect was mediated by inhibiting the transformation of an intermediate product, indole acetic acid, to skatole by 84%. In contrast, rumen microbes exposed to CT from *Lotus pedunculatus* for up to 6 hours are still able to convert L-tryptophan to skatole *in vitro* (Schreurs et al., 2007a) suggesting a varied activity of the CTs from these two forages.

Rumen formation of skatole can also be reduced by substitution of fresh forages with energy-dense feeds such as maize grain (Young *et al.,* 2003). The effect of maize in the

diet is two-fold. Firstly it has a much lower rumen degradable protein content than fresh forages (32 vs. 122 g/kg DM) so its introduction into the diet gives a proportional reduction in skatole formation due to dilution. Secondly its high content of non-structural carbohydrate in the form of starch (Gulmez and Turkmen, 2007) improves the balance of energy and protein substrates and enables increased production of microbial protein with a concomitant decrease in amino acid deamination to ammonia and skatole.

1.3 SKATOLE ABSORPTION

The reticulorumen, comprising the rumen, reticulum and omasum has a smooth muscle wall with a lining of stratified squamous epithelium with a significant absorptive function. The surface of the rumen is covered in specialised papillae that vastly increase its epithelial surface area. The omasum has an intricate lining of internal folds that form a large absorptive surface. The wall of the reticulorumen is highly vascularised from rumen arteries and drained by the gastric vein which delivers blood via the portal vein to the liver.

Skatole is a hydrophobic compound and is poorly soluble in water. Analysis of whole and strained rumen fluid indicates that skatole in the rumen partitions into the particulate fraction. Both *in vitro* and *in vivo* observations undertaken by Carlson *et al.* (1981) demonstrate that rumen fluid obtained by cheesecloth straining of whole rumen digesta contained only 21% of the total skatole present in whole rumen digesta sampled.

The absorption of skatole has been investigated in several ruminant species. Carlson *et al.* (1981) reported the rapid disappearance of skatole from rumen fluid of goats following intrarumen skatole administration. Skatole concentration rapidly decreased and skatole was detected in the peripherial plasma whilst only 0.03% of the dose remained in liquid phase entering the duodenum. Furthermore Roy *et al.*, (2004) report that in ewes fed the tannin containing forage sulla (*Hedysarum coronarium*) and prepared with catheters in the mesenteric, portal and hepatic veins and mesenteric artery, the appearance of skatole in the mesenteric blood represents less than 8% of the portal appearance. These observations indicate that skatole absorption in ruminants

occurs primarily across the rumen epithelium resulting in the appearance of skatole in the portal blood drainage via the gastric vein (Table 1.5).

Table 1.5. Concentration of skatole in the mesenteric vein, portal vein, hepatic vein and mesenteric artery and the effect of polyethylene glycol in lactating ewes fed sulla (Roy et al., 2004).

Skatole concentration (μg/mL)	Treatments		SED	Probability
	PEG, n = 6	Control, n = 5		
Mesenteric vein	10.4	8.2	3.1	0.64
Portal vein	135.1	47.4	23.6	0.03
Hepatic vein	24.8	2.5	11.4	0.03
Mesenteric artery	12.9	1.4	5.5	0.03

The observations of Carlson *et al.* (1981) and Roy *et al.* (2004) demonstrate that the rumen is the primary site of skatole absorption in ruminants and little absorption takes place in the lower digestive tract. Because skatole is a terminal product of tryptophan degradation, skatole disappearance from the rumen can be attributed to the sum of absorption and outflow as it cannot be further metabolised in the rumen (Deslandes *et al.,* 2001). As a result skatole escaping the rumen (most likely associated with particulate material) can be expected to be excreted in the faeces.

Skatole absorption in cattle dosed with an intrarumen bolus of tryptophan (400 mg/kg BW) was measured via catheters placed in the portal vein, mesenteric vein and either femoral or iliac arteries. Net skatole absorption peaked 24 hours after tryptophan dosing at an average of 1.05 g/h. By 48 hours post dosing 29% of the tryptophan dose was accounted for by skatole absorption into the portal drainage (Hammond *et al.,* 1982).

The site of skatole absorption from the gastrointestinal tract of adult cattle was determined by Hammond *et al.*, (1982), using cattle fitted with rumen and duodenal cannula. All cattle were dosed with tryptophan as a single intrarumen bolus of 400 mg/kg body weight (BW). Mean peak concentration of skatole was 152, 16 and 9 μg/mL for rumen fluid, duodenal digesta and peripheral plasma samples respectively.

Carlson *et al.* (1981) investigated the absorption of skatole from the forestomachs of goats dosed with a single intrarumen bolus of skatole. Three measurements were made at two levels (25 and 500 mg/kg BW) of skatole administration. The concentration of skatole in the rumen fluid rapidly decreased after dosing at either level with a half life of approximately two and a half hours between one and six hours post dosing. The skatole concentration in the rumen was minimal by 24 hours after dosing. Skatole rapidly appeared in peripheral plasma and was present in the first sample collected 1 h after dosing. Carlson *et al.* (1975) administered skatole to mature cattle (intrarumen 100 mg/ kg BW) and report that mean plasma skatole concentration peaked (17 μg/mL) just three hours after dosing and decreased thereafter to a mean of 1.6μg/mL by 12 hours. In sheep fed fresh white clover, perennial ryegrass or *Lotus corniculatus* the post prandial skatole concentration in peripheral (jugular) blood peaks 1 to 2 hours following a 2 hour feeding bout (Figure 1.9; Schreurs *et al.*, 2007c).

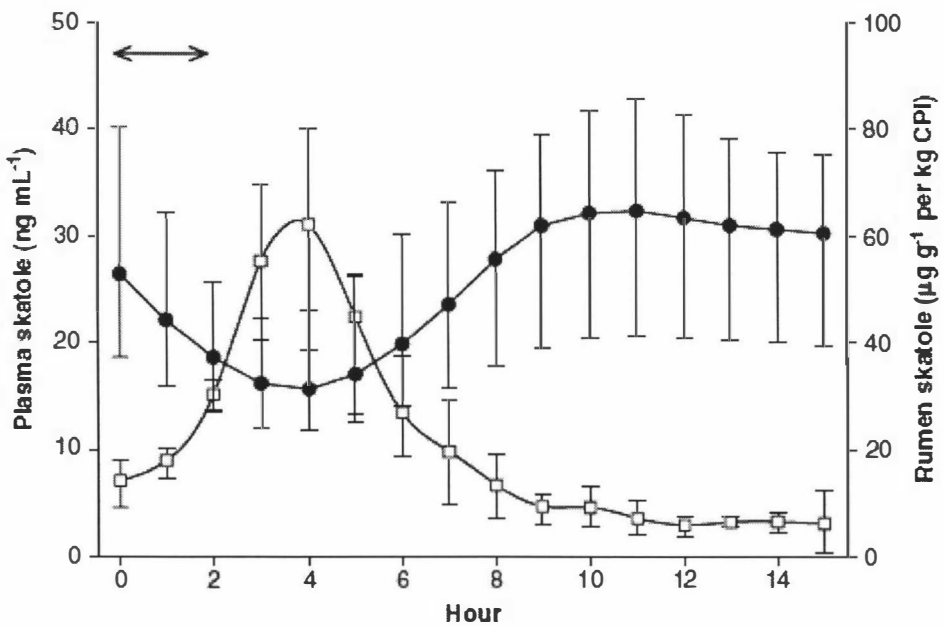

Figure 1.9. Mean concentration of skatole adjusted for crude protein intake in rumen fluid (●) and plasma (□) of sheep (n = 6) fed perennial ryegrass. Error bars indicate the range; the double ended arrow indicates the period over which feeding took place (Schreurs et al., 2007c).

These results indicate that skatole is rapidly and quantitatively absorbed from the forestomachs of ruminants into the portal drainage and losses via outflow to the lower digestive tract are minimal. It is unclear from the literature whether the absorption occurs only in the reticulorumen and what if any additional uptake occurs in the abomasum.

1.4 SKATOLE METABOLISM

Skatole is a potentially toxic rumen metabolite of tryptophan and its entry into the body in the portal blood necessitates its detoxification and subsequent elimination (Figure 1.10). In order for elimination to occur skatole must be metabolised to produce more soluble substrates suitable for urinary excretion (Deslandes *et al.*, 2001). Excluding any metabolic function of the reticulorumen wall, the liver is the first potential site for skatole detoxification. Absorption of skatole at rates greater than the livers' capacity for detoxification results in skatole entry into the peripheral circulation and its deposition into lipidic pools including adipose tissue and milk fat (Babol *et al.*, 1998a).

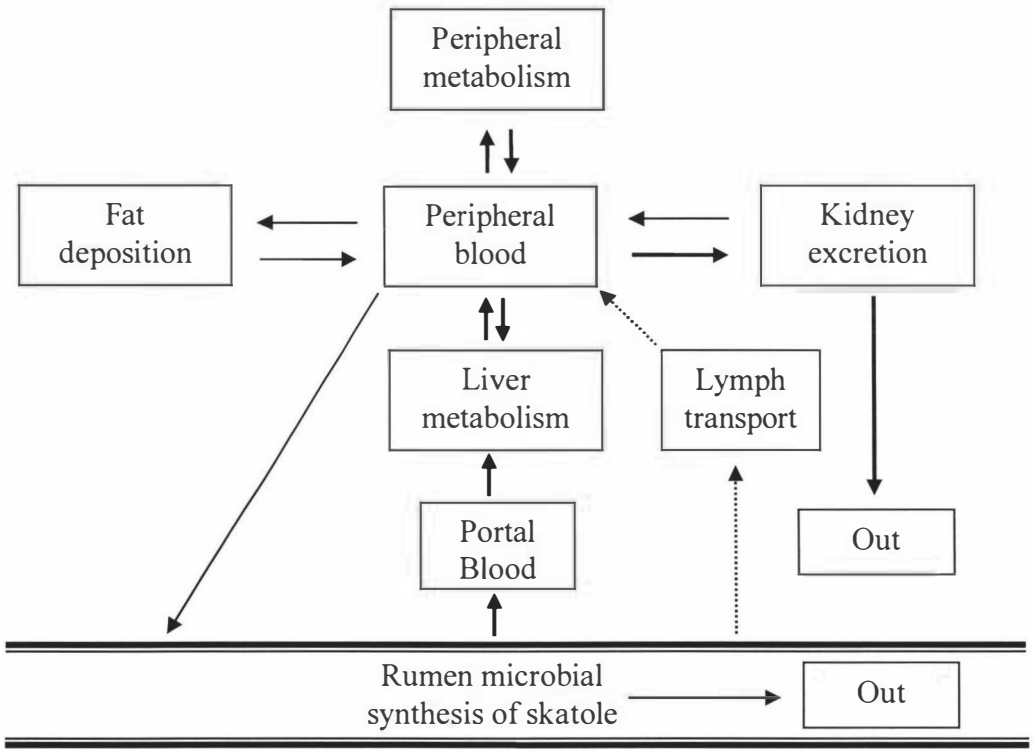

Figure 1.10. Transport routes of skatole within the body (adapted from Agergaard and Laue, 1992).

Hammond *et al.* (1979) used a jugular infusion of radioactive skatole to demonstrate that goats metabolise skatole rapidly and that the metabolites are excreted in the urine within 72 hours (Table 1.6).

Table 1.6. Accumulation of radioactivity at 72 hours in the urine, expired air and faeces of each of two goats given jugular infusions of [Methyl-^{14}C] skatole (Hammond et al., 1979).

	Radioactivity as a percentage of dose[1]			
	Urine	Expired Air	Faeces	Total Excretion
Goat 1	87.4	0.4	ND[2]	87.8
Goat 2	91.9	0.9	<0.1	92.9

[1]Dose: 0.57μCi/kg body weight and 0.04g 3MI/kg body weight, goat 1 or 0.03g 3MI/kg body weight, goat 2.
[2]Not detectable.

The level of skatole in the peripheral circulation can be considered a real-time account of skatole absorption in excess of both metabolism and deposition into lipidic pools. Infusion of skatole into the peripheral circulation of goats has been used to estimate the clearance of skatole from the blood, resulting in an estimated half-life of approximately 25 minutes in this species (Bradley and Carlson 1982). This speed of skatole disappearance indicates that skatole is actively removed from the blood by detoxification processes (Agergaard and Laue, 1992).

Little investigation of the mechanisms of hepatic metabolism of skatole in ruminant species has been reported. The role of the liver in skatole detoxification and the limitation of skatole entry into the peripheral circulation of ruminant animals is uncertain however it is likely to play an important role as it is a major site of xenobiotic detoxification in the body.

In contrast to hepatic metabolism, the pulmonary metabolism of skatole in ruminants has been investigated due to the involvement of skatole in pneumotoxicity. First identified as fog fever and know known as acute pulmonary edema and emphysema the often fatal condition is caused when ruminants, particularly goats and cattle, are exposed to sudden increases in skatole absorption.

The effect of skatole pneumotoxicity is tissue selective and species specific (Deslandes *et al.*, 2001). Skatole is activated in the lung cells via metabolism by cytochrome P450, an enzyme of the mixed function oxidase system. Its activation results in the formation of a cytotoxic alkylating metabolite which covalently binds to cellular macromolecules of the bovine lung (Hanafy and Bogan 1980) causing membrane lipid peroxidation and necrosis. Necrosis of bronchiolar epithelium is a reproducible and consistent effect of lethal doses of skatole in ruminants (Hanafy and Bogan, 1982).

It has been proposed that pulmonary non-ciliated bronchiolar epithelial cells (Clara cells) are the site of skatole activation in the bovine lung as Clara cells are the target for reactive metabolite-forming pneumotoxic compounds whose reactive metabolites are formed by a cytochrome P450 dependant mechanism (Boyd *et al.,* 1980). The high concentration of Clara cells in bronchiole epithelium may explain the sensitivity to these pneumotoxic compounds (Hanafy and Bogan, 1982).

Ruminant animals are more resistant to dietary induced acute pulmonary edema and emphysema when exposed to a gradual increase in skatole absorption however it is not clear if this is due to a lower peak level of skatole absorption or an increase in ability to tolerate skatole. In mice it has been clearly demonstrated that resistance to skatole mediated pulmonary necrosis can be induced. Turk *et al.* (1986) compared the damage

to bronchiolar epithelium 24 hours after an injection of 400 mg skatole/kg BW, in mice either receiving a placebo or a daily pre-treatment injection of skatole at a sub-clinical dose (100 mg skatole/kg BW) over a preceding 72 hour period. Results are illustrated in Figure 1.11. A pre-treatment with skatole dramatically reduced the severity of pulmonary necrosis following a skatole dose indicating probable induction of alternative, non-pneumotoxic skatole detoxification pathways. A similar effect has also been observed in goats where an oral pre-treatment with 40 mg skatole/kg BW/day for five days protected treated animals from pulmonary disease following a subsequent challenge dose of skatole administered as a two hour jugular infusion of 40 mg skatole/kg BW (Leung *et al.*, 1983). These results indicate that short term skatole exposure at non-toxic concentrations may be effective in inducing non-toxic skatole detoxification pathways in ruminants.

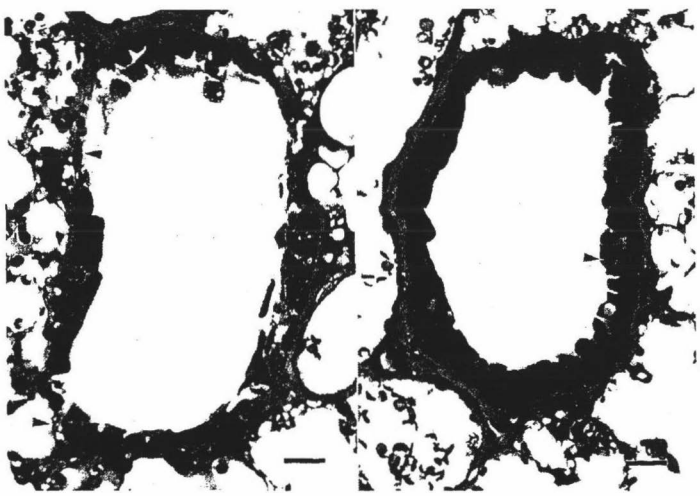

Figure 1.11. Pre-treatment of mice with a small dose of skatole before a challenge dose reduced pulmonary necrosis in the lung at 24 hours. (Left field) Lung of mouse pre-treated with placebo, necrosis and denuded basement membrane; (Right field) lung of mouse pre treated with 100 mg skatole, epithelium intact with rare necrotic cells. Bar = 40 μm (Turk et al., 1986).

In contrast to ruminant species the metabolism of skatole in monogastrics has been thoroughly investigated as skatole is one of the main compounds contributing to boar

taint (an off-odour liberated when pork is cooked) in 5 to 10% of uncastrated male pigs (Lundstrom and Zamaratskaia, 2006). It is not known why only a small percentage of a given population of pigs accumulates skatole to a level that can be detected by humans and it is proposed that this is due to individual differences in the metabolism and rate of skatole elimination from the body (Lundstrom *et al.,* 1994).

Skatole is rapidly metabolised in the pig liver (Agergaard and Laue, 1998) and the rate of skatole elimination by the liver affects skatole concentration in adipose tissue (Babol *et al.,* 1998b). Pigs can metabolise skatole via several pathways, by the formation of oxindoles (Skiles *et al.,* 1989) and aminoacetophenones, by ring hydroxylations in the 5-, 6- and 7-positions and by oxidation of the methyl group and conjugation with glutathione (Bæk *et al.,* 1997). Some of the metabolites are conjugated to sulphate or glucuronic acid (Skiles *et al.,* 1989).

The enzymatic systems responsible for skatole metabolism in the liver involve phase I initial oxidation reactions usually performed by the membrane bound cytochrome P450 system (Squires and Lindstrom 1997; Babol *et al.,* 1998a) resulting in formation of products with hydroxyl, primary amine or sulfhydryl groups that are substrates for phase II metabolism. Phase II reactions are conjugations, predominantly sulphation and glucuronidatation catalysed by transferases (Babol et al 1998b) resulting in reduced biological activity and increased solubility. These reactions are evident in pigs due to the appearance of conjugates of hydroxylated products in the plasma and urine (Bæk *et al.,* 1995).

The detoxifying enzymes of both phase I and phase II metabolism, such as those involved in skatole biodegradation, are regulated by various mechanisms including substrate dependant induction and a variety of dietary factors (Runge-Morris 1997; Zhu

et al., 1995; Yang *et al.,* 1992). In contrast however Babol and Squires (1999) conclude that the overall rates of oxidation reactions carried out by cytochrome P450 are not induced by skatole administration and the conjugation reactions that are involved in skatole metabolism and clearance are not likely to be affected by increased skatole levels in pigs.

The phase I enzymes cytochrome P4502E1 (CYP2E1), cytochrome P4502A6 (CYP2A6) and aldehyde oxidase and the phase II enzyme phenol sulfotransferase (SULT1A1) have all been identified as important in the effective clearance of skatole by pigs (Squires, 2006; Diaz and Squires *et al.,* 2000b; Diaz *et al.,* 1999; Babol *et al.,* 1998a).

Selective chemical inhibitors have been used to define the catalytic specificity of cytochrome P450 enzymes and the extent to which a particular enzyme is responsible for a reaction. In microsomal preparations skatole metabolism was reduced in the presence of inhibitors of CYP2E1 and CYP2A6 confirming the importance of these enzymes in the hepatic metabolism of skatole in pigs (Diaz and Squires, 2000a).

A significant negative correlation between CYP2A6 activity and the concentration of skatole in pig fat further suggests that CYP2A6 is critical for an adequate clearance of skatole in this species. Pigs with high hepatic levels of CYP2E1 activity also have consistently low skatole concentration in their fat depots (Squires and Lundstrom, 1997). However when CYP2A6 and CYP2E1 expression levels are low, skatole levels in fat depots can be either high or low. This is because skatole deposition is independently affected by the amount of skatole absorbed from the gastrointestinal tract (Diaz and Squires, 2000a; Squires and Lundstrom, 1997).

The production of two major metabolites, 3-hydroxy-3-methyloxindole (HMOI) and Hydroxymethylindolenine (HMI), account for more than 64% of the metabolism of skatole by porcine liver microsomes *in vitro* and are unaffected by P450 inhibitors, suggesting significant involvement of other as yet unidentified enzymes in the phase I metabolism of skatole in pigs (Diaz and Squires *et al.*, 2000b).

HMI is rapidly metabolised to HMOI by a cytosolic metalloflavoprotein, aldehyde oxidase (Diaz *et al.*, 1999). The activity of aldehyde oxidase is negatively correlated with the level of skatole in fat. Diaz and Squires (2000b) reported pigs classified as high skatole accumulators had a mean hepatic aldehyde oxidase activity 3.4 times lower than those pigs classified as low accumulators. These results suggest that aldehyde oxidase present in the hepatic cytosol of pigs is a key regulator of skatole metabolism in this species.

Aldehyde oxidase plays an important role in the metabolism of exogenous compounds and is known to catalyse nucleophilic oxidation of a number of aldehydes and a variety of nitrogen containing heterocyclic xenobiotics resulting in different metabolites from those obtained via electrophillic oxidation by the cytochrome P450 system. The enzyme catalyses a unique reaction, involving the oxidative hydroxylation of the substrate, in which the oxygen atom incorporated into the product is derived from water rather than molecular oxygen (Diaz and Squires, 2000b; Kitamura *et al.*, 2006).

There is considerable variability of aldehyde oxidase activity in liver cytosol of mammals as well as inter-individual variation in activity within species (Kitamura *et al.*, 2006). Aldehyde oxidase is a homodimer composed of two identical subunits, each subunit containing FAD, molybdenum and iron (as iron/sulphur clusters) as prosthetic groups in a 1:1:4 ratio (Rajagopalan, 1980). As molybdenum is a co-factor for this

enzyme, its deficiency can lead to reduced aldehyde oxidase activities in the liver (Beedham, 1985). The role of aldehyde oxidase in the metabolism of skatole in sheep has not yet been confirmed however the impact of molybdenum status upon the skatole clearance capacity of rapidly growing pasture fed lambs is of significant interest given the important role of aldehyde oxidase activity in the clearance of skatole in pigs.

Herbage molybdenum concentrations <2 mg molybdenum/kg DM are common in New Zealand pastures (Meat New Zealand, 2001) and the absorption of dietary Mo in ruminants is impeded by the intra-rumen interaction of molybdenum with copper and sulphur (Grace and Suttle, 1979). These interactions at rumen and abomasal pH result in the formation of unabsorbable copper-tetrathiomolybdates thus reducing availability of dietary molybdenum to sheep (Lee *et al.,* 2002). The concentration of molybdenum in the plasma of New Zealand pasture grazed sheep is very low (100 nMol/L) and shows little seasonal variation (Grace and Knowles, unpublished data). Further research is required to investigate if the molybdenum status is sufficiently low to affect the activity of aldehyde oxidase of New Zealand sheep and the effect this has on skatole metabolism in these animals.

SULT1A1 a phase II sulphate conjugation catalyst is an important enzyme in the clearance of skatole from pigs. Its activity is negatively correlated with concentrations of skatole in plasma and fat of pre-pubescent pigs (Lanthier *et al.*, 2007). *In vivo* studies have also demonstrated that an increased ability of pigs to form 6-sulphatoxyskatole corresponds to increased skatole clearance (Bæk *et al.,* 1997). A polymorphism in the porcine SULT1A1 gene has been discovered which results in decreased expression and is related to increased skatole concentration in fat deposits of affected pigs (Lin *et al.,* 2004).

1.4.1 Metabolites of skatole degradation

Skatole metabolites isolated from pigs are; 6-sulfatoxy-3-methylindole a sulphate conjugate of 6-hydroxy-3-methylindole, 3-hydroxy-3-methyloxindole, (3-[N-acetylcystein-S-yl)-methyl]indole (Bæk *et al.,* 1995), conjugated 5-hydroxy-3-methylindole and the glucuronic acid conjugate of 6-hydroxy-3-methylindole. Of these the appearance of 6-hydroxy-3-methylindole and 3-hydroxy-3-methyloxindole are negatively correlated to levels of skatole in fat (Bæk *et al.,* 1995) suggesting their formation may be a rate limiting step in skatole clearance.

Diaz *et al.* (1999) investigated the phase I metabolism of skatole by pig liver microsomes *in vitro.* Seven major skatole metabolites produced in microsomal incubations were identified (Figure 1.12), of which three had already been identified in pigs (Bæk *et al.,* 1997; Babol *et al.,* 1998a), HMOI, 5-hydroxyskatole (5-OH-3MI) and 6-hydroxy skatole (6-OH-3MI). The other four major skatole metabolites identified were, 3-hydroxy-3-methylindolenine (HMI), 3-methyloxindole (3MOI), indole-3-carbinol and 2-aminoacetophenone. The metabolite that was produced in largest amounts was HMI (45%) followed by 3MOI (28%) and HMOI (19%). These results suggest that the main pathway of phase I detoxification of skatole in pigs is via the formation of oxindole derivatives.

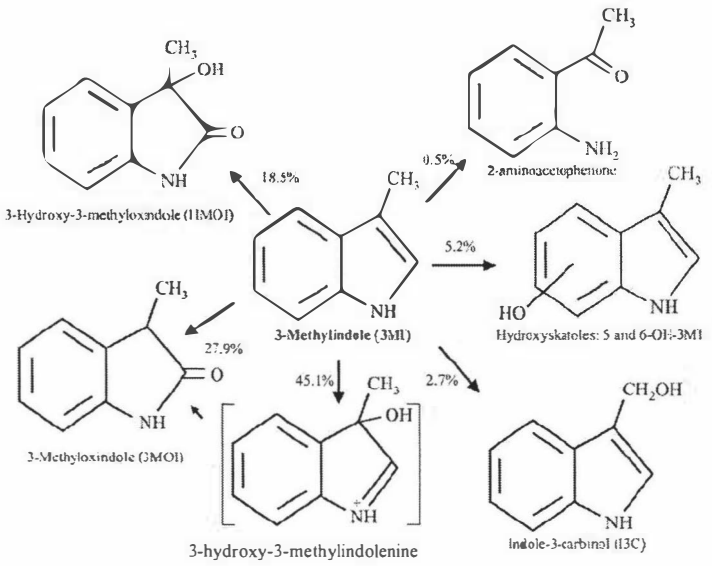

Figure 1.12. Chemical structures and percentages of the major phase I metabolites of skatole produced by pig liver microsomes (Diaz et al., 1999).

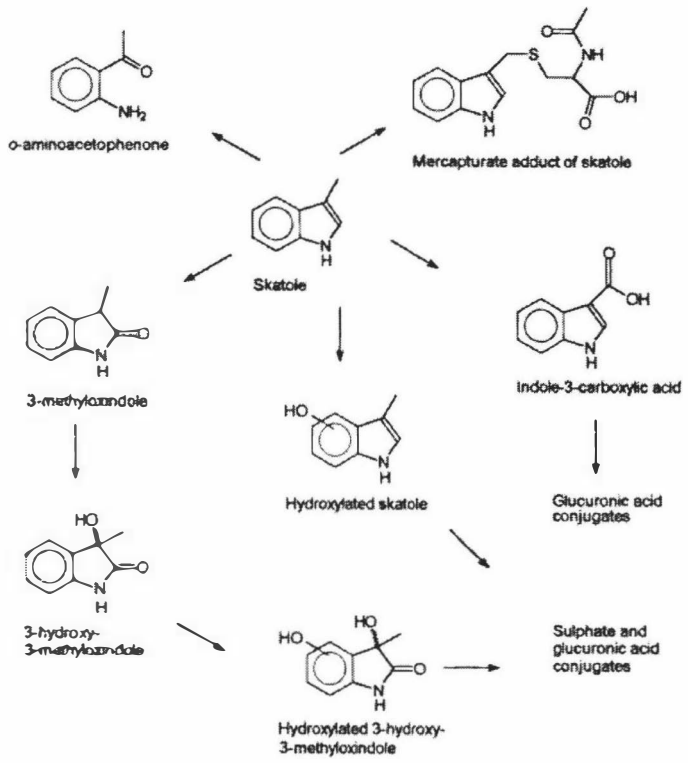

Figure 1.13. Metabolic pathways of skatole detoxification via formation of oxindoles, aminoacetophenones, ring hydroxylations, methyl group oxidation and conjugation with glutathione (Bæk et al., 1997)

6-OH-3MI and HMOI are the major metabolites of skatole seen in the plasma of normal pigs of both sexes (Figure 1.13). However HMOI becomes the major metabolite

appearing in the plasma of male pigs with a diminished capacity to form 6-sulfatoxyskatole. The major skatole metabolite appearing in the urine of male pigs is the excreted form of the 3-methylindole-glutathione adduct, 3-[(*N*-Acetylcystein-*S*-yl)methyl]indole, a mercapturic acid conjugate of skatole (Bæk *et al.*, 1997).

A large inter-individual difference in the rate of production of all skatole metabolites by pigs has been observed is attributed to differences in the level of expression of phase I detoxification enzymes (Diaz *et al.*, 1999).

The extensive conversion of skatole to oxindole derivatives in pigs may explain the lack of skatole pneumotoxicity in this species as compared to ruminants, particularly goats (Dickinson *et al.*, 1976) and cattle (Carlson *et al.*, 1968). Diaz *et al.*, (1999) reported that indole-3-carbinol accounted for less than 3% of the skatole metabolites produced by pig liver microsomes whereas appreciable amounts are produced as the predominant hydration metabolite of the toxic electrophillic intermediate 3-methyleneindolenine that is produced via P450 catalysed dehydrogenation of skatole in susceptible ruminant species such as goats (Skiles and Yost, 1996).

1.5 SKATOLE AND PASTORAL FLAVOUR OF SHEEP MEATS

The accumulation of skatole in the inter-muscular fat of sheep has been correlated with sensory characteristics described as 'barnyard', 'faecal' and 'sheepy'. Collectively these flavours are described as pastoral flavour as they are unique to temperate pastoral production systems.

Consumer preference for food flavours is diverse, at one extreme of preference, pastoral flavour is described as 'full-bodied', an attribute to be valued. While at another, pastoral flavour is perceived to be undesirable and increases the risk of meat rejection on the basis of its sensory qualities. Dislike of pastoral flavour is associated with consumer groups who are either sensitive or unaccustomed to these flavours due to their traditionally low consumption of pasture raised sheep meats (Schreurs *et al.*, 2007a; Young *et al.*, 2003).

Branched chain fatty acids (BCFAs) 4-methyloctanoic acid and 4-methylnonanoic acid are also associated with the flavour of New Zealand sheep meats and confer a 'muttony' flavour (Brennand *et al.*, 1989). Like skatole BCFAs are highly volatile. They arise from the metabolism of fatty acids in sheep and goats (Duncan and Garton, 1979). BCFAs are considered a dominant contributor to flavour variation between species (Young *et al.*, 2003). It has also been reported that pastoral flavour may be exacerbated by interactions between BCFAs and skatole in pasture fed sheep (Young *et al.*, 1997).

As skatole is a volatile compound associated with meat fat, its odour is most obvious when these lipids are heated during cooking (Lundstrom and Zamaratskaia 2006). Using coupled sensory and chemical analyses Young *et al.* (2003) identified volatile

compounds released from heated (100°C) fat from 200 day old lambs raised on either; pasture, maize concentrate or Lucerne pellet diets. Their sensory panel results confirm that skatole (3-methylindole) contributes to the flavour intensity of meat from pasture-fed lambs due to the association of meat skatole concentration and panellist perception of pastoral flavours 'barnyard', 'faecal' and 'sheepy' (Figure 1.14).

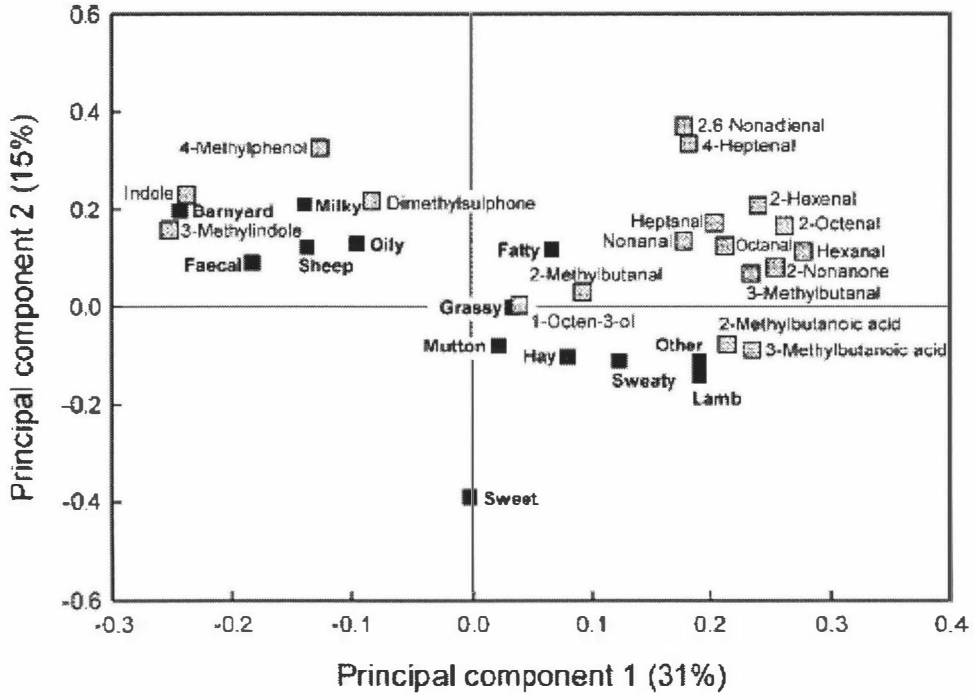

Figure 1.14. Principal component analysis of correlations between frequency of panellist comments (■) *and relative concentrations of odorous compounds* (□) *in headspace of heated subcutaneous fat (Young et al., 2003).*

It has been determined that the sensory detection limit of consumers for skatole in pork fat is between 90 to 250 ppb (Babol *et al.*, 2002) however some individuals may be more sensitive and able to detect skatole at much lower levels under controlled conditions (Annor-Frempong *et al.*, 1997).

The flavour preference of consumers in potential export markets has been studied by comparing the preferences of New Zealand and Japanese consumers for the relative

impact of BCFAs and skatole. In order to undertake a standardised test, cooked lean beef samples containing added BCFAs (0, 5.7 or 17 ppm) and skatole (0, 80 or 240 ppb) were used in place of real sheep meat in a 3 x 3 factorial design. The results of this study indicate that Japanese women are influenced by BCFAs at either 17 ppm or 5.7 ppm concentrations and both Japanese and New Zealand women by skatole at 240 pbb (Figure 1.15). It is considered that BCFAs and skatole also interacted to influence acceptability of the meat. Cluster analysis revealed that the combination of BCFAs and skatole is important for consumer sub-groups and suggest that reductions in BCFAs and skatole may improve the acceptability of sheep meat in Japan and perhaps other North Asian markets (Prescott *et al.*, 2001).

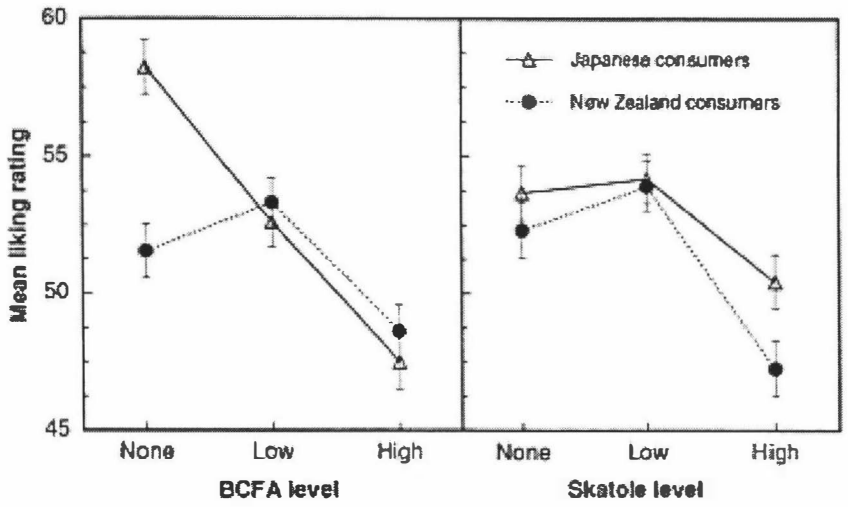

Figure 1.15. Mean (± SEM) liking ratings of the meat samples made by Japanese and New Zealand female consumer panels as a function of the different levels of added branched chain fatty acids and skatole (Prescott et al., 2001).

1.6 Conclusions and Hypotheses

New Zealand sheep meat is associated with a characteristic flavour and cooking odour known as pastoral flavour. Pastoral flavour is perceived as a desirable 'full bodied' taste by traditional consumers however consumer preference for food flavours are diverse and a dislike for pastoral flavours is associated with consumer groups, particularly in potential Asian markets, who are unaccustomed to pasture raised sheep meats.

The extensive use of low input highly digestible pasture species is the basis of the international competitiveness of New Zealand agriculture. Improvements in the nutritive value of pasture cultivars, in particular perennial ryegrass and white clover, through selective breeding, increased use of nitrogen fertilisers and intensive grazing management have both improved productivity and reduced the efficiency with which ingested protein is used by the productive ruminant animal.

In the pasture fed ruminant an asynchrony is believed to exist between the rapid rate of plant and microbial mediated proteolysis relative to energy generation via microbial celluloysis in the rumen. Under these conditions bacterial growth is limited by energy supply and free amino acids released into the rumen fluid are rapidly deaminated to yield ammonia and other non-protein compounds.

The synthesis of skatole in the gastrointestinal tract requires bacterial deamination of the amino acid L-tryptophan, hence its rate of production can be manipulated by reducing rumen degradation of plant proteins or decreasing deamination of free amino acids within the rumen. In grazing ruminants skatole production is positively correlated with the intake of rumen degradable protein. Ruminants grazing New Zealand improved

temperate pasture species, in particular white clover, ingest sufficient rumen degradable protein to allow post-prandial skatole formation at a rate greater than the first-pass detoxification capacity of the liver. Skatole is rapidly absorbed from the rumen into blood entering the gastric vein with little outflow to the lower digestive tract. Unless metabolised in the liver skatole enters the peripheral circulation where it is rapidly deposited into adipose tissue and imparts a pastoral flavour to the meat products of grazing ruminants.

Reducing the formation of skatole has been investigated as a means of reducing the concentration of skatole in meat and milk products. These approaches have included reducing the rumen degradability of forage proteins through the use of condensed tannins, modification of the rumen bacterial population or improving microbial protein synthesis by correcting the protein:energy imbalance within the rumen. All are effective in reducing the extent of tryptophan derived skatole formation. However to date these practices have been of limited practical application in pastoral agriculture. This is due to the cost of growing forages with sufficient tannin content due to their low persistence under grazing relative to pasture, the high cost of energy rich supplements and the opposition of export markets to antibiotic use in food producing livestock.

The mechanisms of skatole metabolism in ruminants are not well understood, however high concentrations of skatole in peripheral circulation precedes the onset of acute pulmonary odema and emphysema in goats, cattle and sheep. Skatole itself is not a pulmonary cytotoxin however it is activated to an alkylating metabolite by cytochrome P450 enzymes in bronchiolar epithelial cells. Skatole mediated pulmonary toxicity can be reduced via pre-treatment with sub-clinical doses of skatole in mice and goats,

indicating that induction of alternative non-toxic skatole detoxification pathways is possible in these species.

The concentration of skatole deposited within the adipose tissue of sheep displays significant inter-individual variation however it is not known if this variation arises due to differences in skatole formation in the rumen or rate of elimination via hepatic or post-hepatic metabolism. Pigs also display significant inter-individual variation in the accumulation of skatole in adipose tissue. In pigs the liver is the primary site of skatole metabolism and hepatic enzyme activity is thought to control skatole accumulation in this species. The role of the liver in ruminant skatole clearance is not known however given the role of the liver in detoxification of hydrophobic xenobiotics and its ability to eliminate compounds prior to their entry into peripheral circulation, its capacity for skatole metabolism is also likely to be a key factor affecting accumulation of skatole in sheep.

Due to the current lack of economic methods to reduce rumen skatole formation in pasture fed sheep the objective of this study was to undertake a transcriptional profile of hepatic tissue using DNA microarrays to identify gene induction in response to skatole treatment in sheep. A second objective was to determine the effect of steady state intrarumen skatole administration upon skatole concentration in rumen and peripheral plasma pools and to determine the rate of skatole elimination in sheep.

It was therefore hypothesised that under conditions of minimal endogenous skatole production a 72 hour controlled administration of exogenous skatole will result in an increase in rumen skatole to a plateau concentration and induce differential gene expression in the ovine liver. These hypotheses have been tested using a continuous

intrarumen infusion of skatole, measurement of skatole concentration in rumen fluid, peripheral plasma and inter-muscular fat and transcriptional analysis of hepatic tissue using DNA microarrays.

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CHAPTER 2

METABOLISM OF RUMEN INFUSED SKATOLE AND EFFECTS ON HEPATIC GENE EXPRESSION IN SHEEP

2.1 ABSTRACT

Skatole contributes to the flavour palate known as pastoral flavour (characterised by 'sheepy' or 'grassy' odours) that is unique to sheep meat products from temperate pastoral production systems. Skatole (3-methylindole) is rapidly produced from L-tryptophan during rumen digestion of soluble proteins. Production of skatole at rates that exceed the primary clearance capacity of the body results in the accumulation of skatole in peripheral adipose tissue. The present experiment was undertaken to test if under conditions of minimal endogenous skatole production (diet low in rumen degradable protein) a continuous rumen infusion of skatole will elevate hepatic exposure and modify hepatic skatole metabolism in sheep. A 72 hour rumen infusion of skatole (140 mg/h; n = 6) dissolved in propane-1,2-diol (PD) or PD media alone (n = 6) was performed. Samples of rumen fluid and blood were collected at 0, 2, 4, 6, 8, 12, 16, 24, 48 and 72 hours for determination of skatole concentration. The 72 hour infusion of skatole increased ($P< 0.01$) levels of skatole in rumen fluid (21 μg/g), peripheral plasma (154 ng/mL) and inter-muscular fat (4240 ng/g). A two-compartment model was fitted to the rumen and plasma skatole concentrations of treated sheep to estimate the rate of clearance from peripheral circulation (2.08 ± 0.35/h). A negative correlation ($P < 0.05$) between the rate of elimination and level of skatole deposition in inter-muscular fat was found. Total hepatic RNA was used for cDNA screening of a 20,736 expressed sequence tag bovine micro array. For each expressed sequence tag a modified t-test was performed and the probability values were then adjusted for multiple testing resulting in a false discovery rate (FDR). Expressed sequence tags with significant ($FDR < 0.01$) differential expression in either direction represented about 14% of those assessed. Amongst these only five had a fold change greater than 2.0. Of these

genes three encode cytosolic phase I oxidoreductase enzymes involved in detoxification; aldehyde dehydrogenase 1 family member A1, NAD(P)H dehydrogenase quinone 1 and leukotriene B_4 12-hydroxydehydrogenase. The metabolic oxidoreductase enzyme stearoyl-CoA desaturase was also induced along with phase II detoxification enzyme glutathione S-transferase. Induction of these genes, specifically those with known catalytic activity towards toxic xenobiotics, indicates that the ovine liver is a site of detoxification for skatole or its intermediary metabolites. Further investigation is required to determine the role of these genes in the regulation of skatole detoxification in the ovine liver and the possibility to reduce pastoral flavour in forage grazing ruminants through modulation of the activity of these genes or enzymes.

2.2 INTRODUCTION

Pastoral flavour compounds arising from ruminant production systems reliant upon forages with high rumen degradable protein content impart flavours and odours to New Zealand meat and milk products that consumers in some international markets are not accustomed to. Flavour and odour preference of consumers varies regionally and is strongly influenced by the characteristics of local or traditional foods (Sanudo *et al.* 2007). Pastoral flavour constrains sheep meat marketability and returns, especially in Asian and European markets (Prescott *et al.*, 2001).

The volatile indolic compound skatole, produced in the rumen (Carlson *et al.*, 1968), contributes to these undesirable meat flavours (Priolo *et al.*, 2001; Young *et al.*, 2003). Strategies to reduce deposition of lipophilic skatole in meat products must also reduce its appearance in peripheral circulation as skatole is rapidly deposited in both intermuscular and intramuscular fat (Whittington *et al.*, 2004).

The major routes of skatole disappearance from the reticulo-rumen are outflow to the intestines, in association with the particulate phase, and absorption into the portal blood. Almost all skatole absorption occurs across the rumen epithelium (Carlson *et al.*, 1981; Hammond *et al.*, 1983; Hammond *et al.*, 1984; Roy *et al.*, 2004). Absorbed skatole arising in the portal blood flows to the liver where its metabolism has the potential to modify the concentration of skatole appearing in the peripheral circulation. Thus the rate of hepatic skatole metabolism affects the potential for accumulation of skatole in body fat (Zamaratskaia *et al.*, 2006).

The present experiment was undertaken to test if under conditions of minimal endogenous skatole production (diet low in rumen degradable protein) a continuous rumen infusion of skatole will elevate hepatic exposure and modify hepatic skatole metabolism in sheep.

In preparation for the experiment sheep were fed a high starch, low rumen degradable protein diet to promote a low rumen pH and reduce availability of the skatole precursor tryptophan to ensure negligible bacterial skatole production. The rate of skatole elimination from the peripheral circulation was estimated by modelling the disappearance of infused skatole from the rumen liquid phase and its concentration in the peripheral blood. Skatole induced changes in gene expression following 72 hours of skatole exposure were determined via microarray analysis of cDNA synthesised from total hepatic RNA.

2.3 MATERIALS AND METHODS

2.3.1 Experimental Design

2.3.1.1 Animals and surgery

All animal procedures were reviewed and approved by the Crown Research Ins Animal Ethics Committee in Palmerston North, New Zealand according to the A Protection Act (1960) and Animal Protection Regulations (1987) and amendments.

Twelve castrate Suffolk x Romney sheep of 10 months of age and from a single sire genetically similar dams were selected following screening for normal liver function u clinical laboratory tests of gamma-glutamyl transferase, glutamate dehydrogenase, protein, albumin and globulin (New Zealand Veterinary Pathology, Palmerston North, N

All sheep were surgically fitted with rumen cannula under general anaesthesia. Sheep w prepared for surgery by withholding food for 12 h. The anterior dorsal abdomen v shaved and the skin marked to show the desired fistula location whilst the sheep v standing. The position was caudal of the last rib and approximately equidistant betwe midline and spine. An area of the neck was prepared for injection into the jugular ve injection by shaving and disinfecting the skin with a solution of Hibitane® (5.0% w chlorhexidine gluconate in 70% v/v alcohol; Dainippon Sumitomo Pharma, Osaka, Japan)

Anaesthesia was induced via an i.v. jugular dose of Diazepam (1 mg/kg BW, 5 mg/ml Parnell Pamlin Injection; Parnell Laboratories, Auckland, NZ) mixed with Ketamine (mg/kg BW, 100 mg/mL; Phoenix Ketamine Injection; Phoenix Pharm Distributor

Auckland, NZ) whilst the sheep was held in a head up position to minimise the likelihood of regurgitation.

A cuffed endotracheal tube (8 mm i.d., 10.9 mm o.d., 30 cm; Mallinckrodt®, Hazelwood, MO, USA) was inserted into the trachea using direct vision with the aid of a laryngoscope (Miller 182 mm blade; Welsh Allyn, Skaneateles Falls, NY, USA), with the head and neck fully extended to align the pharynx and trachea, once in place the 27 mm cuff was inflated to occlude the trachea. The animal was placed in a right lateral recumbency and the endotracheal tube connected to a ventilator (Ohmeda 700; Datex-Ohmeda, Madison, WI, USA). The head was positioned so that the mouth was lower than the pharynx to facilitate drainage of fluids.

Anaesthesia was maintained by intratracheal perfusion of a mixture of oxygen and isoflurane (Isoflurane-vet, Rhodia NZ Ltd, Auckland, NZ) by means of a vaporiser (Fluotec 3, Cyprane Ltd., Keighly, UK) set to administer 2 to 3% isoflurane gas mixture. Once an appropriate depth of anaesthesia was attained anaesthetic was reduced to 1 to 1.5% isoflurane with the oxygen flow rate set between 0.6 to 0.8 L/minute. Respiratory rates were maintained at 10 to 15 breaths per minute via the ventilator and tidal volume of 15 L/minute adjusted to maintain an end–tidal CO_2 of approximately 25 to 30 mm Hg.

Depth of anaesthesia was monitored through eye position and reflexes. The sheep eye rolls rostroventral between light and medium anaesthesia and returns to a central position during deep anaesthesia and the palpebral reflex is weak at a plane of anaesthesia satisfactory for surgery (Hall *et al.* 2001). Oxygen saturation, CO_2 level and pulse rate was monitored via a

pulse oximeter / multigas monitor (Datex-Engstrom, Helsinki, Finland), with the pulse rate probe attached to the tongue. Body temperature (rectal) was monitored using a Datex 3 anaesthesia monitor (Datex-Engstrom, Helsinki, Finland) and maintained between 37°C and 39°C via a T/Pump® thermal circulating pad (Gaymar Industries, Orchard Park, NY, USA) set to 39°C.

The surgical site was washed with surgical soap, disinfected with Hibitane® solution and then draped with sterile surgical sheeting. Systemic antibiotic (2.0 mL Duplocillin LA®; 150 mg procaine benzyl penicillin and 115 mg benzathine benzyl penicillin/mL; Intervet, Auckland, NZ) and Ketofen 1% analgesic (2 mg/kg BW Ketoprofen; Merial, Manukau City, NZ) was administered i.m. prior to the start of the surgical procedure.

An incision 5 cm long was made first through the epidermis, then trough the subcutaneous facia, starting from a point about 1.5 cm caudal from the last rib and from the end of the transverse process of the first lumbar vertebra. Bleeding from the skin was prevented by cauterisation using an electro-surgery device (Sensimatic 600SE; Parkell, Edgewood, NY, USA) and clamping with haemostat forceps. Both sides of the incision were covered with sterilised gauze soaked in warm sterile saline (37°C). Blunt dissection was then made through the abdominal muscle layers and peritoneum to form a 5 cm long opening to the abdominal cavity. A section of the rumen lying beneath the incision was withdrawn and held with forceps. The exposed rumen tissue was kept warm and moist with wet gauze coverings while the peritoneum, abdominal muscle layers, facia and rumen smooth muscle layer were sutured together around the circumference of the incision using sterile absorbable suture (Vicryl 0, polyglactin suture; Ethicon, Somerville, NJ, USA). Antibiotic

powder (1 g Mamyzin; penethamate hydriodide; Boehringer Ingelheim, Ingelheim, Germany) was administered internally to the sutured muscle layers and to the rumen to facia suture line. The externalised rumen was then firmly clamped to inhibit blood flow using a sterilised clamp (Hecker, 1969). The epidermis incision was then closed around the externalised rumen using Michelle clips. Topical antibiotic (Aureomycin powder; 2% chlortetracycline HCl and 1% benzocaine; Bomac Laboratries, Manakau City, NZ) was applied to the incision site. Following closure the ends of the clamp were wrapped in gauze and sutured to the skin to hold the clamp securely in place. Post-operative ventilation rate was increased to 1.5 L/minute oxygen enriched air and maintained until the sheep began to regain consciousness. The endotracheal tube was removed once chewing began and the animals were able to swallow and withdraw their tongue. Water was withheld for 3 hours and food for a further 6 hours.

The surgical site was inspected twice daily following surgery and food intake monitored. Seven to ten days post surgery the exteriorised part of the rumen began to detach along the clamp. The sutures, Michelle clips and clamp were then removed along with the externalised piece of rumen wall and a rumen cannula (25mm o.d.; Beruc Equipment, Benoni West, Republic of South Africa) was inserted into the rumen fistula.

2.3.1.2 Feeding and husbandry

Following surgery the sheep grazed a perennial ryegrass-white clover pasture for two months prior to the commencement of the experiment. In order to minimise endogenous skatole production a pelleted concentrate diet (Table 2.1) with low rumen degradable protein content (13.4 MJ/kg ME; 130 g/kg DM CP; 40 g/kg DM estimated RDP) was

designed to replicate the skatole reducing effect of maize feeding shown by Young *et al.* (2003). Animals were adjusted to this diet during a 14 day period on an outdoor feed pad. They were then housed indoors for a further 14 day period to adapt to individual metabolism crates and hourly feeding (50 g DM/h), prior to commencement of treatments. Water was available *ad libitum.*

Table 2.1. Composition of the pelleted concentrate diet

Component	Dry matter (g)
Maize grain[1]	804
Peas[1]	120
Lucerne meal[1]	108
Barley straw[1]	108
Molasses[1]	36
Limestone[1]	12
Dicalcium Phosphate[1]	<4
Salt[1]	<4
Vitamin and mineral pre-mix (sheep)[2]	<4

[1]Denver Stock Feeds Limited, Palmerston North, New Zealand.
[2]Vitec Nutrition Limited, Auckland, New Zealand.

2.3.1.3 Treatments, infusion and sampling

A completely randomised block design (six sheep per block) was used with period of infusion (n = 2) as a blocking factor. A pre-treatment of the carrier, propylene glycol (propane-1,2-diol; 99.5% purity; Asia Pacific Specialty Chemicals, Seven Hills, NSW, Australia) was administered as a continuous intraruminal infusion to all sheep at a rate of 5 mL/h via a multi channel variable speed peristaltic pump (Watson-Marlow Bredel Pumps, Falmouth, Cornwall, UK) for 72 hours. Control sheep (n = 6) continued to receive an infusion of skatole-free carrier while treated sheep (n = 6) received propylene glycol

containing 28 mg/mL skatole (3-methylindole, 98% purity; Aldrich Chemical Company, Milwaukee, WI, USA), via a common pump, supplying a total skatole dose of 140 mg/h for a period of 72 hours. Skatole infusate was prepared daily to minimise skatole oxidation prior to administration.

Rumen digesta, rumen fluid and jugular blood samples were collected at 0, 2, 4, 6, 8, 12, 16, 24, 48 and 72 hours relative to the start of the treatment infusion. Whole rumen digesta samples were frozen (-20°C) for determination of total nitrogen content. The rumen liquid fraction was obtained by straining using two layers of muslin cheese cloth. For skatole analysis sub-samples of the rumen liquid fraction (4 mL) were snap-frozen in liquid nitrogen, to inhibit microbial activity, and stored at -20°C. Blood samples were kept chilled on ice, plasma was separated via centrifugation (3,300 *g*, 15 minutes, 4°C), snap-frozen in liquid nitrogen and stored at -85°C. All sheep were euthanased 72 h after commencement of the infusion. At 72 h, samples of liver, inter-muscular fat from the hind leg, kidney, muscle and rumen epithelium were collected, frozen immediately in liquid nitrogen and stored at -85°C.

2.3.2 Analytical Methods

2.3.2.1 Rumen fluid skatole determination

Skatole concentration in rumen fluid was determined using the high performance liquid chromatography (HPLC) method of Mattivi *et al.* (1999) as described by Schreurs *et al.* (2003). The method employed a solid phase extraction followed by HPLC analysis. Samples were loaded into a 1 mL column containing 50 mg of ISOLUTE® ENV+ sorbent (International Sorbent Technologies, Hengoed, Mid Glamorgan, UK). The sorbent is a

cross linked polystyrene polymer capable of retaining small hydrophobic analytes such as skatole. The ISOLUTE® ENV+ sorbent was prepared for the rumen fluid sample by conditioning with 1 mL of 100% methanol and equilibrated by rinsing with 1mL of equilibration buffer (potassium dihydrogen orthophosphate 2.36 mg/mL and disodium hydrogen phosphate 3.86 mg/mL, pH 7.0 at 20°C). Samples were prepared for column extraction by adding 100% methanol 1:1 with 0.5 mL of rumen fluid sample and mixed by vortex. The samples were centrifuged (2000 *g* for 5 minutes) and the supernatant collected. The remaining pellet was twice re-suspended in 1 mL of equilibration buffer, mixed using a vortex and centrifuged (2000 *g* for 5 minutes) and the resulting supernatants combined. The combined supernatants were added to the solid phase extraction column and eluted by gravitational flow. Following the sample each column was eluted with 1 mL 20% methanol and 1 mL 55% methanol in equilibration buffer to remove lipophilic contaminants. The column was then eluted with 2 mL methanol to collect the solute-bound skatole (Figure 2.1). An internal standard (50 mL; 2-methylindole, 0.05 μg/μL) was added to the MeOH eluate and the solution transferred into a sealed HPLC vial.

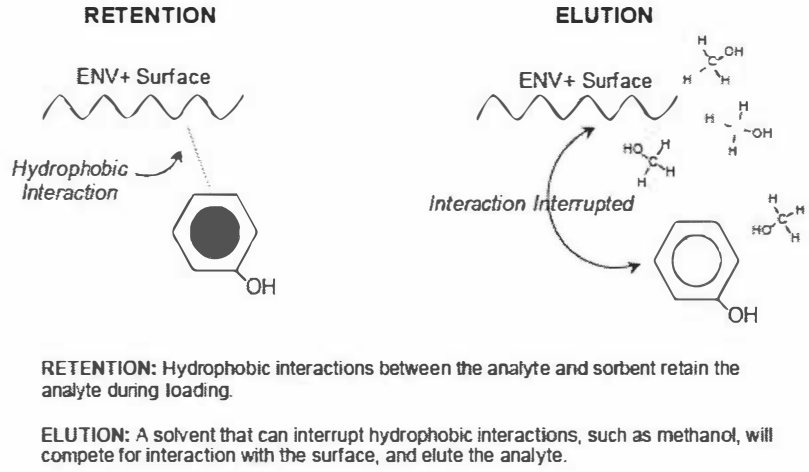


Figure 2.1. Interaction between analyte and ISOLUTE® ENV+ sorbent used in solid phase extraction of skatole from the aqueous rumen fluid admixture (International Sorbent Technology, 1997).

Skatole concentration in the solid phase extraction eluate was measured using an HPLC system comprising a Shimadzu (LC10ADvp) pump, auto injector (SIL-10ADvp) and detector (RF-10Axl; Shimadzu, Kyoto, Japan). The chromatography used an isocratic mobile phase of 70% acetic acid and 30% isopropanol (Hypersolv, BDH Laboratory Supplies, Poole, Dorset, UK) at 1 mL/min. An injection volume of 5 µL was used and chromatographic separation was performed using a reverse-phase C18 column (Platinum, 150 x 4.6 mm; Alltech, Deerfield, IL, USA). The fluorescence excitation was set at 285 nm and emission to 350 nm for the detection of skatole and the internal standard 2-methylindole. An external standard containing 2-methylindole (0.05 µg/µL) and skatole (3-methylindole; 0.1 µg/µL) was analysed concurrently to provide a reference for the calculation of skatole concentration in the samples. Data acquisition and peak analysis was performed using Shimadzu, Class-VP software (version 5.032, Shimadzu, Kyoto, Japan). Peak area for skatole was normalised to the peak area of the internal standard (2-

methylindole) and concentration calculated using peaks areas obtained with the external standard and adjusted for the weight of rumen fluid sample used for solid phase extraction.

2.3.2.2 Plasma skatole determination

Plasma skatole concentration was determined using the ether extraction method of Claus *et al.* (1993) and HPLC analysis (Schreurs *et al.*, 2003). Briefly an internal standard (5 ng 2-methylindole in acetonitrile/water, 75:25, v/v) was added to 0.5 mL of plasma sample and vortex mixed with 2 mL diethylether. The mixture was centrifuged (1200 *g*, 15 minutes) and incubated at -20°C to freeze the aqueous phase. The ether-borne skatole was decanted into tubes containing 1 mL of HPLC mobile phase (0.02 M acetic acid/isopropanol, 60:40, v/v) and the ether was removed by evaporation at 47°C. The resulting mobile phase was passed through a 2 μm filter (Upchurch Scientific, Oak Harbour, USA) and transferred into a sealed HPLC vial. Skatole concentration was measured using the HPLC system described above for rumen fluid samples.

2.3.2.3 Inter-muscular fat skatole determination

The skatole content of inter-muscular fat was determined using gas chromatograph mass spectrometry (GC-MS) following simultaneous distillation-extraction as described by Schreurs *et al.* (2007e). Briefly, a sample of fat (5 to 10 g) was added to a 250 mL round bottom flask containing 40 mL of saturated sodium chloride and internal standard (50 ng/g d_3-skatole). 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.

2.3.3 Microarray Experiment Methods

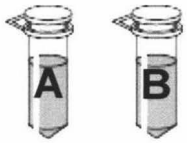
2.3.3.1 RNA isolation and purification

The acid guanidinium thiocyanate-phenol-chloroform extraction method as described by Chomczynski and Sacchi, (1987) was found to be unsuitable for the extraction of RNA from the ovine liver due to precipitation of impurities and subsequent degradation of RNA. A modified method was developed (Figure 2.2) whereby total hepatic RNA was isolated from liver tissue by simultaneously thawing and homogenising the samples in ice-cold TRIzol (Invitrogen, Auckland, New Zealand). Isolated RNA was purified using a RNeasy kit (Qiagen, Valencia, CA, USA) and quantified with a Nanodrop ND-1000 spectrophotometer (NanoDrop Technologies, Wilmington, DE, USA). RNA integrity was assessed using the Agilent 2100 Bioanalyser (Agilent Technologies, Palo Alto, CA, USA). Only total RNA with an OD 260/280 ratio >2.0 and a Bioanalyser 28s/18s peak ratio >1.4 was used for microarray hybridisation.

1

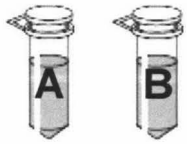
TRIzol extraction of RNA
Thaw and homogenise liver tissue in TRIzol (on ice)
1 mL TRIzol : 100 mg liver tissue
Incubate (10 minutes on ice)
Centrifuge (12000 *g*, 10 minutes, 4°C)
Divide supernatant into two tubes

2



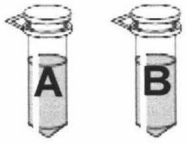
Remove phenol and proteins
+ 200 µL Chloroform/mL TRIzol used
Vortex mix
Centrifuge (12000 *g*, 15 minutes, 4°C)
Collect aqueous fraction

3



Precipitate RNA
+ 500 µL Isopropanol
+ 500 µL salt solution (0.8M sodium citrate & 1.2M NaCl)
Vortex mix
Incubate (10 minutes on ice)
Centrifuge (12000 *g*, 10 minutes, 4°C)
Collect supernatant

4



Wash RNA pellet
Add 500 µL 75% ethanol
Vortex mix
Centrifuge (7500 *g*, 5 minutes, 4°C)
Air dry pellet

5

Dilute pellet and re-precipitate RNA
Combine both pellets in 100 µL RNAse free H_2O
+ 10 µL 3M sodium acetate
+ 1 µL glycogen
+ 278 µL 100% ethanol
Vortex mix
Incubate (-85°C, 30 minutes)
Centrifuge (12000 *g*, 30 minutes, 4°C)
Discard supernatant

6

Wash RNA pellet (repeat 3 times)
+ 1 mL 70% ethanol
Vortex mix
Centrifuge (12000 *g*, 10 minutes, 4°C)
Discard supernatant

7

Re-suspend RNA
Air dry pellet
Re-suspend in 40 µL RNAse free H_2O
Quantify [RNA] (Nanodrop spectrophotometer)

Figure 2.2. A modified method developed to obtain total RNA from sheep liver tissue.

2.3.3.2 Experimental design

The microarray experiment consisted of an 18 array augmented two loop design (Figure 2.3) balanced for dye bias with a treatment comparison efficiency, control vs. skatole of 1. cDNA samples from each sheep were paired for hybridisation against the three sheep of the opposite treatment within the same treatment block (day).

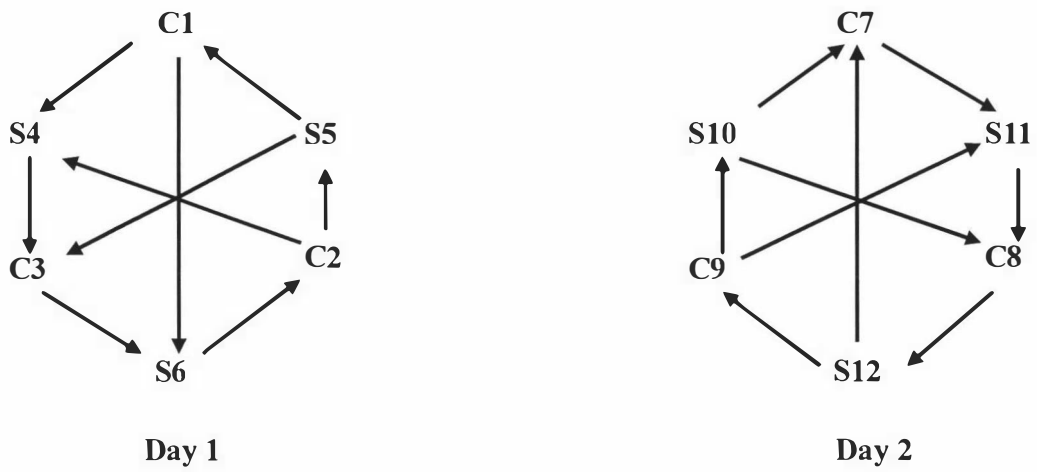


Figure 2.3. Eighteen slide microarray design, each loop represents the array design for three control (C) and three skatole (S) treated sheep blocked by day of treatment. Each arrow represents an array, the direction of each arrow represents cyanine dye allocation (Cy3 →Cy5).

2.3.3.3 Slide design and manufacture

cDNA microarray slides containing 20,736 expressed sequence tags (ESTs) were produced using the AgResearch bovine EST database that represents ESTs from a variety of tissues and approximately 80% of the bovine genome (Diez-Tascón *et al.* 2004). The cDNAs were amplified by PCR in a 50 µL reaction volume in 96-well plates. PCR products were precipitated with 0.2 M sodium citrate and an equal volume of isopropanol, the 96-well plates were centrifuged (3,500 *g* for 1 h), and resulting pellets washed with 70% ethanol and dried. Prior to printing the pellets were re-suspended in water, transferred to 384-well

plates, dried and re-suspended in 3 x SSC printing solution (0.45 M sodium chloride, 0.045 M sodium citrate). The amplified products were printed onto glass microscope slides (Gold Seal Microslides; Erie Scientific Corporation, Portsmouth, NH, USA) coated with poly-$_L$-lysine solution (Baird *et al.*, 2004). Printing was performed using an ESI array robot (Engineering Services Inc., Toronto, ON, Canada) with up to 32 split pinheads depositing 0.6 nL with a 100 μm spot size. After printing the slides were UV irradiated to cross-link the DNA to the polylysine coating.

2.3.3.4 cDNA synthesis, labelling and array hybridisation

cDNA was prepared for microarray screening using the SuperScript Indirect cDNA Labeling System (Invitrogen, Carlsbad, CA, USA) incorporating Cy3 and Cy5 mono-reactive cyanine dyes (GE Healthcare, Buckinghamshire, UK). Microarray slides were prepared by incubation for 20 minutes in 45°C pre-hybridisation buffer 12.5 mL 20x SSC (3.0 M sodium chloride; 0.30 sodium citrate), 38 mL H_2O, 0.25 mL 10% SDS and 12.5 mg bovine serum albumin, rinsed twice in deionised H_2O, once in isopropanol and air dried. The dye labelled cDNA samples were combined and denatured by heating (95°C, 10 minutes) prior to mixing with 60 μL of SlideHyb Buffer #1 (Ambion, Austin, TX, USA) loading buffer at 68°C. The labelled cDNA was hybridised to 20,736 EST cDNA bovine microarrays in microarray hybridisation chambers (Corning, Corning, NY, USA) for 16 hours using a 45°C water bath. Following hybridisation slides were washed by vigorous shaking by hand in three solutions at 45°C over 17 minutes to remove the hybridisation buffer and unbound cDNA and centrifuged (210 *g*, 3 minutes) to remove residual moisture then dried at 37°C and kept in the dark to avoid photo-bleaching.

2.3.3.5 Microarray scanning

The slides were scanned using a GenePix Professional 4200A scanner (Molecular Devices, Sunnyvale, CA, USA) using two photo multiplier tube (PMT) voltages: a high scan of approximately 550 PMT, and a low scan of approximately 450 PMT. Spot identification and quantification was performed with GenePix 6.0 software (Molecular Devices, Sunnyvale, CA, USA).

2.3.4 Pharmacological Model of Skatole Elimination

To determine the rate of skatole elimination by skatole infused sheep, a two compartment pharmacological model (Thomas and Beadle, 1985) was generated for the rumen and plasma concentration data from six skatole-infused sheep using the population study algorithm from WinSAAM (Wastney *et al.*, 1999; Figure 2.4).

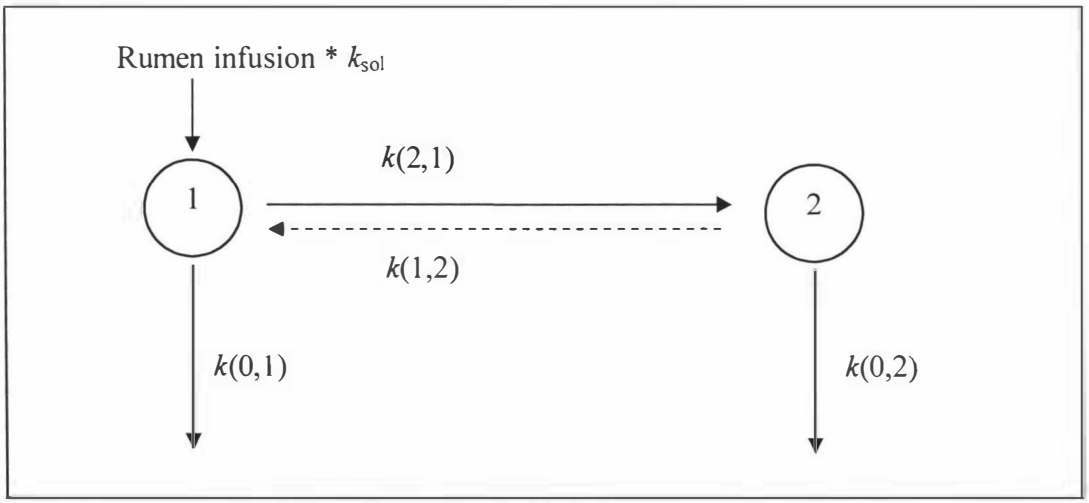


Figure 2.4. A two-compartment model fitted to the rumen liquid phase (compartment 1) and plasma (compartment 2) skatole data of rumen skatole-infused sheep. k_{sol} describes the partition coefficient of infused skatole into the liquid phase. The model provides an estimated rate of transfer from rumen to peripheral blood k(2,1), peripheral blood to rumen k(1,2) and an estimated rate of clearance from the rumen k(0,1) and peripheral circulation k(0,2).

Compartment 1 was assumed to be the rumen liquid phase. Skatole entering into this compartment was calculated as the hourly rate of skatole infusion multiplied by an assumed partition coefficient of skatole into the liquid phase (k_{sol}) of 21% (Carlson *et al.*, 1981), with the remaining skatole becoming associated with the particulate fraction outflow from the rumen. The volume of compartment 1 was assumed to be 10% of the bodyweight of the animal (de Vega *et al.*, 1998). Compartment 2 represents absorbed skatole, assuming an apparent volume of distribution of skatole of 1.3 L/kg BW (Friis, 1993). The apparent volume of distribution is the theoretical plasma volume in which the total absorbed skatole dose would have to be diluted to produce a given plasma concentration. As skatole is lipophilic its rapid deposition into adipose tissues results in a reduced plasma concentration, thus for any given dose skatole has a low plasma concentration and a high volume of distribution. First order kinetics were assumed for the two compartments, with rates of transfer defined as $k(2,1)$ for transfers from rumen to plasma and $k(1,2)$ for transfer from plasma to rumen. Additional loss from the rumen was assumed to occur in the form of passage of liquid to the post rumen compartment: $k(0,1) = 0.07$ (average of Karsli and Russell, 1999 and Uden *et al.*, 1982). The rate of disappearance from compartment 2 (i.e. $k(0,2)$) was assumed to represent the capacity of individual sheep to metabolise skatole and skatole accretion into body tissues. Skatole concentrations measured from control animals (multiplied by rumen liquid and plasma volume) were used to define the initial conditions for the two compartments.

2.3.5 Statistical Analysis

2.3.5.1 Animal experimental data

Rumen and plasma data were analysed using GenStat, version 8.11 (Lawes Agricultural Trust, 2005). As variance of the measurements in the control group was considerably lower than those of the skatole group, a non-parametric Kruskal-Wallis analysis of variance test was used to compare the skatole concentration data in rumen fluid and plasma between the two groups. Tests of rumen and plasma data were done at 6, 16 and 72 hours. Skatole concentration in inter-muscular fat was analysed using the MIXED procedure of SAS (2002, v. 9.1 SAS Institute). Fixed effects included treatment, while heterogeneous variances between groups were dealt with by using the grouping variance option in the "repeated" statement of the procedure, using sheep as the subject (i.e. random effect). A simple covariance matrix (i.e. variance components) was deemed to be the most appropriate structure underlying the data. Significant difference between treatments was declared when the probability was less than 0.05.

2.3.5.2 Microarray experimental data

Microarray data was analysed using the limma package in Bioconductor (Smyth, 2005). The high scan intensities were used for all EST spots except those nearing saturation. This level was optimised for each slide by determining the approximate point at which there was no longer a constant ratio between the high and low scans. The low scan results were used otherwise, having first adjusted these values using the algorithm described by Lyng *et al.* (2004). For each EST a modified t-test was performed and the probability values were then adjusted for multiple testing using the Benjanini and Hochberg correction (Benjamini and Hochberg, 1995), resulting in a FDR. The FDR is the probability that a gene declared to be

significant is in fact not significant. ESTs with FDRs less than 0.01 were considered to be of interest.

2.4 RESULTS

2.4.1 Animals, Intakes and Infusion Rates

There was no significant difference (P = 0.44) between the mean bodyweight of sheep allocated to skatole (40.5 ± 3.6 kg) and control (42.1 ± 3.4 kg) infusions. All sheep consumed 1,200 g DM/day of the concentrate diet. The rate of skatole infusion was 141 mg/h for all treated sheep.

2.4.2 Rumen, Plasma and Fat Skatole

The production of skatole from rumen degradation of dietary tryptophan was minimised by the diet. Mean skatole concentration (±SEM) was 0.02 ± 0.02 μg/g in rumen fluid and 0.74 ± 0.77 ng/mL in plasma of control sheep during the treatment period. There was no significant effect (P = 0.90) of period of infusion (blocks) on rumen or plasma skatole concentrations measured.

Infusion of skatole generated a rapid rise in skatole concentration in both the liquid fraction of the rumen and peripheral blood (Figure 2.5). Mean skatole concentration in the rumen fluid and peripheral circulation of skatole-infused sheep was greater (P < 0.01) than control sheep at all times following the start of infusion. At 72 hours, the mean concentration of skatole (± SEM) in rumen fluid and plasma was 20.6 ± 6.5 μg/g and 154.2 ± 87.3 ng/mL in treated sheep and 0.01 ± 0.03 μg/g and 0.89 ± 0.91 ng/mL in control sheep respectively.

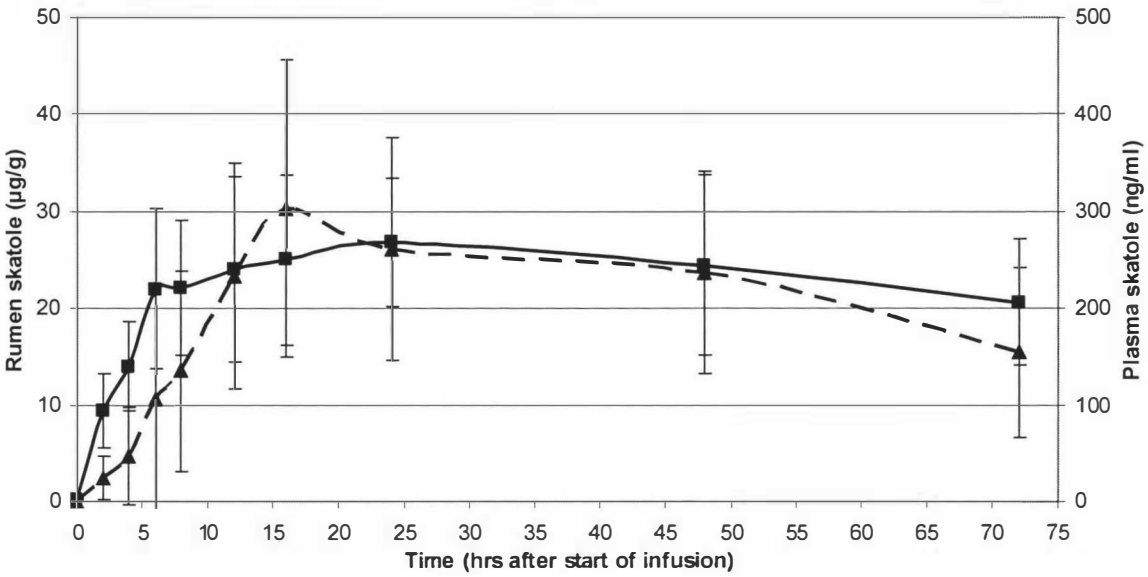


Figure 2.5. Mean skatole concentration in rumen fluid (■; µg/g) and plasma (▲; ng/ml) of sheep receiving a continuous rumen infusion of skatole (141 mg/h; n = 6). Skatole concentrations in rumen fluid (0.02 ± 0.02 µg/g) and plasma (0.74 ± 0.77 ng/ml) of control sheep (n = 6) were unchanged throughout the treatment period (data not shown). Error bars shown are the SEM.

Skatole concentration in inter-muscular fat from the hind leg was increased ($P < 0.001$) by the skatole infusion resulting in a mean (± SEM) concentration of 4,240 ± 1,720 ng/g and 15 ± 15 ng/g in treated and control groups, respectively.

2.4.3 Estimation of Skatole Elimination

Fitting the model described in Figure 2.4 to the rumen and plasma skatole concentration data of treated sheep indicated that the rate of transfer from plasma to rumen ($k(1,2)$) was close to zero and was therefore removed from the model. Fitting the model on this basis resulted in estimates for parameter $k(2,1)$ of 0.23 ± 0.02/hour and for parameter $k(0,2)$ of 2.08 ± 0.35/hour via all routes of elimination (Table 2.2).

Table 2.2. Mean skatole flux in treated sheep (n = 6) as predicted using the two compartment model described in Figure 2.4, using population parameters generated from the WinSAAM population algorithm (Wastney et al., 1999).

Parameter	Rate (g/day)	90% confidence interval [1]
Inflow into rumen liquid	0.71	Not applicable
Outflow from rumen to intestine $k(0,1)$	0.16	0.157 – 0.164
Absorption from rumen $k(2,1)$	0.55	0.545 – 0.553
Clearance from circulation $k(0,2)$ [2]	0.55	0.545 – 0.553

[1] Of mean given a two tailed normal distribution.
[2] Assuming transfer from peripheral blood to rumen $k(1,2)$ to be zero.

Rates of skatole elimination of individual treated sheep ranged from 1.11/hour to 4.47/hour of the total absorbed skatole pool. The rate of skatole elimination from the peripheral circulation was negatively correlated (-0.73, $R^2 = 0.47$, $P < 0.05$) with the concentration of skatole in the intra-muscular fat deposits from the hind leg of treated sheep.

2.4.4 Microarray Expressed Sequence Tag Identification

ESTs with significant differential expression ($FDR < 0.01$) in either direction represented about 14% of those assessed. Of these, five had a fold change greater than 2 in response to skatole treatment (Table 2.3). Genes with a fold change of 1.5 fold in either direction in response to skatole treatment are listed in appendix I (increased expression) and appendix II (decreased expression).

Table 2.3. Genes identified via cDNA microarray analysis with a greater than 2 fold increase in expression in response to the skatole treatment.

Gene name	Symbol	Accession Number	Fold Change	FDR[1]
Aldehyde dehydrogenase 1 family member A1	ALDH1A1	OAU12761	+2.94	6.3E-10
NAD(P)H dehydrogenase quinone 1	NQO1	DY484462	+2.56	7.6E-8
Leukotriene B_4 12-hydroxydehydrogenase	LTB4DH	DY507800	+2.51	9.1E-11
Stearoyl-CoA desaturase	SCD	NM_001009254	+2.05	2.5E-10
Glutathione S-transferase A1	GSTA1	AF140223	+2.08	1.2E-8

[1]False Discovery Rate

Three up-regulated genes encode cytosolic phase I oxidoreductase enzymes involved in xenobiotic metabolism: 1) aldehyde dehydrogenase 1 family member A1 (EC 1.2.1.3) uses NAD^+ as a co-factor to catalyse the clearance of potentially cytotoxic aldehydes that react with thiol and amino groups; 2) NAD(P)H dehydrogenase quinone 1 (EC 1.6.5.2) utilises either NADH or NADPH as a reducing co-factor to catalyse the conversion of toxic quinones to hydroquinones in a single two-electron step thus bypassing the production of potentially toxic semiquinone radical intermediates (Ross *et al.*, 2000); and 3) leukotriene B_4 12-hydroxydehydrogenase (EC 1.3.1.74) catalyses the initial key step of the reductive inactivation of leukotriene B_4 to its less reactive metabolite, 12-oxo-leukotriene B_4 in the presence of $NADP^+$. It also catalyses the detoxification of α,β-unsaturated aldehydes and ketones via reduction of the α,β-carbon-to-carbon double bond (Dick *et al.*, 2001).

In addition to these the expression of the gene encoding metabolic oxidoreductase enzyme stearoyl-CoA desaturase (EC 1.14.19.1) was also induced. Stearoyl-CoA desaturase is a

membrane-bound lipogenic enzyme involved in maintaining cellular lipid homeostasis. It catalyses the Δ9-*cis* desaturation of saturated fatty acyl-CoAs used in the synthesis of monounsaturated fatty acids (Chu *et al.*, 2006). The implication of induced stearoyl-CoA desaturase expression during skatole metabolism is unclear. The fifth gene, glutathione S-transferase (EC2.5.1.18) exists in cytosolic and microsomal isoforms, has been purified from ovine liver tissue and catalyses the phase II conjugation of toxic electrophiles with glutathione (Eaton and Bammler, 1999).

2.5 DISCUSSION

The present experiment successfully prepared a cohort of twelve half-sibling wether sheep for investigation of skatole metabolism. Adaptation of the sheep to an hourly concentrate feeding regime over a 28 day period minimised skatole concentration in rumen fluid, peripheral blood and inter-muscular fat.

Intraruminal skatole infusion produced a rapid rise in skatole concentration in both rumen liquid and peripheral plasma pools. The elevation of skatole concentration in the peripheral circulation indicated that the infusion rate was sufficient to exceed the first pass clearance capacity of the liver and therefore confirms that the liver of skatole treated sheep were exposed to elevated levels of skatole. A significant difference in hepatic skatole exposure between treatment groups was maintained throughout the 72 hour treatment period. As expected from pre-experimental feeding regimes, skatole concentration in control animals remained negligible throughout the experiment ensuring a minimal hepatic exposure to skatole in control sheep.

Rapid appearance of rumen-infused skatole in the jugular blood and inter-muscular fat is evidence of a high rate of both absorption and deposition of skatole in sheep. A two compartment model fitted to the rumen and plasma skatole concentrations of skatole treated sheep enabled estimation of the rate of elimination from peripheral circulation (k = 2.10). Individual sheep displayed a range of elimination rates (k = 1.11 to 4.47) and the rate of skatole elimination was negatively correlated (-0.73, R^2 = 0.47, $P < 0.05$) with the accumulation of skatole in inter-muscular fat. This variation between sheep receiving the

same skatole dose indicates that whole-body metabolism of skatole, rather than rumen skatole production alone, influences the accumulation of skatole and therefore pastoral flavour in sheep meat.

The 20,736 ESTs used for this microarray experiment were selected from AgResearch's proprietary database that represented approximately 80% of the bovine genome. The prospect for successful analysis of the ovine hepatic tissue using bovine ESTs was high since the estimated homology of protein coding sequence between ovine and bovine is greater than 97% (Kijas *et al.*, 2005). Analysis of the microarray data has identified five genes; aldehyde dehydrogenase 1-A1, NAD(P)H dehydrogenase quinone 1, leukotriene B_4 12-hydroxydehydrogenase, stearoyl-CoA desaturase and glutathione S-transferase from the ovine liver with increased expression in response to skatole exposure. Although not previously linked to skatole metabolism their possible roles can be elucidated from their known functions. These include; substrate specific phase I oxidoreductase activity towards toxic xenobiotics (aldehyde dehydrogenase 1-A1, NAD(P)H dehydrogenase quinone 1 and leukotriene B_4 12-hydroxydehydrogenase), oxidoreductase activity towards endogenous metabolic products (stearoyl-CoA desaturase) and phase II conjugation of xenobiotic metabolites (glutathione S-transferase). Several known functions of these genes and enzymes are of potential relevance to the metabolism of skatole by sheep and are discussed below.

The up-regulation of the gene expression levels of three enzymes involved in the reduction (aldehyde dehydrogenase 1-A1, leukotriene B_4 12-hydroxydehydrogenase) and conjugation (glutathione S-transferase) of aldehydes suggest that aldehydes are present in the liver

during skatole metabolism. These enzymes, together, catalyse phase I and II detoxification of aldehydes by increasing their solubility thus limiting their accumulation in lipids and increasing their excretion in urine. This process is accelerated by the ability of glutathione S-transferase conjugated metabolites to be rapidly eliminated from cells via ATP-dependant glutathione S-conjugate efflux pumps (Hayes and Pulford, 1995). Furthermore the pulmonary lesions formed by reactive metabolites of skatole in ruminants are consistent with aldehyde cytotoxicity (Sladek *et al.* 1989; Guan and Weiner, 1990; Dick *et al.*, 2001). Both aldehyde dehydrogenase and glutathione S-transferase A1-1 have been purified from sheep liver (Reddy *et al.*, 1983; Stayner and Tweedie, 1995; Prabhu *et al.*, 2001) indicating the possible role of these enzymes in catalysing the high rate of skatole elimination observed in this study.

Up-regulation of glutathione S-transferase gene expression in skatole treated sheep is consistent with the function of this enzyme in catalysing phase II detoxification of electrophilic compounds via glutathione conjugation (Reddy *et al.*, 1983). Glutathione inhibits covalent binding of skatole metabolites to bovine microsomal proteins (Hanafy and Bogan, 1982) and the pre-treatment of sheep with pulmonary glutathione depletion agent diethylmaleate increases their sensitivity to the pneumotoxic effects of skatole, demonstrating the importance of glutathione conjugation in skatole detoxification.

The research of van Lieshout *et al.* (1998) and Diaz *et al.* (1999) confirms a possible link between phase I hepatic metabolism of skatole (porcine, microsomal) and the hepatic concentration of hepatic glutathione S-transferase (murine, cytosolic). This theory provides a logical mechanism for the induction of glutathione S-transferase expression in skatole-

treated sheep in the current experiment. Indole-3-carbinol (Figure 2.6), a metabolite of phase I skatole metabolism in pig liver microsomes (Diaz *et al.*, 1999), has been shown to increase concentrations of hepatic glutathione S-transferase alpha enzyme levels when fed to male Wistar rats for 14 days (van Lieshout *et al.*, 1998).

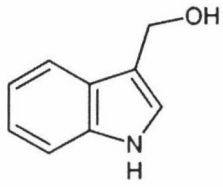


Figure 2.6. Structural formula of indole-3-carbinol

Large inter-individual differences in the rate of production of indole-3-carbinol by porcine liver microsomes exist and are attributed to differences in the activity or level of expression of phase I biotransformation enzymes (Diaz *et al.*, 1999). Induction of glutathione S-transferase via a phase I metabolite (indole-3-carbinol) feedback mechanism could explain the inter-individual variations in skatole elimination rate observed in the current study given the accelerated elimination of glutathione conjugates via efflux pumps (Hayes and Pulford, 1995).

A mediator of glutathione S-transferase and NAD(P)H dehydrogenase quinone 1 induction is the antioxidant response element, which is responsive to dietary phenolic antioxidants (Benson *et al.*, 1980; De Long *et al.*, 1986; Masella *et al.*, 2005). Given this evidence, and the up-regulation of these genes in the current study, further investigation is warranted to establish if the reduction of skatole accumulation in the adipose tissue of sheep fed phenolic containing plants (Meagher *et al.*, 2004; Schreurs *et al.*, 2007e) is solely due to reduced

rumen skatole production (Tavendale *et al.*, 2005; Schreurs *et al.*, 2007d). An alternative explanation is that dietary phenolics might reduce skatole deposition in sheep fat due to a combined effect of reduced rumen protein degradation and an induction of hepatic skatole metabolising enzymes via the antioxidant response element. Further research is required to elucidate the role of dietary phenolic antioxidants in skatole accumulation.

A further interaction between three genes with up-regulated expression in the current study is the ability of dithiolethione leukotriene B_4 12-hydroxydehydrogenase to induce expression of glutathione S-transferase and NAD(P)H dehydrogenase quinone 1 genes (Dick *et al.*, 2001). Skatole exposure has thus triggered the induction of an inter-related group of phase I and II detoxification genes.

In this study it was hypothesised that the controlled administration of skatole to sheep would increase rumen skatole concentrations and induce differential gene expression in the ovine liver. The 72 hour infusion of skatole was effective in raising skatole concentration in rumen fluid, peripheral circulation and inter-muscular fat in addition to inducing the up-regulation of aldehyde dehydrogenase 1-A1, NAD(P)H dehydrogenase quinone 1, leukotriene B_4 12-hydroxydehydrogenase, stearoyl-CoA desaturase and glutathione S-transferase genes. The available evidence suggests that these genes have plausible roles in the elimination of skatole in sheep. Further investigation is required to determine the role of these genes in the elimination of skatole by the ovine liver. Furthermore, the elimination of skatole from the peripheral circulation of treated sheep was negatively correlated with its accumulation in inter-muscular fat indicating that pastoral flavour, associated with skatole concentration of sheep meats, is influenced by skatole metabolism in addition to skatole

formation in the rumen. The between animal variation in fractional rate of skatole elimination (1.11 to 4.47/hour) from the peripheral circulation observed amongst genetically similar sheep in this study suggests that variation in the accumulation of absorbed skatole can be expected within the New Zealand sheep population. This finding is of particular importance to the New Zealand sheep meat industry. Selection of animals for enhanced skatole clearance has the potential to reduce the risk of product rejection by consumers arising from a small number of high skatole accumulating animals. Marker assisted selection or implementation of a sire testing scheme to identify elite rams with a propensity to produce low-skatole accumulating offspring has the potential to reduce pastoral flavour in sheep meats produced under existing pastoral conditions. Such an initiative could enable the New Zealand sheep meat industry to broaden the appeal of its products in export markets where consumers are sensitive to pastoral flavours.

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APPENDIX I

Genes with increased expression of >1.5 fold in response to skatole treatment.

Gene name	Symbol	Accession Number	Fold Change	FDR[1]
Aldehyde dehydrogenase 1 family, member A1	ALDH1A1	NM_000689	2.94	6.3E-10
NAD(P)H dehydrogenase, quinone 1	NQO1	NM_000903	2.56	7.6E-08
Leukotriene B4 12-hydroxydehydrogenase	LTB4DH	NM_012212	2.51	9.1E-11
NAD(P)H dehydrogenase, quinone 1	NQO1	NM_000903	2.13	4.3E-06
Glutathione S-transferase A1	GSTA1	NM_145740	2.08	1.2E-08
Stearoyl-CoA desaturase (delta-9-desaturase)	SCD	NM_005063	2.05	2.5E-10
Stearoyl-CoA desaturase (delta-9-desaturase)	SCD	NM_005063	1.97	6.2E-07
SFRS protein kinase 1	SRPK1	NM_003137	1.89	2.6E-09
Thioredoxin	TXN	NM_003329	1.87	1.1E-08
Glutathione S-transferase A1	GSTA1	NM_145740	1.74	1.3E-08
Thioredoxin	TXN	NM_003329	1.74	2.5E-08
Aldo-keto reductase family 1, member C1	AKR1C1	NM_001353	1.67	9.6E-06
Heat shock 22kDa protein 8	HSPB8	NM_014365	1.66	2.1E-04
3-hydroxy-3-methylglutaryl-Coenzyme A synthase 1 (soluble)	HMGCS1	NM_002130	1.61	1.9E-08
Microsomal glutathione S-transferase 1	MGST1	NM_145764	1.59	2.8E-09
UDP glucuronosyltransferase 2 family, polypeptide B7	UGT2B7	NM_001074	1.58	2.3E-4
Flavin containing monooxygenase 5	FMO5	NM_001461	1.58	1.1E-08
Aminolevulinate, delta-, synthase 1	ALAS1	NM_000688	1.58	8.4E-07
Protein tyrosine phosphatase, receptor type, F	PTPRF	NM_002840	1.57	1.9E-08
Galactose mutarotase (aldose 1-epimerase)	GALM	NM_138801	1.56	5.7E-08
UDP glucuronosyltransferase 2 family, polypeptide B10	UGT2B10	NM_001075	1.56	1.3E-06

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NAD(P)H dehydrogenase, quinone 1	NQO1	NM_000903	1.55	1.9E-06
Protein phosphatase 2A, regulatory subunit B' (PR 53)	PPP2R4	NM_178000	1.55	6.2E-08
3-hydroxy-3-methylglutaryl-Coenzyme A synthase 1 (soluble)	HMGCS1	NM_002130	1.54	1.4E-05
Fatty acid desaturase 1	FADS1	NM_013402	1.52	4.9E-07
Aminolevulinate, delta-, synthase 1	ALAS1	NM_000688	1.52	1.4E-07
UDP-glucose dehydrogenase	UGDH	NM_003359	1.51	1.6E-07
Isopentenyl-diphosphate delta isomerase 1	IDI1	NM_004508	1.51	8.8E-09

[1]False Discovery Rate

APPENDIX II

Genes with >1.5 fold decreased expression in response to skatole treatment.

Gene name	Symbol	Accession Number	Fold Change	FDR[1]
Insulin-like growth factor binding protein 2, 36kDa	IGFBP2	NM_000597	-1.72	7.4E-08
Adhesion molecule, interacts with CXADR antigen 1	AMICA1	NM_153206	-1.70	3.8E-07
Insulin-like growth factor binding protein 1	IGFBP1	NM_001013029	-1.65	4.3E-08
B-cell translocation gene 1, anti-proliferative	BTG1	NM_001731	-1.54	9.4E-08
Cytochrome b5 domain containing 2	CYB5D2	NM_144611	-1.53	1.7E-05

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