METHANE EMISSIONS FROM RUMINANTS FED WHITE CLOVER AND PERENNIAL RYEGRASS FORAGES

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ABSTRACT

Ruminant enteric methane (CH_4) emissions account for ~35% of New Zealand's total greenhouse gas (GHG) emissions and a commitment has been made for their reduction. Previous research suggested lower CH_4 yields (g/kg dry matter intake; DMI) from sheep fed white clover (*Trifolium repens*) compared to perennial ryegrass (*Lolium perenne*; ryegrass), and the initial focus was to account for that difference. However, measurements undertaken here showed little difference between diets in CH_4 yield. The objective of this thesis was amended to better understand causes of variation in CH_4 emissions from ruminants fed white clover and ryegrass forages.

A database analysis showed greater variation in CH_4 yield from sheep fed ryegrass forages with measured intakes using the SF_6 technique, compared to respiration chambers (23.4 ± 5.70 vs. 23.1 ± 2.90 g/kg DMI). The composition of ryegrass fed to sheep predicted <2% and 20% of the variation in CH_4 yield when derived from SF_6 and respiration chamber techniques, respectively. For cattle, the database of CH_4 yields determined by SF_6 found ryegrass composition accounted for 13% of the variation.

Measurements in respiration chambers of CH_4 yield from sheep in three experiments reported here, had similar values for white clover and ryegrass (22.6 g/kg DMI), despite higher concentrations of fibre and less crude protein in ryegrass. Feed composition predicted less than 19% of variation in CH_4 yield. Measurements of CH_4 emissions from sheep fed white clover or ryegrass at multiples of 0.8 to 2.5 the metabolisable energy requirements for maintenance (ME_m) showed a decline in CH_4 yield of 3.47 g/kg DMI for each multiple of ME_m intake above maintenance. Measurements of rumen function and digesta kinetics, suggested the rate of liquid flow through the gastro-intestinal tract, and molar percentages of propionate were the main drivers of a change in CH_4 yield with intake.

This research has shown minor effects of forage composition on CH_4 yield, and has highlighted the importance of digestive function to account for effects of intake and individual variation on methanogenesis. The benefits of high feed intakes for production will be complemented by a low CH_4 yield and low emissions per unit of production.

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Dedicated in loving memory to my Grandad

Colin James Hammond

31st December 1935 to 5th September 2011

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A

(A + B)/P	acetate + butyrate/propionate ratio
ADF	acid detergent fibre
$AgNO_3$	silver nitrate
A:P	acetate to propionate ratio
ATP	adenosine triphosphate
ADP	adenosine diphosphate

B

bp	base pair

C

CH_4	methane
CH_4-E	methane energy
CH_4-E/GEI	methane energy relative to gross energy intake
cm	centimetres
Co	cobalt
CO_2	carbon dioxide
CO_2-eq	carbon dioxide equivalents (weight basis)
CP	crude protein
Cr	chromium
CT	condensed tannins
CV	coefficient of variation

D

d	day
DDM	digestible dry matter
DDMI	digestible dry matter intake
DGF	dry gas flow
DGGE	denaturing gradient gel electrophoresis
DM	dry matter
DMI	dry matter intake
DNDF	digestible neutral detergent fibre
DNDFI	digestible neutral detergent fibre intake
DOM	digestible organic matter
DOMI	digestible organic matter intake

E

EDTA	ethylenediamine tetraacetic acid
Ei	emissions intensity
Eq.	equation

F

FAD^+	flavin adenosine dinucleotide oxidised
FADH	flavin adenosine dinucleotide reduced

Fe^{3+}	iron
FOR	fractional outflow rate

G

g	gram
GC	gas chromatography
GE	gross energy
GEI	gross energy intake
GHG(s)	greenhouse gas(es)
GIT	gastro-intestinal tract
GWP(s)	global warming potential(s)

H

h	hour
H_2	hydrogen gas
H_2S	hydrogen sulphide
H^+	hydrogen ion
HCl	hydrochloric acid
HFC(s)	hydrofluorocarbon(s)
HP	hewlet packard
HWSC	hot water soluble carbohydrates

I

ICP-OES	inductively coupled plasma optical emissions spectrometry

K

kg	kilogram
KJ	kilojoule

L

L	litre
LCA	life cycle analysis
LCFA	long chain fatty acids
ln	natural logarithm
LW	live weight

M

M	moles
m	metres
ME	metabolisable energy
MEI	metabolisable energy intake
ME_m	metabolisable energy requirements for maintenance
MF	methanofuran
mg	milligram
min	minute
MJ	megajoule
ml	millilitre
mM	millimole
mm	millimetre

MRT	mean retention time
MW	molecular weight

N

N	nitrogen
no.	number
n	nano
Na	sodium
NAD^+	nicotinamide adenosine dinucleotide oxidised
NADH	nicotinamide adenosine dinucleotide reduced
$NADP^+$	nicotinamide adenosine dinucleotide phosphate oxidised
NADPH	nicotinamide adenosine dinucleotide phosphate reduced
NDF	neutral detergent fibre
NDFI	neutral detergent fibre intake
NFC(s)	non-fibre carbohydrate(s)
NH_2	amino group
NH_3	ammonia
NIRS	near infrared reflectance spectroscopy
NO_2^-	nitrite
NO_3^-	nitrate
N_2O	nitrous oxide

O

OM	organic matter
OMI	organic matter intake

P

Pa	Pascal
PCR	polymerase chain reaction
PEG	polyethylene glycol
PFC(s)	perfluorocarbon(s)
ppm	parts per million
PPS	protein precipitate solution
P-value	probability-value

R

R	correlation coefficient
R^2	coefficient of determination
REML	Restricted Maximum Likelihood
RFC	readily fermentable carbohydrates
RFC:NDF	readily fermentable carbohydrates to neutral detergent fibre ratio
RFI	residual feed intake
RG	ryegrass

S

SD	standard deviation
SED	standard error of the difference of the mean
SF_6	sulphur hexafluoride
STP	standard temperature and pressure

SOP(s)	standard operating procedure(s)
SO_4^{2-}	sulphate
SSS	soluble sugars and starch
U	
UNFCCC	United Nations Framework Convention on Climate Change
V	
V	volts
VFA(s)	volatile fatty acid(s)
VFI(s)	voluntary feed intake(s)
vs.	versus
W	
WC	white clover
°C	degrees Celsius
ΔG	free energy change
µ	micro
%	percentage
~P	high energy phosphate bond

CHAPTER 1

GENERAL INTRODUCTION

CHAPTER 1: GENERAL INTRODUCTON

1.1 INTRODUCTION

Over the last decade there has been growing international interest in emissions of greenhouse gases (GHG). Greenhouse gases are atmospheric gases that absorb and re-emit long-wave radiation back to the earth's surface. The main GHG's are carbon dioxide (CO_2), methane (CH_4) and nitrous oxide (N_2O). Faced with increasing evidence that the world's climate is getting warmer (IPCC, 2007), there is now an international effort to reduce anthropogenic GHG emissions to the atmosphere, with the United Nations Framework Convention on Climate Change (UNFCCC) and the Kyoto Protocol (Clark *et al.*, 2005) being the most important organisations. New Zealand ratified the Kyoto Protocol in early 2005 and committed to reduce GHG emissions to the 1990 levels during the 2008 to 2012 period (Ministry for the Environment, 2010).

Ruminants have the unique advantage of converting otherwise indigestible cellulose-rich plant material into meat, milk, wool and other products, whilst not competing directly with humans for food (Buddle *et al.*, 2011). However, farming ruminant livestock is associated with an environmental impact, and CH_4 emissions from enteric fermentation are a major contributor to New Zealand's GHG emissions. The principal source of CH_4 from ruminants is enteric fermentation arising mainly from digestion processes in the rumen and, to a lesser extent, the large intestine (Clark *et al.*, 2005, Waghorn and Woodward, 2006).

Agriculture is a major component of the New Zealand economy. In 2007, agricultural products comprised over 56% of total merchandise exports from New Zealand, valued at NZ$15.25 billion dollars per annum (Ministry for the Environment, 2010). Consumers today are becoming increasingly sensitive to issues such as food safety, food miles, environmental management and animal welfare, as well as demanding a higher level of food quality and traceability (i.e. what is in it, where it is from, and how it is made). To maintain international competitiveness, the New Zealand agricultural industry must attend to consumer expectations, including the GHG costs associated with production.

New Zealand has a temperate climate devoid of climatic extremes, which allows ruminant livestock to graze forages, predominately perennial ryegrass (*Lolium perenne*)-dominated pastures, all year round (Clark *et al.*, 2011). The New Zealand grazing system for production has fewer inputs, lower costs and fewer options for GHG mitigation than intensive animal production industries (Eckard *et al.*, 2010), so an accurate inventory is required, against which reductions can be measured. In addition to the importance of lowering GHG emissions, CH_4 represents a substantial loss of energy from ruminant feeds, which is not used for production. The percentage of gross energy (GE) loss to CH_4 (CH_4-E), expressed in relation to GE intake (GEI), is typically 5 to 7% from ruminants grazing temperate forages (Lassey *et al.*, 1997, Pinares-Patiño *et al.*, 2003d).

Methane yield, expressed as g CH_4 per kg of dry matter intake (g CH_4/kg DMI), varies substantially between individual animals and between diets, with lower values reported from legumes than grasses (Waghorn *et al.*, 2002, Krause, AgResearch Report). This variation in CH_4 emissions has been attributed to the effects of diet chemical composition, feed intake, and intrinsic animal factors, and provides opportunities for mitigation. However, the causes of the variation have not been fully defined and need to be elucidated to provide a basis for manipulation. Digestive factors and mechanisms likely to affect CH_4 yield (e.g. rumen pH, digesta kinetics) have not been well established, and possible influences of measurement techniques on CH_4 emissions need to be assessed. Defining the potential to achieve measureable differences in CH_4 emissions from ruminants fed the most common forage species in New Zealand pastures (white clover; *Trifolium repens* and perennial ryegrass) may offer practical opportunities for CH_4 mitigation.

1.2 OBJECTIVES

A series of trials were undertaken to better understand the variation in CH_4 emissions from ruminants fed either white clover or perennial ryegrass (ryegrass) forages. The objectives of this research were:

- To evaluate an existing CH_4 database comprising of data obtained using the sulphur hexafluoride (SF_6) and respiration chamber techniques, to determine whether variations in emissions from sheep and cattle can be predicted by the chemical composition of ryegrass forage (Chapter 4).
- To measure CH_4 emissions from sheep fed white clover or ryegrass forages, and compare respiration chamber measurements with previous reports based on the SF_6 tracer technique (Chapter 5).
- To measure and establish relationships between feed intake and CH_4 emissions from sheep fed white clover and ryegrass forages (Chapter 6).
- To examine the effects of feed intake, rumen fill (using intra-ruminal water balloons), and diet on digesta kinetics (flow rates and residence times) in relation to CH_4 emissions from sheep fed white clover or ryegrass forages (Chapter 7).

1.3 FORMAT OF THE THESIS

This Thesis is presented in nine chapters. This General Introduction (Chapter 1) is followed by a Review of Literature (Chapter 2) in which the importance of CH_4 emissions from ruminants is indicated, the principles of SF_6 and respiration chamber methods for determining CH_4 emissions are summarised, and sources of variation in emissions from ruminants are discussed. Chapter 3 outlines the main experimental materials and methods used in the study. Chapter 4 utilised the New Zealand CH_4 emission database; involving data collected between 1995 and 2008 from sheep and cattle fed fresh ryegrass-based diets with measured feed intakes. The effect of diet chemical composition on CH_4 emissions was evaluated, based on measurements using the SF_6 tracer and respiration chamber techniques.

A series of four animal trials were carried out feeding either white clover or ryegrass forages to sheep to address CH_4 emissions in relation to diet composition, feed intake and digestive function. All CH_4 measurements were made in respiration chambers and the information from these trials has been combined to evaluate sources of variation in CH_4 emissions. Chapter 5 summarises the effect of diet (white clover and ryegrass) on CH_4 emissions from sheep. Chapter 6 defines the relationship between intake of white clover and ryegrass forages on emissions and establishes a relationship between yield (g

CH_4/kg DMI) and feed intake. Chapter 7 integrates the three main variables of interest; diet (white clover or ryegrass), feed intake (and rumen fill) and digesta kinetics, and the effects these have on CH_4 emissions from sheep. Chapter 8 summarises the main findings from this Thesis, discusses the implications of this work, and identifies further research opportunities. Annex A summarises a pilot trial that investigates the effect of intra-ruminal infusions to alter rumen pH on CH_4 emissions from sheep. Chapter 9 is the bibliography.

CHAPTER 2

REVIEW OF LITERATURE

CHAPTER 2: REVIEW OF LITERATURE

2.1 GREENHOUSE GAS EMISSIONS

2.1.1 Global greenhouse gases

Greenhouse gas (GHG) emissions have become an increasingly important topic worldwide due to their effects on global warming and climate change. Since the industrial revolution in the 1750's, there has been a global increase in atmospheric concentrations of GHGs. Evidence that the global temperature is increasing (IPCC, 2007) has resulted in an international effort to reduce anthropogenic GHG emissions to the atmosphere. New Zealand endorsed the Kyoto Protocol in 2002 and in early 2005 New Zealand committed to ensure that average emissions of GHGs over the first commitment period (2008 to 2012) would be less than or equal to emissions in 1990 (Ministry for the Environment, 2010).

Global GHG emissions due to human activities have increased 70% between 1970 and 2004, and there are a variety of contributing sources (Figure 2.1). Sources of GHG emissions include fossil fuel use, enteric fermentation from livestock and manure management, rice agriculture, biomass burning, and waste management. The remaining emissions come from natural sources including wetlands, gas hydrates, permafrost, termites, oceans, freshwater bodies, non-wetland soils, volcanoes and wildfires (IPCC, 2007, Sejian *et al.*, 2011). The largest growth in GHG emissions between 1970 and 2004 has come from energy supply (+145%) and transport and industry (+120%), while emissions from residential and commercial building and agriculture sectors have been growing at a slower rate (+26% and +27%, respectively) (IPCC, 2007).

Agricultural lands occupy about 40 to 50% of the Earth's land surface (IPCC, 2007) and agriculture accounts for an estimated 10 to 14% of total global anthropogenic GHG emissions (IPCC, 2007). Greenhouse gas sources within the agriculture sector release into the air significant amounts of carbon dioxide (CO_2), methane (CH_4) and nitrous oxide (N_2O) (IPCC, 2007). Carbon dioxide is largely released from microbial decay or burning of plant litter and soil organic matter (OM) (Janzen, 2004). Methane is produced when OM decomposes in oxygen deprived conditions (anaerobiosis), mainly

from fermentative digestion in ruminant livestock, from stored manures, and from rice grown under flooded conditions (Mosier *et al.*, 1998). Nitrous oxide is generated by the microbial transformation of nitrogen (N) in soils and manures, and is often increased when available N exceeds plant requirements, especially under wet conditions (Oenema *et al.*, 2005).

Global CH_4 sources are well established, but the absolute values for emissions are less well defined (IPCC, 2007). Methane has a strong infrared absorbance and a short atmospheric residence time (12 years) when compared to CO_2 (around 100 years). Methane's 'global warming potential' is estimated to be 21 times that of CO_2 (weight basis) (IPCC, 2007), so each kg of CH_4 in the atmosphere absorbs the same amount of infrared energy from Earth's outgoing radiation spectrum as 21 kg of CO_2 over a 'time horizon' standardised at 100 years (IPCC, 2007).

2.1.2 New Zealand greenhouse gas emissions

In contrast with other developed countries, the New Zealand agricultural sector is responsible for a high percentage of total GHG emissions (47%) (Figure 2.2). Agriculture is a major component of the New Zealand economy, contributing 56% of total merchandise exports, earning NZ$15.25 billion dollars in the year 2007 (Ministry for the Environment, 2010). Between 1990 and 2007, GHG emissions from New Zealand agriculture increased by 12.1% (Figure 2.2), with CH_4 accounting for about 35% of total GHG emissions in 2007 (Ministry for the Environment, 2010).

Within the New Zealand agricultural sector, CH_4 emissions from enteric fermentation in ruminant livestock has increased by 6.9% since 1990, and in the year 2007, accounted for 64% of agricultural GHG emissions (Figure 2.3). Enteric CH_4 arises as a by-product of feed fermentation in the rumen and, to a lesser extent, the large intestine (Clark *et al.*, 2005, Waghorn and Woodward, 2006).

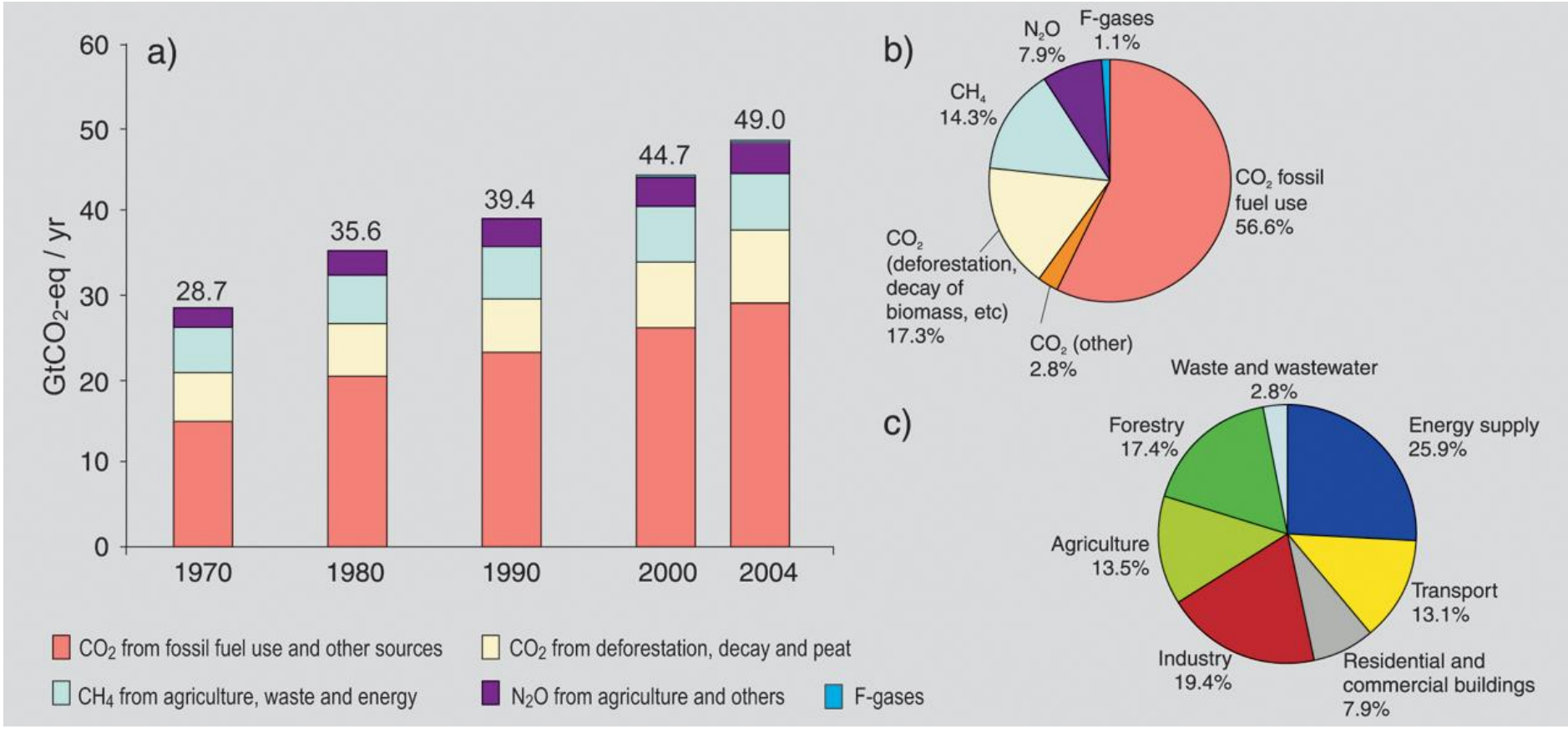


FIGURE 2.1 Anthropogenic global greenhouse gases (GHG) expressed as carbon dioxide equivalents (CO_2-eq). (a) Increasing emissions from 1970 to 2004[1]. (b) The gases contributing to global GHG. (c) Sector contributions to global GHG in 2004. Sourced from IPCC (2007).

[1]Includes only carbon dioxide (CO_2), methane (CH_4) nitrous oxide (N_2O), and F-gases including hydrofluorocarbons (HFCs), perfluorocarbons (PFCs) and sulphur hexafluoride (SF_6), whose emissions are covered by the United Nations Framework Convention on Climate Change (UNFCCC). These GHGs are weighted by their 100-year Global Warming Potentials (GWPs), using values consistent with reporting under the UNFCCC. NB forestry includes deforestation.

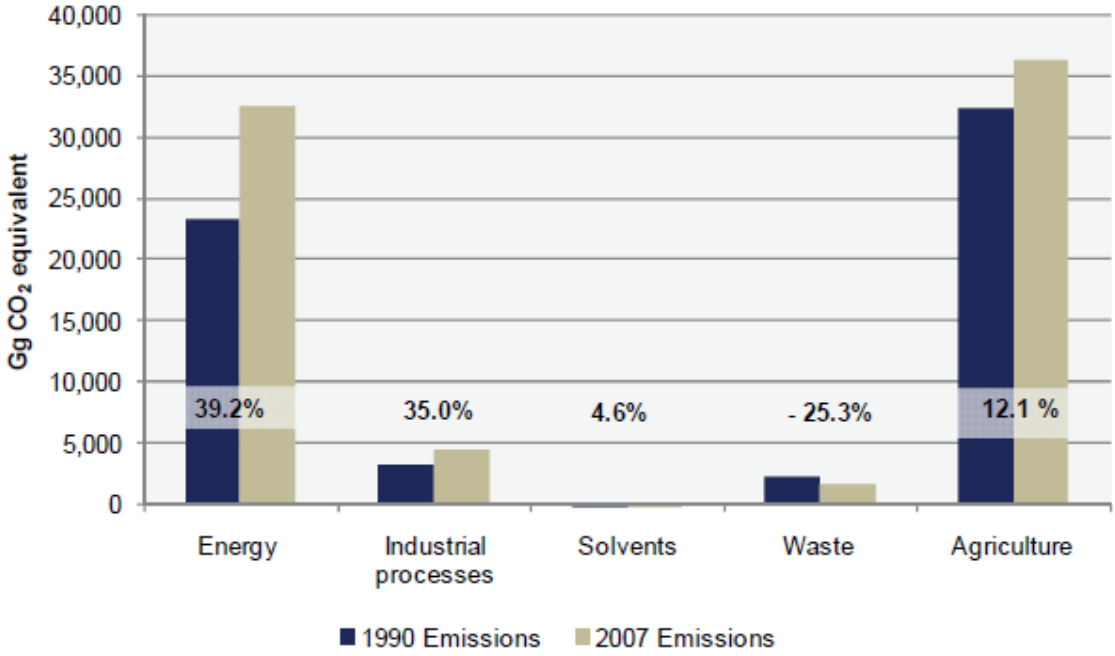


FIGURE 2.2 New Zealand's greenhouse gas contributions from different sectors and the change in emissions from 1990 to 2007 (%; across bars). Sourced from the Ministry for the Environment (2010).

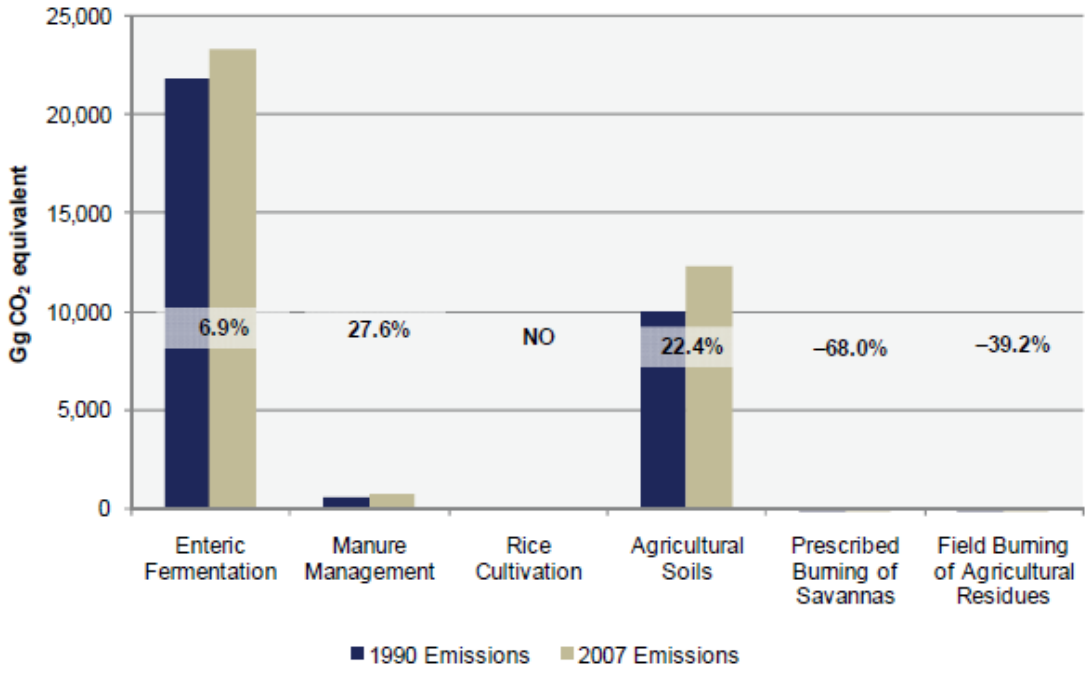


FIGURE 2.3 New Zealand agricultural sector sources of greenhouse gases and the change from 1990 to 2007 (%; across bars). NO, not occurring. Sourced from the Ministry for the Environment (2010).

2.1.3 Ruminants and the environment

Methane formation in the rumen is important for the ruminant animal because it removes hydrogen (H_2) arising from the fermentation of feed that would otherwise accumulate and have adverse effects for rumen function (Moss *et al.*, 2000). The methanogens in the rumen maintain low H_2 concentrations (Wolin *et al.*, 1997), which allows the primary fermentation of feed to proceed (Buddle *et al.*, 2011). The net effect of CH_4 formation is that four moles of H_2 are removed for each mole of CO_2 reduced to CH_4 (Table 2.1). The free energy change (ΔG) associated with CH_4 production phosphorylates adenosine diphosphate (ADP) to form adenosine triphosphate (ATP), which provides energy for maintenance and growth of the methanogenic archaea (Russell and Wallace, 1997).

Methane production from ruminant digestion not only contributes to the global greenhouse effect (Rossi *et al.*, 2001), but it also represents a substantial waste of feed energy (Waghorn *et al.*, 2007). As a percentage of the gross energy (GE) consumed by ruminants, 2 to 12%, is lost as CH_4 (Johnson and Johnson, 1995). This variation is associated with factors including diet quality, and losses from ruminants grazing temperate forages are typically 5 to 7% of GE intake (GEI) (Lassey *et al.*, 1997, Pinares-Patiño *et al.*, 2003d). In general, CH_4 emissions are closely related to the digestible OM intake (DOMI). About 55 to 65% of digestion occurs in the rumen (Moss *et al.*, 2000, Waghorn *et al.*, 2007), but the rumen does not account for all of the CH_4 produced by the animal. It has been estimated that 10 to 30% of OM digestion occurs in the hindgut (Moss *et al.*, 2000), and while its contribution may be relatively small, the hindgut does produce and contribute to overall CH_4 emissions (Ellis *et al.*, 2008).

A primary objective of commercial livestock farming is the generation of edible animal product. There is now greater emphasis on the importance of reducing CH_4 per unit of food produced i.e. CH_4 emissions intensity (Ei) (Waghorn and Hegarty, 2011). Gill *et al.* (2010) showed a range of energy efficiencies associated with conversion of animal feed into product. Waghorn and Hegarty (2011) calculated the efficiency of feed energy capture into milk, beef, pork and poultry meat was about 0.25, 0.06, 0.21, and 0.20, respectively. Efficiencies were lowest for beef production, and energy captured in milk was similar to that for pork and poultry. However, when animal diets were separated

into human edible (e.g. grain) and inedible components (e.g. forages), ruminants were up to 10 times more efficient in converting inedible to edible products than pig and poultry (Gill *et al.*, 2010). With human populations and global food demand increasing, the value of ruminants grazing forages and their contribution to food supply relative to global GHGs is emphasised (Waghorn and Hegarty, 2011).

Increased demand for global supply of animal products will drive an increase in livestock populations, resulting in higher total emissions of CO_2, CH_4 and N_2O, and result in greater use of N fertilisers. This is a concern for the environment and as a consequence research is now re-focussed on increased use of legume forages in grazing pastures to overcome some environmental issues. Previous research (Waghorn *et al*., 2002; Krause, AgResearch Report) has shown sheep fed fresh forages such as white clover (*Trifolium repens*), lotus major (*Lotus pedunculatus)*, and other legumes (Waghorn *et al.*, 2002), had much lower CH_4 yields (12 to 16 g CH_4/kg dry matter intake (DMI)) compared to perennial ryegrass (*Lolium perenne*; 20.9 g/kg DMI; Ministry for the Environment, 2010). The lower CH_4 yields from sheep fed alternative forage species presents an opportunity for GHG mitigation whilst increasing animal productivity and decreasing Ei, which is applicable under grazing where there are limited opportunities for nutritional manipulation.

2.2 METHANE EMISSIONS FROM RUMINANTS

Methane from ruminants is produced when feed macromolecules are fermented by microorganisms in the gastro-intestinal tract (GIT). The catabolism yields volatile fatty acids (VFAs), CO_2, ammonia (NH_3), H_2 and heat. Volatile fatty acids and NH_3 are absorbed via the rumen wall, whereas CO_2 is both absorbed and eructated (Preston and Leng, 1987). Methane production is the last step of the fermentation process and is carried out by methanogenic archaea (methanogens), which in the rumen predominately utilise H_2 as an energy source to reduce CO_2 to CH_4 (Table 2.1). The CH_4 produced by methanogens accounts for about 25% of ruminal gases (Moate *et al.*, 1997) and it is absorbed and eructated with CO_2. Cattle produce about 150 to 420 L of CH_4 per day (107 to 300 g CH_4/d) and sheep about 25 to 55 L per day (18 to 39 g CH_4/d), depending on intake (Czerkawski, 1969, Holter and Young, 1992, McAllister *et al.*, 1996).

Methanogens comprise a range of species and populate the rumen at 10^8 to 10^9 cells/ml of rumen fluid (Stewart, 1991, Kumar *et al.*, 2009). Although H_2 and CO_2 are preferred substrates, formate, acetate, methanol and mono-, di- and tri-methylamine can also be utilised as substrates for CH_4 formation (Wolin *et al.*, 1997). Cleavage of methyl groups from compounds such as pectin, methylamines and methylated sulphides, can also serve as precursors for CH_4 formation; as well as breakdown products of methylated amino compounds and methionine (Ellis *et al.*, 2008). Short chain alcohols can also serve as electron donors for CO_2 reduction; where secondary alcohols are oxidised to ketones, and primary alcohols are reduced to carboxylic acids (Widdel, 1986, Zellner and Winter, 1987).

Methanogens are unique because they have a high affinity for very low H_2 concentrations (Stewart, 1991). They are consistently more competitive for H_2 compared to other H_2 utilising microbes (i.e. sulphate reducing bacteria and acetogenic bacteria) because they use pathways with a more negative change in free energy (ΔG) (Janssen, 2010) (Table 2.1). For example, an alternative H_2 utilising pathway is the reduction of CO_2 to acetate (acetogenesis), which is thermodynamically less favourable (ΔG = -72.2 KJ) than the reduction of CO_2 to CH_4 (ΔG = -134.9 KJ) (Table 2.1) (Kohn and Boston, 2000). A negative ΔG enables a reaction to proceed, so in conditions where species are using fermentation pathways with a large negative ΔG, they will dominate the microbial community (Janssen, 2010).

TABLE 2.1 Key reactions in the rumen and the free energy (ΔG) change that is available for doing work. Adapted from Kohn and Boston (2000).

Reaction	Formula	ΔG (KJ/M)
Glucose to acetate	$C_6H_{12}O_6 + 2H_2O \rightarrow 2C_2H_4O_2 + 4H_2 + 2CO_2$	-142.4
Glucose to propionate	$C_6H_{12}O_6 + 2H_2 \rightarrow 2C_3H_6O_2 + 2H_2O$	-303.9
Glucose to butyrate	$C_6H_{12}O_6 \rightarrow C_4H_8O_2 + 2H_2 + 2CO_2$	-233.1
Glucose to lactate	$C_6H_{12}O_6 \rightarrow 2C_3H_6O_3$	-116.8
Lactate to propionate	$C_3H_5O_3 + H_2 \rightarrow C_3H_5O_2 + H_2O$	-93.6
Methanogenesis	$CO_2 + 4H_2 \rightarrow CH_4 + 2H_2O$	-134.9
Acetogenesis*	$2CO_2 + 4H_2 \rightarrow C_2H_4O_2 + 2H_2O$	-72.2
ATP generation	$ADP + P_i \rightarrow ATP + H_2O$	-10.4

* not demonstrated in the rumen

H_2O, water; H_2, hydrogen; CO_2, carbon dioxide; CH_4, methane; P_i, high energy phosphate; ADP, adenosine diphosphate; ATP, adenosine triphosphate; M, mole

2.2.1 Digestion and fermentation in the rumen

Forage digestion, especially of plant cell walls, arises from a symbiotic association between the host ruminant and gut microflora (bacteria, archaea, protozoa and fungi) (Akin, 1993). The majority of digestion takes place in the reticulo-rumen (termed rumen), which is the primary source of CH_4, and the remainder in the lower gut (mainly caecum and colon). In general, 55 to 65% of the apparent OM digestion takes place in the rumen, about 20 to 30% in the small intestine, and 5 to 15% in the large intestine (Waghorn *et al.*, 2007).

The rumen functions as a large anaerobic fermentation vat buffered with bicarbonate from saliva to maintain the pH between 5.6 to 6.8, and temperature is about 39°C (Hungate, 1966, Kolver and de Veth, 2002). A pH between 6.0 and 6.8 provides an ideal environment for the microflora and enzymes from rumen microbes responsible for fermentation of the feed (Leng, 1984, Fisher *et al.*, 1995). The large quantity of digesta in the rumen of sheep and cattle (10 to 20% of body weight) (Merchen, 1993), as well as mechanisms involved in feed retention (e.g. chewing and particle size reduction), allows ingested feed to be retained for an extended period. This enables extensive digestion by microbial enzymes, and is assisted by cell rupture and breakdown through mastication and rumination (Leng, 1984). Rumen contractions move and mix the contents ensuring contact between microorganisms and feed particles, and facilitate eructation of gases (Leng, 1984).

During the fermentation process, energy is conserved in the form of ATP and utilised for the maintenance and growth of the microbial population (France and Dijkstra, 2005). Dietary carbohydrates such as cellulose, hemicellulose, pectin, starch and soluble sugars are degraded to hexoses and pentoses before being fermented to VFAs via pyruvate (Figure 2.4) (France and Dijkstra, 2005). The products of fermentation are primarily acetate, propionate and butyrate, and NH_3 from proteolysis, with CO_2, and H_2 (Janssen, 2010) (Table 2.1). Acetyl-Co A is an intermediate in the formation of both acetate and butyrate from pyruvate, whilst propionate formation occurs mostly via the succinate pathway from pyruvate (Figure 2.4).

In addition to production of microbial biomass, small concentrations of formate, ethanol, lactate and succinate are produced during fermentation. Proteins are

hydrolysed to amino acids and peptides; each amino acid is then deaminated to NH_3 and a fatty acid (Wallace *et al.*, 1997). Dietary lipids are hydrolysed by bacterial lipases into glycerol and their constituent long-chain fatty acids (LCFAs), which are hydrogenated by the microflora (Drackley, 2000). Thus, the products from digestion of different plant components result in varying amounts of H_2 formation and consequently CH_4 (Janssen, 2010).

The proportions of VFAs produced from digestion are important for ruminant production because they differ in their end uses and in the efficiency of energy capture. The ratio of glucogenic (propionate) to non-glucogenic (acetate and butyrate) VFAs will affect the energetic efficiency and composition of the products (milk and meat) from the ruminant (Bannink and Tamminga, 2005). When host energy needs are met, surplus acetate and butyrate must be stored as fat (Waghorn *et al.*, 2007). Propionate is more versatile and may be converted to glucose or to glycogen for storage, as well as to fatty acids (McDonald *et al.*, 2002). Propionate production in the rumen represents a 7% increase in the efficiency of energy capture from hexoses relative to acetate (Beever, 1993) and results in a net utilisation of H_2, whereas production of acetate and butyrate yield H_2 (Table 2.1 and Figure 2.4).

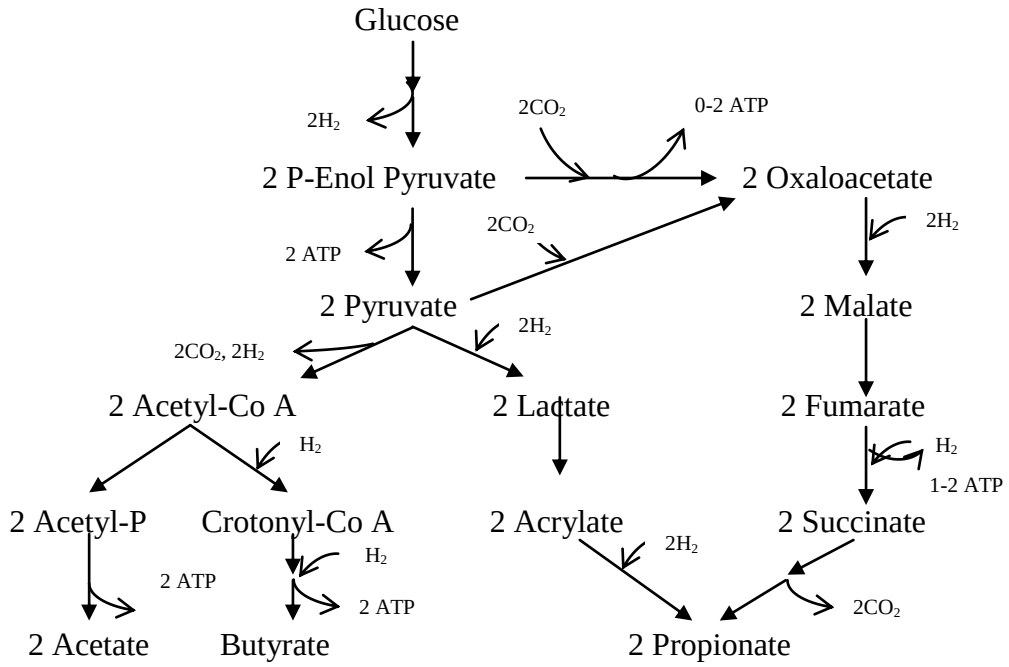


FIGURE 2.4 Fermentation pathways in the rumen. Methane is formed from carbon dioxide (CO_2) and hydrogen (H_2). Diagram sourced from Ungerfeld and Kohn (2006).

2.2.2 Hydrogen and methanogenesis

Anaerobic fermentation reactions are controlled by reduced cofactors (nicotinamide adenosine dinucleotide, NADH; nicotinamide adenosine dinucleotide phosphate, NADPH; and flavin adenosine dinucleotide, FADH), which are oxidised (NAD^+, $NADP^+$, FAD^+) by the donation of electrons to hydrogen ions (H^+) to form H_2 (Hino and Asanuma, 2003, Martin *et al.*, 2010, Kittelmann and Janssen, 2011). These co-factors are required for energy generation (~P, as ATP) for microbial growth, and most of the H_2 produced in the rumen is used for methanogenesis (Janssen, 2010).

The total pool of H_2 in the rumen is small and the concentration of dissolved H_2 is 0.1 to 50 µM (Janssen, 2010). The rate of CH_4 formation is determined by the rate at which H_2 enters the dissolved pool, because the partial pressure of H_2 drives methanogenesis (Janssen, 2010). Methanogens maintain a low H_2 partial pressure in the rumen, which prevents oxidation of NADH to form products such as ethanol or lactate (Miller, 1995), with the release of H_2. If H_2 accumulates, the oxidation of NADH is inhibited because it is thermodynamically unfavourable and because of feedback inhibition. Microbial growth, forage digestion, and the associated production of acetate, butyrate and propionate is inhibited when NAD^+, $NADP^+$ and FAD^+ accumulate (Joblin, 1999) and this will stop fermentation.

Many of the enzymes involved in the methanogenesis pathway are found only in the methanogens. An understanding of the mechanisms driving the methanogenesis pathway for CH_4 formation is improving (Leahy *et al.*, 2010). The process of energy capture by methanogens involves four reductive intermediates, in association with six coenzymes, which enable the reduction of CO_2 to CH_4 under strict anaerobiosis (McAllister *et al.*, 1996). In brief, methanogenesis commences with the fixation of CO_2 with methanofuran (MF) to produce a stable intermediate, formyl-MF (McAllister *et al.*, 1996, Leahy *et al.*, 2010). The formyl group is transferred to the carrier molecule, tetrahydromethanopterin, which is reduced, and the methyl group is transferred to a coenzyme, which is again reduced to CH_4. This reaction completes the cycle and the MF is available for further fixing CO_2 and repeating the process.

2.2.3 Fermentation in the hindgut

In ruminants, large amounts of OM pass from the rumen to be digested in the small intestine and hindgut. In the hindgut, methanogens are also able to use H_2 arising from fermentation to reduce CO_2 to CH_4 (Miller and Wolin, 1986), as in the rumen. Methane arising from fermentation diffuses through the gut wall into the blood stream and most is expelled via the lungs, but acetogenic bacteria appear to utilise some of the H_2 to form acetate (discussed in Section 2.5.9) (Moss *et al.*, 2000). The majority of CH_4 from hindgut fermentation is absorbed and respired (89%), with up to 11% lost via the flatus (Murray *et al.*, 1976).

The importance of methanogenesis from the hindgut is still open to debate, with early reports from Murray *et al*. (1976) showing that the hindgut accounted for less than 11% of total CH_4 production in sheep. However, hindgut methanogenesis appears to be greater under conditions of high rumen outflow rates (Pinares-Patiño and Clark, 2008). Increased passage rates can shift methanogenesis to the hindgut because more undigested material is available for fermentation (Hindrichsen *et al.*, 2006). Kennedy *et al.* (1976) showed that cold conditions resulted in up to 32% of the total CH_4 production from hindgut fermentation in sheep.

2.3 DETERMINATION OF METHANE EMISSIONS FROM RUMINANTS

Measurements of CH_4 emissions from ruminants became a major focus of research in the 1950's and 60's to understand the bases of energy metabolism for animal maintenance and growth (Blaxter, 1964, Blaxter and Clapperton, 1965, Czerkawski *et al.*, 1966). There are a number of methods available for determining CH_4 emissions from ruminants and the three main methods discussed here are tracer, enclosure, and prediction equations.

2.3.1 Tracer techniques

Both isotopic and non-isotopic tracer techniques can be used to estimate CH_4 production from animals. Isotopic methods involve the use of hydrogen or carbon labelled CH_4 (Murray *et al.*, 1976) with ruminally cannulated animals, to label the pools through which all CH_4 passes. The labelled gas is infused into the rumen, and the rumen headspace gas is sampled to measure the specific activity of gas to calculate total CH_4 production (Johnson and Johnson, 1995). The technique is most easily applied to animals in 'steady state' conditions. The limitation of this technique is the very low solubility of CH_4, whereas its diffusibility is high (Hegarty *et al.*, 2007b). Thus, the infused CH_4 may not mix into the rumen CH_4 pool before being eructated (Johnson and Johnson, 1995). Murray *et al.* (1976) avoided this problem by preparing the isotopes in solution so that infusions could be made into the primary pool.

Since the mid-1990's, most CH_4 emission values have been derived using the sulphur hexafluoride (SF_6) tracer technique (Johnson *et al.*, 1994). This is a relatively low cost method compared to enclosure techniques, and has been used to estimate CH_4 emissions from large groups of individual animals under grazing conditions (Vlaming *et al.*, 2007). Methane emissions using the SF_6 technique are estimated by placing a source of SF_6 gas with a known release rate (termed a permeation tube) in the rumen. The release rate of the gas from permeation tubes (typically 1 to 4 mg SF_6/d for sheep) is determined gravimetrically by incubating tubes at 39ºC for at least eight weeks before its insertion into the rumen. A halter is placed on the animal's head and the sampling point situated above the nose is connected to an evacuated sampling canister (Johnson *et al.*, 1994). A flow restriction ensures that a 0.5 to 1.5 L sample of air around the mouth and nose is accumulated, usually over 24 h into collection canisters. The ratio of CH_4 to SF_6 in the breath sample is determined by gas chromatography (GC) and CH_4 emissions are calculated, after correction for background CH_4 and SF_6 concentrations (Ulyatt *et al.*, 1999). This technique eliminates the necessity to restrain or enclose animals, and it is not necessary to sample directly from the animal's rumen or throat, as per other tracer techniques (Johnson and Johnson, 1995).

2.3.2 Enclosure techniques

Direct measurements of CH_4 from animals require total or partial enclosure. These methods can be classified according to their operating principles as open-circuit and closed-circuit systems (Pinares-Patiño and Clark, 2008).

The most common form of the open-circuit system is the whole-animal enclosure chamber (respiration chamber), but the same principles apply to partial enclosures, such as head boxes and masks (Pinares-Patiño and Clark, 2008). The whole animal enclosure requires animals to be housed in sealed chambers with outside air circulated through the chamber at known rates. Methane emissions are determined from the total air flow through the system and the difference in CH_4 concentrations of air entering and leaving the chamber (Johnson and Johnson, 1995).

Respiration chambers enable accurate measurements of CH_4 emissions, and feed intakes, but animal behaviour and diet selection can be restricted (Pinares-Patiño *et al.*, 2008c). Accurate measurement of feed intake is an important advantage of the respiration chambers, compared with CH_4 estimates in grazing situations using the SF_6 technique. This is because the amount of feed eaten is the most important determinant of CH_4 production (van Zijderveld *et al.*, 2011), and intakes from grazed pasture cannot be measured accurately.

Systems have been developed which extend the chamber principles to the grazing situation, where animals are grazed in a tent on pasture with airflow and gas measurements made (termed the tunnel method) (Lockyer and Jarvis, 1995). Another method is the use of hoods, enclosing the animal's head, but measurements need to be made over the entire eating/resting cycles (e.g. 24 h) and this is not always possible with hoods. The dilemma associated with the hood method is that CH_4 emission rates may have a two to three fold variation during a day, associated with eating and resting (Figure 2.5) (Pinares-Patiño *et al.*, 2011b), and it is essential that samples be representative of the animals overall behaviour.

Closed circuit systems have been used as an inexpensive method for calculating CH_4 emissions, where animals are placed in a sealed container, usually for short periods, and CH_4 production is determined (Goopy *et al.*, 2009). However, oxygen concentration

will diminish over time, CO_2 will increase and CH_4 emissions will vary in relation to time since feeding.

2.3.3 Prediction equations

Methane production can be estimated using prediction equations. These are based on the production of VFAs, or feed characteristics, to predict the amount of CH_4 produced (Johnson and Johnson, 1995). Unfortunately, VFA production is rarely measured *in vivo* and there are inherent weaknesses in prediction equations based on the composition of dietary components, especially if the stoichiometry is determined by *in vitro* methods. Although prediction equations are useful tools when the feed characteristics consumed by the animal are known, values require validation and it is unlikely that simple equations will predict CH_4 production under all conditions (Johnson and Johnson, 1995).

2.4 QUANTIFYING METHANE EMISSIONS FROM RUMINANTS

2.4.1 Expressing methane emissions

Methane emissions can be expressed in a number of ways depending on the end use, whether it be inventory, assessment of efficiency of feed utilisation, or losses to digestion. All forms of expression have relevance to national and on-farm mitigation, but it is essential that the boundaries and assumptions associated with the different expressions of CH_4 are clearly defined to avoid misinterpretation (Waghorn and Clark, 2006).

Methane can be expressed on an absolute basis i.e. production of CH_4 per animal per day (g CH_4/d), or yield of CH_4 per unit of feed intake, such as per kg of DMI or OMI (g CH_4/kg DMI or g CH_4/kg OMI, respectively), or relative to GEI (CH_4-E/GEI), or per unit of the digestible portion of feed (g CH_4/kg DDMI or g CH_4/kg DOMI). Alternatively, emissions of CH_4 can be expressed as emission intensity (Ei), i.e. emissions per unit of animal product output.

Emissions intensity is a more recent expression of CH_4 emissions (Leslie *et al.*, 2008). It enables a balance to be achieved between the demand for food and either CH_4 or all GHG costs associated with its production (Waghorn and Hegarty, 2011). Emissions intensity can be calculated in terms of carcass, edible cuts, energy, protein etc and is attractive for producers, as well as consumers, because efficient production (high productivity) lowers Ei and is usually most profitable. Calculations may focus on CH_4 but can be extended to a range of emissions, including estimates determined by lifecycle analysis (LCA).

2.4.2 Methane emissions from ruminants in pasture-based systems

New Zealand's livestock production systems are based on temperate pastures that comprise about 80 to 85% perennial ryegrass (ryegrass) and 15 to 20% white clover (Harris *et al.*, 1998). The New Zealand Greenhouse Gas Inventory is based on emission values that have involved ryegrass-dominant pasture forages (pasture forage), with CH_4 yields of 20.9 and 21.6 g/kg DMI for sheep and cattle, respectively (Ministry for the Environment, 2010). However, some of the data were derived from trials where feed intake has been calculated on the basis of energy requirements. Table 2.2 summarises data available for fresh cut pasture forage, whereas those using alternative fresh forages to pasture forage are presented in Table 2.3.

Mean CH_4 yields (g/kg DMI) from trials with sheep fed fresh pasture forages, with measured intakes, and estimated using the SF_6 technique were 25.7 (Waghorn *et al.*, 2002), 25.9 (Cosgrove *et al.*, 2008), 23.8 and 21.9 for ewes and lambs, respectively (Knight *et al.*, 2008), and 23.7 and 22.9 from reproductive and vegetative pasture forages, respectively (Molano and Clark, 2008). Values (g CH_4/kg DMI) from respiration chambers ranged from 23.1 to 25.4 for dry, pregnant and lactating sheep (Muetzel *et al.*, 2009), and 22.5 to 23.8 for sheep fed a range of feed intakes (Hammond *et al.*, 2011, Sun *et al.*, 2011).

More data are available from cattle (Table 2.2), mostly from trials using the SF_6 technique. Values are similar to those from sheep, with mean CH_4 yields (g/kg DMI) for cattle fed fresh pasture forages, ranging from 16.3 to 35.1 for lactating dairy cows

(Woodward *et al.*, 2001, Woodward *et al.*, 2002, Lee *et al.*, 2004, Woodward *et al.*, 2004, Van Vugt *et al.*, 2005, Waugh *et al.*, 2005, Woodward *et al.*, 2006, Grainger *et al.*, 2007, Waghorn *et al.*, 2008), and averaging 26.8 from steers (Pinares-Patiño *et al.*, 2007). Values (g CH_4/kg DMI) from respiration chambers averaged 22.6, 26.9 and 27.6 from calves fed primary, re-growth and secondary re-growth pasture forages, respectively (Beever *et al.*, 1985), 20.7 from steers, and 20.8 to 24.6 for lactating dairy cows (Bruinenberg *et al.*, 2002, Grainger *et al.*, 2007).

2.4.3 Quantifying methane from other fresh forages

Animals fed legume or herb forages may emit less CH_4 compared with emissions from animals fed pasture forage (Beever *et al.*, 1985, McCaughey *et al.*, 1999, Krause, AgResearch Report), although van Dorland *et al.* (2007) did not find differences in CH_4 emissions from dairy cows fed a mixture of fresh white clover, red clover (*Trifolium pratense*) or ryegrass. Methane yields from sheep and cattle fed alternative fresh forages with measured feed intakes are shown in Table 2.3. Reports based on estimates using the SF_6 technique (Table 2.3) showed an average CH_4 yield of 13.1 g/kg DMI when sheep were fed lotus major, as well as low CH_4 yields from sheep fed lucerne (*Medicago sativa*; 19.6), sulla (*Hedysarum coronarium*; 17.5), chicory (*Cichorium intybus*; 16.2) and white clover (12.3) (Woodward *et al.*, 2001, Waghorn *et al.*, 2002, Krause, AgResearch Report), compared to pasture forages (24.0; Table 2.2). For fresh forage mixtures (50:50) of sulla plus lucerne, chicory plus sulla, and chicory plus red clover, respective CH_4 yields, estimated by the SF_6 technique, were 19.0, 16.9 and 19.7 g/kg DMI. Recent measures (Hammond *et al.*, 2011, Sun *et al.*, 2011) using respiration chambers have shown substantially higher CH_4 yields when white clover and chicory were fed to sheep (22.6 and 22.8 g/kg DMI, respectively), relative to the values reported using the SF_6 technique.

Studies with cattle using the SF_6 technique reported average CH_4 yields (g/kg DMI) of 23.4 when fed Birdsfoot trefoil (*Lotus corniculatus*) (Woodward *et al.*, 2001, Woodward *et al.*, 2004), 19.5 when fed sulla (Woodward *et al.*, 2002), and a decrease in CH_4 yield when percentages of white clover in pasture increased from 15% to 30% to 60% (20.9, 18.6 and 18.1 g/kg DMI, respectively) (Lee *et al.*, 2004). Methane yield

from cattle fed white clover in respiration chambers ranged from 21.0 to 24.9 g/kg DMI (Beever *et al.*, 1985, Cammell *et al.*, 1986). Fresh forage mixtures (60:40) of pasture plus white clover, and pasture plus red clover yielded 22.6 and 21.7 g CH_4/kg DMI, respectively, when fed to cattle in respiration chambers (van Dorland *et al.*, 2007).

TABLE 2.2 Methane yield (g CH_4/kg DMI), using either the sulphur hexafluoride (SF_6) tracer or respiration chamber methods, from sheep or cattle fed fresh ryegrass-based pasture forage with measured feed intakes.

Animal Species	Details	Number of animals	Method	Methane yield	Reference
Sheep	Lambs	6	SF_6	25.7	Waghorn *et al.* (2002)
Sheep	Lambs	2	SF_6	25.9	Cosgrove *et al.* (2008)
Sheep	Dry ewes	13	SF_6	23.8	Knight *et al.* (2008)
Sheep	Lambs	13	SF_6	21.9	Knight *et al.* (2008)
Sheep	Lambs	16	SF_6	23.7	Molano and Clark (2008)
Sheep	Lambs	16	SF_6	22.9	Molano and Clark (2008)
Sheep	Dry ewes	10	Chamber	25.4	Muetzel *et al.* (2009)
Sheep	Pregnant ewes	10	Chamber	23.3	Muetzel *et al.* (2009)
Sheep	Lactating ewes	10	Chamber	23.1	Muetzel *et al.* (2009)
Sheep	Wethers	8	Chamber	22.5	Hammond *et al.* (2011)
Sheep	Wethers	16	Chamber	23.6	Hammond *et al.* (2011)
Sheep	Wethers	8	Chamber	23.8	Sun *et al.* (2011)
Cattle	Calves	6	Chamber	22.6	Beever *et al.* (1985)
Cattle	Calves	6	Chamber	26.9	Beever *et al.* (1985)
Cattle	Calves	6	Chamber	27.6	Beever *et al.* (1985)
Cattle	Steers	4	Chamber	20.8	Cammell *et al.* (1986)
Cattle	Steers	4	Chamber	20.6	Cammell *et al.* (1986)
Cattle	Lactating cows	6	SF_6	35.1	Woodward *et al.* (2001)
Cattle	Lactating cows	63	Chamber	21.3	Bruinenberg *et al.* (2002)
Cattle	Lactating cows	20	Chamber	19.9	Bruinenberg *et al.* (2002)
Cattle	Lactating cows	13	Chamber	24.6	Bruinenberg *et al.* (2002)
Cattle	Lactating cows	8	SF_6	24.6	Woodward *et al.* (2002)
Cattle	Lactating cows	8	SF_6	21.7	Lee *et al.* (2004)
Cattle	Lactating cows	16	SF_6	24.2	Woodward *et al.* (2004)
Cattle	Lactating cows	15	SF_6	16.9	Van Vugt *et al.* (2005)
Cattle	Lactating cows	8	SF_6	16.3	Waugh *et al.* (2005)
Cattle	Lactating cows	8	SF_6	18.5	Woodward *et al.* (2006)
Cattle	Lactating cows	16	SF_6	17.1	Grainger *et al.* (2007)
Cattle	Lactating cows	16	Chamber	20.8	Grainger *et al.* (2007)
Cattle	Dry cows	3	SF_6	24.9	Pinares-Patiño *et al.* (2007)
Cattle	Dry cows	3	SF_6	29.6	Pinares-Patiño *et al.* (2007)
Cattle	Dry cows	3	SF_6	25.8	Pinares-Patiño *et al.* (2007)
Cattle	Lactating cows	16	SF_6	19.5	Waghorn *et al.* (2008)

TABLE 2.3 Methane yield (g CH_4/kg DMI), using either the sulphur hexafluoride (SF_6) tracer or respiration chamber methods, from sheep or cattle fed alternative fresh forages, or a mix of alternative fresh forages and ryegrass pasture forage, with measured feed intakes.

Animal species	Diet	Details	Number of animals	Method	Methane yield	Reference
Sheep	Lotus major (*Lotus pedunculatus*)	Wethers	3	SF_6	14.5	Woodward *et al.* (2001)
Sheep	Lucerne (*Medicago sativa*)	Wethers	3	SF_6	18.6	Woodward *et al.* (2001)
Sheep	Lucerne	Lamb trial 1	6	SF_6	20.6	Waghorn *et al.* (2002)
Sheep	Sulla (*Hedysarum coronarium*)	Lamb trial 1	6	SF_6	17.5	Waghorn *et al.* (2002)
Sheep	Sulla + lucerne (50:50)	Lamb trial 1	6	SF_6	19.0	Waghorn *et al.* (2002)
Sheep	Chicory (*Cichorium intybus*)	Lamb trial 2	6	SF_6	16.2	Waghorn *et al.* (2002)
Sheep	Red clover (*Trifolium pratense*)	Lamb trial 2	6	SF_6	17.7	Waghorn *et al.* (2002)
Sheep	Sulla	Lamb trial 2	6	SF_6	17.5	Waghorn *et al.* (2002)
Sheep	Lotus major	Lamb trial 2	6	SF_6	11.5	Waghorn *et al.* (2002)
Sheep	Chicory + sulla (50:50)	Lamb trial 2	6	SF_6	16.9	Waghorn *et al.* (2002)
Sheep	Chicory + red clover (50:50)	Lamb trial 2	6	SF_6	19.7	Waghorn *et al.* (2002)
Sheep	White clover (*Trifolium repens*)	Wethers	8	Chamber	19.8	Hammond *et al.* (2011)
Sheep	White clover	Wethers	16	Chamber	25.3	Hammond *et al.* (2011)
Sheep	Chicory	Wethers	8	Chamber	22.8	Sun *et al.* (2011)
Sheep	White clover	Indoor housing	6	SF_6	11.6	Krause (AgResearch Report)
Sheep	White clover	Outdoor housing	6	SF_6	12.9	Krause (AgResearch Report)
Cattle	White clover	Fed mid-season	6	Chamber	24.1	Beever *et al.* (1985)
Cattle	White clover	Fed late-season	6	Chamber	24.9	Beever *et al.* (1985)
Cattle	White clover	Fed mid-season	4	Chamber	21.0	Cammell *et al.* (1986)
Cattle	White clover	Fed late-season	4	Chamber	22.3	Cammell *et al.* (1986)
Cattle	Birdsfoot trefoil (*Lotus corniculatus*)	Lactating	6	SF_6	26.9	Woodward *et al.* (2001)
Cattle	Sulla	Lactating	8	SF_6	19.5	Woodward *et al.* (2002)
Cattle	Pasture + 15% white clover	Lactating	8	SF_6	20.9	Lee *et al.* (2004)
Cattle	Pasture + 30% white clover	Lactating	8	SF_6	18.6	Lee *et al.* (2004)
Cattle	Pasture + 60% white clover	Lactating	8	SF_6	18.1	Lee *et al.* (2004)
Cattle	Birdsfoot trefoil	Lactating	16	SF_6	19.9	Woodward *et al.* (2004)
Cattle	Pasture + white clover (60:40)	Lactating	3	Chamber	22.6	van Dorland *et al.* (2007)
Cattle	Pasture + red clover (60:40)	Lactating	3	Chamber	21.7	van Dorland *et al.* (2007)

2.5 SOURCES OF VARIATION IN RUMINANT METHANOGENESIS

New Zealand's livestock production systems are based on temperate pastures and most livestock are grazed on hilly terrain with infrequent handling, so options for CH_4 mitigation are limited. However, variation in CH_4 yield between individual animals provides opportunities for selecting low emitting animals, provided that the differences are persistent and heritable (Waghorn and Hegarty, 2011). The variation in CH_4 yield between individuals is well established (e.g. Hammond *et al.* (2009), (Robertson and Waghorn, 2002), Pinares-Patiño *et al.* (2003c), Vlaming *et al.* (2008)), and can be affected by individual digestive physiology and interactions with diet, physiological state and feed intake (Pinares-Patiño *et al.*, 2011b). However, the size of the differences is often confounded by effects of feed intake.

This discussion is based on data derived from both SF_6 and respiration chamber techniques, but care has been taken to consider only values where feed intakes have been measured. Table 2.3 summarises CH_4 yields from sheep and cattle fed legume and herb forages (McCaughey *et al.*, 1999, Waghorn *et al.*, 2002). The values are often lower than pasture (Table 2.2), which is unexpected because legumes (especially white clover) usually have a higher digestibility than grasses (Ulyatt and Egan, 1979), which should lead to greater H_2 and CH_4 production. However the poor relationship between digestibility and CH_4 yields were confirmed in an analysis of several data sets by Johnson *et al.* (1994). Differences between fresh grasses and legumes in CH_4 yield may be due to the effects of diet chemical composition and ruminal fermentation patterns, combined with digesta passage rates, mean retention times (MRT) and feed intakes (McCaughey *et al.*, 1999, O'Mara *et al.*, 2008).

2.5.1 Diet composition

The association between CH_4 yield and diet composition has been based on fermentation processes which affect proportions of VFAs, and the availability of H_2 for methanogenesis. Carbohydrates are the major source of digestible energy for ruminants, and the type of carbohydrate has been reported to affect CH_4 production (Johnson and

Johnson, 1995). Readily fermentable carbohydrates (RFC) can lower rumen pH, causing a shift in VFA fermentation patterns towards increased lactate and propionate production (Moss *et al.*, 2000). This influences CH_4 production because lactate is metabolised to propionate (Figure 2.4) with a net incorporation of H_2 (Ungerfeld and Kohn, 2006).

However, forages comprise a large amount of fibre (neutral detergent fibre; NDF); mainly cellulose and hemicellulose, which are more slowly degraded in the rumen than RFC. They also result in a higher CH_4 yield compared with non-cell wall components (Moe and Tyrrell, 1979, Johnson and Johnson, 1995). Diets containing high fibre (NDF) and low starch (RFC) (e.g. forages) generally result in higher enteric CH_4 emissions than low fibre, high starch diets (e.g. concentrates) (Moss *et al.*, 1995, Hindrichsen *et al.*, 2004, Beauchemin *et al.*, 2008). Johnson and Johnson (1995) reported a CH_4-E loss of 6 to 7% of GEI when forages were fed and this was reduced to 2 to 3% when high grain concentrates (>90%) were offered.

Some components of forages that contribute to H_2 sinks include proteins and LCFA. When dietary crude protein (CP) exceeds requirements, for example pasture or legume forages typical of spring growth in New Zealand, ruminal proteolysis and de-amination yields amino groups (NH_2) and carbon skeletons that are catabolised to VFAs (Waghorn *et al.*, 2007). The amino groups are converted to NH_3 for absorption and excretion in the urine, so formation of NH_3 could utilise H_2 leaving less available for CH_4 formation (Waghorn *et al.*, 2006).

Plant secondary compounds, typically comprising less than 5% of DM (McMahon *et al.*, 2000) have been linked to effects on digestion, especially *in vitro* incubation where reductions in CH_4 production have been claimed (Patra, 2011), but rarely demonstrated *in vivo*. Condensed tannins may (Woodward *et al.*, 2004, Grainger *et al.*, 2009) or may not (Beauchemin *et al.*, 2007) affect methanogenesis and they do reduce rumen proteolysis (Waghorn, 2008), but they are restricted to the flower of white clover, accounting for about 5.2% of flower DM (Burgraaf *et al.*, 2008). Concentrations of condensed tannins in white clover forage are always less than 1% of DM because flowers are usually less than 10% of the DM (Stockdale and Dellow, 1995).

Lipids are a minor component of forages, comprising 3 to 6% of the DM, and about half are LCFA (Waghorn *et al.*, 2007). Most are unsaturated and rumen hydrogenation will utilise small quantities of H_2. For example, the 3 double bonds in linolenic acid ($C_{18}H_{30}O_2$) would utilise 3 moles of H_2 in the formation of stearic acid. In contrast, some lipids can affect a greater reduction in methanogenesis by reducing the activity of methanogens as summarised by Grainger and Beauchemin (2011). Other dietary H_2 sinks (sulphate and nitrate) are discussed in Section 2.5.9.

Improving quality of forage diets, either through lowering NDF and increasing RFC, or grazing less mature pastures, can reduce CH_4 emissions from ruminants when fed similar intakes (Ulyatt *et al.*, 2002b, Beauchemin *et al.*, 2008). Improving forage quality also tends to increase voluntary feed intake (VFI) and reduces the MRT of digesta in the rumen (Eckard *et al.*, 2010), reducing CH_4 yields. Legume forages tend to have a lower fibre content and result in a high VFI and faster rate of digesta passage from the rumen, compared with grasses (Table 2.5) (Ulyatt, 1969, Moseley and Jones, 1984, Beever *et al.*, 1986). If the high RFC:NDF ratio in white clover, compared to ryegrass, decreases the rumen acetate to propionate ratio (Burke *et al.*, 2006), this may also lower CH_4 yield (Janssen, 2010) because propionate production results in the net uptake of H_2.

Up to 51% of variation in CH_4 yields from sheep fed fresh ryegrass pasture (in unrelated trials) was predicted on the basis of chemical composition, but no relationships could be established for cattle (Waghorn and Woodward, 2006). It appears that the relationships associated with diets containing high concentrations of either NDF or RFC do not account for variation in CH_4 production associated with temperate forages, so the kinetics of degradation and passage may affect the variation in methanogenesis associated with diets and individuals.

2.5.2 Feed intake and its measurement

Measurements of CH_4 yield in relation to intake of conserved diets fed to sheep or cattle have been summarised and reviewed (Blaxter and Clapperton, 1965, Moe and Tyrrell, 1979, Johnson and Johnson, 1995, Harper *et al.*, 1999, Sauvant and Giger-Reverdin,

2009, Yan *et al.*, 2010). All show a declining CH_4 yield as feed intake increases, and the trend is greater with diets containing concentrates, or pelleted diets, compared to conserved roughages (Blaxter and Clapperton, 1965).

Increasing intakes of concentrate diets from one to two times metabolisable energy (ME) requirements for maintenance (ME_m) reduced the percentage of dietary GE lost as CH_4 by 1.6% in cattle (Johnson and Johnson, 1995) and by 1.5% in sheep (Moss *et al.*, 1995). Yan *et al*. (2010) showed a decline of 3.03 g CH_4/kg DMI for each multiple of intake above ME_m in cattle fed fresh grass and grass silage, which was similar to findings reported by Sauvant and Giger-Reverdin (2009). Although Robinson *et al.* (2010) found intakes above ME_m was positively correlated ($R=0.87$) with CH_4 production from sheep, the yield was reduced. In theory, an increase in feed intake decreases the MRT in the rumen (Mertens, 1993); promotes more energetically efficient post-ruminal digestion; as well as a reduction in the dietary energy lost to CH_4 (Blaxter and Clapperton, 1965, Moseley, 1981).

New Zealand livestock are fed mainly pasture forages, and there are relatively few data defining relationships between intake of pasture forages and CH_4 emissions (Hammond *et al.*, 2009). It is not possible to measure feed intakes of individual animals grazing mixed pasture with a high degree of accuracy, so defensible relationships between intakes and CH_4 must be determined from indoor trials where intakes are measured (i.e. in pens, metabolism crates and respiration chambers). Estimated feed intakes based on energy requirements will not account for daily variations in intake (and CH_4 emissions), which are likely under grazing, especially when using the SF_6 technique. Halters and collection canisters placed on the animal for CH_4 measurement can interfere with grazing (Pinares-Patiño *et al.*, 2008b), especially with young animals, and a lower than expected feed intake will underestimate CH_4 yield. In addition to the requirement for accurate measurement of feed intakes to determine CH_4 yield, the frequency of feeding may also contribute to variation and affect CH_4 production (Figure 2.5), particularly with infrequent feeding. There is also a trend for propionate production to increase relative to other VFAs with increased frequency of feeding (Van Nevel and Demeyer, 1996).

Estimates of feed intake by individual animals can be made using either ‘external’ markers (chromium (Cr) oxide, titanium oxide, rare earths) and/or ‘internal’ markers

(lignin, acid-insoluble ash, cellulase indigestible acid detergent fibre (ADF), indigestible NDF and alkanes) (Tamminga *et al.*, 1989). External markers rely on a known measure of digestibility, which reduces their accuracy because digestibility varies substantially between individual animals (Titgemeyer, 1997). Internal markers can be affected by their recovery and reliable feed intake estimates using markers is difficult (Waghorn and Hegarty, 2011). The alkane method (Dove and Mayes, 1991) accommodates individual animal variation in digestibility but alkane concentrations differ widely between plant species, so this marker may only be appropriate when monocultures are fed. The recovery of alkanes in the faeces is also variable (Waghorn and Clark, 2004) which further prejudices the accuracy of feed intake measurements from grazing ruminants (Waghorn and Hegarty, 2011).

2.5.3 Animal species

Determinations of CH_4 yield have been undertaken with a range of ruminant species. Table 2.4 shows similar values for some cattle breeds (Munger and Kreuzer, 2008), whereas substantial differences amongst sheep breeds have been reported by Blaxter and Wainman (1964). When comparing animal species, CH_4 yields from deer (Galbraith *et al.*, 1998, Semiadi *et al.*, 1998) tended to be lower than both cattle and sheep, but this may have been a consequence of pelleted diets fed to the deer. This variation in CH_4 emissions between species and breeds within species is likely to be affected by diet, feed intake, eating patterns, the rumen microflora, and their interactions.

Variations in CH_4 emissions occur in animals fed at the same intake. For example, Grainger *et al.* (2007) reported a coefficient of variation (CV) of 4.3% within individuals and 17.8% between lactating dairy cows, which may be attributed to variation in rumen function. There is good evidence that individual animals harbour their own specific microflora with differences in rates of *in sacco* degradation among animals fed the same diet (Weimer *et al.*, 1999). Variations may include the extent and duration of digestion in the rumen (Titgemeyer, 1997), which can influence quantities and proportions of VFAs, proteolysis and deamination of amino acids, microbial growth, and methanogenesis (Waghorn and Hegarty, 2011). Seasonality can have

pronounced effects on the VFI of different ruminant species, which changes in response to fluctuations in body weight and liveweight gain (Sibbald and Milne, 1993). The change in VFI with seasonality will have associated effects on digestibility, rumen digesta load and pool sizes, contributing to further variation between ruminant species and having possible consequences on methanogenesis.

TABLE 2.4 Methane production (g CH_4/d) and yield (g CH_4/kg DMI) determined using the sulphur hexafluoride (SF_6) and respiration chamber techniques from ruminants fed a range of diets with measured dry matter intakes (kg/d).

Experiment and species	Method	Diet	Dry matter intake	Methane production	Methane yield	Reference
Deer species						
Sambar	Chamber	Pelleted mixed ration	1.28	26.8	20.7	Semiadi *et al.* (1998)
Red deer	Chamber	Pelleted mixed ration	1.52	23.2	15.4	Semiadi *et al.* (1998)
Deer vs. Cattle						
Bison	Chamber	Alfalfa pellets	4.07	86.8	21.3	Galbraith *et al.* (1998)
Wapiti	Chamber	Alfalfa pellets	3.87	62.3	16.1	Galbraith *et al.* (1998)
White tailed deer	Chamber	Alfalfa pellets	1.32	23.6	17.9	Galbraith *et al.* (1998)
Deer vs. Sheep						
Red deer	Chamber	Pelleted + dried grass	1.00	17.9	17.9	Simpson *et al.* (1978)
Sheep	Chamber	Pelleted + dried grass	0.75	13.7	18.2	Simpson *et al.* (1978)
Deer vs. cattle vs. Sheep						
Red deer	SF_6	Lucerne chaff	1.95	31.5	16.5	Swainson *et al.* (2008)
Cattle	SF_6	Lucerne chaff	6.50	141	20.6	Swainson *et al.* (2008)
Sheep	SF_6	Lucerne chaff	0.99	18.5	18.4	Swainson *et al.* (2008)
Breeds of cattle						
Holstein Friesian	SF_6	Grass hay	8.40	170	20.3	Boadi and Wittenberg (2002)
Charolais x Simmental	SF_6	Grass hay	8.20	164	20.0	Boadi and Wittenberg (2002)
Breeds of cattle						
Holstein Friesian	SF_6	Fresh pasture + sulla	12.9	228	23.2	Woodward *et al.* (2002)
Jersey	SF_6	Fresh pasture + sulla	11.0	285	20.3	Woodward *et al.* (2002)
Breeds of cattle						
Holstein Friesian	Chamber	Mixed ration			24.6	Munger and Kruezer (2008)
Simmental	Chamber	Mixed ration			25.3	Munger and Kruezer (2008)
Jersey	Chamber	Mixed ration			25.6	Munger and Kruezer (2008)
Breeds of cattle						
Holstein Friesian	Chamber	Silage + 30% conc.	14.6	342	23.4	Xue *et al.* (2011)
Holstein Friesian	Chamber	Silage + 60% conc.	16.6	333	20.1	Xue *et al.* (2011)
Jersey-Holstein	Chamber	Silage + 30% conc.	15.2	360	23.7	Xue *et al.* (2011)
Jersey-Holstein	Chamber	Silage + 60% conc.	17.8	360	20.2	Xue *et al.* (2011)
Alpaca vs. Sheep						
Alpaca	SF_6	Lucerne hay	0.84	14.9	17.7	Pinares-Patiño *et al.* (2003d)
Sheep	SF_6	Lucerne hay	1.25	18.8	15.0	Pinares-Patiño *et al.* (2003d)
Sheep vs. Cattle						
Suffolk cross sheep	Chamber	Hay + maize	0.81	19.2	24.3	Blaxter and Wainman (1964)
Blackface sheep	Chamber	Hay + maize	0.87	16.0	18.7	Blaxter and Wainman (1964)
Cheviot sheep	Chamber	Hay + maize	0.72	21.9	32.5	Blaxter and Wainman (1964)
Ayrshire x shorthorn cattle	Chamber	Hay + maize	4.29	101	24.4	Blaxter and Wainman (1964)
Aberdeen angus cattle	Chamber	Hay + maize	5.26	133	25.3	Blaxter and Wainman (1964)

2.5.4 Methane measurement technique

Some variation in CH_4 emissions is associated with the measurement method. For example, studies using the SF_6 technique reported between-animal variations (CV) of 12 to 25% (Lassey *et al.*, 1997, Boadi and Wittenberg, 2002, McNaughton *et al.*, 2005, Vlaming *et al.*, 2008), whereas respiration chamber CV's were 7 to 18% (Blaxter and Clapperton, 1965, Grainger *et al.*, 2007). However, recent evaluations have challenged the precision of the SF_6 technique for estimating CH_4 emissions (Vlaming *et al.*, 2005, McGinn *et al.*, 2006, Vlaming *et al.*, 2007, Pinares-Patiño and Clark, 2008, Pinares-Patiño *et al.*, 2011a). It appears that the SF_6 technique contributes to the between-animal variation, although the mean values for CH_4 yield from sheep fed fresh pasture determined using the SF_6 technique (Clark *et al.*, 2005) are similar to those from respiration chambers using similar diets (Hammond *et al.*, 2009). Simultaneous determinations of CH_4 emissions using the SF_6 and respiration chamber techniques with cattle fed either grain or forage diets have also shown good agreement of the means (McGinn *et al.*, 2006, Grainger *et al.*, 2007).

An issue associated with the SF_6 technique is the growing evidence that CH_4 estimates are influenced by the SF_6 gas permeation rate from the tubes (McNaughton *et al.*, 2005, Vlaming *et al.*, 2007, Pinares-Patiño and Clark, 2008). Pinares-Patiño and Clark (2008) showed 6 to 13% of the variation in CH_4 emissions were predicted by SF_6 gas release rates. Vlaming *et al.* (2007) compared SF_6 tubes releasing at a low (2.88 mg SF_6/d) or high (7.34 mg SF_6/d) mean permeation rates and showed significantly ($P<0.001$) different estimates for CH_4 yields (18.3 and 21.8 g CH_4/kg DMI, respectively). Hegarty *et al.* (2007b) also concluded that problems relating to the SF_6 technique were likely to be related to low SF_6 release rates.

The duration of permeation tube activity is another potential problem associated with the tracer technique. After 150 d from when the tubes were filled there appears to be a decline in SF_6 release rate, resulting in an over-estimation of CH_4 emissions (Lassey *et al.*, 2001). For example, Pinares-Patiño *et al.* (2008b) placed permeation tubes in the rumen 250 d before the SF_6 estimations were made, and CH_4 emissions were 39% higher using SF_6 compared with respiration chambers. The decline in SF_6 release rate as tubes age beyond 150 days seems independent of the normal curvilinear decline in release rate (Lassey *et al.*, 2001).

Inconsistent equilibration of the SF_6 gas with rumen headspace gases (Lassey *et al.*, 2001) may contribute to variation in CH_4 emissions, especially as SF_6 gas volumes are small relative to rumen pool size and gas production. If the SF_6 gas were released as small bubbles, these may be entrapped, and later released from particulate matter in the rumen. This is likely to result in variable concentrations of SF_6 in respired air, so that an under-emission of SF_6 into the rumen headspace would over-estimate CH_4, and vice versa.

Additional weaknesses associated with the SF_6 technique include positioning of the sampling port relative to the animals nose; experimental conditions (poor ventilation of buildings leading to increased background concentration of SF_6 and CH_4 gases); damage to gas collection tubes; and mishandling of samples prior to analyses of gases (Ulyatt *et al.*, 1999, Vlaming *et al.*, 2005, Vlaming *et al.*, 2007, Pinares-Patiño and Clark, 2008, Pinares-Patiño *et al.*, 2008a, Pinares-Patiño *et al.*, 2008b). Many of these problems result in gas sample concentrations that are not sufficiently different from background, and can be addressed by appropriate halter manufacture to maintain correct location of the sample above the nostrils, and adequate ventilation in buildings.

Respiration chambers remove uncertainties associated with markers, and enable accurate measurement of both CH_4 emissions and feed intakes. However, respiration chambers are expensive compared to marker methods, so the numbers of animals that can be evaluated are limited. The feed offered may also differ in chemical composition from grazed pasture, because cut grass is usually either longer than optimal, or harvested closer to ground level than in the field. Choice is removed and eating patterns are likely to be determined by the chosen feeding regime.

Figure 2.5 shows the CH_4 emissions from a cow confined to a respiration chamber over 48 h and fed pasture forage. Methane production has a two to three fold variation over a 24 h period, with highest rates during or soon after eating, and lowest production during the evening and night time. This highlights the importance of eating and other activity upon methanogenesis, and the need for measurements over 24 h periods to obtain meaningful data on CH_4 yield.

Goopy *et al.* (2009) has proposed short term (1 to 2 h) measurements of CH_4 emissions as a means for screening large numbers of animals, and several studies have used hoods

for CH_4 determination (Nkrumah *et al.*, 2006, Hegarty *et al.*, 2007a). Very short term measurements of CH_4 may be appropriate for screening because it allows for processing of large numbers of animals, particularly if the objective is to find individuals with extreme CH_4 emissions. The work of Nkrumah *et al.* (2007) was undertaken with steers held in hoods for 16 h of CH_4 measurement. These authors suggested a 25% reduction in CH_4 yield identified for steers with efficient use of feed for growth (low residual feed intake; RFI), but measurements appeared to have been made after the animals had been fed. The low CH_4 emissions reported in that study may have been a consequence of measurement during a period of low emissions relative to eating, and could have contributed to the different CH_4 yields between high and low RFI animals. Neither Hegarty *et al.* (2007a) or Waghorn and Hegarty (2011) found relationships between RFI in cattle and CH_4 yield, so conclusions based on short periods of measurement could be incorrect and misleading. Placing animals in hoods for short periods can be valid provided that animals are able to access feed.

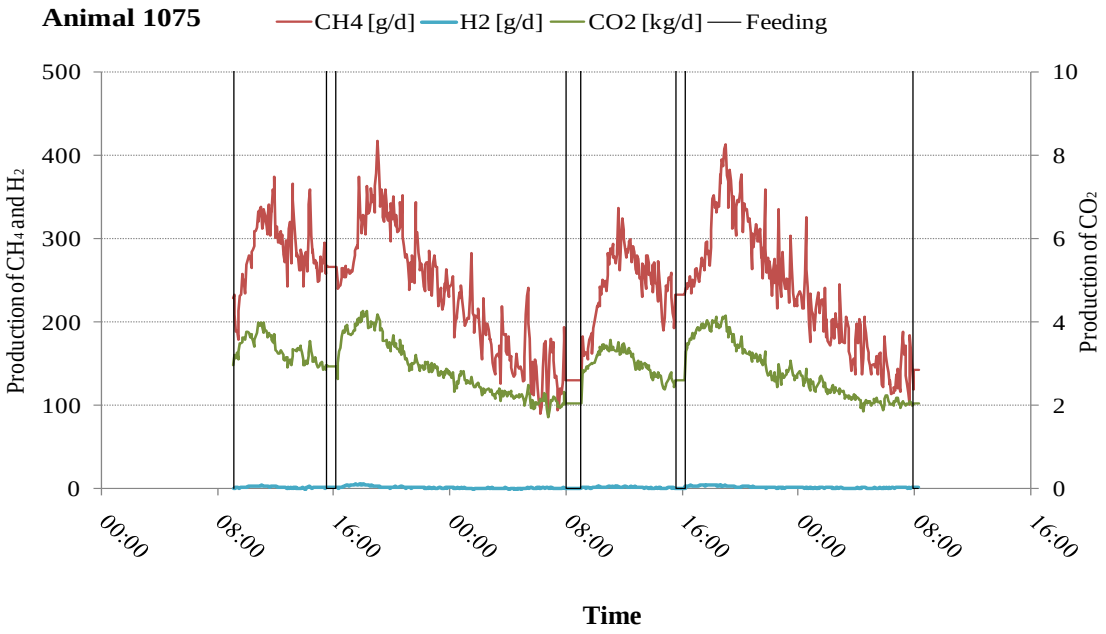


FIGURE 2.5 Production of methane (g CH_4/d), hydrogen (g H_2/d) and carbon dioxide (kg CO_2/d) from a cow fed ryegrass-based pasture forage in a respiration chamber for two days (Waghorn, unpublished).

2.5.5 Particle breakdown

Ingested feed enters the rumen with saliva, and its passage from the rumen is determined by particle size and rates of breakdown. The breakdown and passage of feed are affected by plant composition, structure (Wilson, 1993) and chewing. Clearance of food residues from the rumen can affect feed intake and depends on the process of digestion, including the physical reduction in particle size and subsequent microbial digestion. Forage composition and maturity affects the proportion of DM in the solid (particulate) and liquid (fluid) fractions of digesta, which in turn affects the efficiency of energy utilisation for both the microbes and the ruminant itself.

Feed particles can be reduced in size by four processes: chewing during eating; chewing during ruminating; microbial attack; and the action of rumen contractions (Ulyatt *et al.*, 1986). The reduction of particle size is a process that is partly responsible for the delay in transit of material through the rumen (Ulyatt *et al.*, 1986). Thus, particle size of rumen contents may indirectly influence methanogenesis because it is one of the key determinants for rate of passage (Lechner-Doll *et al.*, 1991).

Chewing during eating and ruminating can reduce forage structural DM to a size able to leave the rumen (threshold of 1 mm for sheep and 2 mm for cattle; Poppi *et al.*, 1987). The effectiveness of chewing during eating and rumination is influenced by the diet. Diets that are fresh and have a high nutritive value are more effectively chewed than diets that are dry and of lower nutritive value (Ulyatt *et al.*, 1986). Furthermore, rumination time can reach 10 h/d on high intakes of forages with low feeding value, but this time can decrease with increasing digestibility and decreasing fibre content. Breakdown of plant components is associated with lignin content (Thornton and Minson, 1972) because it affects toughness and resistance to breakage. Hence, chemical and physical composition (Minson and Wilson, 1994) affect physical breakdown and digestion, which in turn affect feed intake, because the capacity of the rumen is limited (Lechner-Doll *et al.*, 1991). There is little reduction in the size of particulate DM after it leaves the rumen (Waghorn *et al.*, 1989).

Up to 95% of the microbial biomass in the rumen of forage-fed ruminants is associated with particles (Hungate, 1966, Czerkawski, 1986) and so, the influence of particle size on the passage of particulate matter from the rumen may affect the ability of

methanogens to adhere to particles and grow (Janssen, 2010). When methanogens are unable to adhere to particles they must grow at a higher rate to maintain themselves in the rumen as the passage rate of the liquid phase is faster than the passage rate of solids (Czerkawski, 1986, Owens and Goetsch, 1986).

2.5.6 Disappearance of feed from the rumen

Feed disappearance is a collective term referring to the process of particle breakdown, fermentation, absorption, and the passage of digesta from the rumen. Mean retention time is a measure of the time digesta is retained in the rumen and applies to both liquid and solid fractions. The MRT is usually longer for solids than liquids and is affected by diet type. For example, for sheep fed ryegrass forages, Ulyatt (1969) reported the MRT of OM in the rumen to be 10.0 h, compared to only 6.0 h for sheep fed white clover (Table 2.5). However, MRT is also controlled to some extent by the animal and there are variations between individuals (Pinares-Patiño *et al.*, 2003c).

A short MRT is associated with high intakes, a rapid passage rate of particles from the rumen, and/or rapid physical breakdown of fibre, whereas a long MRT is associated with low intakes and passage rates, and physically tough cell walls (lignified). Okine *et al*. (1989) reduced the digesta volume in steers by adding 24 kg weights to the rumen, and this increased the rate of fibre passage through the rumen by 63%, and decreased CH_4 production by 29%. They suggested that the decrease in CH_4 production associated with increased passage rates may indicate a partial shift to propionate formation in the rumen.

A short MRT can be associated with a lower extent of digestion, possibly resulting in lower CH_4 production as less substrate is fermented. However, at low feed intakes, intake and CH_4 production are not necessarily related and dependant on feed type (Pinares-Patiño *et al.*, 2003c). As mentioned previously, white clover contains less structural carbohydrates compared to ryegrass (Moseley, 1981), and undigested material passing from the rumen is subject to digestion in the small intestine and microbial degradation in the hindgut, with associated production of CH_4. The material flowing from the rumen includes microbes, potentially digestible feed residues, and lignified

fibre, and faecal DM comprises bacterial and plant cell walls and endogenous matter (Van Soest, 1982).

TABLE 2.5 Summary of published comparisons on the differences in digestion and digesta kinetics of white clover and ryegrass forages fed to sheep.

Parameter	White clover	Ryegrass	Reference
Digestibility of dry matter (DM) (%)	72	80	Moseley and Dellow (1985)
Percentage (%) of feed digested in rumen	67	66	Ulyatt (1969)
Time eating + ruminating (min/100 g)	10	18	Moseley and Dellow (1985)
Time spent ruminating (min/100 g)	13	33	Moseley and Dellow (1985)
Rate of eating (g DM/min)	11	5	Ulyatt (1984)
Percentage energy (kcal/24h) digested in:			Ulyatt and MacRae (1974)
rumen	57	60	
small intestine	28	29	
large intestine	15	12	
Percentage digested hemicellulose in:			Ulyatt and MacRae (1974)
rumen	73	91	
small intestine	14	-5	
large intestine	14	14	
Percentage digested cellulose in:			Ulyatt and MacRae (1974)
rumen	92	90	
small intestine	-3	-4	
large intestine	11	14	
Percentage digested pectin in:			Ulyatt and Egan (1979)
rumen	96	100	
hindgut	4	0	
Mean rumen pH	6.40	6.40	Burke (2004)
Volatile fatty acid (VFA) (mM/L)	96	79	Burke (2004)
VFA (mM/L)			Burke (2004)
acetate	61.7	55.1	
propionate	18.9	13.4	
butyrate	10.1	7.2	
Methane yield (g CH_4/kg DM intake)	12.3	23.4	Krause (AgResearch Report); Molano and Clark (2008)
Fractional digestion/d	2.11	1.46	Ulyatt (1984)
Outflow rate (at same DM digestibility)/d	1.97	1.54	Ulyatt (1984)
Mean retention time of feed in the rumen (h)	6.00	10.0	Ulyatt (1969)

2.5.7 Rumen pH

The pH of the rumen declines soon after feeding and is driven by VFA production (Robinson and Sniffen, 1985, Shabi *et al.*, 1999). Although rumen pH is lower with grain- compared to forage-fed ruminants (e.g. 5.7 vs. 6.6, respectively; Lana *et al.*, 1998), high quality forages that are degraded rapidly in the rumen can also result in lower pH values (Kolver and de Veth, 2002). Intake of feed which has been processed

to make it more digestible can also result in a rapid decline in rumen pH, which may be due to VFA formation in combination with lower saliva production (Janssen, 2010).

Forage diets typically result in rumen pH ranging between about 5.6 to 6.8 (Lana *et al.*, 1998, Christophersen *et al.*, 2008), and the optimum pH for methanogen growth is 6.0 to 6.4 (Jarvis *et al.*, 2000, Rea *et al.*, 2007). Thus, methanogen growth rates will decline as pH decreases (Janssen, 2010), which may explain the low CH_4 yields reported from ruminants fed diets containing high proportions of grain (Lana *et al.*, 1998, Christophersen *et al.*, 2008).

There is an association between low rumen pH and diets high in concentrate causing a decrease in CH_4 emissions (Burrin and Britton, 1986, Van Kessel and Russell, 1996, Lana *et al.*, 1998). *In vitro* experiments have demonstrated a lower CH_4 production when the rumen pH was less than 6.0 (Lana *et al.*, 1998), whilst Van Kessel and Russell (1996) showed that methanogens in rumen fluid from animals fed on a roughage-based diet were unable to utilise H_2 at pH of 5.5, giving rise to free H_2 in the gas phase. This suggests animals fed diets containing a high proportion of grain (Nkrumah *et al.*, 2006) inhibit CH_4 production in response to a low rumen pH, as it is likely pH will be below 5.5 some of the time (Erfle *et al.*, 1982).

2.5.8 Rumen microbial populations

The rumen ecosystem contains a diversity of microflora including methanogens, protozoa, bacteria, and fungi; all of which can be further categorised according to their functions (Hobson, 1997). Methanogens are ultimately responsible for CH_4 production within the ruminant GIT. However, other microbes also have an influence on CH_4 production because they either are involved in H_2 metabolism, or because they affect the numbers of methanogens or other members of the microbiota (Morgavi *et al.*, 2010).

Of the 66 species of methanogens isolated from a variety of anaerobic habitats, seven have been isolated from the rumen (Janssen and Kirs, 2008). Analysis of rumen samples from ruminants fed differing diets around the world suggest the majority of methanogens fall into three main groups: *Methanobrevibacter*, *Methanomicrobium*, and a large, as yet uncultured, group of rumen archaea referred to as 'rumen cluster C'

(Janssen and Kirs, 2008). Rumen methanogens have a lower threshold for H_2 partial pressure than other H_2 utilising microbes, a fast doubling time that can be as short as 1 h, and optimal conditions appear to be close to 39°C and near neutral rumen pH (Thauer *et al.*, 2008). Most methanogens are attached to feed particles but some are also found in rumen liquid and are also associated with the rumen epithelium and protozoa (Morgavi *et al.*, 2010).

There is a strong association between protozoa and methanogens (Finlay *et al.*, 1994). Removal of protozoa from the rumen (defaunation) has lowered CH_4 emissions by 13%, but this varies with diet (Hegarty, 1999b). Rumen protozoa numbers do not exceed 10^7 cells per ml but they can account for half of the microbial biomass (Russell, 2002). Morgavi *et al.* (2010) suggested the interspecies H_2 transfer between protozoa and their methanogen colonies could facilitate methanogenesis through H_2 supply.

Of the estimated 400+ species of rumen bacteria (Kim *et al.*, 2011), the fibre degraders are the major functional group, with about ~10^{10} cells/g of ruminal contents (Russell, 2002). Rumen bacteria vary greatly in their substrate specificity, with fibrolytic bacteria generally degrading components of the plant cell wall (i.e. cellulose), whereas non-fibrolytic bacteria ferment RFCs such as sugars and starch (Chesson and Forsberg, 1997). Although most fibrolytic bacteria produce H_2 as an end product, there are non-H_2 producing fibrolytic bacteria (e.g. *Fibrobacter). Fibrobacter succinogenes* produces succinate from cellulose degradation which is further decarboxylated to propionate and CO_2 (Wolin *et al.*, 1997). Chaucheyras-Durand *et al.* (2010) suggested that increasing *Fibrobacter* species in the rumen would limit H_2 supply for methanogens and possibly lower CH_4 production.

The rumen fungi are able to degrade fibre, but contribute less than 8% of the total microbial biomass (Theodorou and France, 2005). The presence of active fungal populations in the rumen has been accompanied by increased feed digestibility *in vivo* (Gordon and Phillips, 1993) and their removal from the rumen of sheep fed low quality feed decreased OM digestibility by 3 to 7% (Gordon and Phillips, 1998). Anaerobic fungi produce a variety of end products including CO_2 and H_2 (Gordon and Phillips, 1998) which contribute to CH_4 production.

2.5.9 Alternative hydrogen sinks and methane sources

The absence of clear relationships between CH_4 yield and plant composition (Waghorn and Woodward, 2006) and variation between animals and diets in CH_4 emissions has prompted a brief examination of alternative H_2 sinks in the rumen. These include nitrate (NO_3^-), iron (Fe^{3+}), sulphate (SO_4^{2-}), oxygen, and the presence of acetogens, but many of these reactions are dependent on the H_2 partial pressure in the rumen. Methanogens are abundant in habitats where alternative electron acceptors such as NO_3^-, Fe^{3+}, SO_4^{2-} and O_2 are limiting (Hegarty, 1999a). For the electron transfer between H_2 producing (acetate and butyrate oxidation) and H_2 utilising (acetogens, methanogens and sulphate-reducers) to occur, conditions in the rumen must be energetically favourable.

When electron acceptors other than CO_2 are present, methanogens are outcompeted by denitrifying bacteria, iron-reducing bacteria, and sulphate-reducing bacteria (Weimer, 1998, Anderson *et al.*, 2000, Simon, 2002, Liu and Whitman, 2008) because the reactions are more thermodynamically favourable (i.e. more negative ΔG) compared to the reduction of CO_2 to CH_4 (Liu and Whitman, 2008). Although denitrifying, iron-reducing and sulphate-reducing bacteria are not dominant members of the rumen microbiota, numbers can increase if the appropriate electron acceptor is present in the diet (Morgavi *et al.*, 2010). Sulphate-reducers have a lower minimum H_2 threshold than methanogens meaning that they have a more competitive advantage for H_2 use in the rumen (Ellis *et al.*, 2008). However, the reduction of SO_4^{2-} is not desirable as an end product because hydrogen sulphide (H_2S) is toxic to the host animal (Gould *et al.*, 1997). It has also been demonstrated that iron-reducing bacteria can outcompete both sulphate-reducing bacteria and methanogens for H_2 as an energy source (Fredrickson and Gorby, 1996).

The utilisation of NO_3^- as an electron acceptor is preferable, since the end product of NO_3^- metabolism by rumen microbes is NH_3. The thermodynamics of utilising NO_3^- as a electron acceptor is more favourable than the formation of CH_4 from CO_2 and can replace methanogenesis if NO_3^- is available (Morgavi *et al.*, 2010). In a recent study by Hulshof *et al.* (unpublished), cattle consuming a sugar cane-based diet were fed 2.2% NO_3^- in diet DM and this reduced CH_4 yield by 27% compared to control animals, with no effect on feed intake. However, under some nutritional conditions/feed management,

NO_3^- becomes toxic to the host animal because of the accumulation of nitrite (NO_2^-) in the rumen (Morgavi *et al.*, 2010). This can cause deaths in ruminants fed temperate pastures with high N concentrations (Bolan and Kemp, 2003).

Methanotrophy, oxidation of CH_4, has also been reported to account for a minor amount (<0.5%) of rumen CH_4 production *in vitro* (Kajikawa *et al.*, 2003).

An intermediate competitive pathway to methanogenesis is the reduction of dicarboxylic acids, including aspartate, malate and fumerate to propionate. These organic acids and their metabolites are reduced by rumen microbes that use either H_2 or formate as electron donors, to produce propionate via succinate (Figure 2.4) (Ellis *et al.*, 2008). Formation of propionate from the reduction of formate is also thermodynamically more favourable (ΔG = -303.9 KJ) than methanogenesis (ΔG = -134.9 KJ) (Table 2.1), within the H_2 partial pressures of the rumen (Ungerfeld and Kohn, 2006).

Joblin (1999) reported that autotrophic acetogenic bacteria use H_2 as an energy source to reduce CO_2 to acetate and suggested acetogenesis as a way of increasing feed-use efficiency. This is because the energy losses to CH_4 would be reduced, in conjunction with an increase in substrate supply. Although homoacetogens are present in the rumen, they are not dominant H_2 users and cannot compete effectively with methanogens (Hungate, 1966). The methanogenesis pathway is thermodynamically more favourable than that of acetogenesis (ΔG = -134.9 KJ and -72.2 KJ, respectively; Table 2.1); therefore acetogenesis is negligible (Moss *et al.*, 2000). Acetogenesis can occur in the hindgut; however the extent of this is variable and depends on animal species and the type and nature of the diet (Váradyová *et al.*, 2000).

2.6 HYPOTHESES

The focus of this study is to further investigate factors which may better explain the variation in CH_4 emissions from ruminants fed either white clover or ryegrass forages; the two main species grazed in the New Zealand pastoral livestock system. A review of the literature has shown that the variation in CH_4 emissions can be attributed to a number of factors including the diet chemical composition, digestibility, feed intake,

digesta kinetics, the microbial population, and the accuracy of CH_4 measurements. To further address these factors, a number of hypotheses, based on the review of literature were designed and these are as follows:

1. The yield of CH_4 from sheep fed white clover is significantly lower than that from sheep fed fresh ryegrass forages at similar feed intakes
2. Methane measurements determined from animals using the SF_6 technique are similar to values measured in respiration chambers
3. Variation in CH_4 yield from sheep and cattle can be explained by the chemical composition of fresh ryegrass-based diets
4. Increasing feed intakes of white clover and ryegrass forages affect a decrease in CH_4 yield from sheep
5. Methane yield can be reduced by an increased FOR or decreased MRT of solid and liquid digesta fractions. It is hypothesised that effects would be brought about by increased intakes or by reducing rumen digesta volume (through inserting an intra-ruminal water-filled balloon) or feeding white clover which is expected to degrade more rapidly than ryegrass forages.

2.7 CONCLUSIONS AND FUTURE RESEARCH

Farming of ruminant livestock is a major source of GHG emissions in New Zealand, contributing almost all of the CH_4 emissions. Methane is a by-product of feed fermentation in the ruminant GIT, especially in the rumen.

It is well documented that rumen fermentation results in different amounts of H_2 formation. Hydrogen supply, in combination with thermodynamics of CH_4 production and disappearance of H_2 to alternative sinks, contributes to a large amount of the variation in CH_4 produced. Furthermore, recent analyses of VFA yields from fermentation of plant components have shown poor relationships between predicted and actual propionate production, so the theoretical stoichiometric relationships between VFA production from substrate fermentation and CH_4 emissions seem to have little application *in vivo*.

Methane production (g/d) has a strong positive association with feed intake, however effects of feed intake on CH_4 yield (g/kg DMI) are less clear, and there is large variation between animals and between diets. The low and variable CH_4 yields from animals fed fresh forage legumes, compared to ryegrass dominant pasture forages is poorly understood and will be affected by the fermentation processes that determine proportions of VFAs, as well as H_2, CO_2 and microbial mass.

Rumen function and factors that affect digesta retention times are well defined. An increased feed intake can increase the outflow rate of ruminal digesta and reduce the time available for microbial fermentation. This will reduce fermentation in the rumen and consequently decrease CH_4 formed per unit of feed eaten. Faster passage rates, as a consequence of increased feed intake, are also associated with fermentation pathways that lead to higher proportions of propionate, which in turn decreases the availability of H_2 for methanogenesis. An understanding of CH_4 emissions from ruminant animals requires the interaction between effectors, such as microbes, particle breakdown, H_2 sinks, and rumen kinetics, to be understood so their contribution to variation in CH_4 emissions from ruminants fed fresh forage diets can be determined.

Future recommendations are to ensure that determinations of CH_4 emissions from ruminant animals are robust and feed intakes should be measured to ensure accurate and reliable interpretation of CH_4 data.

The strategy to address these hypotheses is to firstly examine available CH_4 data from New Zealand sheep and cattle fed fresh ryegrass diets with measured intakes, and establish relationships between CH_4 emissions and ryegrass chemical composition. Secondly, a number of trials are to be undertaken where animals are fed either white clover or ryegrass forages at several intakes and measure CH_4 emissions using respiration chambers. Additional measurements would include components of rumen function, such as rumen pH, digesta kinetics, and rumen fill.

CHAPTER 3

EXPERIMENTAL METHODS

CHAPTER 3: EXPERIMENTAL METHODS

3.1 INTRODUCTION

This Chapter provides details of the methodology (except statistical analyses) used in subsequent chapters. Detailing methods here will lessen repetition and allow subsequent chapters to focus on experimental design, statistical analyses, results and discussion.

3.2 EXPERIMENTAL OVERVIEW

Five experiments were undertaken and are summarised in Table 3.1. 'Experiment 1' was a database analysis, to determine if the chemical composition of fresh ryegrass-based pastures could explain variation in methane (CH_4) emissions from sheep and cattle, measured using the sulphur hexafluoride (SF_6) tracer and respiration chamber techniques. Details of the database and the analyses are presented in Chapter 4. Experiments 2, 3, 4 and 5 were *in vivo* sheep trials and all involved similar procedures (i.e. forages from the same paddocks, CH_4 measured in respiration chambers, sample collection, processing and laboratory analyses) and these details are the main focus of this Chapter.

The experimental plans are summarised in Table 3.1. Experiment 2 measured CH_4 emissions from sheep fed either fresh white clover (*Trifolium repens*) or perennial ryegrass (*Lolium perenne;* ryegrass) forages, as well as apparent digestibility and rumen parameters (rumen pH, volatile fatty acids (VFAs), and ammonia (NH_3)). In Experiment 3, sheep were fed white clover and ryegrass forages at two feed intakes, and CH_4 emissions were determined. For Experiment 4, CH_4 emissions from sheep fed white clover and ryegrass forages was measured over two periods, with rumen fill manipulated by insertion and removal of a 1 L water balloon, and determination of rumen digesta kinetics. In Experiment 5, sheep were fed ryegrass forages at five different feed intakes and gastro-intestinal tract (GIT) digesta kinetics was determined in association with CH_4 emissions.

TABLE 3.1 Summary of experiments undertaken during this PhD programme.

	Experiment 1	**Experiment 2**	**Experiment 3**	**Experiment 4**	**Experiment 5**
Date	1995 to 2008	May to June 2009	Oct to Nov 2009	Nov to Dec 2009	April to May 2010
Number of periods	N/A	1	1	2	1
Number of animals	552 (sheep & cattle)	16 (8 with rumen fistulae)	28 (all intact)	8 (all with rumen fistulae)	30 intact
Diet	RG	WC or RG	WC or RG	WC or RG	RG
Feed offered (x ME_m)	0.5 to 4.7	1.6	0.8 & 2.0	1.6	0.8, 1.2, 1.6, 2.0 & 2.5
Treatment	SF_6 & Chamber	Diet	Diet and feed intake	Diet ± water balloon[a]	Feed intake
Animals/treatment	195 SF_6 cattle 196 SF_6 sheep 161 chamber sheep	8 WC & 8 RG	16 WC & 12 RG	4 WC & 4 RG 4 Balloon & 4 Control	6 per feed intake
Feeding regime	Twice daily	Twice daily	Twice daily	Hourly	Twice daily
Measurements	Dry matter intake, emissions of methane				
Other		Feed digestibility Rumen pH, VFA, NH_3		Feed digestibility Rumen pH, VFA, NH_3 Digesta kinetics	Feed digestibility Rumen VFAs Digesta kinetics

ME_m, metabolisable energy requirements for maintenance, SF_6, sulphur hexafluoride tracer technique; WC, white clover; RG, ryegrass; VFA, volatile fatty acid; NH_3, ammonia; N/A, not available

[a]Sheep were with or without a 1 L water-filled balloon in the rumen

3.3 FORAGES

The two forages used were fresh white clover and ryegrass (Photograph 3.1). Both forages were grown at AgResearch Aorangi Research Farm, Manawatu, New Zealand (40º20;'S, 175º28'E; 15 m above sea level), about 15 km from the feeding facility at the AgResearch Grasslands Research Centre.

PHOTOGRAPH 3.1 Left: White clover (*Trifolium repens*). Right: Perennial ryegrass (*Lolium perenne*).

3.3.1 Quality

Plant composition is affected by its maturity. Advancing plant maturity is largely regulated by seasonal temperatures, with a cool-season vegetative growth phase in autumn (March to May); growth quiescence in winter (June to August); primary growth in spring (September to November); and re-growth in summer (December to February) (Ayres *et al.*, 1998). To lessen the effects of seasonality on quality, white clover and ryegrass were harvested in a vegetative state with no reproductive material (minimal flowers for white clover) or seed heads present. This was achieved by grazing at 4 to 6 week intervals for six months prior to experiments commencing, and allowing about 4 weeks of re-growth before the first cutting, so that an adequate sward height (~30 cm) of vegetative forage was available for harvesting.

Pure swards of white clover and ryegrass were required for this study. To achieve this, the white clover (*cv.* Kopu II) was sown as a pure sward, with ryegrass and other weeds

removed with a herbicide containing 100 g/L haloxyfop-R methyl ester ('Gallant', Dow AgroSciences New Zealand Limited), which was applied at 2.5 L/hectare four months prior to harvesting. The ryegrass (*cv.* Quartet) was a three year old sward, with white clover and other weeds removed with a herbicide containing 100 g/L dimethylamine salt (Kamba®, Nufarm Australia Limited), which was applied at 0.6 L/hectare four months prior to harvesting.

3.3.2 Harvesting

Forages were harvested daily in the morning using a sickle bar mower (1995 S.E.P, San Martino in Rio, Italy), set to a cutting height of about 8 cm, and delivered to the AgResearch Grasslands Research Centre (Palmerston North, New Zealand) by 14:00 h. Occasional weeds were removed by hand at harvest.

When the forages were delivered to the trial site, the dry matter (DM) content was estimated by drying in a microwave oven, and meals were weighed and stored at 4ºC until feeding. The determination of DM content involved weighing 3 x 200 g of each forage, which were dried on the 'high' microwave setting for 2 to 3 min, weighed and reheated for about 1 min until weights were constant. The DM content was calculated and averaged over the three samples, but the microwave tended to overestimate DM, especially of white clover. So, 3 x 200 g samples of each forage was oven-dried at 105°C for 16 h and feed allowances were adjusted on the basis of 105ºC oven DM.

3.3.3 Sampling

Daily forage samples included 3 x 200 g for DM determination (oven-dried at 105°C for 16 h) and an additional 200 g sample was oven-dried at 65°C for 48 h during digestibility and CH_4 measurement periods. The latter samples were ground in a Wiley mill (IKA-Werke, model MF10, Staufen, Germany) to pass a 1 mm sieve (aperture size) for analysis of chemical composition by Near Infrared Reflectance Spectroscopy (NIRS) and wet chemistry (Section 3.9).

3.4 EXPERIMENTAL ANIMALS

Animals were sourced and selected from two mobs of sheep from AgResearch Grasslands Research Centre, and when not being used for experiments, they were grazed on a ryegrass-dominant sward. Mob one consisted of two year old wethers; including eight that had been previously rumen-fistulated and fitted with a 30 mm (o.d.) rumen cannulae (Beruc Equipment Ltd, Benoni, South Africa). The second mob was made up of intact (non-fistulated) one year old wethers.

Two weeks prior to experiments, sheep were shorn if needed and drenched with an oral anthelmintic containing 80 mg/ml levamisole hydrochloride and 45.3 mg/ml oxfendazole ('Scanda', 1 ml/10 kg live weight (LW), Schering-Plough Animal Health Limited, Upper Hutt, New Zealand) to remove gastro-intestinal parasites. Animals were weighed prior to morning feeding at the start of each experiment and fortnightly thereafter until the experiment was finished.

3.4.1 Animal ethics

For all animal experiments, procedures were reviewed and approved by the AgResearch Palmerston North Animal Ethics Committee. Any animal manipulations, such as stomach tubing and gas measurements were done according to specific AgResearch Grasslands Standard Operating Procedures (SOPs) (GMSOP04_04 and GMSOP06_07, respectively). For Experiments 2, 3, 4, and 5, the respective Animal Ethics numbers were 11935, 11918, 11912, and 11918.

3.4.2 Selection and acclimatisation

More animals were available than required for experiments and this enabled selection based on their ability to acclimatise to handling and to the diet. The criteria for selection included adaptation to indoor feeding, appropriate LW, body condition, and absence of any clinical disease or health ailments, including dags.

Acclimatisation of animals to handling involved daily interaction and included weighing and feeding, as well as moving from paddock to pen to metabolism crates. Animals were fed the selected diet for about two weeks prior to the experiment.

The timeline for each experiment, depicted in Photograph 3.2 enabled two weeks grazing appropriate diets (Experiment 2); a minimum of 7 d for acclimatisation to feeds and indoor group feeding in pens (Experiments 2 to 5); 3 d in individual metabolism crates (Experiments 2 to 5); 7 d apparent digestibility period in individual metabolism crates (Experiments 2, 4 and 5); and 2 d in respiration chambers (Experiments 2 to 5). Following measurements in respiration chambers animals were released to pasture.

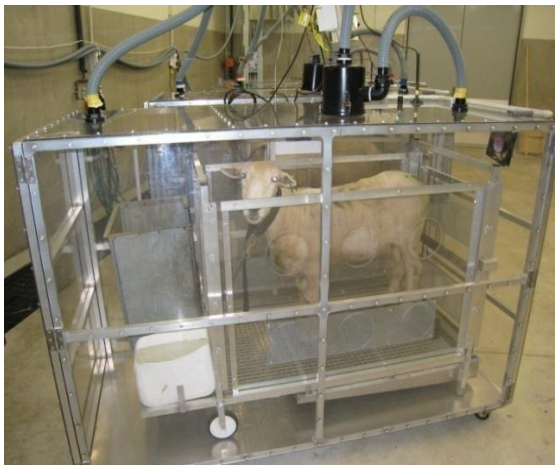
The indoor pen feeding period allowed observations of animal behaviour, and any that were reluctant to eat were removed from the trial before undertaking measurements. The time in metabolism crates (3 to 15 d, depending on the trial) allowed feed intakes to be measured, and this confinement facilitated acclimatisation to respiration chambers (Cammell *et al.*, 1986, Dawson and Steen, 1998, Yan *et al.*, 2000), where gas exchange was determined over 48 h.

PHOTOGRAPH 3.2 Timeline of acclimatisation for Experiments 2 to 5.

(a) Grazing of diets in paddock for up to 2 weeks.

(b) Fed diets in pens indoors for a minimal 7 d.

(c) Indoors in individual metabolism crates for a minimal 3 d adaptation and/or 7 d digestibility.



(d) Individual respiration chambers for 48 h.

3.4.3 Feeding and its frequency

For the majority of the experiments, animals were fed diets at restricted intakes, and feed requirements to meet metabolisable energy (ME) needs for maintenance (ME_m) were based on the Australian Feeding Standards (Australian Agricultural Council, 1990). This was about 7 MJ ME/d for a sheep weighing 40 to 50 kg.

For Experiments 2, 3 and 5, sheep were fed two times daily at 09:00 and 16:00 h, with feed allowance divided into two equal meal portions. However, in Experiment 4 total

daily feed allowance was fed hourly from overhead belt feeders during the digestibility period and twice daily whilst in respiration chambers.

Feed refusals were collected from individual animals once daily during the 3-d metabolism crate adaptation (Experiments 2 to 5) and *in vivo* digestibility periods (Experiments 2 and 5), and twice daily whilst in respiration chambers (Experiments 2 to 5). Refusals were weighed and a 100 g sub sample taken if the refusal was larger than 200 g for oven-drying at 65°C for 48 h. The dried refusal was ground in a Wiley mill to pass a 1 mm sieve aperture. Composites were collected for individual sheep during the digestibility (Experiments 2 and 5) and respiration chamber periods and pooled to give one refusal sample per sheep for chemical composition analysis by wet chemistry.

3.5 FEED DIGESTIBILTY

The measurement of whole tract feed digestibility was determined by the collection of individual feed refusals and faeces over 7 d (Experiments 2, 4 and 5). Fresh faeces were collected once daily in the morning before feeding from all animals, and more frequently if markers for measuring digesta kinetics were used (Experiments 4 and 5). Faeces were collected by two methods. Samples from intact animals were obtained from stainless steel collection trays located below the metabolism crate. These faecal trays were covered in mesh to separate faeces from urine. Fresh faeces were collected from fistulated animals into bags attached to a harness which prevented contamination of faeces with rumen fluid leaking from the cannulae.

Faeces collected from each animal were weighed and a 10% aliquot was sampled. The aliquot was frozen at -20°C and pooled over the 7 days for each animal. After the conclusion of the trial, faeces were thawed, mixed and a 120 g sub sample taken. Samples were dried at 65°C for 48 h to determine DM percentage, and ground in a Wiley mill in preparation for analysis by wet chemistry.

3.6 RESPIRATION CHAMBERS

3.6.1 Description

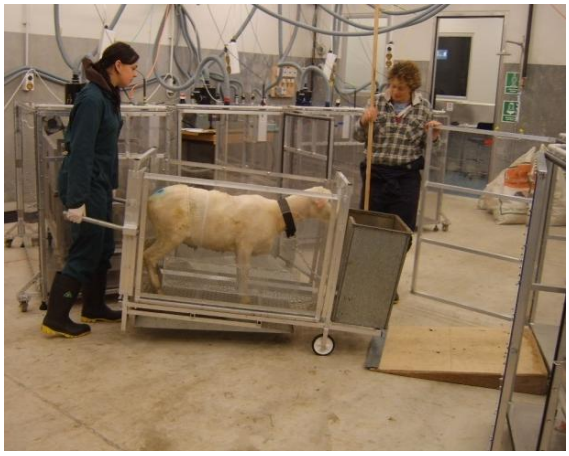
Methane emissions were measured using the eight chamber sheep respiration facility of AgResearch Grasslands Research Centre (Photograph 3.3). One sheep was placed in each chamber, which measured 1.8 m long, 0.85 m wide and 1.2 m high (1.83 m^3). The walls and roof were made of 6 mm clear polycarbonate sheet, fixed to an aluminium frame using silicone sealant and rubber seated screws to form an air tight seal (Pinares-Patiño *et al.*, 2008c). The sheep were restrained in the chambers in modified metabolism crates with a feed bin, drinking water container and separate tray to collect urine and faeces. Crates containing individual sheep were wheeled into the chambers through the back door and sheep were fed through the front door of the chambers.

Chamber operation (Pinares-Patiño *et al.*, 2008c) involved circulation of air through each set of four chambers, controlled by two vacuum pumps which drew up about 250 L of air per min from the room through each chamber. The outlet hose for each chamber was connected to a diaphragm gas meter (AL425, American Meter Company) for wet gas flow measurement. The chambers were constantly monitored for temperature, relative humidity and pressure. A sub sample of the outlet air (0.5 L/min) was collected via a micro diaphragm pump (NMP 09L, KNF Neuberger Inc, Freiburg, Germany) into the drying unit. Before entering the analyser each sample was filtered through a 0.5 µm pore filter and conditioned using a heated gas drier (MDH-110-96, Perma Pure, New Jersey).

Gas analysis was by a non-dispersion infrared analyser (ZKJ-1, Fuji Electric Systems Co., Ltd., Tokyo, Japan), able to detect CH_4 in the range of 0 to 1000 ppm. Carbon dioxide (CO_2) concentrations in the chambers were separately monitored for animal safety reasons. If CO_2 exceeded the threshold concentration of 5000 ppm the doors of the chambers opened automatically and an operator was called. Every morning when respiration chambers were opened the analyser was calibrated using a zero gas (pure nitrogen) and a spam gas (250-2500 ppm CO_2).

In all experiments, gas measurements were carried out over about 48 h, commencing at 09:00 h. Sheep were placed in the chambers and fed at about 08:30 h, and chambers were only opened again at 16:00 h (feeding and refusal collection), 09:00 h the next day (feeding, refusal collection and cleaning), and 16:00 h (feeding and refusal collection), before sheep were released at 08:30 h. Opening the chambers prevented CH_4 measurements for about 30 min each morning and 20 min in the afternoon. Respiration chamber measurements were completed over 4 d for Experiment 2, 8 d for Experiment 3, 4 d for Experiment 4, and 8 d for Experiment 5.

PHOTOGRAPH 3.3 Left: AgResearch Grasslands, whole animal enclosure sheep respiration chambers. Right: Sheep were restrained inside the chambers using modified metabolism crates.



3.6.2 Calculation of methane emissions

Daily CH_4 production by sheep was calculated from the mean concentration (ppm) over 24 h from the chamber outlet (measured every 5 min) and an inlet air source (measured hourly) (Figure 3.1). Daily CH_4 production (g) was calculated as follows:

Total wet air flow (L/min) was calculated from gas meter readings, and converted to dry gas flow (DGF) (Equation (Eq.) 1) and adjusted to standard temperature and pressure gas flow (STP) (Eq. 2):

$$DGF = 100 - ((((a1 + a2 + a3 \times T^2 + a4 \times T^3 + a5 \times T^4 + a6 \times T^5 + a7 \times T^6) \times H/100) \times 100/P) \times 100/T)/100 \times F \times 1000 \quad \text{(Eq. 1)}$$

$$STP = (DGF \times P/(T + 273.15))^* 273.15/1013.25) \quad \text{(Eq. 2)}$$

Where: a1 to a7, are coefficients of water vapour (a1 = 6.11, a2 = 4.44×10^{-1}, a3 = 1.43×10^{-2}, a4 = 2.65×10^{-4}, a5 = 3.02×10^{-6}, a6 = 2.04×10^{-8}, a7 = 6.39×10^{-9}); and the following parameters are P = Pressure (mbar), H = relative humidity (%), T = temperature (°C) and F = flow (m^3/min).

The net CH_4 concentration (CH_4 above background) was then determined by CH_4 concentration (ppm) in the outlet air less background CH_4 (ppm), and this was converted into CH_4 expressed as g/d (Eq. 3):

$$\text{Net } CH_4 \text{ (g/d)} = \text{Net } CH_4 \text{ (ppm)}/10^6/22.414 \times 16.042 \times (STP \times 1440) \quad \text{(Eq. 3)}$$

Where: the constants for molar gas volume = 22.414l L/M; CH_4 molar mass = 16.042 g/M; and 1440 is 60 min x 24 h.

The amount of CH_4 measured for each sheep was averaged for a 24 h period (g/d) (Figure 3.1) and interpolated for the time when doors were opened for feeding.

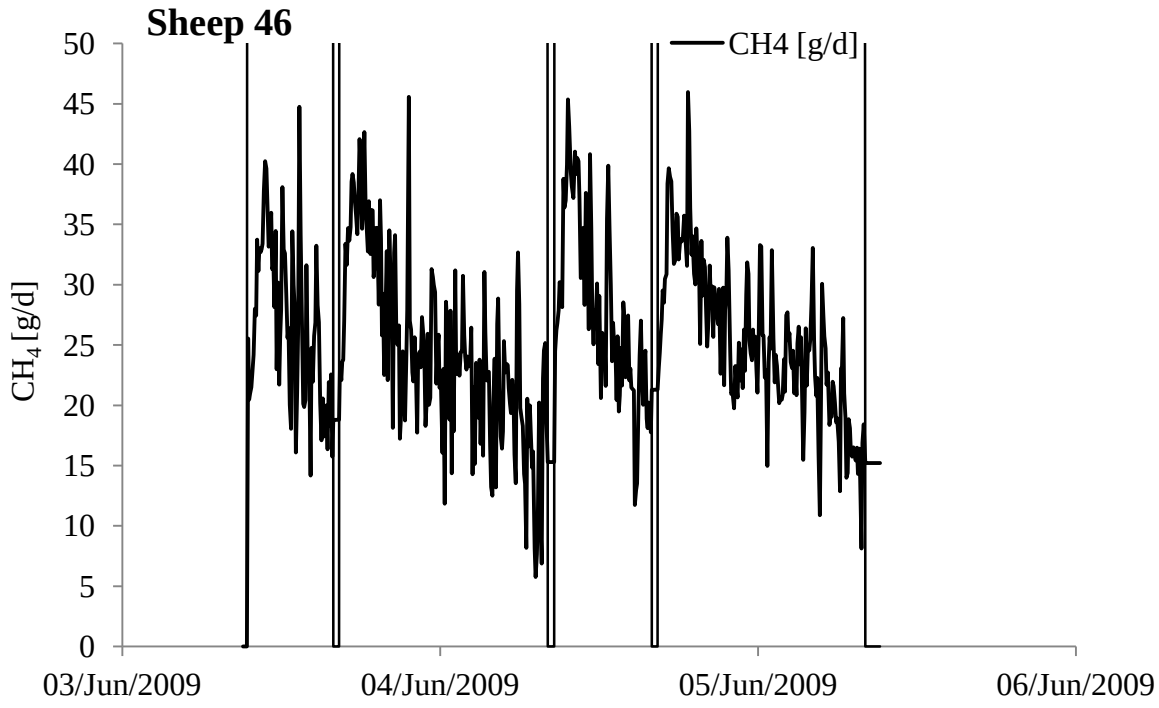


FIGURE 3.1 Daily gas emissions of methane (CH_4; g/d) from sheep 46. Bars indicate door opening and closing in relation to feeding**.**

3.7 RUMEN SAMPLING

Rumen contents were obtained by either direct sampling from the rumen of fistulated animals (Experiments 2 and 4) or by stomach tubing intact animals (Experiment 5).

When intact animals were used, rumen samples were collected by oral stomach tubing (lavage). A 100 cm long flexible plastic tube with a blunt perforated fitting on one end was inserted via the mouth and oesophagus, into the rumen and a syringe was used to obtain around 20 ml of rumen sample by suction. Care was taken to limit saliva contamination by quick and effective operation, because saliva dilutes the sample as indicated by an elevated pH (Geishauser and Gitzel, 1996).

Rumen fistulae were installed in two year old sheep in March 2009. Sampling was via a stainless steel probe (about 110 mm long by 18 mm diameter, and holes were 5 mm) in the rumen and attached to tubing that passed through the cannula stopper. A syringe and gentle suction was used to obtain approximately 20 ml of fluid from the rumen.

If required, pH was measured in the digesta immediately at the time of rumen sampling using a MeterLab® pH probe (PHM210, Radiometer Pacific Limited, Copenhagen, Denmark). Rumen sampling times differed between experiments and are indicated in appropriate chapters.

Rumen samples were analysed to measure concentrations of VFAs (acetate, propionate, butyrate, isobutyrate, isovalerate, and valerate) and NH_3. Sample preparation involved pipetting 1.8 ml of rumen fluid into 2 ml eppendorf microcentrifuge tubes, centrifuging for 10 min at 21,000 x g, and transferring 900 µl of the supernatant into a fresh tubes containing 100 µl of internal standard (2-Ethylbutyric acid (19.87 mM); phosphoric acid (20% v/v)) which were frozen at -20°C. The thawed sample was centrifuged for 10 min at 21,000 x g and approximately 800 µl of supernatant was collected into a gas chromatography (GC) vial for VFA analysis. The remaining supernatant was collected into a 1.1 ml tube and stored at -20°C for NH_3 analysis.

3.8 MARKERS FOR THE ESTIMATION OF DIGESTA KINETICS

Digesta kinetics, such as fractional outflow rate (FOR), dilution rate, mean retention time (MRT) and rumen fill, was estimated using markers in Experiments 4 and 5. Kinetics of solid fractions were estimated using chromium mordanted neutral detergent fibre (Cr-mordanted NDF) and liquids with the sodium salt of cobalt ethylenediamine tetraacetic acid (Co-EDTA) (Uden *et al.*, 1980). A range of measurements were carried out, including rumen (and faecal) sampling in Experiment 4, but mainly faecal sampling in Experiment 5, with some rumen measurements by stomach tubing. Samples for analyses were collected from the rumen or the faeces prior to marker administration and at different times after the marker was given, to calculate rumen and whole tract digesta kinetics.

Digesta kinetics was measured in Experiment 4 when sheep did or did not have a water balloon installed in the rumen. Details of these procedures are given in Chapter 7.2.2.

3.8.1 Marker preparation

Preparation of Cr-mordanted NDF was according to Uden *et al.* (1980). This involved the preparation of a neutral detergent solution (30 g/L Na lauryl sulphate, 4 g/L Na hydroxide, 14.6 g/L free acid EDTA, 6.8 g/L Na borate, 4.6 g/L Na phosphate, and 10 ml/L triethylene glycol dissolved in 18 L distilled water) and the extraction of NDF from the forage offered (i.e. white clover or ryegrass). To extract the NDF, 600 g of dried and chopped forage was added to 18 L of neutral detergent solution and boiled for 2 h (Uden *et al.*, 1980). Approximately 5 L of acetone was used to rinse excess detergent solution and the residue (NDF) was oven dried at 65°C for 48 h. The NDF was mordanted with Cr by adding to it 344 g of Na dichromate dissolved in 5 L of water and baking at 100°C for 24 h. The material was then rinsed with ascorbic acid (5 g of ascorbic acid per kg dry fibre and dissolved in approximately 15 L of water) to remove excess Na dichromate. The Cr-mordanted NDF was oven dried at 65°C for 48 h. The concentration of Cr in the mordanted NDF was determined by inductively coupled plasma optical emissions spectrometry (ICP-OES) (New Zealand Laboratory Services (NZ LABS), Hamilton, New Zealand) and was 33 g Cr/kg DM in the white clover NDF and 37 g Cr/kg DM in the ryegrass NDF.

The Co-EDTA was prepared as the Na salt of the monovalent Co-EDTA anion. The procedure was according to Uden *et al.* (1980), with 146 g/L free acid EDTA, 125 g/L Co II acetate.$4H_2O$ and 20 g/L Na hydroxide added to 800 ml distilled water. The solution was cooled to room temperature before adding 80 ml of 30% hydrogen peroxide (final concentration of 2.7%). To this, 600 ml of ethanol (95% v/v) was added and the mixture left to stand overnight to form insoluble NaCo-EDTA crystals. The resulting crystals were filtered through a buckner funnel with Whatman No. 1 paper by repeated washing with ethanol. A precipitate was collected and dried in an oven at 65ºC for 48 h to yield NaCo-EDTA. Prior to animal application the Na Co-EDTA was dissolved in water.

3.8.2 Marker administration

The recommended Cr dose for measuring digesta kinetics is 1-2 mg Cr/kg LW (De Smet *et al.*, 1992, Hummel *et al.*, 2008) so 50-100 mg Cr (3-6 g Cr-mordanted NDF) was needed for each sheep, and in Experiment 4 sheep were given 5 g Cr-mordanted NDF (165-190 mg Cr/sheep) via the rumen fistulae. However, the minimum detection limit for Cr concentration in the faeces (by ICP-OES) was 5 mg/kg (NZLABs, Hamilton, New Zealand), and in Experiment 5 GIT kinetics were determined by faecal analysis up to 120 h post dosing, when Cr concentrations were likely to be below this limit. Thus, when intact animals were used in Experiment 5 they were given an oral dose of 10 g Cr-mordanted NDF (390 mg Cr/sheep) to maintain Cr concentrations above a detectable level.

Oral administration of Cr-mordanted NDF was achieved by training animals to consume a dried grass mixture mixed with a small amount of molasses. During acclimatisation, 10 g of dried grass, 4 ml molasses and 1 teaspoon rock salt were mixed and offered to each animal prior to morning feeding. Animals had around 5 min to consume the fibre before feeding.

The recommended Co-EDTA marker dose is around 55 mg Co/kg DMI (Hummel *et al.*, 2008). For Experiments 4 and 5, dry matter intake (DMI) ranged from 0.50 to 1.50 kg/d. To cover the range of DMI and allow for a similar dosage of Co-EDTA for all animals, each were given 55 ml of 23.4 mM Co-EDTA (molecular weight (MW) = 428.11) solution; providing approximately 76 mg of Co per sheep. The syringe used to administer the Co-EDTA solution was weighed before and after dosing to determine the exact amount given to each sheep.

For determination of faecal Cr and Co concentrations, samples were collected from each sheep, before marker administration (background) and at 6 to 10 h intervals after marker administration. Approximately 15 faecal samples were collected from each animal in Experiments 4 and 5 were collected over the 120 h time period. Each faecal collection was weighed, and 120 g sub sampled and oven-dried at 65°C for analysis by ICP-OES.

For determination of rumen digesta kinetics, samples were taken prior to Co-EDTA administration for background measurement and liquor was sampled from individual

sheep commencing approximately 30 min post Co-EDTA dosing and until 24 h. Up to 13 rumen samples were collected over the 24 h sampling period from sheep in Experiments 4 and two treatment groups in Experiment 5. Times of sampling are given in Chapters 5 and 7, and liquor was centrifuged for 10 min at 21,000 x g to obtain about 5 ml of supernatant to determine Co concentration by ICP-OES.

3.8.3 Calculation of marker concentrations

3.8.3.1 Disappearance of marker from the rumen

The fraction of marker lost per unit time is known as a fractional rate constant or rate constant and given the symbol *k* (Shipley and Clark, 1972). The liquid dilution rate (*k*) of Co-EDTA in the rumen was determined by the decrease in Co concentration over time. The *k* value was calculated as the slope of the exponential plot of Co concentration against time and can be described by Eq. 4 in Shipley and Clark, 1972:

$$q = D\, e^{-k\,t} \qquad \text{(Eq. 4)}$$

Where: *q* is the amount of marker (mg) in the rumen observed later at time t (h); *D*, is the initial amount of marker dosed into the rumen (mg); e is the base for natural logarithms, and *k*, is rate constant (i.e. the fraction of the content of the pool that is being replaced per unit of time; %/h).

Equation 4 can be expressed in terms of concentration (c) by dividing by a constant volume (V) to give units of the marker (Co) per L (Eq. 5):

$$q/V = D/V \times e^{-k\,t} \quad \text{or} \quad c = c_0 e^{-kt} \qquad \text{(Eq. 5)}$$

Expression of concentrations by natural log (ln) transformation results in a linear relationship for a single pool system, and rearrangement enables the degradation rate to be determined as *k* (Eq. 6) (López, 2008):

$$k = (\ln c_0 - \ln c)/t \qquad \text{(Eq. 6)}$$

Where: *k* is the rate constant (%/h); ln is the natural logarithm; c_0 is the marker concentration in the rumen at zero time (mg/L); c is the marker concentration (mg/L) in the rumen at time t (h).

The rumen liquid pool is estimated by plotting marker concentrations over time (Eq.7) and extrapolating to zero time, so the intercept on the *y* axis is the predicted concentration of the marker at zero time (c_0). The rumen liquid pool was then estimated by dividing the dose of marker given by the concentration of the marker at time zero (Eq. 7) (Shipley and Clark, 1972):

$$V = D/k \qquad \text{(Eq. 7)}$$

Where: V, is the rumen liquid volume (L); *D*, is the amount of marker dosed into the rumen (mg) at time zero; and *k* is the rate constant (i.e. slope of the line).

The outflow of liquid from the rumen (ml/h) was calculated by multiplying the estimated volume of ruminal liquid by the dilution rate constant (*k*) (Eq. 8):

$$\text{Outflow} = (V \times 1000) \times k \qquad \text{(Eq. 8)}$$

Where: outflow, is ml/h; V, is the rumen liquid pool (L); and *k*, is the rate constant (%/h).

3.8.3.2 Digesta passage rates based on faecal excretion

Mathematical modelling of faecal excretion data using indigestible markers is generally non-linear and represents the time-course of marker concentration determined from the faeces (López, 2008). To estimate solid (Cr) and liquid (Co) FOR (*k*), a multi-compartmental model, based on Dhanoa *et al.* (1985), was used to fit faecal marker concentrations as shown in Eq. 9:

$$Yt = Ae^{-(k \times t)} \exp\left[-(N - 2)\, e^{-k \times t}\right] \qquad \text{(Eq. 9)}$$

Where: *Y*t, represents the faecal marker concentration at time = t; *N*, denotes the number of compartments; *k*, resembles the FOR; and *A*, forms a scalable parameter dependant on *N* and *k*.

This model uses a multiplicative equation containing a single exponential term and a double exponential term for describing faecal outflow rate and particle kinetics. The multi-compartmental model assumes an unspecified number of sequential compartments with a constant increase of outflow from each compartment to the next one, without allowance for by-pass fluxes. The success of any fit of data to the model was judged on the basis of three criteria (Dhanoa *et al.*, 1985):

- the ability to describe data without systematically over- or under-estimating any section of the curve;
- biologically acceptable parameter estimates;
- convergence to a solution.

3.9 SAMPLE ANALYSIS

3.9.1 Near infrared reflectance spectroscopy

A sub sample of forages was sent to FEEDTech, AgResearch, Palmerston North, New Zealand, for analysis by NIRS to determine chemical composition. Analyses provided crude protein (CP), NDF, acid detergent fibre (ADF), lignin, soluble sugars and starch (SSS), lipid and ash as well as estimates of digestibility and ME concentration of the DM. The samples were scanned using an FT-NIR spectrophotometer (Bruker Optics, model MPA, Ettlingen, Germany), and spectra were collected from 780 to 2500 nm. Analysis of NIRS data, including spectra collection, calibration, prediction and validation, were conducted using OPUS Software version 5.0.53. Calibration curves for each component were based on principal component analysis of samples of known composition which had been previously scanned.

3.9.2 Wet chemistry

Following each experiment samples of feed offered, refusals and faeces were analysed for chemical composition by wet chemistry (Massey University Nutrition Laboratory, Palmerston North, New Zealand). The laboratory used the following methodology: neutral detergent fibre, ADF, and lignin were determined using the Tecator Fibretec

System (Leco Corporation, St. Joseph, MI, USA) following the procedures of Robertson and Van Soest (1981). Cellulose content was calculated as ADF less lignin and hemicellulose content calculated as NDF less ADF. Nitrogen content was determined by total combustion (Leco CNS 2000 model, USA) according to Method 1968.06 (AOAC, 2005) and converted to CP by a factor of 6.25. Crude fat (lipid) was determined by Soxtec extraction (Soxtec System AT1043 Extraction Unit, Foss, Höganäs, Sweden) using Method 920.39 (AOAC, 2000). Organic matter (OM) was measured by ashing in a furnace (Ceramic Engineering, Sydney, Australia) at 500°C for 16 h (Method 942.05, AOAC, 1990) and hot water soluble carbohydrate (HWSC) and pectin were extracted by reflux in boiling water and in 0.5% ammonium oxalate, respectively (Blumenkrantz and Asboe-Hansen, 1973). Gross energy (GE) was measured by combustion using an adiabatic bomb calorimeter (AC350, Leco Corporation, St Joseph, MI, USA).

3.9.3 Volatile fatty acids

Volatile fatty acid analysis was carried out with a Hewlett-Packard (HP) 6890 series GC system with an auto-injector and flame ionisation detector. The procedure is a modification of Tavendale *et al.* (2005) as follows: a 800 µl sample was used for analysis, with VFA separation in a nitroterephthalic acid modified polyethylene glycol column (DB – FFAP, 30 m x 0.53 mm x 1 µm film thickness; J and W Scientific, Ca, USA). The detector temperature was held at 240°C and the oven temperature (85°C) increased at 10°C/min to 180°C and held for 5 min. The carrier gas was helium at 5.5 ml/min. Volatile fatty acids were identified from their retention time and quantified from chromatograph peak areas (Figure 3.2) using the program ChemStation Rev.A.06.03[509] (HP 1990-1998).

Volatile fatty acid peak areas were calibrated using an aqueous solution containing 143, 43.7, 20.0, 1.82, 1.73 and 1.63 mM/L of acetate, propionate, butyrate, valerate, isobutyrate and isovalerate, respectively. This external standard was diluted 2, 4 and 10 fold to yield a four point calibration curve and a linear regression was used to convert peak areas into their respective VFA concentrations. Generally, the R^2 of the calibration was greater than 0.9995. External standards were run at the beginning of a

series of 40 samples with blanks (internal standard in water) injected after every 10 samples.

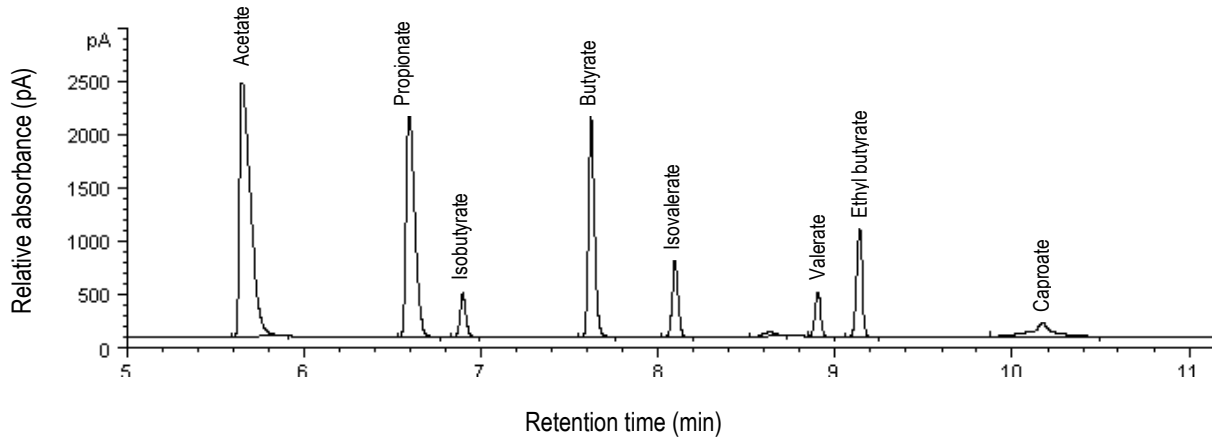


FIGURE 3.2 The elution sequence and separation of volatile fatty acids determined by gas chromatography.

3.9.4 Ammonia

The determination of NH_3 concentration was based on a downscaled version of the Weatherburn (1967) method. This is a colorimetric procedure using a combination of reagents for the catalysed indophenol reaction for the determination of NH_3. Ammonia concentrations were determined by pipetting 300 µl of phenol nitroprusside solution (10 mg/ml phenol, 50 ug/ml Na nitroprusside) into a 1.0 ml deep well plate and 15 µl of sample was added. To this, 300 µl of alkaline hypochlorite solution (10 mg/ml Na hydroxide, 0.84% (v/v) Na hypochlorite) was added and then incubated for 30 min at 37°C. Three aliquots of 100 µl were collected of the reaction and transferred into a microtitre plate where the absorbance was read at 625 nm using a SpectraMAX 250.

A standard curve containing 1, 2, 4, 6, 9, 12 and 15 mM/L of NH_3 prepared from $(NH_4)_2SO_4$ was used. Ammonia concentration was calculated from a linear regression with absorbance read at 625 nm. Samples that were outside of the calibration curve were diluted and the procedure was repeated.

3.10 DETERMINATION OF METHANOGENIC POPULATIONS

3.10.1 DNA extraction

In Experiment 5, two treatment groups of sheep had rumen samples obtained by stomach tube and total DNA was isolated from these using a FastDNA® SPIN Kit for soil and a FastPrep® Instrument (MP Biomedicals, Santa Ana, CA). The protocol provided by the kit manufacturers was as follows: samples were thawed and 100 µl transferred to a tube containing 1.4 mm ceramic spheres, 0.1 mm silica spheres, and one 4 mm glass bead. The microorganisms were homogenised in a FastPrep® Instrument for 40 seconds at a speed setting of 6.0 with Na phosphate buffer (978 µl) and MT buffer (122 µl), which are reagents developed to protect and solubilise nucleic acids and proteins upon cell lysis. Following lysis, samples were centrifuged (14,000 x g; 10 min) to pellet debris and 850 µl of supernatant was transferred to a clean 2.0 ml microcentrifuge tube. To this, 250 µl PPS (Protein Precipitation Solution) was added and mixed thoroughly. The reaction was centrifuged at 17,000 x g for 5 min to pellet precipitate and 1 ml of the supernatant was transferred to a 5 ml tube where 1 ml of Binding Matrix suspension was added.

The DNA was given approximately 2 min to bind and then a further 3 min was allowed for the settling of the silica matrix. About 800 µl of the supernatant was removed and discarded taking care to avoid the Binding Matrix, which was resuspended in the remaining supernatant, and 600 µl of the mixture was transferred to a Spin Filter where it was centrifuged at 14,000 x g for 1 min. The catch tube containing the Spin Filter was emptied and the remaining mixture was added and centrifuged, then emptied again, after which 500 µl of SEWS-M was added to re-suspend the pellet. This was centrifuged at 14,000 x g for 1 min and the process was repeated with the addition of a further 500 µl of SEWS-M. Without the addition of any liquid, the catch tube containing the Spin Filter was centrifuged for a further 2 min at 14,000 x g, then discarded and replaced with a new one. After 5 min of air drying at room temperature the Binding Matrix above the Spin Filter was resuspended in 100 µl of DES (DNase/Pyrogen-Free Water). This was centrifuged at 14,000 x g for 1 min to bring down eluted DNA into the clean catch tube. Extracted DNA was stored at -20°C.

The concentration and quality of the extracted DNA were determined at absorbencies of 260 and 280 nm using an ND-1000 spectrophotometer (NanoDrop Technologies, Wilmington, DE). If the 260/280 absorbency ratio was greater than 1.80 then the sample was judged to be of high quality, suggesting proteins, phenols or alcohol have been removed. The length of DNA (~20 KB) was determined on a 1% agarose gel (3 g agarose with 300 ml 0.5 x TAE buffer) which was run at 200 V for 30 min. After electrophoresis, the gel was stained with ethidium bromide (10 µg/ml), viewed with ultra-violet transillumination, and photographed.

3.10.2 Polymerase chain reaction amplification of methanogenic DNA

This procedure is based on a modified version of Jeyanathan *et al.*, 2011. Briefly, for amplification of a 470 bp product covering the V6-V8 region of the 16S rRNA gene of methanogenic archaea, the DNA was diluted 10-fold, and the methanogen-specific primers used are listed in Table 3.2. Forward primers included a GC-clamp at the 5'-terminus. The polymerase chain reaction (PCR) was performed in a Px2 thermal cycler (ThermoElectron, Milford, MA). The PCR master mix solution (25 µl) contained 20 pM of each primer, each deoxynucleoside triphosphate at a concentration of 0.2 mM, 5 U *Taq* polymerase, 1 x PCR buffer, 1.5 mM $MgCl_2$, 0.67 mg/ml BSA, and 1 µl DNA template. The amplification was performed using the following cycling parameters: an initial denaturation for 3 min at 95°C; 35 cycles of denaturation at 95°C for 30 seconds; annealing at 59°C for 30 seconds; extension at 72°C for 60 seconds; and final elongation for 7 min at 72°C. The resultant products were analysed for DNA on a 1.6% agarose gel (5 g agarose with 300 ml 0.5 x TAE) before denaturing gradient gel electrophoresis (DGGE) was performed.

TABLE 3.2 Primers used to target 16S rRNA genes of total methanogenic archaea

Primer	Sequence (5' to 3')	Reference
915af*	5'AG GAA TTG GCG GGG GAG CAC	Watanabe *et al.* (2004)
GC clamp*	CGC CCG CCG CGC GCG GCG GGC GGG GCG GGG GCA CGG GGG G	Watanabe *et al.* (2004)
1386r	5'GCG GTG TGT GCA AGG AGC	Skillman *et al.* (2004)

*GC clamp was added to the 5' terminus of the forward primer

3.10.3 Denaturing gradient gel electrophoresis

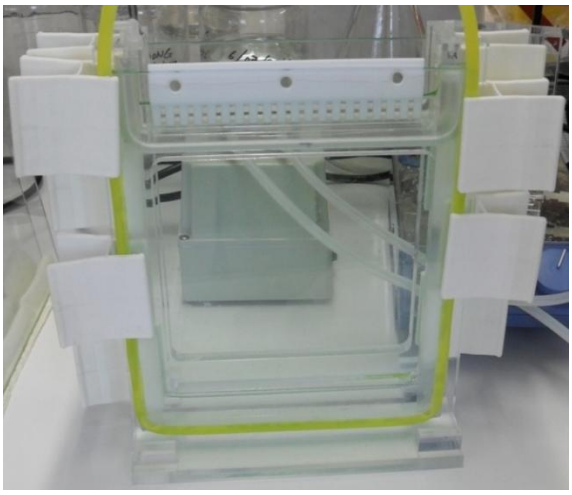
Denaturing gradient gel electrophoresis was performed using a CAB scientific DGGE gel system (C.B.S. Scientific Company, Del Mar, CA) at 150 V at 60°C for 6 h (Photograph 3.4). The procedure is as follows:

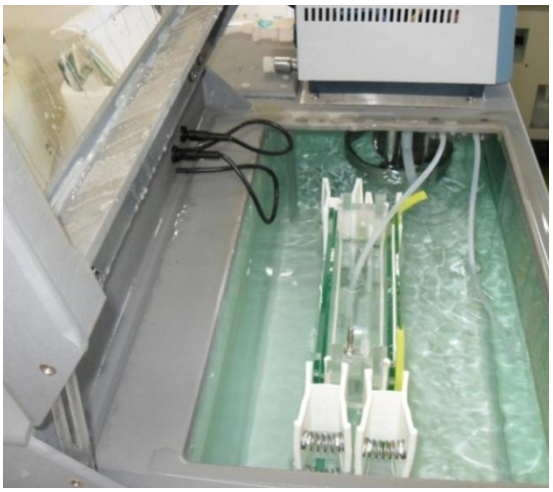
Two work solutions of 100% (6% acrylamide (37:5:1), 7.0 M urea, 40% formamide, 0.5 x TAE, 2% glycerol, made up to 200 ml with DH_2O) and 0% (6% acrylamide (37:5:1), 0.5 x TAE, 2% glycerol, made up to 82 ml with DH_2O) were prepared. A polyacrylamide gel (6% w/v) was prepared from two separating gel solutions of low (30%) and high (55%) denaturation, which were made from the 100% and 0% solutions, in addition with TEMED (0.1%) and APS (0.1%). The denaturation solutions were placed separately in a gradient maker which had a tube attached. The tube was run through a peristaltic pump with a needle on the front end inserted between the DGGE plates. Once the gel had run through the system into the DGGE plates, a comb was place on top of the gel and left to polymerise for 30 to 45 min.

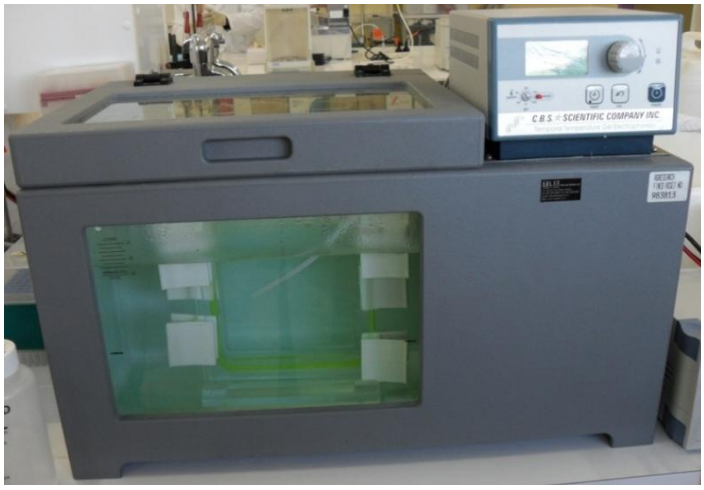
Once the gel had set it was assembled as a cassette. The comb was removed and the resulting wells were washed with 1 x TAE buffer. Approximately 10 µl of PCR product containing 3 µl of DGGE loading dye (0.05% (w/v) bromophenol blue, 0.05% (w/v) xylene cyanol, 70% (w/v) glycerol, in water, pH 8.0) were loaded into the wells of the gel within the cassette. A reference standard (Marker II; Nippongene, Tokyo, Japan) was loaded on either side of 12 wells containing PCR products. The cassette was then placed into the DGGE tank containing 1 x TAE buffer (40 mM Tris, 20 mM acetic acid, 1 mM EDTA, pH 8 with NaOH) (Photograph 3.4) and run for 6 h.

When the gel was finished polymerising, the cassette was removed from the DGGE tank and dissembled. The gel was removed from the plates and prepared for silver ($AgNO_3$) staining. The gel was placed in a staining tray and 200 ml of 1 x fixation solution (8 x fixation solution made up of 80% ethanol, 4% acetic acid and made up to 1 L with dH_2O) was added. After approximately 3 min the fixation solution was removed and 200 ml of staining solution (200 ml of 1 x fixation solution and 2 mg/ml $AgNO_3$) was added. After approximately 10 min the staining solution was removed from the tray and the gel was rinsed with 200 ml of water for a further 2 min. The gel was placed in a developing tray to be scanned and analysed with the Geldoc Quantity One Software (Bio-Rad Laboratories, Hercules, CA, USA). The positioning of individual bands on the gel, relative to those of the standard, was identified.

PHOTOGRAPH 3.4 Denaturing gradient gel electrophoresis (DGGE) method for examining methanogenic populations. Top left: Casting of polyacrylamide gel. Top right: Cassette containing gel and samples in buffer tank with lid open. Bottom: Front view of the cassette in the buffer tank whilst gel is polymerising.







CHAPTER 4

VARIATION IN METHANE EMISSIONS FROM SHEEP AND CATTLE FED FRESH RYEGRASS-BASED FORAGES

Chapter published in-parts in:

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CHAPTER 4: VARIATION IN METHANE EMISSIONS FROM SHEEP AND CATTLE FED FRESH RYEGRASS-BASED FORAGES

ABSTRACT

This analysis ('Experiment 1') was undertaken with data collected from 1995 to 2008, by which time over 3000 records (each 2 to 4 days of measurements/animal) of methane (CH_4) emissions had been used to define the New Zealand Greenhouse Gas Inventory for sheep and cattle fed a range of diets. Most values had been derived from the sulphur hexafluoride (SF_6) tracer technique, but since 2007 direct measurements from sheep have become available from respiration chambers. Of the 3000 records, about 500 were from sheep and cattle fed perennial ryegrass (*Lolium perenne*)-based (ryegrass) forages as a sole diet.

A comparison has been made of CH_4 emission data generated by the SF_6 and respiration chamber methods from sheep fed fresh ryegrass diets, and the extent to which variation in emissions could be predicted by the chemical composition of the diet was determined. Data for cattle were all derived using the SF_6 technique and were analysed to determine the amount of variation in CH_4 emissions that could be predicted by diet chemical composition. All analyses were based on measurements with fresh ryegrass and measured feed intakes. Diets containing less than 10% crude protein (CP) or more than 68% neutral detergent fibre (NDF) were excluded as atypical. A total of 196 SF_6 records were available for sheep and 195 for cattle, and 161 sheep respiration chamber records (no chamber data were available for cattle at the time of analysis).

Both techniques showed similar CH_4 yields (CH_4 per unit of dry matter intake; g CH_4/kg DMI) for sheep, but there was less variation in respiration chamber data (23.1 ± SD 2.89 g/kg DMI) than with SF_6 (23.4 ± 5.73 g/kg DMI). Cattle CH_4 yields estimated using SF_6 averaged 19.1 ± 3.70 g/kg DMI. Multiple regression analysis of sheep data showed ryegrass chemical composition accounted for <2% of variation in CH_4 yield determined by SF_6 and 20% from respiration chambers. Only 13% of the variation in CH_4 yield from cattle was accounted for by ryegrass composition.

This study suggests the remaining (true) variance in CH_4 emissions to be a consequence of differences in feed intakes, digestive characteristics of individual animals, and possible interactions between forage physical and chemical components, rather than chemical composition of fresh ryegrass.

4.1 INTRODUCTION

Methane accounts for about 35% of New Zealand's greenhouse gas (GHG) emissions, with about 87% derived from enteric fermentation (Ministry for the Environment, 2010). New Zealand is committed to mitigating GHG emissions in accordance with the Kyoto Protocol, and the New Zealand Greenhouse Gas Inventory has been created as part of this process (Ministry for the Environment, 2010). Inventory calculations are based on animal numbers, production, calculated feed intakes and CH_4 yield (g CH_4/kg DMI). Measurements collected to create the inventory for sheep and cattle in New Zealand had resulted in over 3,000 records of CH_4 emissions by 2008, mostly using the SF_6 tracer technique as described by Johnson *et al.* (1994).

The SF_6 technique itself has been evaluated, and its accuracy for measuring CH_4 emissions has been questioned (Vlaming *et al.*, 2005, McGinn *et al.*, 2006, Vlaming *et al.*, 2007, Pinares-Patiño and Clark, 2008). There is evidence that CH_4 emission estimates can be affected by the SF_6 gas permeation rates (Vlaming *et al.*, 2007, Pinares-Patiño and Clark, 2008), which could exacerbate variation in CH_4 yield estimates (Pinares-Patiño *et al.*, 2008b). More precise measurements of CH_4 emissions can be obtained by respiration chambers, but chambers are relatively expensive, and are restrictive for the animal, compared with SF_6 estimates made at grazing (Pinares-Patiño *et al.*, 2008c). With respiration chambers animals are usually housed individually in sealed chambers which allow measurement of total digestive tract CH_4 emissions, and enable feed intakes to be determined accurately (Pinares-Patiño *et al.*, 2008c).

Methane yield from forage digestion in sheep and cattle averages about 20 g/kg DMI (Ministry for the Environment, 2010), with CH_4 derived from digestion of organic matter (OM), primarily in the rumen. Methane yield appears to be lower when forage legumes are fed, compared to ryegrasses (Waghorn *et al.*, 2002), even though more dry

matter (DM) is digested from a legume diet, and there are significant variations in CH_4 yield when perennial ryegrass cultivars are fed to sheep and cattle (McNaughton *et al.*, 2005, Waghorn and Woodward, 2006). The variation in CH_4 yield has been attributed to effects of feed intake, diet chemical composition, intrinsic animal factors such as the rumen microflora (Pinares-Patiño *et al.*, 2009), and more recently, the SF_6 technique itself (Pinares-Patiño and Clark, 2008). Previous attempts (Waghorn and Woodward, 2006) to predict variation in CH_4 yield from sheep in unrelated trials showed diet chemical composition accounted for up to 51% of the variation in yield when ryegrass pastures were fed to sheep. However, no relationships between CH_4 yield and chemical composition could be established when legumes and herbs were fed to sheep, or when ryegrass-based pasture diets were fed to cattle (Waghorn and Woodward, 2006).

The bases for variation in CH_4 yield are important because they could offer opportunities for mitigation. The objective of this study ('Experiment 1' in this thesis) was to test the hypotheses that 1) CH_4 measurements determined from animals using the SF_6 technique were similar to values measured in respiration chambers, and 2) variation in CH_4 yield could be explained by the chemical composition of fresh ryegrass-based forages.

4.2 METHOD

Data for analysis was compiled between 1995 and 2008, and comprised a total of 1,190 records from sheep and 1,880 from cattle, with each record based on 2 to 4 d of measurements. This study used records which included only animals fed ryegrass forages with measured feed intakes from indoor feeding trials. Evaluations were based on both CH_4 production (g/d) and yield (g/kg DMI) and included a comparison between SF_6 and respiration chamber techniques for CH_4 measured from sheep. No respiration chamber data were available for cattle. Values were excluded when diets contained less than 10% CP or more than 68% NDF, because these were not representative of New Zealand ryegrass forages. Aberrant CH_4 yields of <10 and >39 g/kg DMI were also excluded because they were likely to be incorrect; 10 g/kg DMI is less than half values used in the New Zealand Inventory (Ministry for the Environment, 2010), and 39 g/kg DMI exceeds the maximum from forages fermentation (Bannink *et al.*, 2005).

Application of these selection criteria (>90% ryegrass, measured feed intakes, exclusion of aberrant values) to the SF_6 database resulted in 196 records for sheep and 195 for cattle. Respiration chamber data were only available for sheep, with a total of 472 records in the database. Application of the selection criteria to the respiration chamber data resulted in 161 records from sheep.

Overall, there were 391 animal records using SF_6, and 161 using respiration chambers that fitted the criteria. These were derived from 14 experiments, of which six had been published in peer reviewed journals. Published SF_6 sheep data (from six experiments) were from Swainson *et al.* (2007), Knight *et al.* (2008) and Molano and Clark (2008). The SF_6 cattle data were from four studies and included two publications by Pinares-Patiño *et al.* (2007) and Waghorn *et al.* (2008); and the sheep respiration chamber database were from four studies with one publication by Muetzel *et al.* (2009).

The SF_6 sheep database consisted of measurements from male (26%) and female (74%) animals of which 41% were aged one year or more (mature) and 59% less than one year of age. In all studies, fresh ryegrass was cut in the morning and offered twice daily at various restricted intakes. The cattle SF_6 data were collected from females, with the majority lactating (>80%) and the remainder dry (<20%). Feed was cut and offered twice daily, with the majority fed at *ad libitum* intakes. Sheep housed in respiration chambers for CH_4 measurements comprised 43% males (11% castrated) and 56% females (31% dry, 10% lactating and 15% pregnant). Ryegrass was cut in the morning and fed twice daily with intakes ranging from restricted to *ad libitum*.

The chemical composition of diets fed to animals used for CH_4 measurements was an important part of this study. Composition, and predicted digestibility of organic matter (DOM; g/kg DM) and metabolisable energy (ME) values, were determined by Near Infrared Reflectance Spectroscopy (NIRS) at FEEDTech, AgResearch, Palmerston North, New Zealand. Feed intakes were expressed as kg DM/d and also as a multiple of ME requirements for maintenance (x ME_m), based on the Australian Feeding Standards (Australian Agricultural Council, 1990). Components of the feed were expressed as either intakes (kg/d) or concentrations (g/kg DM) of lipid, CP, NDF and non fibre carbohydrate (NFC). The NFC was defined as 1 minus proportions of ash + lipid + NDF + CP.

Methane emission is a generic term that refers to either: production (g/d); or yield (g/kg DMI). Multiple regressions were used to determine the extent that the variation in CH_4 emissions (g/d and g/kg DMI) could be explained by intakes of dietary components and chemical composition.

Statistical models to examine the extent that DMI, DMI above ME_m, DOM intake (DOMI) and chemical components could predict variation in CH_4 production (g/d) and yield (g/kg DMI) used single and multiple regressions. Relationships were determined separately for sheep and cattle, and for the sheep SF_6 and respiration chamber datasets. All subsets of up to six variables (DMI, intakes above ME_m, DOM(I), CP, lipid, NDF, NFC), were assessed by multiple regression to predict the variance in CH_4 production or yield. The 'all subsets regression' procedure in GenStat software, version 10.2 (Payne *et al.*, 2010), was used for analysis and variables which predicted the most variation in CH_4 emissions were identified.

4.3 RESULTS

4.3.1 Variation in methane production and yield

Mean (± SD) CH_4 production from the SF_6 database for sheep averaged 19.4 ± 7.03 g/d, with a yield of 23.4 ± 5.70 g/kg DMI, whereas the corresponding values from respiration chambers were 20.4 ± 5.30 g/d and 23.1 ± 2.90 g/kg DMI. The greater variability of the SF_6 estimates for both CH_4 production and yield, compared with those determined by respiration chambers, are illustrated in Figures 4.1 and 4.2.

Data from cattle were all based on the SF_6 technique and estimates of CH_4 production averaged 294 ± 76 g/d, with an average yield of 19.1 ± 3.70 g/kg DMI (Figure 4.3). The large range of CH_4 production values from cattle was associated with the different intakes and energy requirements for lactation.

For the sheep SF_6 and respiration chamber databases, diet chemical composition and intake of the ryegrass diets covered a wide range of values, summarised in Table 4.1. The dietary CP concentrations ranged from 103 to 247 g/kg DM, whilst NDF concentrations were from 397 to 626 g/kg DM. Intakes of sheep ranged from below

ME_m to more than twice ME_m, so there was a four to five fold range in intakes of dietary constituents in the data used for analysis (Table 4.1).

The fresh ryegrass diets fed to cattle contained 151 to 225 g CP/kg DM and 396 to 569 g NDF/kg DM (Table 4.1). As with the sheep data, diet chemical composition and intake covered a wide range of values and thus provided good opportunities to identify relationships with CH_4 production and yield.

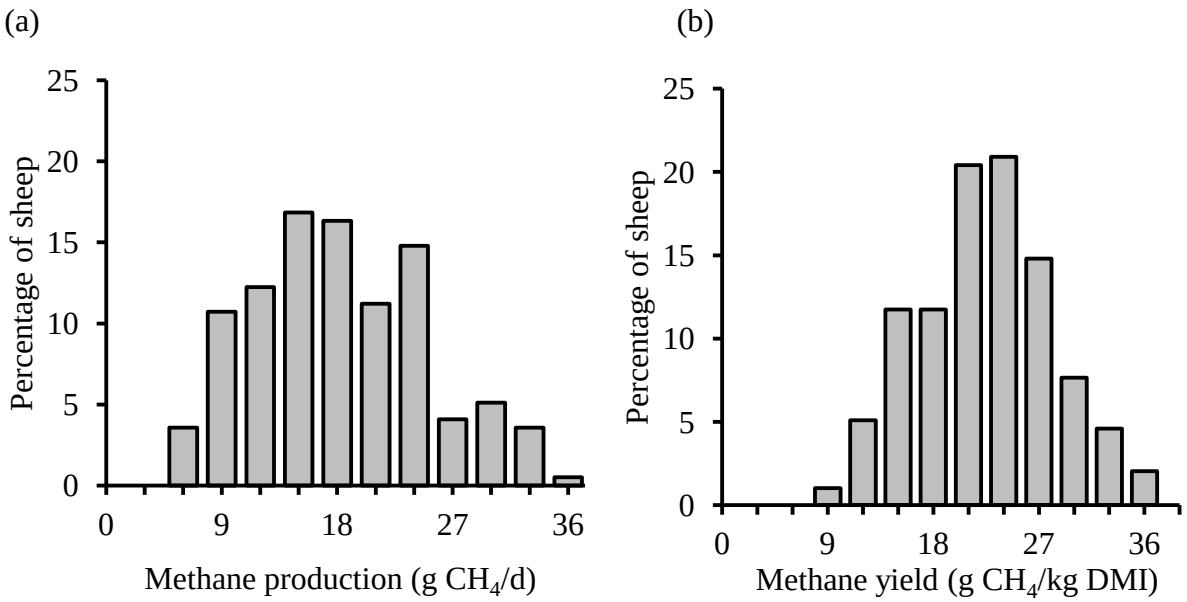

FIGURE 4.1 Methane production (a) and yield (b) from sheep fed fresh ryegrass, estimated using the sulphur hexafluoride (SF_6) tracer technique.

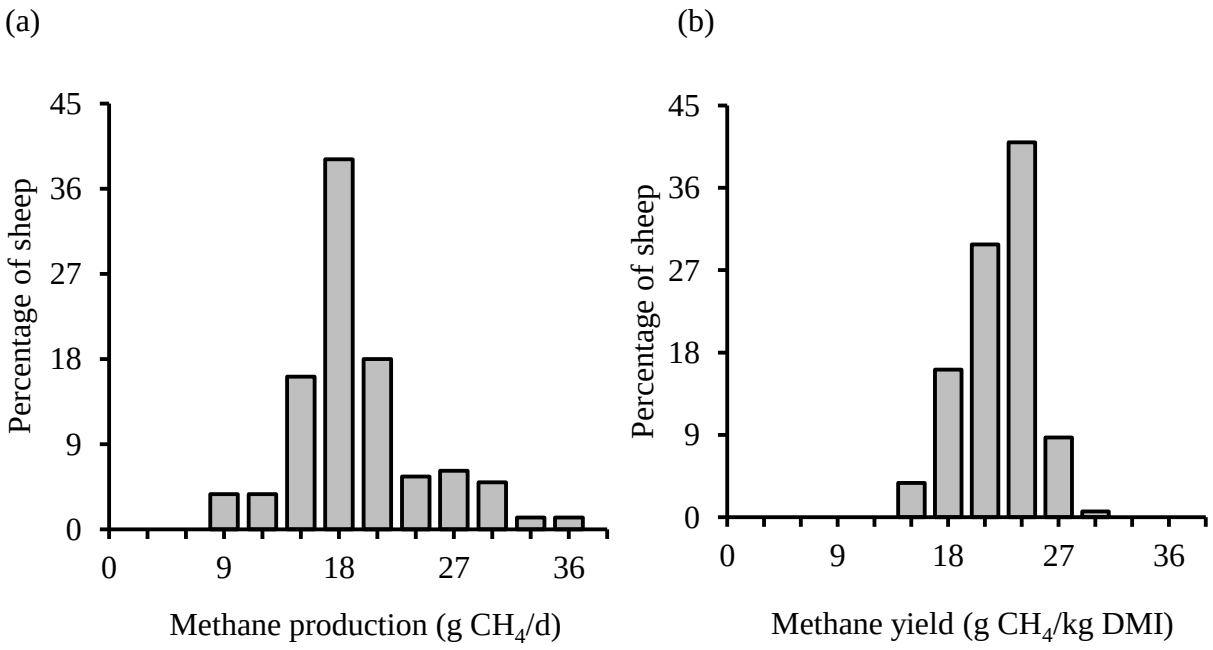

FIGURE 4.2 Methane production (a) and yield (b) from sheep fed fresh ryegrass, measured using respiration chambers.

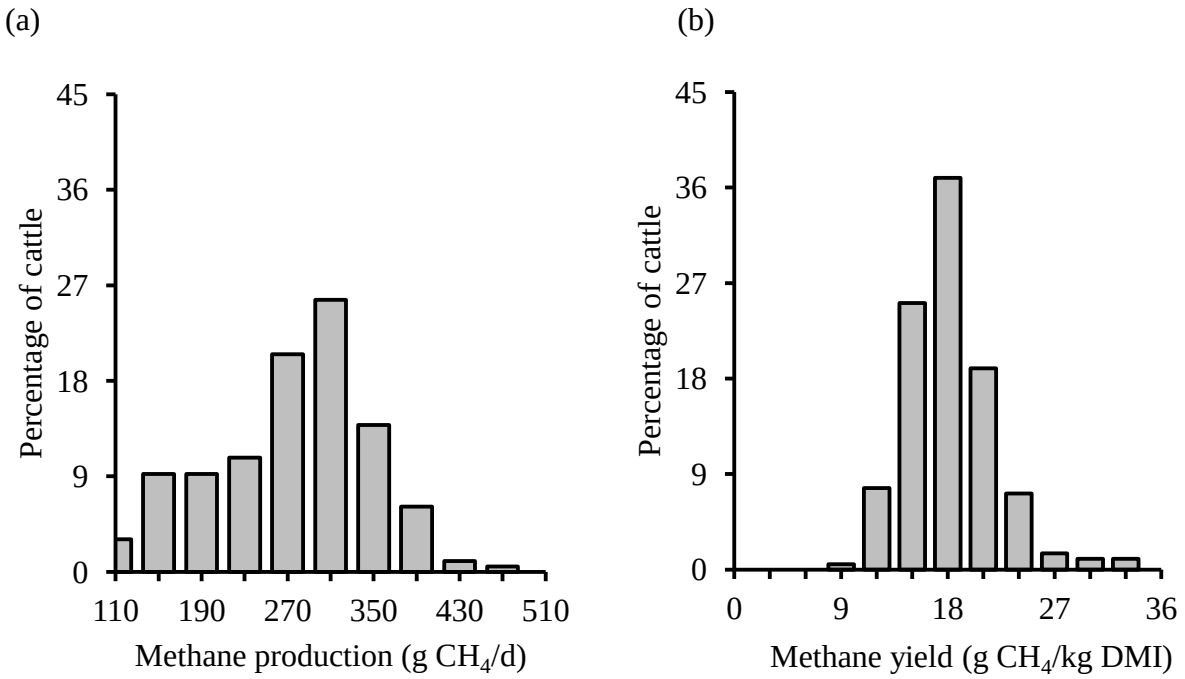

FIGURE 4.3 Methane production (a) and yield (b) from cattle fed fresh ryegrass, estimated using the sulphur hexafluoride (SF_6) tracer technique.

TABLE 4.1 The range of concentrations and intakes of chemical components in fresh ryegrass diets fed to sheep and cattle used to determine methane emissions using respiration chamber (Chamber) and sulphur hexafluoride (SF_6) tracer techniques.

Component	Sheep				Cattle	
	Chamber		SF_6		SF_6	
	Range	Average	Range	Average	Range	Average
Component composition (g/kg DM)						
Crude protein	103 - 174	144	108 - 247	180	151 - 225	194
Lipid	16 - 35	27.5	24 - 50	37	33 - 42	37.4
Neutral detergent fibre	431 - 626	494	397 - 609	504	396 - 569	495
Non-fibre carbohydrate	175 - 398	261	123 - 225	180	71 - 305	174
Digestible organic matter	487 - 821	739	636 - 800	724	694 - 808	752
Component intake (kg/head/d)						
Dry matter	0.32 - 1.72	0.91	0.38 - 1.69	0.84	8.17 - 20.81	15.4
Crude protein	0.05 - 0.25	0.13	0.05 - 0.42	0.15	1.77 - 4.58	2.97
Lipid	0.01 - 0.05	0.02	0.01 - 0.07	0.08	0.31 - 0.87	0.58
Neutral detergent fibre	0.14 - 0.80	0.45	0.20 - 0.79	0.42	4.50 - 11.69	7.55
Non-fibre carbohydrate	0.11 - 0.48	0.23	0.06 - 0.39	0.15	0.58 - 5.51	2.79
Digestible organic matter	0.25 - 1.41	0.66	0.26 - 1.30	0.61	5.77 - 15.75	11.6
Dry matter intake above ME_m[1]	0.57 - 2.59	1.26	0.66 - 2.40	1.36	1.69 - 4.74	3.27

[1]expressed as multiples of ME_m

ME_m, metabolisable energy requirements for maintenance

4.3.2 Methane emissions in relation to feed intake

For the purposes of this Chapter, CH_4 emissions have been given as production (g/d) and yield (g/kg DM). Data have been interpreted in terms of diet chemical composition, either as intakes of chemical constituents (kg/d) or chemical composition (g constituent/kg DM), and their relationship with emissions of CH_4 has been summarised in Table 4.2. Dry matter intake has also been included in the analyses, as either kg/d, or as intakes as a multiple of ME_m.

4.3.2.1 Sheep

When CH_4 production (g/d) from sheep was estimated by the SF_6 tracer technique, DMI predicted 51% ($P<0.001$) of the total variation (Figure 4.4). When CH_4 production was measured in respiration chambers, the single component best able to account for the variance was again DMI, but it predicted 81% ($P<0.001$; Figure 4.4). The relationship of DMI with CH_4 production (i.e. the slope and intercept of data in Figure 4.4) was not significantly different between measurement techniques (SF_6 and respiration chambers).

When CH_4 yield (g/kg DMI) estimated using the SF_6 technique was plotted against DMI, only 2% of the variation from sheep was predicted, but with respiration chamber data 36% ($P<0.001$) of the variation was predicted (Figure 4.5). The respiration chamber data suggested a decline in CH_4 yield of 6.18 g CH_4/kg DMI for every 1 kg increase in DMI (Figure 4.5).

When intakes were expressed as multiples of ME_m, 23% and 67% ($P<0.001$) of the variation in CH_4 production was predicted by DMI of sheep, when using the SF_6 and respiration chamber techniques, respectively (Table 4.3).

4.3.2.2 Cattle

Dry matter intake accounted for 52% ($P<0.001$) of the variation in CH_4 production (g/d) from cattle (Figure 4.6). However, there was no relationship between CH_4 yield and DMI (kg/d or as a multiple of ME_m) for cattle (Figure 4.7).

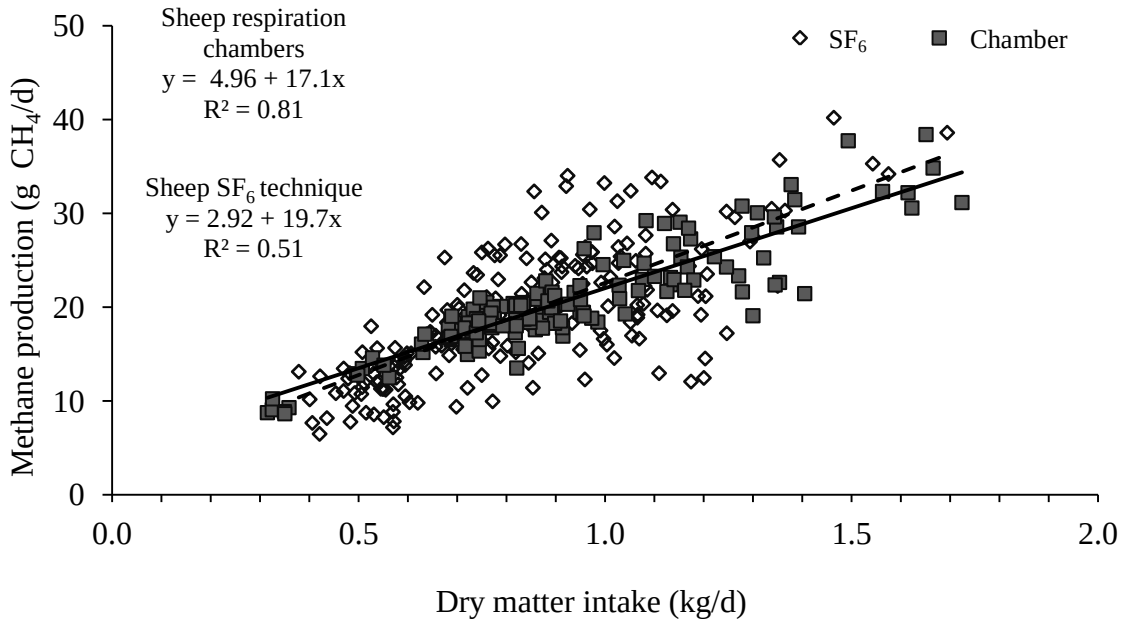

FIGURE 4.4 Relationship between methane production and dry matter intake from sheep fed fresh ryegrass, measured in either respiration chambers (solid line) or using the sulphur hexafluoride (SF_6) tracer technique (dashed line).

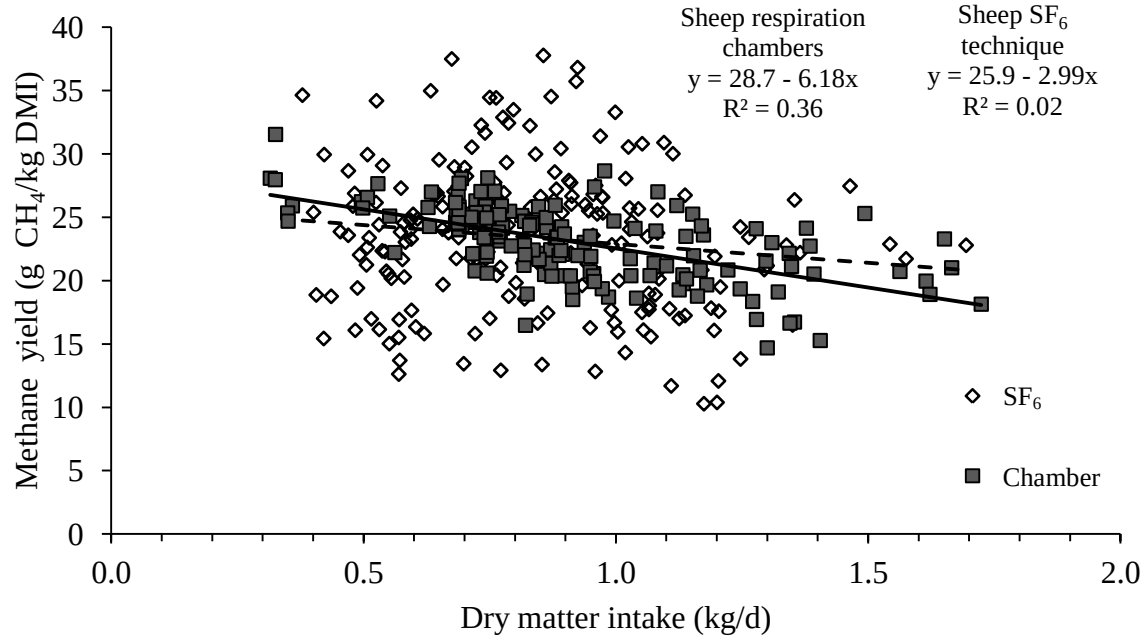

FIGURE 4.5 Relationship between methane yield and dry matter intake from sheep fed fresh ryegrass, measured in either respiration chambers (solid line) or using the sulphur hexafluoride (SF_6) tracer technique (dashed line).

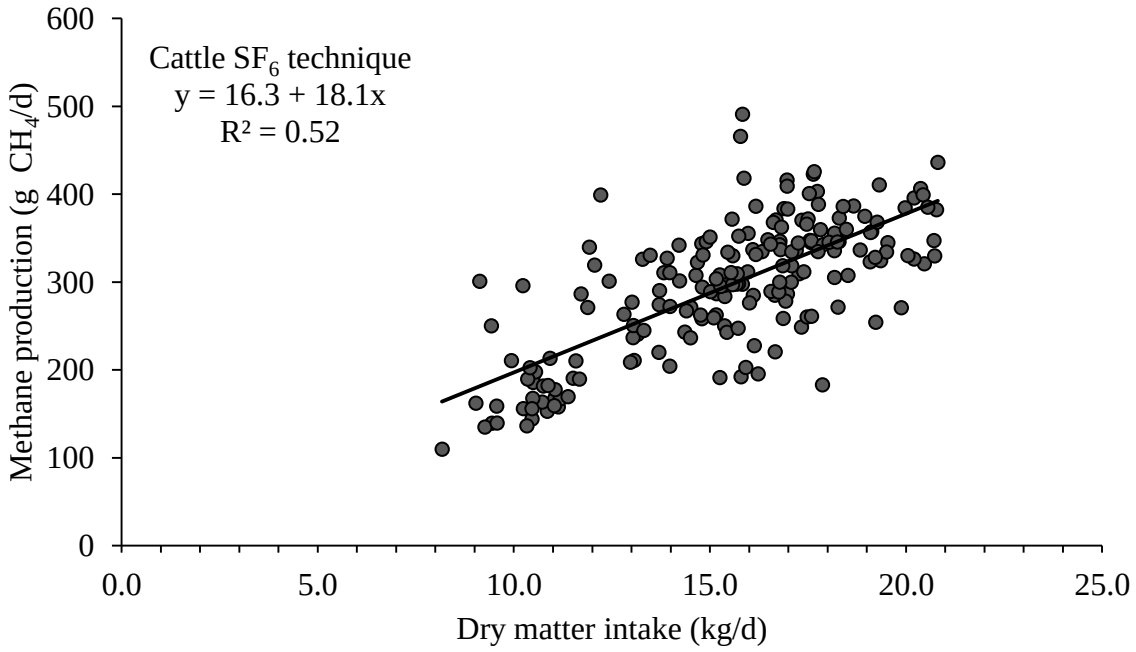

FIGURE 4.6 Relationship between methane production and dry matter intake from cattle fed fresh ryegrass estimated using only the sulphur hexafluoride (SF_6) tracer technique.

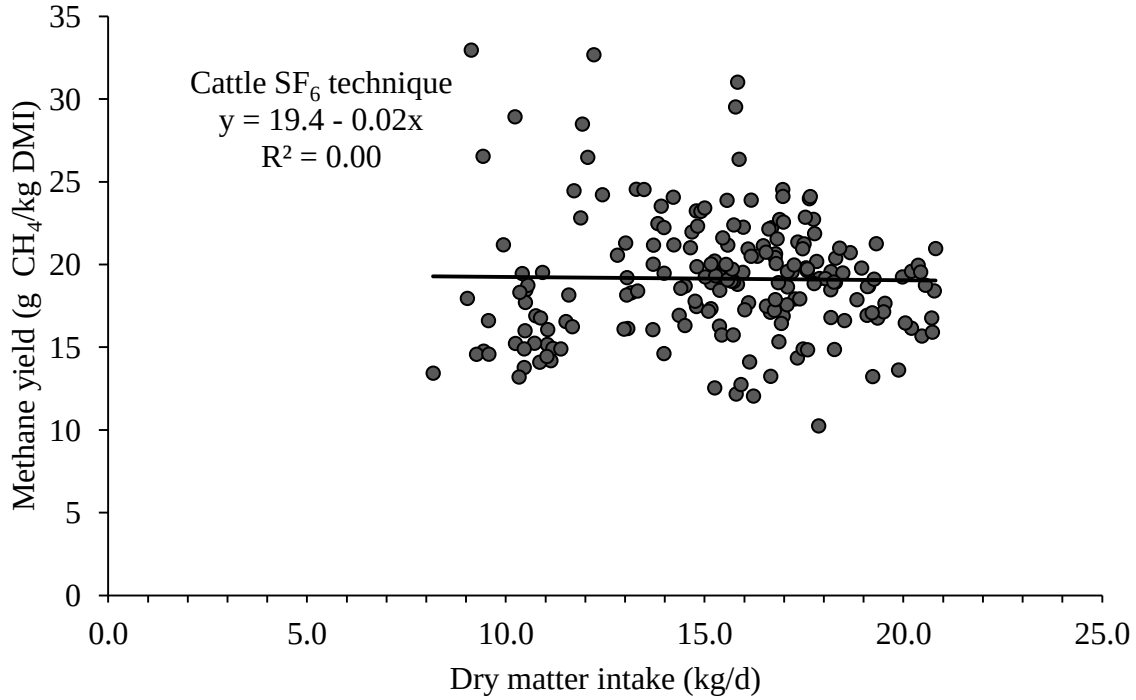

FIGURE 4.7 Relationship between methane yield and dry matter intake from cattle fed fresh ryegrass estimated using only the sulphur hexafluoride (SF_6) tracer technique.

TABLE 4.2 Summary of the relationships derived by linear regression between intakes of dry matter and dietary components (intake and concentration) of fresh ryegrass fed to sheep and cattle, and methane (CH_4) emissions. Data are for CH_4 production (g/d) and yield (g/kg DMI) and are based on the sulphur hexafluoride (SF_6) tracer and respiration chamber techniques.

Variable	SF_6			Respiration chamber		
	Intercept	Slope	Variation predicted (%)	Intercept	Slope	Variation predicted (%)
SHEEP						
methane production (g)						
Intake (kg DM/d)	2.92	19.7	51	4.96	11.4	81
Intake as a multiple of ME_m	6.01	9.77	23	6.00	11.4	67
Component (kg/d)						
Digestible organic matter	5.03	23.6	46	5.66	22.2	78
Crude protein	12.3	46.0	25	6.55	107	68
Neutral detergent fibre	2.99	39.0	49	6.56	29.8	71
Lipid	10.1	292	35	8.17	499	61
Non-fibre carbohydrate	11.8	50.9	21	7.51	56.0	62
SHEEP						
methane yield (g)						
Intake (kg DM/d)	25.9	-2.99	2	28.7	-6.18	36
Intake as a multiple of ME_m	28.6	-3.94	6	27.1	-3.19	18
Component (g/kg DM)						
Digestible organic matter	28.8	-0.013	1	13.3	0.01	14
Crude protein	24.7	-0.008	1	19.3	0.03	3
Neutral detergent fibre	18.8	0.009	0	34.9	-0.02	13
Lipid	23.0	0.012	0	18.4	0.16	7
Non-fibre carbohydrate	23.0	0.002	0	18.7	0.02	12
CATTLE						
methane production (g)						
Intake (kg DM/d)	16.3	18.1	52			
Intake as a multiple of ME_m	58.0	72.2	44			
Component (kg/d)						
Digestible organic matter	29.9	22.8	49			
Crude protein	121	58.4	25			
Neutral detergent fibre	72.7	29.4	30			
Lipid	64.0	401	41			
Non-fibre carbohydrate	230	23.2	16			
CATTLE						
methane yield (g)						
Intake (kg DM/d)	19.4	-0.020	0			
Intake as a multiple of ME_m	19.2	-0.009	0			
Component (g/kg DM)						
Digestible organic matter	10.1	0.012	1			
Crude protein	27.7	-0.044	8			
Neutral detergent fibre	24.4	-0.010	2			
Lipid	20.8	-0.044	0			
Non-fibre carbohydrate	16.4	0.015	8			

DM, dry matter; ME_m, metabolisable energy requirements for maintenance

4.3.3 Methane production and chemical component intake

When CH_4 production from sheep was estimated using the SF_6 tracer technique, the dietary chemical component intakes best able to predict the variation in CH_4 production were intakes (kg/d) of NDF (49%), DOM (46%), lipid (35%), and CP (25%), whilst intake of NFC predicted only 21% of variation (Table 4.2). No combination of component intakes (kg/d) could account for more of the variation in CH_4 production than DMI. Intakes of NDF and lipid together predicted 51% (Table 4.3), and the addition of other chemical component intakes into the model had little effect on the amount of variation in CH_4 production predicted.

When CH_4 production from sheep was measured in respiration chambers, compared to the SF_6 technique, a higher percentage of variation in CH_4 production was predicted by chemical component intakes. The DOMI accounted for 78% of the variation in CH_4 production, with intakes of NDF, CP, NFC and lipid predicting 71%, 68%, 62% and 61% of variation, respectively (Table 4.2). The combination of chemical components best able to predict the variation in CH_4 production were intakes of lipid, NDF and NFC, which together predicted 80% of the variation (Table 4.3). The addition of other chemical component intakes had minor effects on the prediction of CH_4 production.

Dry matter intake predicted the most variation in CH_4 production for cattle (52%), closely followed by DOMI (49%), and intakes of lipid (41%), NDF (30%), CP (25%), and NFC (16%) (Table 4.2). The combination of intakes of lipid, CP and NDF were able to predict 51% of the variation in CH_4 production from cattle (Table 4.3). The addition of intakes of other individual dietary constituents had no significant relationship to CH_4 production.

4.3.4 Methane yield and chemical component concentration

Despite the range in diet chemical composition (Table 4.1), less than 2% of the variation in CH_4 yield (g/kg DM) from SF_6 determinations was predicted by the concentration of any individual chemical component (Table 4.2). Furthermore, multiple regression analysis showed incorporation of additional constituent concentrations into the model did not account for more variance in CH_4 yield (Table 4.3).

Evaluation of the respiration chamber database showed that DOM concentration predicted up to 14% of the variation in CH_4 yield. Individual chemical component concentrations predicted 13% (NDF), 12% (NFC), 7% (lipid) and 3% (CP) of the variation in CH_4 yield (Table 4.2). Up to 20% ($P<0.001$) of the variation in CH_4 yield was predicted by a combination of concentrations of lipid, NDF, CP and NFC (Table 4.3). Additional chemical component concentrations did not significantly improve the prediction.

The cattle data showed the concentration of individual dietary components predicted very little of the variation in CH_4 yield estimated by the SF_6 technique. The largest variation in CH_4 yield was predicted by NFC and CP concentrations, each at 8%, whereas NDF concentration accounted for 2%, and DOM concentration 1% (Table 4.2). The combination of component concentrations best able to predict the variation in CH_4 yield for cattle were concentrations of CP, NFC and lipid, which together predicted 13% ($P<0.001$) (Table 4.3).

TABLE 4.3 Intakes of dietary components and concentrations best able to account for variation in methane production and yield from sheep using the sulphur hexafluoride (SF_6) tracer and respiration chamber techniques, and from cattle using the SF_6 tracer technique.

Technique	Components best able to account for variation in the model and their formula for prediction	Variation predicted (%)
Methane production (g CH_4/d)	**Component intake (kg/d)**	
Sheep respiration chambers	117Lipid + 18.4NDF + 18.9NFC + 4.62	80
Sheep SF_6	30.9NDF + 108Lipid + 2.99	51
Cattle SF_6	685Lipid – 77.2CP + 14.7NDF + 19.2	51
Methane yield (g CH_4/kg DMI)	**Component concentration (g/kg DM)**	
Sheep respiration chambers	-0.07Lipid – 0.02NDF + 0.01CP + 0.01NFC + 33.1	20
Sheep SF_6	No significant effects	<2
Cattle SF_6	- 0.07CP + 0.002NFC +0.36Lipid + 18.9	13

CH_4, methane; NDF, neutral detergent fibre; NFC, non-fibre carbohydrate; CP, crude protein

4.4 DISCUSSION

The first hypothesis for this Chapter was only partly proven; the variation in CH_4 emissions determined by the database analyses showed similar means for the two techniques when applied to sheep but the variation in estimates of CH_4 yield was about twice the magnitude for data derived by the SF_6 method, compared to respiration chambers. The second hypothesis was not proven; the variation in chemical composition of ryegrass was poorly associated with yield and did not account for more than 20% of the variation. The principal findings from this study were that DMI predicted up to 81% of the variation in CH_4 production (g/d) and up to 36% of the variation in yield (g/kg DMI). The higher CH_4 yield from ryegrass forages fed to sheep (about 23.3 g/kg DMI) compared to cattle (19.1 g/kg DMI) is unlikely to be affected by diet composition and may result from differences in digestive physiology.

4.4.1 Methane emissions and feed intake

Regression analysis of CH_4 yields from sheep against DMI, based on SF_6 estimations (Figure 4.5) showed no relationship, but the analysis based on respiration chamber data suggested a decline of 6.18 g CH_4/kg DMI as DMI increased (Table 4.2). This relationship was derived from the four-fold range in feed intakes, from less than 1 x ME_m to about 2.5 x ME_m (Table 4.1). The adult sheep used for CH_4 measurements and evaluated in this study weighed around 40 to 50 kg, so their ME_m requirements were about 7 MJ ME/d (Australian Agricultural Council, 1990), which is about 0.6 kg DM/d of good quality ryegrass pasture (11.5 MJ ME/kg DM). When the decline in CH_4 yield was expressed in relation to multiples of ME_m intakes, above maintenance, the decline was 3.19 g CH_4/ME_m intake (Table 4.2). This is equivalent to a reduction in CH_4 energy (CH_4-E) of about 176 KJ/multiple of ME_m (11.2 MJ GE) or 0.016 CH_4-E as a proportion of gross energy intake (GEI). Expression in terms of ME_m lessens the effects of variation in animal size on rumen fill and digesta turnover.

There is a consensus that increasing feed intake reduces CH_4 yield (Blaxter and Clapperton, 1965, Johnson *et al.*, 1993, Hart *et al.*, 2009, Sauvant and Giger-Reverdin, 2009, Yan *et al.*, 2010). Although the relationship appears to be variable (Table 4.4),

some reports suggest a smaller reduction in animals fed roughage/silage diets (Blaxter and Clapperton, 1965, Yan *et al.*, 2010) compared to concentrates in some situations (Johnson *et al.*, 1993) but not others (Sauvant and Giger-Reverdin, 2009).

There are relatively few published evaluations of CH_4 yields in relation to feed intake from sheep. Johnson *et al.* (1993) summarised data from 159 sheep fed concentrate diets from several studies and reported a decline of 0.016 CH_4-E/GEI per multiple of maintenance intakes above ME_m (Table 4.4). This decline was similar to that reported by Muetzel *et al.* (2009) for dry, pregnant and lactating ewes fed fresh pasture, where CH_4 yield decreased by 5.3 g/kg DMI for every increase in ME_m (about 0.016 CH_4-E/GEI). These values are also similar to that derived from sheep fed fresh ryegrass in respiration chambers reported here (Table 4.4). However, Sun *et al.* (2011) and Hammond *et al.* (2011) reported a smaller decrease in CH_4 yield from 25.5 to 21.5 g/kg DMI (about 0.009 CH_4-E/GEI for each multiple of ME_m) when intakes of sheep fed fresh ryegrass were increased from 0.8 to 2.2 x ME_m. Molano and Clark (2008) also found no relationship between CH_4 yield and intake when ewes were fed fresh pasture at about 0.8 to 2.0 x ME_m (estimated with the SF_6 technique).

The results from cattle evaluated here were similar to the SF_6 estimates from sheep, where there was no relationship between CH_4 yield and feed intake. However, published data sets based on respiration chamber measurements, summarised in Table 4.4, have demonstrated a decline in CH_4 yield when intakes of cattle are increased. When concentrate-based diets were fed to either beef or lactating cows, the reduction in CH_4 yield ranged from about 0.008 to 0.018 CH_4-E/GEI for every increase in feed intake above ME_m (Yan *et al.*, 2000, Beauchemin and McGinn, 2006, Sauvant and Giger-Reverdin, 2009, Yan *et al.*, 2010) (Table 4.4).

It appears that the extent of the decrease in CH_4 yield is less with roughage diets than concentrates (Table 4.4), but the relationships are variable and may be less robust when CH_4 emissions have been determined using SF_6 tracer technique.

TABLE 4.4 Effect of increasing intake by one multiple of maintenance energy requirements (ME_m) on energy lost to methane (CH_4) emissions; a summary of respiration chamber studies of CH_4 emissions from sheep and cattle fed a variety of diets. Data are expressed as a change in energy loss (CH_4-E/GEI per increase in feed intake by 1 x ME_m) unless indicated.

Animal	Diet	CH_4-E/GEI change	No. of animals	R^2	Reference
Sheep and cattle	Pelleted[a]	-0.0205	N/A	N/A	Blaxter and Clapperton (1965)
Sheep and cattle	Roughage[b]	-0.0079	N/A	N/A	Blaxter and Clapperton (1965)
Sheep	Concentrate	-0.0160	159	0.43	Johnson *et al.* (1993)
Sheep	Fresh pasture	-0.0160	20	0.53	Muetzel *et al.* (2009)
Sheep	Fresh ryegrass	-0.0090	24	N/A	Hammond *et al.* (2011) & Sun *et al.* (2011)
Sheep	Fresh ryegrass[c]	-0.0160	161	0.36	This study
Beef cattle	Concentrate	-0.0180	118	0.17	Johnson *et al.* (1993)
Dairy and beef cattle	Silage/grass	-0.0078	322	0.85	Yan *et al.* (2000)
Lactating dairy cows	Concentrate[d]	-0.0077	161	N/A	Sauvant and Giger-Reverdin (2009)
Lactating beef cattle	Silage/grass	-0.0091	579	0.56	Yan *et al.* (2010)
Beef cattle	Barley silage	-0.0080	8	<0.20	Beauchemin and McGinn (2006)
Cattle	Fresh ryegrass[c]	0.0000	195	0.00	This study

[a]Based on CH_4-E/GEI = 0.059 DE/GE -0.0267; assume DE = 80% for pelleted diets with a GE of 19.0 MJ ME/kg DM
[b]Based on CH_4-E/GEI = 0.028 DE/GE -0.0103; assume DE = 65% for roughage diets with a GE of 18.4 MJ ME/kg DM
[c]Based on a GE of 18.4 MJ ME/kg DM; sheep data based on respiration chamber measurements of CH_4, cattle data based on SF_6 estimates of CH_4
[d]Based on 4.10 g CH_4/DOMI; assume DOM = 65% and GE 19.0 MJ ME/kg DM
DE/GE, digestible energy (DE) of the feed (GE) at maintenance; CH_4-E/GEI, methane energy as a proportion of gross energy intake; DOMI, digestible organic matter intake; R^2, regression coefficient, N/A, data not available; no., number

The cause of the reduction of CH_4 yield with increasing feed intake has been attributed to shorter rumen residence times and less digestion in the rumen, which can also affect end products of fermentation (Janssen, 2010). Methane is an end product of microbial fermentation and can be affected by animal factors such as chewing, salivation, and solid and liquid phase retention times (Wilson and Kennedy, 1996, Varga and Kolver, 1997, Faichney, 2005), as well as proportions and yields of volatile fatty acids (VFAs). When feed intakes increase to a greater extent than rumen pool size, the residence time in the rumen is reduced, so there is less time available for microbial fermentation. Reduced fermentation lowers CH_4 formation per unit of feed (Yan *et al.*, 2000) and higher passage rates have been associated with fermentation pathways that lead to more propionate and less hydrogen (H_2) formed per unit of feed fermented, and less CH_4 production, as reviewed by Janssen (2010). These principals apply to all diets, but the lower NFC:NDF ratios in roughages compared to concentrates, limits the extent of propionate production (France and Dijkstra, 2005). There is some evidence that high intakes of roughage diets result in an increase in rumen volume (Waghorn *et al.*, 1986) to a greater extent than high intakes of concentrates (Mertens, 1987). So, the change in rumen residence time for animals fed roughages may be less than from concentrate feeding (Baumgardt, 1970, Dado and Allen, 1995), and in combination with substrate suitability for propionate production, could affect the extent of the reduction in CH_4 yield with increasing feed intakes.

4.4.2 Diet composition and methane yield

Despite large variations in the chemical composition of the ryegrass diets evaluated here (Table 4.1), concentration of ryegrass chemical components accounted for only 20% or less of the variation in CH_4 yield from respiration chamber measurements (Table 4.3). Expectation of a greater association between CH_4 yield and chemical composition was based on previous measurements suggesting lower CH_4 emissions from sheep fed a range of legume and herb forages. These measurements were based on the SF_6 technique and CH_4 yields (g/kg DMI) from various diets included: lotus major (*Lotus pedunculatus*), 11.6 or 14.5; lucerne (*Medicago sativa*), 19.6; sulla (*Hedysarum coronarium*), 17.5; chicory (*Cichorium intybus*), 16.2; and white clover (*Trifolium repens*), 12.3 (Table 2.3) (Woodward *et al.*, 2001, Waghorn *et al.*, 2002, Krause,

AgResearch Report), compared to pasture (24.0; Table 2.2). However, the lower CH_4 yields for white clover and chicory have not been supported by respiration chamber measurements undertaken since the database analysis (Chapter 5 and Sun *et al.* 2011).

Expectations of causative relationships between CH_4 yield and diet chemical composition were based on variations in products of fermentation which affect proportions of VFAs as well as H_2. The theoretical yield of H_2 from fermentation is greatest when acetate is produced, followed by butyrate, with propionate resulting in a net uptake of H_2 (Kohn and Boston, 2000). Based on the end products of fermentation, Beever (1993) estimated CH_4 yields (mole CH_4/mole hexose) of 0.61 from roughage and 0.38 from concentrate diets. Hence, immature pasture containing high concentrations of NFC fed to sheep may have resulted in a lower CH_4 yield compared to more mature, fibrous pasture, but the data analysis reported here (and Molano and Clark, 2008) suggest only minor effects of ryegrass quality on CH_4 yield. Only diets containing 70 to 90% grain result in substantially lower CH_4 yields compared to forages (2-3% vs. 6-7% GEI, respectively) (Lovett *et al.*, 2003, Beauchemin and McGinn, 2006, Martin *et al.*, 2007) and these diets also result in high proportions of propionate in the total VFA (Johnson and Johnson, 1995). However, high grain diets also result in lactate production and are not comparable with forage digestion.

The absence of a strong correlation between forage components and CH_4 yield from this database analysis suggests the variation in yield is a consequence of several interacting aspects of digestion, rather than specific substrates. For example, variation in the extent and efficiency of chewing will affect digesta presented to the rumen microflora, as well as rumen pH regulation, digesta turnover, and rumen residence time. Furthermore, reports from the literature have not shown differences in CH_4 emissions with forages types, particularly with legume compared to grasses, despite large differences in chemical composition (Beever *et al.*, 1985, Cammell *et al.*, 1986, van Dorland *et al.*, 2007). The interactions between different aspects of fermentation may account for some of the variation in CH_4 yield from sheep fed both similar and contrasting forages, but these are not simple relationships with diet chemical composition.

4.4.3 Importance of measurement technique

The respiration chamber technique is considered to be more accurate than the SF_6 tracer dilution (Pinares-Patiño *et al.,* 2008c) because measurements are direct and not dependant on marker efficacy for representing CH_4 emissions. Weaknesses associated with any marker (Shipley and Clark, 1972) suggest that some of the variation associated with SF_6 estimates (Figures 4.1 and 4.2) may have been a consequence of the technique itself (Chapter 2.5.4). Mean values were not affected by technique, but the much lower variation between data measured using respiration chambers suggests the SF_6 tracer technique affected the precision of measurements.

Both the respiration chamber and SF_6 tracer techniques have strengths and weaknesses, but in situations where animals are confined (to enable accurate measurement of feed intake), there is good evidence that CH_4 emission estimates are influenced by the SF_6 gas permeation rate from the tubes (McNaughton *et al.,* 2005, Vlaming *et al.,* 2007; Pinares-Patiño *et al.,* 2008a). Vlaming *et al.* (2007) found a positive correlation between SF_6 permeation tube release rate and estimates of CH_4 yield, whilst Lassey *et al.* (2001) showed that the greater the time between CH_4 determinations and calibration of SF_6 permeation tubes, the higher the risk of error. The losses of CH_4 in the flatus is probably minor (less than 2% of total CH_4; Murray *et al.,* 1976), but it is not included in SF_6 estimations of CH_4. The extent of the variation with measurement technique has been investigated by Pinares-Patiño *et al.* (2008b), who reported a coefficient of variation (CV) of 18.4% (SF_6 technique) compared to 6.7% (respiration chamber technique) for CH_4 production from sheep fed chaffed lucerne hay.

The CH_4 emissions from cattle presented here are all based on the SF_6 technique and variations in CH_4 emissions are likely to be over-estimated. However, the extent of the error is uncertain because reports of differences between the two methods appear much smaller for cattle. Grainger *et al.* (2007) reported CV's of 19.6% with SF_6 and 17.7% using respiration chambers with lactating cows. McGinn *et al*. (2006) showed that SF_6 estimates of CH_4 from cattle were more accurate than the chamber technique, especially when forages were fed *ad libitum* (CV = 5.9% vs. 7.3%, respectively).

An advantage of SF_6 methodology is that CH_4 measurements can be made from grazing animals, which can select their diet in a manner representative of farmed livestock,

whereas animals confined in chambers are fed harvested (often long) forages in relatively few meals. The SF_6 technique enables estimates of CH_4 to be made on large numbers of animal's relative to respiration chambers, but feed intakes cannot be measured accurately at grazing (Waghorn *et al.*, 2007). The choice of technique for the determination of CH_4 emissions from ruminant animals should be based on experimental objectives, but respiration chamber measurements are more appropriate for determining the cause in variation of CH_4 emissions.

4.4.4 Evaluation of analyses performed

The analyses presented here took advantage of accumulated records from New Zealand studies, and provided an opportunity for familiarisation with information derived from a number of independent trials. Criteria were developed to identify data suitable for the analyses, and the expectation was that some relationships associated with CH_4 production and feeding might have been identified. The clear demonstration of greater variation in CH_4 yield when determined with the SF_6 technique compared with respiration chambers, and the weak relationships between CH_4 yield and ryegrass chemical composition, were unexpected. In retrospect, a meta-analysis of the data may have been more appropriate and have provided a more robust interpretation of the data. However, the quantity of appropriate data available for analysis from SF_6 trials and respiration chamber measurements was limited at the time this study was undertaken and would have constrained a more detailed evaluation.

The use of information from the database did highlight the need for careful scrutiny of information prior to inclusion into the database. It will be important to ensure complete records are obtained, as well as information linking this to the source and persons responsible for the data. A high quality database would add value to the information generated from CH_4 measurements and a meta-analysis of currently available data from respiration chambers would contribute to our understanding of factors affecting CH_4 production from sheep fed fresh forages.

4.5 CONCLUSION

The three main findings from this analysis were that both production and yield of CH_4 measured using respiration chambers were closely associated with feed intake; that CH_4 yield was not affected by forage chemical composition; and the variation in CH_4 yield was reduced when measurements from sheep were made in chambers, relative to the SF_6 technique. When fresh ryegrass diets were fed to sheep, DMI predicted 81% of the variation in CH_4 production and 36% of the variation in CH_4 yield. Comparative values for cattle fed fresh ryegrass, based on the SF_6 technique with measured feed intakes, suggested that DMI was able to account for 52% of the variation in CH_4 production, but did not account for any of the variation in CH_4 yield. None of the chemical components (individually or combined) were able to account for more variation in CH_4 emissions from sheep and cattle than DMI.

Measurements from sheep showed a greater percentage of variation in CH_4 emissions was predicted from chamber measurements, compared to SF_6 estimates. This implies that some of the variation in CH_4 emissions between animals is a consequence of the SF_6 technique itself.

Analyses from this study and reports from the literature have highlighted the importance of feed intake on CH_4 emissions. The effect of feed intake on CH_4 production is a consequence of substrate supply, but effects on yield are less easily explained. It is possible that when feed intakes increase to a greater extent than rumen pool size, passage rates increase, there is less time for microbial fermentation, and the end products of fermentation change with the formation of more propionate, less H_2, and consequently less CH_4 per unit of feed eaten.

Diet chemical composition was able to predict only 20% of the variation in CH_4 yield from respiration chamber measurements. Expectations of a greater association between CH_4 yield and chemical composition were based on previous CH_4 estimates made using the SF_6 technique, which varied between 11.6 and 19.6 g CH_4/kg DMI from sheep fed fresh legumes (lotus major, lucerne, sulla, and white clover) that differed markedly in chemical composition. However, the relationships between diet composition and CH_4 yield in this and other studies are weak and inconsistent.

Further investigations are warranted to define factors affecting CH_4 emissions in relation to feed intake. Interpretation should include expression on an OM basis, as well as DMI because the ash fraction cannot contribute directly to methanogenesis. It is postulated that interactions between plant structural and chemical characteristics affect microbial colonisation, communities, growth and ultimately methanogenesis, in association with feed intakes. These characteristics differ between individual animals and may account for some of the variation in CH_4 yield when similar or contrasting forages are fed.

CHAPTER 5

EFFECT OF WHITE CLOVER (*Trifolium repens*) OR PERENNIAL RYEGRASS (*Lolium perenne*) FORAGES ON METHANE EMISSIONS FROM SHEEP

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CHAPTER 5: EFFECT OF WHITE CLOVER (*Trifolium repens*) OR PERENNIAL RYEGRASS (*Lolium perenne*) FORAGES ON METHANE EMISSIONS FROM SHEEP

ABSTRACT

Enteric methane (CH_4) contributes about one third of New Zealand's greenhouse gas (GHG) emissions, and measurements from our laboratory using the sulphur hexafluoride (SF_6) tracer technique suggested much lower CH_4 yields (g CH_4 per kg of dry matter intake; g/kg DMI) from sheep fed white clover (*Trifolium repens*) compared with perennial ryegrass (*Lolium perenne*; ryegrass) forages. Thus, white clover offers a potential opportunity to reduce CH_4 emissions from pastoral-based ruminant livestock systems, subject to confirmation of a lower CH_4 yield using respiration chambers.

Three experiments (Experiments 2, 3 and 4) were undertaken where good quality, freshly harvested white clover and ryegrass forages were fed to sheep and CH_4 emissions were measured in respiration chambers (for two consecutive days). There were 16 sheep in Experiment 2 (8 per diet), 28 sheep in Experiment 3 (16 fed white clover and 12 fed ryegrass), and 8 sheep measured over two periods in Experiment 4 (4 per diet). Prior to each experiment, sheep had a 10-d acclimatisation period to respective diets. Apparent digestibility was measured over 7-d from animals in Experiments 2 and 4, along with the collection of rumen digesta samples for rumen pH, and ammonia (NH_3) and volatile fatty acid (VFA) concentrations.

Methane yield (g CH_4/kg DMI) was 12% lower for white clover (19.8) compared with ryegrass-fed sheep (22.5) in Experiment 2 (P=0.035), but in Experiment 3, CH_4 yield was 6% higher from white clover (25.2) compared with ryegrass-fed sheep (23.6), although this was not significant. There was no effect of diet on CH_4 yield from sheep in Experiment 4 (22.5 vs. 22.0 for white clover and ryegrass forages, respectively). Analysis of the combined data from all three experiments showed that there were no

dietary effects on emissions of CH_4. Analysis of the combined data from Experiments 2 and 4 showed that there was no effect of diet on apparent dry matter (DM) digestibility (723 g/kg), rumen pH (6.37), NH_3 (17.2 mM), total VFA concentrations (91.0 mM), or molar proportions of propionate or butyrate (0.20 and 0.11, respectively). The molar proportion of acetate was higher in sheep fed ryegrass (0.67) than white clover (0.64; P=0.002).

The use of white clover as an alternative fresh forage to ryegrass does not seem to provide an opportunity to reduce CH_4 emissions from sheep.

5.1 INTRODUCTION

New Zealand's agricultural system is pastoral-based, and animals graze mixed swards consisting predominately of ryegrass, with white clover being 10 to 20% of the herbage (Waghorn and Clark, 2004). Although ryegrass is the dominant species in New Zealand pastoral systems, white clover and other legume forages provide advantages of nitrogen (N) fixation and have a high feeding value, which can promote higher levels of ruminant production (Burke *et al.*, 2002, Waghorn *et al.*, 2007).

Environmental concerns over the use of N fertilisers are re-focusing research towards increased use of legumes in grazing pastures, and previous research (Waghorn *et al.*, 2002) has shown sheep fed white clover have much lower CH_4 yields (i.e. 12 to 16 g/kg DMI), compared to sheep fed ryegrass (20.9 g/kg DMI). These data, as well as those used for the New Zealand Greenhouse Gas Inventory (Ministry for the Environment, 2010), were derived using the SF_6 tracer technique described by Ulyatt *et al.* (1999) and Pinares-Patiño and Clark (2008).

It has been difficult to explain the low CH_4 yields from legumes (McCaughey *et al.*, 1999, Waghorn *et al.*, 2002), because they usually have a higher digestibility than grasses (Ulyatt and Egan, 1979), which leads to a higher production of H_2 and consequently CH_4. However, high digestibility enables high feed intakes, which can contribute to a low CH_4 yield. A decline of ~3.0 g CH_4/kg DMI for each increase in feed intake as a multiple of metabolisable energy (ME) requirements for maintenance (x

ME_m) has been shown in sheep fed ryegrass forages (Chapter 4) and cattle fed grain/silage diets (Yan *et al.*, 2010).

This Chapter summarises three experiments for the effect of feeding sheep white clover or ryegrass forages on CH_4 emissions measured in respiration chambers, to confirm previous comparisons using the SF_6 tracer technique. It was hypothesised that sheep fed white clover would have lower CH_4 emissions compared with sheep fed ryegrass at similar feed intakes.

5.2 MATERIALS AND METHODS

Experiment 2 (May to June 2009); Experiment 3 (October to November 2009); and Experiment 4 (November to December 2009) were used in this study (Table 5.1). Principal measurements for all three experiments were DMI and emissions of CH_4, hydrogen (H_2) and carbon dioxide (CO_2) from sheep in respiration chambers fed white clover or ryegrass forages (Table 5.1). Experiments 2 and 4 had additional measurements of apparent digestibility and collection of rumen digesta samples from fistulated sheep for pH, NH_3, and VFA concentrations. Experiment 4 included measurements of rumen solid and liquid fractional outflow rates (FOR) with and without water-filled balloons in the rumen. Outflow and rumen water balloon results were not the objective of this chapter and are discussed in Chapter 7.

In each experiment, sheep had a minimum 10-d period of acclimatisation to feeds and indoor feeding before entering respiration chambers where CH_4 emissions were determined for two consecutive days (Table 5.2). For Experiments 2 and 4, *in vivo* digestibility was measured over 7 days prior to animals entering respiration chambers.

A schedule of events for Experiments 2, 3 and 4 is given in Table 5.2. All procedures were reviewed and approved by the AgResearch Palmerston North Animal Ethics Committee.

TABLE 5.1 Overview of experiments used to compare the effects of white clover (WC) or perennial ryegrass (RG) diets on methane emissions from sheep.

	Experiment 2	**Experiment 3**	**Experiment 4**
Date	May to June 2009	Oct to Nov 2009	Nov to Dec 2009
Number of periods	1	1	2
Number of animals	16 (8 with rumen fistulae)	28 (all intact)	8 (all with rumen fistulae)
Diet	WC or RG	WC or RG	WC or RG
Feed offered (x ME_m)	1.6	0.8 & 2.0	1.6
Treatment	Diet	Diet and feed intake	Diet ± water balloon[a]
Animals/treatment	8 WC & 8 RG	16 WC & 12 RG	4 WC & 4 RG
Feeding regime	Twice daily	Twice daily	Hourly
Measurements	DMI and gas emissions (CH_4, H_2, CO_2) measured in respiration chambers		
Other	Digestibility Rumen samples		Digestibility Rumen samples Digesta kinetics

ME_m, metabolisable energy requirements for maintenance; DMI, dry matter intake; CH_4, methane; H_2, hydrogen; CO_2, carbon dioxide
[a]Sheep were with or without a 1 L water-filled balloon in the rumen

5.2.1 Animals and diets

Wether sheep aged 1 to 2 years were used in all experiments and were fed white clover or ryegrass forages over a range of intakes. The effect of feed intake on CH_4 emissions is discussed in Chapter 6. The white clover (*cv*. Kopu II) and ryegrass (*cv.* Quartet) were grown near Palmerston North and harvested daily using a sickle bar mower. Forages were delivered by 14:00 h, weighed into meal allocations, and stored at 4ºC prior to feeding. For more details of the forages see Chapter 3.3.

For Experiments 2 and 3, forages were provided as equal sized meals twice daily (09:00 and 16:00 h), and for Experiment 4 total daily feed allowance was divided into several portions and fed intermittently every 1 h over a 24 h period. Water was available *ad libitum*.

Experiment 2 used 16 wethers with an average live weight (LW) ± SD of 45.3 ± 1.71 kg. Eight had been previously rumen-fistulated and fitted with a 30 mm (o.d.) rumen cannulae (Beruc Equipment Ltd, Benoni, South Africa). Sheep were randomly allocated into two groups of 8, with 4 rumen-fistulated sheep per group, and fed white clover or ryegrass forages at 1.6 x ME_m (Table 5.2).

Experiment 3 used 28 intact sheep with 16 (45.7 ± 1.20 kg LW) fed white clover and 12 (48.0 ± 1.20 kg LW) fed ryegrass. Within each diet animals were split into two feeding treatments of 0.8 or 2.0 x ME_m (Table 5.2).

Experiment 4 used the same 8 rumen fistulated sheep from Experiment 2. Four sheep (50.6 ± 4.40 kg LW) were maintained on a white clover diet over two periods (giving a total of 8 measurements for white clover), and 4 sheep (51.3 ± 6.50 kg LW) were maintained on a ryegrass diet over two periods (giving a total of 8 measurements for ryegrass). Experiment 4 animals were subject to the treatment of a water balloon in the rumen (Balloon) or without (Control) which was swapped over between Periods 1 and 2. Sheep were fed at 1.6 x ME_m for both diets (Table 5.2).

5.2.2 Gas measurements

Emissions of CH_4, H_2 and CO_2 gas were measured over 48 h for each sheep using the eight chamber sheep respiration facility of AgResearch Grasslands Research Centre, described by Pinares-Patiño *et al.* (2008c). Sheep were placed in individual chambers before 09:00 h and the chambers were opened for feeding and collection of feed refusals (09:00 and 16:00 h) and cleaning (09:00 h). Details of the respiration chambers and calculation of CH_4 emissions are described in Chapter 3.6. Respiration chamber measurements were completed for all sheep over 4 d for Experiment 2, 8 d for Experiment 3, and 4 d for Experiment 4.

TABLE 5.2 Schedule of events for Experiments 2, 3 and 4.

Experiment 2 (11th May 2009 to 7th June 2009)	
Pre-trial	
Day – 1	16 sheep (8 rumen-fistulated) fed white clover (*n* = 8) or ryegrass (*n* = 8).
Adaptation	
1 – 7	Graze white clover or ryegrass in paddock.
8 – 14	Indoor pens and fed twice daily at 1.6 x ME_m.
15 – 17	Individual metabolism crates; fed twice daily with feed refusals collected prior to feeding.
In vivo digestibility	
18 – 24	Apparent digestibility measured. Fed twice daily with refusals collected prior to morning feeding. Rumen samples taken from fistulated sheep.
Gas measurements	
24 – 27	Individual respiration chambers for 48 h. Feed refusals collected prior to feeding.
Experiment 3 (27th October 2009 to 14th November 2009)	
Pre-trial	
- 1	28 intact sheep fed white clover (*n* = 16) or ryegrass (*n* = 12) and split into two groups of feed intake for each diet (0.8 or 2.0 x ME_m).
Adaptation	
1 – 7	Indoor pens and fed diets at 0.8 or 2.0 x ME_m.
8 – 10	Individual metabolism crates; fed twice daily with feed refusals collected prior to feeding.
Gas measurements	
11 – 18	Individual respiration chambers for 48 h. Feed refusals collected prior to feeding.
Experiment 4 (10th November to 22nd December 2009)	
Pre-trial	
- 1	8 rumen-fistulated sheep fed white clover (*n* = 4) or ryegrass (*n* = 4). Two animals within each diet with a 1 L water-filled balloon in the rumen.
Adaptation	
1 – 3	Indoor pens and fed diets twice daily at 1.6 x ME_m.
4 – 10	Individual metabolism crates and fed hourly.
In vivo digestibility and digesta kinetics	
11	Solid marker given via rumen cannulae at 22:00 h.
12	Liquid marker given via rumen cannulae at 10:00 h.
12 – 18	Apparent digestibility and digesta kinetics measured. Diets fed hourly with faecal and rumen sample collections for digestibility and also more frequently for marker measurement.
Gas measurements	
19 – 22	Individual respiration chambers for 48 h. Feed refusals collected prior to feeding.
Water balloon swapped over between treatment groups. Period 2 adaptation starting 2nd December 2009 with the same routine as above.	

ME_m, metabolisable energy requirements for maintenance

5.2.3 Sample collection, processing and laboratory analysis

Details for sample collection, processing and laboratory analyses of feed offered, refused, faeces and rumen fluid are given in Chapter 3 and a brief overview is presented here.

Each day of the acclimatisation, digestibility and respiration chamber periods, the forages on offer were sampled for DM determination by oven-drying 200 g triplicates at 105°C for 16 h. An additional 200 g sample (65°C for 48 h) was sub sampled for chemical composition determination by Near Infrared Reflectance Spectroscopy (NIRS; Chapter 3.9), and during the digestibility period, were pooled for each diet within each experiment, for chemical composition analysis by wet chemistry (Chapter 3.9).

Forage refusals from each sheep were collected and weighed once daily for DM determination during the digestibility period in Experiment 2 and twice daily during the respiration chamber period (Experiments 2, 3 and 4). Composite samples of the refusals were prepared for individual sheep of Experiment 2 during the digestibility period, and sheep of Experiments 3 and 4 during the respiration chamber period, for chemical composition analysis by wet chemistry (Chapter 3.9).

For Experiments 2 and 4, faeces were collected and weighed from each sheep during the 7-d digestibility period. In Experiment 2, total faecal collection was by sheep wearing harnesses with bags attached, and collection occurred every morning of the digestibility period before feeding. For Experiment 4, sheep also wore harnesses with faecal bags, and more frequent faecal sampling occurred when markers were used to determine digesta kinetics. Details of this sampling are given in Chapter 7. For all experiments, a 10% aliquot was taken from each faecal sample, frozen at -20°C, and pooled over the 7-d collection period for each animal. At the end of each experiment the faeces were thawed, mixed, a 120 g sub sample taken and oven-dried (65°C for 48 h) for wet chemistry analysis (Chapter 3.9) and determination of faecal DM percentage, enabling apparent digestibility to be calculated.

Rumen fluid was collected from fistulated sheep (Chapter 3.7) during the *in vivo* digestibility period in Experiments 2 and 4, for measurement of pH, NH_3 and VFAs. In Experiment 2, approximately 10 rumen samples were collected from each sheep over

two days, with two samples collected prior to morning feeding (-2 and -1 h), and thereafter up until 10 h after feeding (0, 1, 2, 3, 4, 6, 8 and 10 h). For Experiment 4, approximately 14 rumen samples were collected per animal over 2 d at 1-2 h intervals, relative to marker dosing at 09:00 h (-1, 0.5, 1, 1.5, 2, 3, 4, 5, 6, 7, 8, 10, 12, 22 h) to determine marker concentrations for the calculation of rumen FOR, described in Chapter 7, as well as for VFA and NH_3 analyses.

Rumen pH was determined immediately after sampling using a MeterLab® (PHM210, Radiometer Pacific Limited, Copenhagen) and a sample of rumen fluid was frozen for later analysis. Samples were thawed and centrifuged at 21,000 g for 10 min, and a 900 µl aliquot of the supernatant was obtained for VFA analysis by gas chromatography (GC) (Chapter 3.9) and for NH_3 analysis by a colorimetric procedure (Chapter 3.9).

5.2.4 Statistical analyses

Data were statistically analysed for each of the three experiments separately (Appendices 5.1 and 5.2) then all data were combined for analysis (Tables 5.4 and 5.5).

In Experiment 2, the effects of diet (i.e. white clover or ryegrass) and fistulation (i.e. fistulated or intact animals) were compared in relation to the following:

- intake: DM intake, DMI; organic matter intake, OMI; neutral detergent fibre intake, NDFI;
- digestibility: digestible DM, DDM; digestible OM, DOM; digestible NDF, DNDF;
- digestible intake: DM, DDMI; OM, DOMI; and NDF, DNDFI;
- emissions of CH_4: g CH_4/d; g CH_4/kg DMI; g CH_4/kg OMI; g CH_4/kg DDMI; g CH_4/kg DOMI; and CH_4 energy in relation to gross energy intake, CH_4-E/GEI;
- emissions of H_2: g H_2/d; g H_2/kg DMI;
- emissions of CO_2: g CO_2/d;
- rumen parameters: pH; NH_3; total VFAs; and individual VFA molar proportions.

Treatment effects were determined by ANOVA (Payne *et al.*, 2010) and the fixed model was expressed as:

Variable = Diet + Fistulation + Diet x Fistulation

where: Variable, is the variable of interest (i.e. intake, digestibility, digestible intake, gas emissions and rumen parameters); Diet, is white clover or ryegrass; and Fistulation, is fistulated or intact sheep.

A similar analysis was applied to Experiment 3, but the model did not include the effect of fistulation since all animals in this experiment were intact. Digestibility, digestible intake and rumen parameters were not measured in this experiment.

In Experiment 4, the effect of diet (white clover or ryegrass), period (1 or 2) and treatment (Balloon or Control) and their interaction on intake, digestibility, digestible intake, gas emissions, and rumen parameters were determined by ANOVA. The blocked effects were animal and period within animal. The treatment was compared between periods within sheep as each sheep was measured with a Balloon and without (Control). The fixed model can be expressed as:

Variable = Diet + Treatment + Diet x Treatment

and the random (blocking structure) model as:

Variable = Animal + Animal x Period

where: Variable, is the variable of interest (i.e. intake, digestibility, digestible intake, gas emissions and rumen parameters); Diet, is white clover or ryegrass; Treatment, is Balloon or Control; Animal, is the animal ID (i.e. ear tag number); and Period, is 1 or 2.

Results of the individual experimental analyses are presented in Appendices 5.1 and 5.2, as means ± standard errors of the difference of the mean (SED) and p-values. Data were derived from individual animals and means presented within each table will not always appear compatible.

Data from all three experiments were combined and subject to a mixed model analysis to determine the overall effect of diet on variables of intake, digestibility, digestible intake, gas emissions, and rumen parameters. The similarity of dietary treatments and experimental structure enabled a combined analysis of data from all three experiments

using the Restricted Maximum Likelihood (REML) method in GenStat software (Payne *et al.*, 2010). The fixed model may be expressed as:

Variable = C + Diet

and the random model as:

Variable = Expt. + Expt. x Diet

where: C, is a constant; Variable, is the variable of interest (i.e. intake, digestibility, digestible intake, gas emissions, and rumen parameters); Diet, is white clover or ryegrass; and Expt., is Experiments 2, 3, or 4.

Where certain variables were not measured in Experiment 3 (i.e. digestibility, digestible intake and rumen parameters), only Experiment 2 and 4 were included in the REML analysis. The random model included experiment effects and interactions between experiment and treatment terms (i.e. Expt. x Diet). Thus, in effect, each treatment term was compared against its interaction within the experiment. In this model, a significant treatment effect implies that the effect was consistent and large compared with its variation across experiments.

Both the individual experimental analysis by ANOVA and the REML analysis used data from Experiment 2 that were adjusted to remove the overall effect of fistulation (i.e. intact vs. rumen fistulated animals). No adjustments were made to data from Experiment 3 (animals all intact). For Experiment 4, data were adjusted to remove the overall effect of Period (i.e. 1 vs. 2) and Treatment (i.e. Balloon vs. Control). Data adjustment for variability was done for the two experiments by performing a one- and two-way ANOVA, respectively; incorporating only the term whose effect was to be removed (i.e. fistulation for Experiment 2, and period and treatment for Experiment 4). Residuals were obtained for each of the variables (e.g. intake, digestibility, digestible intakes, gas emissions, and rumen parameters) and added to a grand mean to give an adjusted mean value for each variable.

By using REML to do a combined analysis of several related experiments, the 'best' estimate (based on data from all 3 experiments) of diet (white clover vs. ryegrass) on the variables are obtained. The REML model takes into account the effect of the

experiment and produces one overall test. The different experiments are likely to have different levels of variability and these are estimated in the separate residual terms for each experiment. This gives an overall diet effect which is largely based on the consistency of the diet across all experiments. The results of the REML analysis are expressed in Tables 5.4 and 5.5 as means ± SED and p-values.

5.3 RESULTS

5.3.1 Chemical composition of white clover and perennial ryegrass

Dry matter values (g/kg wet matter) were similar during digestibility periods (Table 5.3), averaging 162 for white clover and 173 for ryegrass over the three experiments. The main difference between white clover and ryegrass forages fed during the digestibility period was the lower concentration of NDF (g/kg DM) in white clover, averaging 287 compared to 449 in the ryegrass. The crude protein (CP) concentration (g/kg DM) was higher in white clover (235) compared to ryegrass (147). The higher concentration of readily fermentable carbohydrate (RFC; hot water soluble carbohydrate (HWSC) + pectin; g/kg DM) in white clover (179) compared to ryegrass (144) resulted in a two-fold difference between forages in the RFC:NDF ratio.

The differences in diet chemical composition between experiments (Table 5.3) were the lower concentration of NDF in forages fed during Experiment 3 (hence the higher RFC:NDF ratio), lower concentration of CP in forages fed during Experiment 4 (222 and 128 g/kg DM for white clover and ryegrass, respectively), and the higher ash content (140 g/kg DM) in the ryegrass harvested in Experiment 2, which was possibly due to soil contamination as it was a winter harvest.

TABLE 5.3 Chemical composition (g/kg DM[b]), determined by wet chemistry, of white clover and perennial ryegrass forages offered to sheep in Experiments 2 and 4 during the digestibility period, and whilst in respiration chambers for Experiment 3.

Chemical composition (g/kg DM)	Experiment 2		Experiment 3		Experiment 4	
	White clover[a]	Perennial ryegrass[a]	White clover[a]	Perennial ryegrass[a]	White clover[a]	Perennial ryegrass[a]
Gross energy (MJ/kg DM)	19.0	17.6	19.0	18.0	18.9	17.8
Dry matter (g/kg wet matter)	163	173	162	163	160	184
Organic matter	901	860	908	901	903	898
Crude protein	262	196	222	128	220	117
Lipid	24.7	32.0	23.1	28.8	23.4	27.1
HWSC	102	117	129	168	106	126
Pectin	67.6	7.50	67.8	7.10	62.8	7.30
RFC	170	125	197	175	169	133
NDF	286	455	280	425	296	466
ADF	190	235	190	226	203	237
Cellulose	101	197	101	207	134	219
Lignin	88.6	38.1	89.5	18.9	68.6	18.1
RFC:NDF	0.59	0.27	0.70	0.41	0.57	0.29

DM, dry matter; HWSC, hot water soluble carbohydrate; RFC; readily fermentable carbohydrate (pectin + HWSC); NDF, neutral detergent fibre; ADF, acid detergent fibre

[a]*n* = 1, with 6 samples per forage pooled to make one sample for analysis

[b]units unless stated otherwise

5.3.2 Diet, intake, and gas emissions

In Experiment 2, CH_4 production (g/d) was lower (P<0.001) for sheep fed white clover, compared to sheep fed ryegrass (Appendix 5.1). When adjusted for intake, CH_4 yield (g/kg DMI) and CH_4 expressed in terms of OMI (g/kg OMI) was about 12% (P=0.035) and 15% (P=0.002) lower, respectively, for sheep fed white clover compared to those fed ryegrass. The CH_4 energy (CH_4-E) relative to GEI (CH_4-E/GEI) was also lower (P=0.003) for white clover (0.059) compared to ryegrass (0.067). Hydrogen emissions were higher (P=0.012) and CO_2 (g CO_2/d) was lower (P=0.003) from white clover-fed sheep compared with those fed ryegrass.

In Experiment 3 there were no differences in CH_4 production or CH_4-E/GEI between sheep fed white clover or ryegrass (Appendix 5.1). The DMI was similar for both forages, however NDFI was lower for white clover compared to ryegrass. When adjusted for intake, CH_4 yield (g/kg DMI) and CH_4 expressed in terms of OMI (g/kg OMI) were about 6% and 5% higher, respectively, for sheep fed white clover compared to those fed ryegrass, but these were not significant. Sheep fed white clover also had greater H_2 and CO_2 emissions compared to ryegrass diets.

Results from Experiment 4 showed similar trends to those in Experiment 3, and there were no differences between forages in CH_4 production (g/d), yield (g/kg DMI) or CH_4-E/GEI (Appendix 5.1). Intakes of DM and OM were also similar, but NDFI was lower (P<0.001) for white clover compared to ryegrass-fed sheep. Again, H_2 and CO_2 emissions were greater for white clover compared with ryegrass-fed sheep.

A combined analysis of the data from all three experiments showed that overall there was no effect of diet on CH_4 emissions, averaging 23.0 g/d, 22.6 g/kg DMI, and 27.8 g/kg OMI for both diets (Table 5.4). There was no interaction of diet with DMI or OMI, which averaged 0.94 and 0.84 kg/d, respectively for both diets. Although there were large differences between diets in their chemical composition (296 g NDF/kg DM and 235 g CP/kg DM for white clover compared to 466 g NDF/kg DM and 147 g CP/kg DM for ryegrass-fed sheep), this had no net effect on CH_4 emissions. However, there were differences in both H_2 production (P=0.019) and yield (P=0.022), with values highest for sheep fed white clover (0.08 g/d and 0.09 g/kg DMI) compared to sheep fed ryegrass (0.03 g/d and 0.03 g/kg DMI). There was no effect of diet on CO_2 production.

5.3.3 *In vivo* digestibility

Digestibility of white clover and ryegrass were similar in Experiments 2 and 4, with an average DMD of 728 and 722 g/kg for white clover and ryegrass, respectively (Table 5.4). Organic matter digestibility followed the same pattern, and values were similar with each experiment. Although not significant, the NDF was less digestible from white clover than ryegrass (550 vs. 662 g/kg DM), and the lower NDF concentration in white clover DM resulted in a two-fold difference ($P<0.001$) in intakes of DNDF between the two diets (Table 5.4).

TABLE 5.4 Intakes, digestibility and gas emissions from sheep fed either white clover or perennial ryegrass forages. Based on data[b] combined from Experiments 2, 3 and 4 (see Appendix 5.1 for individual experimental results).

Parameter	Combined Experiments[a]		P-value	SED
	White clover	Perennial ryegrass		
No. of animals	32	28		
Intake (kg/d)				
DMI	0.90	0.97	0.279	0.046
OMI	0.82	0.86	0.317	0.026
NDFI	0.25	0.42	<0.001	0.003
Digestibility (g/kg)				
DDM	728	722	0.485	8.39
DOM	782	758	0.344	19.00
DNDF	550	662	0.075	23.16
Digestible intake (kg/d)				
DDMI	0.67	0.71	0.318	0.034
DOMI	0.65	0.65	0.835	0.033
DNDFI	0.14	0.29	<0.001	0.005
Methane emissions				
g CH_4/d	22.5	23.4	0.648	1.907
g CH_4/kg DMI	22.6	22.5	0.925	0.968
g CH_4/kg OMI	27.9	27.6	0.877	1.57
CH_4-E/GEI	0.077	0.077	0.938	0.003
Hydrogen emissions				
g H_2/d	0.08	0.03	0.019	0.008
g H_2/kg DMI	0.09	0.03	0.022	0.012
Carbon dioxide emissions				
g CO_2/d	988	991	0.941	37.96

DMI, dry matter intake; OMI, organic matter intake; NDFI, neutral detergent fibre intake; DDM, digestible dry matter; DOM, digestible organic matter; DNDF, digestible neutral detergent fibre; DDMI, digestible dry matter intake; DOMI, digestible organic matter intake; DNDFI, digestible neutral detergent fibre intake; CH_4, methane; CH_4-E/GEI, methane energy in relation to gross energy intake; H_2, hydrogen; CO_2, carbon dioxide; SED, standard error of differences of means

[a] Combined data from Experiments 2, 3 and 4

[b] Means have been derived from individual animals and values presented here may not appear compatible

5.3.4 Rumen pH, ammonia and volatile fatty acids

In Experiment 2, rumen pH was similar for both white clover and ryegrass forages at all sampling times (Figure 5.1) and there was no overall difference in rumen pH (Appendix 5.2). Concentrations of NH_3 were initially similar between diets, for 4 h after feeding, but between 4 and 10 h post-feeding white clover diets had higher NH_3 concentrations than ryegrass (Figure 5.2). When averaged for all time points (Appendix 5.2) there was no difference in NH_3 concentration with diet. Total VFA concentrations (mM) were higher for white clover (93.5) compared to ryegrass (84.4; P=0.006). Figure 5.3 shows the molar proportions of major VFAs (acetate, butyrate and propionate) for white clover and ryegrass forages at the various times after feeding for sheep in Experiment 2. There were no differences between forages in proportions of the major VFAs (Appendix 5.2), but concentrations of minor VFAs (isobutyrate and isovalerate) were higher for white clover compared to ryegrass-fed sheep.

In Experiment 4, there was no effect of diet on rumen pH, but NH_3 and total VFA concentrations were higher for sheep fed white clover (18.6 mM and 96.9 mM, respectively) compared to ryegrass (5.50 and 88.0 mM, respectively) (Appendix 5.2). The lower NH_3 concentration of ryegrass compared to white clover is likely to have been a result of the low CP concentration of the ryegrass that was offered in Experiment 4. Molar proportions of acetate were lower (P=0.009), and minor VFAs (valerate, isobutyrate and isovalerate) were higher for white clover compared to animals fed ryegrass.

Combined analysis of data from both Experiments 2 and 4 (Table 5.5) showed that there was no effect of diet on rumen pH, NH_3 and total VFA concentrations. Of the major VFAs, diet had a significant (P=0.002) effect on acetate (64.4 vs. 66.7% for white clover and ryegrass forages, respectively), with no effect on propionate or butyrate. Of the minor VFAs, diet had a significant effect on valerate and isobutyrate; with both being greater for white clover compared to ryegrass.

TABLE 5.5 Measurements of rumen pH, ammonia and volatile fatty acids (VFAs) from sheep fed either white clover or perennial ryegrass forages.

Parameter	Combined Experiments		P-value	SED
	White clover	Perennial ryegrass		
Number of animals	16	16		
Number of samples	200	200		
Rumen pH	6.38	6.35	0.829	0.093
Ammonia (mM)	22.3	12.1	0.081	2.992
Total VFA (mM)	95.5	86.5	0.178	5.223
% of total VFA:				
Acetate (A)	64.4	66.7	0.002	0.625
Propionate (P)	19.6	19.9	0.633	0.575
Butyrate (B)	11.6	11.1	0.197	0.396
Valerate	1.15	0.84	<0.001	0.051
Isobutyrate	1.33	0.80	0.050	0.114
Isovalerate	1.40	0.71	0.063	0.170
Ratios:				
A:P	3.32	3.38	0.623	0.121
A + B/P	3.92	3.94	0.922	0.141

SED, standard error of the difference

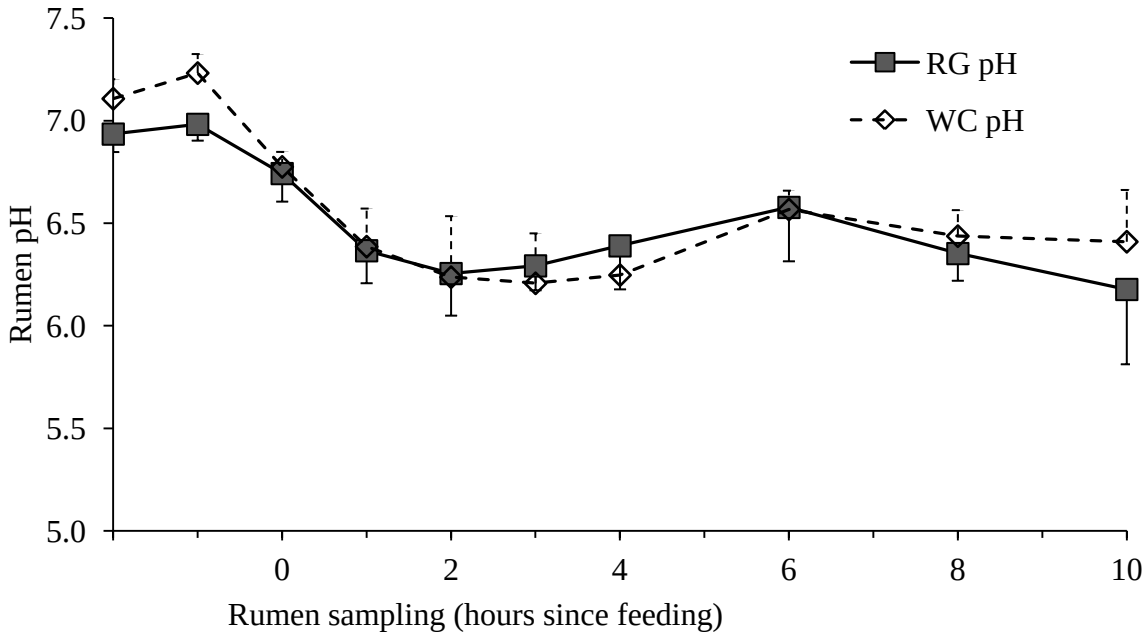


FIGURE 5.1 Rumen pH at each sampling time from pre-feeding until 10 h after feeding, for sheep fed white clover (WC; dashed line) (n = 4) or perennial ryegrass (RG; solid line) (n = 4) in Experiment 2.

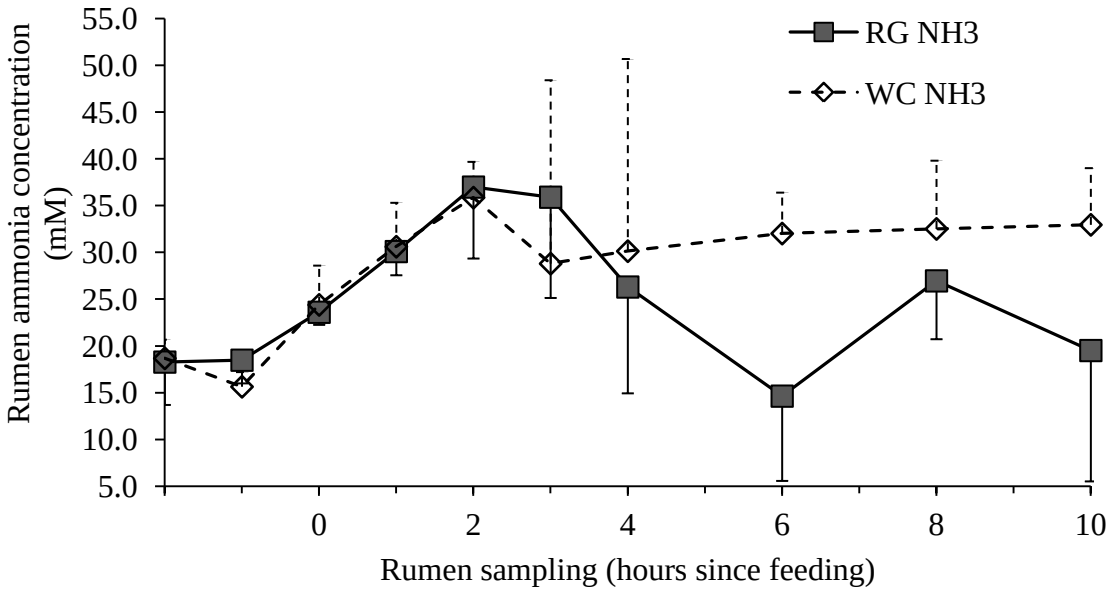


FIGURE 5.2 Rumen ammonia concentration at each sampling time from pre-feeding until 10 h after feeding, for sheep fed white clover (WC; dashed line) (n = 4) or perennial ryegrass (RG; solid line) (n = 4) in Experiment 2.

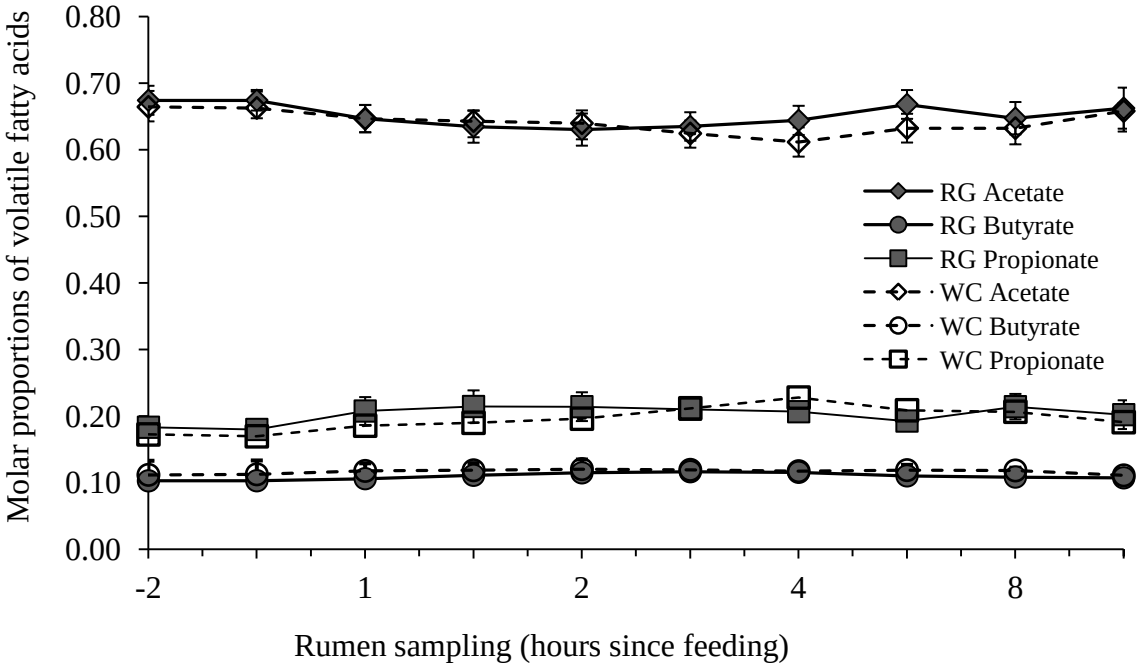


FIGURE 5.3 Molar proportions of volatile fatty acids; acetate, butyrate and propionate at each sampling time from pre-feeding until 10 h after feeding, for sheep fed white clover (WC, dashed line) (n = 4) or perennial ryegrass (RG, solid line) (n = 4) in Experiment 2.

5.4 DISCUSSION

The hypotheses that CH_4 yield from sheep fed white clover would be lower than from ryegrass was not proven and yields were similar from both diets.

5.4.1 Emissions of methane and hydrogen

The combined analysis of all three experiments showed no overall difference in CH_4 yield from sheep fed white clover or ryegrass forages, despite marked differences in chemical composition.

Average CH_4 yields of 22.5 g/kg DMI from sheep fed white clover in this study were similar to values from steers fed white clover measured in respiration chambers (21.0 to 24.5 g/kg DMI; Beever *et al*., 1985; Cammell *et al.*, 1986). Those authors also reported CH_4 yields of 20.8 to 25.7 g/kg DMI when ryegrass was fed to steers; values similar to those for cattle fed ryegrass pasture measured with the SF_6 technique (Waghorn and Woodward, 2006).

Despite similar CH_4 yields from sheep fed white clover and ryegrass forages, high growth rates of lambs fed white clover (Ulyatt, 1981) will reduce CH_4 emissions per animal product (emissions intensity, Ei) relative to ryegrass (Waghorn and Hegarty, 2011). A higher proportion of feed intake from legume diets when fed *ad libitum* is directed toward animal growth, with less to ME_m, so that animals reach slaughter LW more quickly compared to ryegrass diets. Across all three experiments there was a 2.5 fold difference in feed intakes. Although feed intake is an important aspect of CH_4 emissions from ruminants, the effect of different white clover and ryegrass intakes is the focus of Chapter 6 and will be discussed in detail there.

Fermentation of carbohydrates and protein in the rumen leads to the production of H_2; of which is largely utilised by methanogens to produce CH_4 (Benchaar *et al.*, 1998). According to the descriptive model of Janssen (2010), it would be expected that the high fibre (NDF) content of ryegrass forages would result in lower H_2 concentrations. Therefore, pathways favouring the formation of H_2 would be used by bacteria resulting in more acetate and butyrate, and less propionate production, with an associated

increase in CH_4 yield, compared to white clover. This study found sheep fed ryegrass had lower H_2 emissions (both g/d and g/kg DMI), compared with sheep fed white clover; however, there was no associated difference in CH_4.

5.4.2 Diet chemical composition

The overall lack of difference in CH_4 yield from sheep fed white clover and ryegrass forages was unexpected as the anticipated relationship between diet chemical composition and CH_4 emissions was based on results from previous studies. For example, when sheep were fed pasture, lucerne (*Medicago sativa*), chicory (*Cichorium intybus*) or red clover (*Trifolium pratense*) forages, Waghorn *et al.* (2002) reported respective CH_4 yields estimated using the SF_6 technique, of 25.7, 20.6, 16.2 and 17.7 g/kg DMI. However, when these values were adjusted for the reduction in CH_4 yield associated with intakes above maintenance (based on the reduction of 3.19 g CH_4/kg of intake above ME_m, Chapter 4; and assuming a ME requirement of 7 MJ ME/d; Australian Agricultural Council, 1990), values were 28.2, 24.6, 19.1 and 23.3 for the respective feeds at ME_m. Sun *et al.* (2011) reported CH_4 yields from sheep fed chicory forages at 1.3 x ME_m in respiration chambers to be 22.8 g/kg DMI, which is about 23.8 g/kg DMI at ME_m intakes. Muetzel *et al.* (2009) fed sheep pasture forages at 2.2 x ME_m in respiration chambers and reported CH_4 yields of 20.4 g/kg DMI, equivalent to about 24.2 g/kg DMI at ME_m intakes.

These data suggest the SF_6 technique underestimated CH_4 yields from sheep fed chicory, but not pasture, lucerne, or red clover forages. Results from Chapter 4 showed that mean CH_4 yields determined from sheep fed fresh ryegrass were not affected by measurement technique of SF_6 or respiration chambers.

Waghorn *et al.* (2002) also used the SF_6 technique to estimate CH_4 yields from sheep fed forages containing condensed tannins (CT; sulla, *Hedysarum coronarium*; and lotus major, *Lotus pedunculatus*). After correcting to intakes expressed at ME_m, CH_4 yields from two trials with sulla and one from lotus major were 21.8, 19.4 and 12.8 g/kg DMI above ME_m, respectively. Administration of polyethylene glycol (PEG) to sheep fed lotus major to remove the effects of CT increased CH_4 yield by 16%. Both Hess *et al.*

(2006) and Grainger *et al*. (2009) have shown that added CT, obtained from the bark of *Acacia mearnsii*, to ryegrass, red clover and lucerne forages fed to sheep, and a ryegrass/barley grain diet fed to cows, reduced CH_4 yield (measured in respiration chambers) by 10 to 20%. Hess *et al.* (2006) also showed a reduction ($P<0.01$) in CH_4 production relative to OM digested, and suggested CT as a possible avenue for CH_4 mitigation.

It is well established that feeding lambs white clover forages will result in high growth rates (Burke *et al.*, 2002, Waghorn *et al.*, 2007), which is thought to be due in part to the nutrients derived from digestion, its lower NDF content, and higher voluntary feed intake (VFI), compared to grasses (Ulyatt, 1969, Moseley and Jones, 1984, Beever *et al.*, 1986). The low NDF concentration in white clover may lead to rapid ruminal degradation and passage rates, which should lower CH_4 emissions (Okine *et al.*, 1989, Pinares-Patiño *et al.*, 2003a). Therefore, the higher nutritive value of white clover, compared to ryegrass, had theoretically provided an opportunity for reducing CH_4 emissions and lowering Ei from ruminants.

However, the similar CH_4 yields from sheep consuming either white clover or ryegrass forages in the combined results suggests that there are no simple relationships between chemical components of fresh forages and CH_4 yield, as ryegrass contained 50% more NDF and 80% less pectin in the DM than white clover. Molano and Clark (2008) also reported similar CH_4 yields (22.9 and 23.7 g/kg DMI) from sheep fed fresh immature and mature ryegrass containing 246 and 469 g NDF/kg DM, respectively, and Pinares-Patiño *et al.* (2003a) showed CH_4 emissions from steers grazing Timothy (*Phleum pratense*) at four stages of maturity accounted for 6.4% GEI, despite large variations in forage composition (i.e. 546 to 754 g NDF/kg DM). When CH_4 measurements from sheep fed ryegrass forages with widely differing composition (i.e. 431 to 626 g NDF/kg DM) were measured in respiration chambers, only 20% of the variation in CH_4 yield could be predicted by chemical composition (Chapter 4). An evaluation of experiments involving cattle fed a variety of fresh forages (i.e. 357 to 551 g NDF/kg DM) also failed to demonstrate relationships between dietary composition and CH_4 yield (Waghorn and Woodward, 2006).

The physical structure of the forage, rather than its chemical composition *per se*, is likely to have greater influence on CH_4 emissions. White clover has been well

documented to be associated with more rapid rumen degradation (Ulyatt, 1971, MacRae and Ulyatt, 1974), and less affected by maturation, compared to ryegrass (Burke, 2004). Burke *et al.* (2000) reported that degradation kinetics for white clover were faster (DM FOR of 21.1%/h), and had a shorter lag time (0.9 h), compared to ryegrasses (FOR of 10.6%/h and a lag time of 4.6 h). This can result in more substrate and nutrients available for microbial colonisation with white clover, and a greater proportion of small particles in the rumen to provide a larger surface area available for microbial attachment and colonisation (Hungate, 1966, Akin, 1982). If white clover is rapidly degraded in the rumen, this would mean that H_2 concentrations would be high, as fermentation would be rapid, and so pathways favouring a decrease in H_2 (and formation of propionate) would occur, ultimately resulting in a decrease in CH_4 per unit of feed fermented (Janssen, 2010). Although a difference with CH_4 yield between white clover and ryegrass forages was not reported here, results showed that, compared to sheep fed ryegrass, those fed white clover had greater fermentation rates (total VFA concentrations of 95.5 vs. 86.5 mM for white clover and ryegrass, respectively) and more H_2 in the breath (0.08 vs. 0.03 g H_2/d, respectively).

The different physical structure between legumes and grasses is thought to be responsible for faster rates of particle breakdown and passage out of the rumen, rapid degradation, and/or a faster conversion of particulate to soluble material in the rumen for legumes compared to grasses (Ulyatt, 1969, Moseley, 1981, Moseley and Jones, 1984, Beever and Thorp, 1996). Furthermore, due to the higher NDF content in the ryegrass DM, time spent ruminating has been shown to be longer for sheep fed ryegrass (33 min/100 g) compared to white clover (13 min/100 g) (Moseley and Dellow, 1985), and as a consequence, saliva production is likely to be greater for ryegrass diets. The results of this study support a more rapid fermentation of white clover compared to ryegrass (elevated VFA concentrations), but the similar rumen pH suggests that saliva production was greater for ryegrass forages, and this may have been a possible explanation for lower VFA concentrations. The overall differences between white clover and ryegrass forages appear to be too small to cause differences in CH_4 yield.

5.4.3 Digestibility and rumen parameters

The lack of association between CH_4 yield and chemical composition of fresh forages in this study and reports of Pinares-Patiño *et al.* (2003a), Waghorn and Woodward (2006), Molano and Clark (2008), Sun *et al.* (2011), and Chapter 4 emphasise the balance between digestive parameters contributing to methanogenesis. Methane emissions are closely related to the amount of DOM, since about 55 to 65% of OM digestion occurs in the rumen (Moss *et al.*, 2000, Waghorn *et al.*, 2007). However, there were no consistent differences in apparent digestibility of white clover or ryegrass forages in this study. An analysis of 118 studies by Johnson and Johnson (1995) showed that an increasing digestibility of feed was associated with a higher variation in energy loss to CH_4, but there was no relationship between the digestibility and CH_4 (expressed as CH_4-E/GEI, %). They suggested feed intake, particle size, and the presence of alternative H_2 acceptors in the rumen, were likely to have important effects on methanogenesis. Hence, the similar digestibilities of white clover and ryegrass forages in this study minimised the source of variation in CH_4, and previous analyses (Johnson and Johnson, 1995) also suggested digestibility to have minor effects on CH_4 yield.

The chemical constituents of the diet fed, especially the type of carbohydrate, can affect CH_4 production as they are able to influence ruminal pH and subsequently alter the microbiota (Johnson and Johnson, 1995). Janssen (2010) has summarised previous work that indicated that fermentation of large quantities of RFC (i.e. white clover) result in a decrease in ruminal pH, which was positively correlated with increased solid passage rates, less CH_4 formation, a greater proportion of propionate as an end product, and higher H_2 concentrations. However, in this study there were no differences in rumen pH between white clover and ryegrass forages, and this in combination with adequate adaptation to the diets, meant that the rumen environment maintained normal physiological function at a pH of around 5.8 to 6.5 for both forages (Waghorn *et al.*, 2007).

Although there was a difference in total VFA concentrations, this was not significant, and there were no differences in molar proportions of propionate and butyrate between white clover and ryegrass forages. This was unexpected as legumes tend to result in higher molar proportions of propionate in the rumen, relative to grasses (Burke, 2004, Dewhurst *et al.*, 2009). The percentage of acetate was slightly higher for ryegrass

compared to white clover forages. In addition, the low RFC:NDF ratio reported in this study for ryegrass compared to white clover should have increased the A:P ratio, which is also expected to lower CH_4 emissions (Janssen, 2010). However, in these experiments the difference in the RFC:NDF ratio for the two forages had no effect on VFA ratios or CH_4 yields.

5.5 CONCLUSION

Methane yield (g/kg DMI) measured from sheep in respiration chambers was similar for sheep when fed forages of white clover (22.6) or ryegrass (22.5). Both white clover and ryegrass forages had a similar apparent DM digestibility (72%) and similar rumen pH (6.37), NH_3 and VFA concentrations (17.2 and 91.0 mM, respectively).

Despite a marked difference in chemical composition between white clover and ryegrass forages, the CH_4 yield was similar, suggesting no simple relationship between the two variables. Chapter 4 investigated variation in CH_4 emissions from sheep fed ryegrass forage widely differing in chemical composition and found composition predicted less than 20% of the variation in CH_4 yield.

There is evidence that rapid fermentation favours high H_2 concentrations and this would alter pathways to form propionate and decrease CH_4 yield. The increased VFA concentration and higher H_2 in emissions of sheep fed white clover, compared to ryegrass found here, supported this theory. Despite these aspects of white clover and its digestion that show there should be lower CH_4 emissions, compared to ryegrass, a difference was not observed between the two forages. Rapid particle breakdown characteristic of white clover is thought to be associated with increased substrate availability and surface area for microbial fermentation, and increased passage rates from the rumen. However, these parameters were not measured in this study. Therefore, it is unknown if the absence of a difference in CH_4 yield between white clover and ryegrass is a consequence of counteracting processes of digestion or of insufficient differences between forages.

Feeding white clover does not present an opportunity to reduce CH_4 yields from sheep, but high feed intakes and rapid LW gain will lower CH_4 emissions per animal product

from sheep fed white clover relative to ryegrass. It is suggested that factors affecting rumen function and components of digestion, such as feed intake, plant structure characteristics, digesta volume, passage rate and particle breakdown, have an influence on methanogenesis and warrant further investigation.

APPENDIX 5

APPENIDIX 5.1 Intakes, digestibility and gas emissions[b] from sheep fed fresh white clover or perennial ryegrass in Experiments 2, 3 and 4.

Parameter	Experiment 2		P-value	SED	Experiment 3		P-value	SED	Experiment 4		P-value	SED
	White clover	Perennial ryegrass			White clover	Perennial ryegrass			White clover	Perennial ryegrass		
No. of animals	8	8			16	12			8	8		
Intake (kg/d)												
DMI	0.94	1.12	<0.001	0.032	0.81	0.83	0.887	0.141	0.91	0.93	0.026	0.006
OMI	0.85	0.96	0.022	0.042	0.74	0.75	0.931	0.128	0.82	0.84	0.008	0.005
NDFI	0.27	0.47	<0.001	0.024	0.22	0.35	0.014	0.048	0.25	0.43	<0.001	0.002
Digestibility (g/kg)												
DDM	727	714	0.253	10.85	N/A	N/A			725	727	0.915	15.40
DOM	755	764	0.530	14.44					795	751	0.011	12.18
DNDF	570	641	0.021	26.80					532	674	<0.001	20.80
Digestible intake (kg/d)												
DDMI	0.69	0.80	<0.001	0.025	N/A	N/A			0.66	0.67	0.425	0.016
DOMI	0.64	0.74	0.033	0.040					0.65	0.63	0.092	0.012
DNDFI	0.15	0.30	<0.001	0.022					0.13	0.29	<0.001	0.006
Methane emissions												
g CH_4/d	18.4	24.5	<0.001	1.090	19.8	19.0	0.784	2.880	25.7	24.5	0.338	1.070
g CH_4/kg DMI	19.8	22.5	0.035	1.123	25.2	23.6	0.140	1.034	22.5	22.0	0.563	0.857
g CH_4/kg OMI	21.5	25.5	0.002	1.027	27.6	26.2	0.203	1.143	31.3	29.3	0.149	1.199
g CH_4/kg DDMI	26.8	30.8	0.013	1.355	N/A	N/A		N/A	39.0	36.5	0.045	1.005
g CH_4/kg DOMI	28.6	33.4	0.012	1.633	N/A	N/A		N/A	39.4	39.0	0.765	1.069
CH_4-E/GEI	0.059	0.067	0.013	0.003	0.074	0.070	0.202	0.003	0.087	0.085	0.548	0.003
Hydrogen emissions												
g H_2/d	0.10	0.03	<0.001	0.012	0.06	0.02	<0.001	0.012	0.12	0.05	0.026	0.020
g H_2/kg DMI	0.10	0.03	<0.001	0.013	0.08	0.02	<0.001	0.013	0.09	0.04	0.026	0.018
Carbon dioxide emissions												
g CO_2/d	901	996	0.003	25.30	876	859	0.863	94.3	1077	1037	0.032	14.30

DMI, dry matter intake; OMI, organic matter intake; NDFI, neutral detergent fibre intake; DDM, digestible dry matter; DOM, digestible organic matter; DNDF, digestible neutral detergent fibre; DDMI, digestible dry matter intake; DOMI, digestible organic matter intake; DNDFI, digestible neutral detergent fibre intake, CH_4, methane; H_2, hydrogen; CO_2, carbon dioxide; CH_4-E/GEI, methane energy in relation to gross energy intake; SED, standard error of differences of means; N/A, data not available

[b] Data have been derived from individual animals and values presented here may not appear compatible

APPENDIX 5.2 Measurements of rumen pH, ammonia and volatile fatty acids (VFAs) from rumen-fistulated sheep fed either fresh white clover or perennial ryegrass in Experiments 2 and 4. Approximately 10 samples were analysed from each sheep in Experiment 2 and 14 samples from each sheep in Experiment 4.

Parameter	Experiment 2		P-value	SED	Experiment 4		P-value	SED
	White clover	Perennial ryegrass			White clover	Perennial ryegrass		
Number of animals	8	8			4	4		
Number of periods	1	1			2	2		
Total number of samples	80	80			112	112		
Rumen pH	6.54	6.50	0.391	0.054	6.31	6.23	0.439	0.095
Ammonia (mM)	29.7	25.6	0.100	2.130	18.6	5.50	<0.001	1.800
Total VFA (mM)	93.5	84.4	0.006	2.150	96.9	88.0	0.047	3.550
% of total VFA:								
Acetate (A)	63.9	64.9	0.288	0.879	64.7	67.3	0.009	0.682
Propionate (P)	19.9	20.4	0.414	0.609	19.4	19.4	0.987	0.863
Butyrate (B)	11.7	11.0	0.104	0.361	11.9	11.3	0.404	0.579
Valerate	1.20	1.09	0.337	0.109	1.13	0.81	0.001	0.057
Isobutyrate	1.49	1.21	0.018	0.087	1.25	0.60	<0.001	0.049
Isovalerate	1.57	1.24	0.025	0.110	1.32	0.46	<0.001	0.050
Ratios:								
A:P	3.22	3.20	0.901	0.139	3.37	3.49	0.533	0.175
A + B/P	3.81	3.74	0.662	0.146	3.99	4.08	0.713	0.218

VFAs, volatile fatty acids SED, standard error of differences of means

CHAPTER 6

EFFECT OF FEED INTAKE ON METHANE EMISSIONS FROM SHEEP FED WHITE CLOVER (*Trifolium repens*) AND PERENNIAL RYEGRASS (*Lolium perenne*) FORAGES

CHAPTER 6: EFFECT OF FEED INTAKE ON METHANE EMISSIONS FROM SHEEP FED WHITE CLOVER (*Trifolium repens*) AND PERENNIAL RYEGRASS (*Lolium perenne*) FORAGES

ABSTRACT

Published analyses of methane (CH_4) emissions from sheep and cattle show an inverse relationship between feed intake and CH_4 yield (g CH_4 per kg dry matter intake; g/kg DMI), and this relationship provides opportunities for reducing emissions from feed eaten and per unit of animal production. In fact, higher intakes reduce the proportion of feed energy associated with animal maintenance requirements. This is important because ruminants are usually fed to produce human edible food and farmers often need to maximise feed intakes for improved profitability. However, under New Zealand pastoral grazing systems feed allowance (and therefore intake) is often limited in order to maintain pasture quality. Thus, the effect of feed intake on CH_4 emissions needs to be determined at intakes above maintenance. Most relationships between feed intake and CH_4 yield have been developed from measurements with animals fed conserved feeds, especially silages and grains, and the work presented here is an evaluation with white clover (*Trifolium repens*) and perennial ryegrass (*Lolium perenne*; ryegrass) forages fed to sheep.

This study comprised four experiments where good quality, freshly harvested white clover and ryegrass were fed to sheep at a four-fold range in feed intake, and CH_4 emissions were measured in respiration chambers for two consecutive days from each sheep. Measurements were made from 16 sheep in Experiment 2 (fed at 1.6 x metabolisable energy requirements for maintenance; ME_m), 28 sheep in Experiment 3 (at 0.8 and 2.0 x ME_m), 8 sheep and two measurement periods in Experiment 4 (at 1.6 x ME_m), and 30 sheep in Experiment 5 were fed at 0.8, 1.2, 1.6, 2.0 and 2.5 x ME_m. Prior to each experiment, sheep had a 10-d acclimatisation period to either white clover or

ryegrass forages, and analyses were based on 32 two-day CH_4 measurements with white clover and 58 two-day CH_4 measurements with ryegrass. Apparent digestibility was measured over 7-d from animals in Experiments 2, 4 and 5, along with the collection of rumen digesta samples for volatile fatty acid (VFA) determination, as reported in Chapter 5. Rumen samples were obtained from sheep fed at 0.8 and 2.5 x ME_m in Experiment 5 for determination of methanogen composition by polymerase chain reaction-denaturing gradient gel electrophoresis (PCR-DGGE).

The lack of a difference between white clover and ryegrass forages on CH_4 emissions, and similar experimental structure, enabled a combined analysis of data from all four experiments using the Restricted Maximum Likelihood (REML) method, which gave an estimate of the effect of feed intake on digestibility, digestible nutrient intake, gas emissions, and rumen parameters. The REML analysis of combined data showed that when DMI increased from 0.40 to 1.60 kg/d, the predicted responses were an increase in CH_4 production (g/d) of 187% (12.4 to 35.6; $P<0.001$), and a yield (g/kg DMI) decline of 21% (from 25.6 to 20.2; $P<0.001$). Higher feed intakes were associated with predicted increases in molar percentages of propionate from 17.3 to 21.4% ($P=0.038$).

To better understand the effect of feed intake on CH_4 emissions, single and multiple regressions were performed on the data from all experiments. Organic matter (OM) intake predicted 87% of the variation in CH_4 production but there was no relationship with OM digestibility. Molar percentage of propionate predicted 60% of the variation in CH_4 yield. Increasing feed intakes by 1 kg/d of DM reduced CH_4 yield by 4.12 g/kg DMI. Plant composition was weakly related to CH_4 yield.

High intakes of fresh forages will lower CH_4 yield from fermentation, and especially lower CH_4 emissions per unit of animal product. Effects of feed composition on CH_4 emissions were minor and the interaction between effects of feed intake on rumen function require further investigation to understand the relationship with CH_4 emissions.

6.1 INTRODUCTION

Ruminants are farmed in New Zealand for food production; mainly milk and meat. Animal production is maximised when high quality feed is available *ad libitum* to

animals with a high genetic merit for production. Under New Zealand pastoral grazing, the prime constraint to animal production is energy intake (Popova *et al.*, 2011) while the concentration of CP is usually in excess of requirements (Waghorn *et al.*, 2007). Intakes of forages are affected by feed availability and quality. Availability is often restricted in dairy farming because highly productive systems require pasture to be grazed to a low residual dry matter (DM) in order to maintain pasture quality (Waghorn and Clark, 2004). However, even under these circumstances feed intakes of cows are frequently three or more times their ME_m (Holmes *et al.*, 2002).

A similar situation is apparent for lambs, where rapid growth requires high feed intakes and high quality feeds (Kenyon and Webby, 2007). Ulyatt (1981), and more recently Burke *et al.* (2002), have ranked feeds for their capacity to achieve high lamb growth rates. The ranking was based on actual daily live weight (LW) gains achieved, which ranged from the national average of about 120 g/day (Hess *et al.*, 2006) to about 300 g/day with legumes such as sulla (*Hedysarum coronarium*) or white clover (Burke, 2004).

It is important to determine the effect of feed intakes on CH_4 emissions to obtain a true measure of inventory and to identify the benefits of increasing intakes for reducing CH_4 yield in productive animals. Future application of these measurements may include emissions associated with edible food production (Gill *et al.*, 2010). Waghorn and Hegarty (2011) summarised published data showing the emissions of CH_4 and nitrous oxide (N_2O) from lactating cows (per MJ of energy in milk expressed as carbon dioxide equivalent; CO_2-eq) was similar to that for pig and poultry meat production. However, accurate measurements of CH_4 yield in relation to feed intake will enable refinement and improve the accuracy of their predictions.

A number of studies have been undertaken to determine relationships between feed intake and CH_4 emissions. The first was Blaxter and Clapperton (1965) who showed reductions in CH_4 yield with increasing feed intakes for sheep and cattle. However, as with most studies, the work was based on dried feeds and silages. There is a good agreement that increasing feed intake reduces CH_4 yield (Blaxter and Clapperton, 1965, Johnson *et al.*, 1993, Pinares-Patiño *et al.*, 2003a, Pinares-Patiño *et al.*, 2003c, Hart *et al.*, 2009, Yan *et al.*, 2010), although much of these analyses have been undertaken with cattle. The database analysis of CH_4 emissions from sheep measured in respiration

chambers in Chapter 4, showed that the DMI of ryegrass forages predicted up to 81% of the variation in CH_4 production (g/d) and 36% of the variation in CH_4 yield (g/kg DMI). However, published data on the effect of feed intake on CH_4 emissions from animals fed ryegrass forages are scarce and equivocal. This is partly because analyses have been made previously using the sulphur hexafluoride (SF_6) technique, which has resulted in more variation and lower associations between CH_4 yield, feed intake and diet composition (Chapter 4). Also, measurements of CH_4 emissions from sheep and cattle grazing at pasture often do not include measured intakes (Lassey *et al.*, 1997, Ulyatt *et al.*, 1997, Ulyatt *et al.*, 2002a and 2002b, Pinares-Patiño *et al.*, 2003a, Pinares-Patiño *et al.*, 2003d, Ulyatt *et al.*, 2005, Molano *et al.*, 2006, Cavanagh *et al.*, 2008), so estimates of CH_4 yield were less accurate than indoor experiments with measured intakes.

The causes of the reduction in CH_4 yield as feed intakes increase have not been clearly defined, although most suggestions are that higher intakes result in a shorter duration of digesta in the rumen, with less fermentation, compared to low intakes (Ulyatt *et al.*, 1984, Johnson *et al.*, 1993, Dewhurst *et al.*, 2003, Pinares-Patiño *et al.*, 2003c, Pinares-Patiño *et al.*, 2007). When feed intake increases to a greater extent than rumen capacity, the residence time in the rumen must be reduced. Janssen (2010) suggested that the reduced time available for microbial fermentation at high feed intakes may affect end products of fermentation towards more propionate. This would lead to a decrease in hydrogen (H_2), and consequently less CH_4 formed per unit of feed eaten.

The relationship between feed intake and CH_4 yield offers an opportunity to lower emissions whilst increasing animal productivity. The main objective of this study was to confirm published research based on dried feeds, that showed increasing feed intake decreased CH_4 yield (g/kg DMI), and to measure the extent of this effect in sheep fed white clover and ryegrass forages. The second objective was to gain a better understanding of the interactions between feed intake, digestibility and aspects of rumen function such as VFAs, on CH_4 emissions from sheep fed fresh forage diets. Lastly, a preliminary investigation measured the diversity of rumen methanogen populations in sheep fed ryegrass forages at low and high feed intakes.

It was hypothesised that increasing feed intakes of white clover and ryegrass forages would result in a decrease in CH_4 yield, and that digestibility and fermentation end

products would be able to account for the effect of feed intake on CH_4 emissions from sheep.

6.2 MATERIALS AND METHODS

This Chapter summarises four experiments: Experiment 2 (May to June 2009); Experiment 3 (October to November 2009); Experiment 4 (November to December 2009); and Experiment 5 (April to May 2010) (Table 6.1), as described in 6.2.4. Principal measurements from all four experiments were DMI and emissions of CH_4, H_2 and CO_2 from sheep fed white clover and ryegrass forages over a range of feed intakes (Table 6.1). Additional measurements in Experiments 2, 4 and 5 were apparent digestibility, and rumen digesta samples were collected from both fistulated and intact (non-fistulated) sheep for measurements of VFA concentrations. Experiments 4 and 5 included measurements of rumen solid and liquid fractional outflow rates (FOR), including the use of rumen water-filled balloons in Experiment 4 (results are presented in Chapter 7). A preliminary investigation of rumen methanogen populations was undertaken using rumen samples collected before morning feeding from sheep fed ryegrass at low or high feed intakes in Experiment 5.

The data collected from sheep in all four experiments were related to the effect of feed intake. A table of predictions was generated which used set DMI values (which ranged from 0.40 to 1.60 kg/d) and an analysis predicted the responses of different variables, based on data collected from all four experiments (Table 6.3).

A schedule of events for Experiments 2, 3, 4 and 5 are given in Table 6.2. All procedures were reviewed and approved by the AgResearch Palmerston North Animal Ethics Committee.

TABLE 6.1 Overview of experiments investigating the effect of dry matter intake (DMI) on methane (CH_4) emissions from sheep fed white clover (WC) or perennial ryegrass (RG) forages.

	Experiment 2	**Experiment 3**	**Experiment 4**	**Experiment 5**
Date	May to June 2009	Oct to Nov 2009	Nov to Dec 2009	April to May 2010
Number of periods	1	1	2	1
Number of animals	16 (8 with rumen fistulae)	28 (all intact)	8 (all with rumen fistulae)	30 intact
Diet	WC & RG	WC & RG	WC & RG	RG
Feed offered (x ME_m)	1.6	0.8 & 2.0	1.6	0.8, 1.2, 1.6, 2.0 & 2.5
Treatment	Diet	Diet, feed intake	Diet ± water balloon[a]	Feed intake
Animals/treatment	8 WC, 8 RG	16 WC, 12 RG	4 WC, 4 RG	6 per feed intake
Feeding regime	Twice daily	Twice daily	Hourly	Twice daily
Measurements	DMI and gas emissions (CH_4, H_2 and CO_2) measured in respiration chambers			
Other	Digestibility Rumen VFAs		Digestibility Rumen VFAs Digesta kinetics	Digestibility Rumen VFAs Digesta kinetics

ME_m, metabolisable energy requirements for maintenance; H_2, hydrogen; CO_2, carbon dioxide; VFAs, volatile fatty acids
[a]Sheep were with or without a 1 L water-filled balloon in the rumen

6.2.1 Animals and diets

Wether sheep aged 1 to 2 years were used in all of the experiments. In Experiments 2, 3 and 4, sheep were fed white clover or ryegrass forages but only ryegrass was fed in Experiment 5. The white clover (*cv.* Kopu II) and ryegrass (*cv.* Quartet) were grown near Palmerston North and harvested daily using a sickle bar mower. Forages were delivered by 14:00 h, weighed into meals, and stored at 4ºC prior to feeding. More details of the forages and feeding are given in Chapter 3.3.

Feeding was twice daily (equal size meals at 09:00 and 16:00 h) for Experiments 2, 3 and 5, and for Experiment 4, the total daily feed allowance was fed hourly from overhead belt feeders. Water was available *ad libitum*.

The sheep used for these experiments have been described in Chapter 5.2.1; brief descriptions are given here. The 16 wethers used in Experiment 2 included 8 with small

(30 mm o.d.) rumen cannulae, and these sheep were also used in Experiment 4. In Experiment 2, 8 sheep were fed white clover, and 8 fed ryegrass, all at 1.6 x ME_m. Experiment 3 used 28 intact sheep with 16 fed white clover and 12 fed ryegrass and there were two feeding treatments (0.8 and 2.0 x ME_m) for each diet. In Experiment 4, white clover was fed to 4 sheep and the other 4 received ryegrass, all at 1.6 x ME_m. Experiment 4 compared two measurement periods, with and without an intra-ruminal 1 L water balloon (Table 6.2). Experiment 5 used 30 intact sheep (including the 28 from Experiment 3) which had an average LW ± SD of 51.4 ± 4.52 kg, and were fed fresh ryegrass, with 6 sheep per feeding treatment fed at 0.8, 1.2, 1.6, 2.0 and 2.5 x ME_m.

6.2.2 Gas measurements

Emissions of CH_4, H_2 and CO_2 gas were measured using the eight chamber sheep respiration facility of AgResearch Grasslands Research Centre, described in Chapter 3.6. All measurements for individual sheep were carried out over a 48 h period, commencing at 09:00 h, and chambers were only opened 16:00 h (feeding and refusal collection), 09:00 h (feeding, refusal collection and cleaning), and 16:00 h (feeding and refusal collection), before sheep were released at 08:30 h. Respiration chamber measurements for all sheep were completed over 4 d for Experiment 2, 8 d for Experiment 3, 4 d for Experiment 4, and 8 d for Experiment 5.

TABLE 6.2 Schedule of events for Experiments 2, 3, 4 and 5. NB information for Experiments 2, 3 and 4 are abbreviated from Table 5.2.

Experiment 2 (11th May 2009 to 7th June 2009)	
Adaptation	
Day 1 – 18	Adaptation to white clover and ryegrass forages.
In vivo digestibility	
18 – 24	Faecal and feed refusal collection each morning before feeding. 10 rumen samples collected from each fistulated sheep, relative to feeding at -2, -1, 0, 1, 2, 3, 4, 6, 8, and 10 h.
Gas measurements	
24 – 27	Individual respiration chambers for 48 h.
Experiment 3 (27th October 2009 to 14th November 2009)	
Adaptation	
1 – 11	Adaptation to white clover and ryegrass diets, and feed intake treatments of 0.8 and 2.0 x ME_m.
Gas measurements	
11 – 18	Individual respiration chambers for 48 h.
Experiment 4 (10th November to 22nd December 2009)	
Adaptation	
1 – 12	Adaptation to white clover and ryegrass forages, continuous feeding, and 1 L intra-ruminal water balloon treatment.
In vivo digestibility	
12 – 18	Faecal collection (more frequently for marker measurements, see Chapter 7). 14 rumen samples collected from each fistulated sheep, relative to marker dosing (see Chapter 7).
Gas measurements	
19 – 22	Individual respiration chambers for 48 h.
Water balloon treatment swapped over between sheep. Period 2 adaptation starting 2nd December 2009 with the same routine as above.	
Experiment 5 (12th April to 9th May 2010)	
Adaptation	
1 – 11	Adaptation to ryegrass diets, feed intakes of 0.8, 1.2, 1.6, 2.0 and 2.5 x ME_m, and marker consumption for measuring digesta kinetics.
In vivo digestibility and digesta kinetics	
12 – 20	Administration of markers for measurement of digesta kinetics (Chapter 7). Collection of feed refusals each morning before feeding. Collection of faeces (collected more frequently for marker measurements). Rumen samples stomach tubed from all sheep 1 h before and 1 h after morning feed offered.
Gas measurements	
20 – 27	Individual respiration chambers for 48 h.

ME_m, metabolisable energy requirements for maintenance

6.2.3 Sample collection, processing and laboratory analysis

Details of sample collection, processing and laboratory analyses of feed offered, refused, faeces and rumen fluid are given in Chapter 3, and a brief overview has been given in Chapter 5.2.3.

Feed was sampled each day of all trials from the acclimatisation periods onwards, for DM determination (oven-drying 200 g triplicates at 105°C for 16 h). Additional samples were oven-dried (65°C for 48 h) for analysis of chemical composition by Near Infrared Reflectance Spectroscopy (NIRS). Wet chemistry analyses of feeds offered was undertaken during digestibility measurements for Experiments 2, 4 and 5, and during respiration chamber measurements for Experiment 3 (Chapter 3.9).

Feed refusals from each sheep were collected and weighed once daily for DM determination during the digestibility period (Experiments 2 and 5), and twice daily during the respiration chamber period (all four experiments). Composite refusal samples were accumulated for individual sheep during the digestibility period (Experiments 2 and 5), and during the respiration chamber period (Experiments 3 and 4) for analysis by wet chemistry (Chapter 3.9).

During the 7 d digestibility period, faeces from individual sheep were collected into bags attached to harnesses in Experiments 2 and 4, but in Experiment 5 faecal collection was into trays placed under each animal's metabolism crate. The stainless steel trays had a grating that separated faeces from urine. During Experiments 4 and 5, more frequent faecal sampling took place when markers were used for determining digesta flow (see Chapter 7 for sampling times). A 10% aliquot of faeces was taken from each sample and pooled over the 7-d collection for each animal, after which sub samples were taken from each sheep for wet chemistry analysis and DM determination (Chapter 3.9), enabling apparent digestibility to be calculated.

Rumen fluid was collected from fistulated sheep in Experiments 2 and 4, and from intact sheep in Experiment 5 (Table 6.2). Rumen fluid was initially frozen before being thawed and centrifuged for collection of supernatant and determination of VFA concentrations (Chapter 3.9).

A preliminary investigation of rumen methanogen populations was undertaken using rumen fluid samples collected before morning feeding from sheep fed at 0.8 and 2.5 x ME_m in Experiment 5. The PCR-DGGE method was used to show differences in methanogen community composition in sheep fed either low or high intakes of ryegrass forage. PCR-DGGE is a molecular fingerprinting method that separates PCR DNA products based on the guanine-cytosine base pair (bp) content and sequence. DNA fragments of the same size but different sequence composition are separated in denaturing gradient gels, based on their melting points, resulting in a pattern of bands, each theoretically representing a different methanogen strain in the community (Nicholson *et al.*, 2007). The compositional diversity of the methanogenic community can be analysed through this technique by comparing the various band patterns. Details of the PCR-DGGE process are given in Chapter 3.10.

6.2.4 Statistical analysis

Data were analysed by ANOVA for each of the four experiments independently and are presented in Appendices 6.1 and 6.2. Feed intake was the principal treatment effect and before data from all four experiments were combined, a single regression examining the slope and intercept between DMI and CH_4 yield for white clover and ryegrass forages were compared (Figure 6.1). This was to confirm that there was no effect of diet type on CH_4 emissions. Because of the similar responses for both white clover and ryegrass forages, and similar experimental structure, a combined analysis of data from all four experiments could be undertaken. Interpretation of the white clover and ryegrass chemical composition was based on the range and average of values for each component concentration (g/kg DM) and intake (kg/d) (Table 6.3).

For Experiments 2, 3, 4 and 5, to better understand the effect of DMI on CH_4 emission, DMI was related to the following parameters:

- digestibility: digestible DM, DDM; digestible OM, DOM; digestible neutral detergent fibre, DNDF;
- digestible intake: DM, DDMI; OM, DOMI; NDF, DNDFI;

- emissions of CH_4: g CH_4/d; g CH_4/kg DMI, g CH_4/kg OMI; g CH_4/kg DDMI; g CH_4/kg DOMI; and CH_4 energy (CH_4-E) in relation to gross energy intake (GEI), CH_4-E/GEI;
- emissions of H_2: g H_2/d, g H_2/kg DMI;
- emissions of CO_2: g CO_2/d;
- rumen parameters: total VFAs; and individual VFA molar proportions.

Because DMI is a covariate, in order to determine the effect of DMI on the above parameters, an analysis using the REML method in GenStat software (Payne *et al.*, 2010) was used. Set DMI values (0.4, 0.6, 0.8, 1.0, 1.2, 1.4, and 1.6 kg/d) were used to predict values of the response variables, based on data from all four experiments. The prediction responses are given in Table 6.3. By using REML for a combined analysis of several related experiments, the 'best' estimate (based on data from all experiments) of DMI on the effect of the variables was obtained. The REML model takes into account the effect of the experiment and produces one overall test.

The fixed REML model was expressed as:

Variable = C + DMI

and the random model as:

Variable = Expt. + Expt. x DMI

Where: C, is a constant; Variable, is the variable of interest (i.e. digestibility, digestible intake, gas emissions, and rumen parameters); DMI, is dry matter intake (kg/d); and Expt., is Experiments 2, 3, 4 or 5.

The random model included 'experiment' and interactions between experiment and treatment terms (e.g. Expt. x DMI). Thus, in effect, each treatment term was compared against its interaction within the experiment, and a significant treatment effect implied that the effect was consistent and large compared with its variation across experiments. Because REML used set DMI values (0.4, 0.6, 0.8, 1.0, 1.2, 1.4, and 1.6 kg/d) to predict values of the response variables, the prediction model is expressed as:

Response variate = Constant + Diet + DMI + DMI x Diet + Expt.

where: C, is a constant; Response variate, is the prediction value of the variable of interest (i.e. digestibility, digestible intake, gas emissions, and rumen parameters) at each set DMI value; Diet, is white clover or ryegrass; DMI, is dry matter intake (kg/d); and Expt., is Experiments 2, 3, 4 or 5.

The results of the REML analysis are expressed as prediction means ± standard errors of the difference of the mean (SEM), and p-values (Table 6.3).

The different experiments are likely to have different variability and these are estimated in the separate residual terms for each experiment to give an overall feed intake effect which is largely based on the consistency of the effect across all experiments. In Experiments 2, 3 and 4, sheep were fed white clover and ryegrass but because there were no differences in CH_4 emissions between the two diets (Chapter 5), data were combined for both diets. Both the individual experiment analysis by ANOVA and the REML analysis of predicted responses used data from Experiments 2, 3 and 4 that were adjusted to remove the overall effect of diet. Additional adjustments were made to Experiment 2 data to remove the effect of animal fistulation, and to Experiment 4 data for the effects of measurement period (1 vs. 2) and rumen water balloon treatment (Balloon vs. Control) because these variables did not affect DMI (Chapter 7). No adjustments were made to data from Experiment 5 (no fistulated sheep and only ryegrass forages fed).

Data adjustments to each experimental dataset were done by performing an ANOVA which incorporated only the term(s) whose effect was to be removed (i.e. fistulation and diet for Experiment 2; diet for Experiment 3; and period, treatment and diet for Experiment 4). Residuals were obtained for each of the variables (e.g. digestibility, digestible intake, gas emissions, and rumen parameters) and added to a grand mean to give an adjusted mean value for each variable.

For individual experiment analyses, data can be located at the end of this Chapter in Appendices 6.1 and 6.2. Data from both the individual experiments and combined REML predictions were generated from individual animals and the means presented within tables will not always appear compatible.

Single and multiple regression analyses were conducted on data from all four experiments (Appendices 6.1 and 6.2) (Payne *et al.*, 2010) to investigate the relationship

between CH_4 emissions (g/d and g/kg DMI) and diet composition, feed intake, digestibility, digestible intake, and rumen VFAs (Tables 6.5 and 6.6). All subsets of up to 13 variables were assessed by multiple regression for their ability to predict CH_4 production or yield. The 'all subsets regression' procedure in GenStat software, version 10.2 (Payne *et al.*, 2010), was used for analysis and the model which predicted the most variation was identified.

6.3 RESULTS

6.3.1 Dry matter intake and methane yield

The relationship between DMI and CH_4 yield from sheep fed white clover and ryegrass forages is shown in Figure 6.1 and is based on data in Appendix 5.1. The effect of DMI on CH_4 yield was significant ($P<0.001$) and for white clover and ryegrass diets, an increase in DMI of 1 kg/d resulted in a decrease in CH_4 yield of 6.75 and 3.45 g/kg DMI, respectively. Although white clover-fed sheep had a higher intercept (29.2 g CH_4/kg DMI) than those fed ryegrass (27.6 g CH_4/kg DMI), the slope and intercept for each of the diets were not significantly different from each other (Figure 6.1).

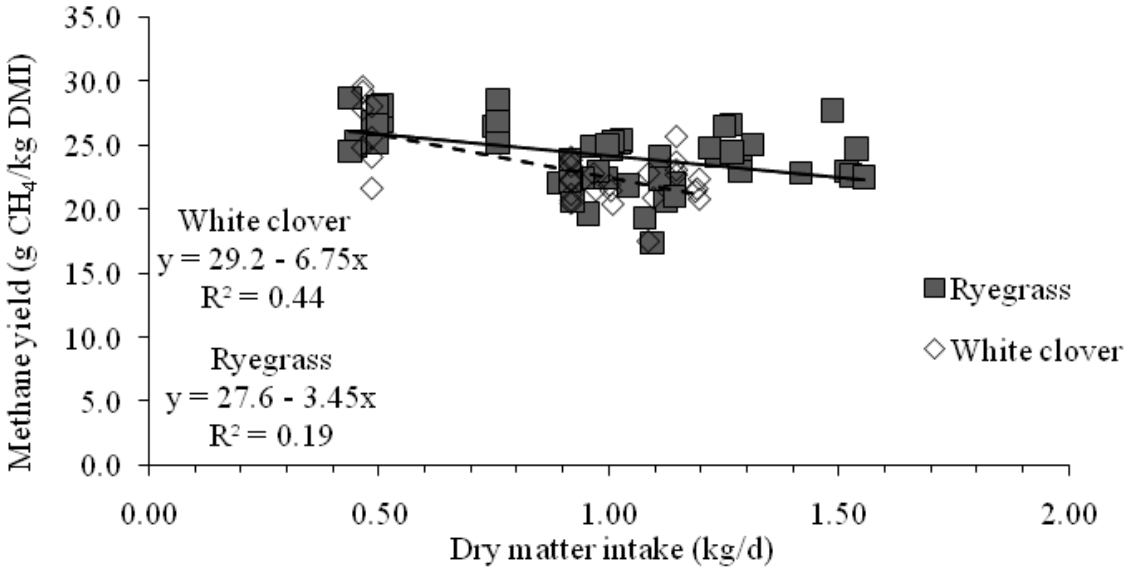


FIGURE 6.1 Dry matter intake versus methane (CH_4) yield for white clover (dashed line) and perennial ryegrass (solid line) fed to sheep in Experiments 2, 3, 4 and 5. Raw data for white clover is presented in Appendix 5.1

6.3.2 Dry matter intake and predicted variable responses

Feed intakes were set to values of 0.4, 0.6, 0.8, 1.0, 1.2, 1.4 and 1.6 kg/d and values for digestibility, gas emissions and rumen parameters were predicted using REML (Table 6.3), based on data measured in all four experiments (Appendices 6.1 and 6.2).

Despite the four-fold range in feed intake, there was only a minor effect on digestibility of DM, OM and NDF (702, 747, and 610 g/kg, respectively) (Table 6.3). However, as expected with an increase in feed intake, there was a significant ($P<0.001$) increase in digestible intakes of the respective components.

As feed intake increased from 0.40 to 1.60 kg/d, CH_4 production (g/d) increased by 187% ($P<0.001$) and CH_4 yield (g/kg DMI) decreased by 21% ($P<0.001$) (Table 6.3). When expressing CH_4 in relation to OMI (g/kg OMI), CH_4 decreased by 25% ($P<0.001$) when intake increased from 0.40 to 1.60 kg/d. Methane yield, expressed relative to digestible DM or OM intakes (i.e. g/kg DDMI and g/kg DOMI), decreased by 30% ($P<0.001$) over the range of intakes. The increase in feed intakes also resulted in a reduction CH_4-E/GEI from 0.084 to 0.065 ($P<0.001$) at predicted intakes of 0.40 and 1.60 kg/d, respectively.

There was a significant ($P<0.001$) increase in H_2 production (g/d) with increasing intake but values were similar when expressed per unit of feed eaten (0.054 g/kg DMI). The production of CO_2 (g/d) increased ($P=0.014$) more than two-fold with increasing feed intakes (Table 6.3).

The amount of feed consumed had no effect on the prediction of total VFA concentrations. Effects of intake on molar percentages of acetate, butyrate, valerate, isobutyrate or isovalerate were not statistically significant (Table 6.3). However, propionate increased from 17.3 to 20.7% of total VFA ($P=0.038$) when feed intake increased from 0.40 to 1.60 kg/d. The change in propionate affected a decrease in the acetate to propionate ratio (A:P) ($P=0.046$) and acetate + butyrate/propionate (A + B/P) ($P=0.041$) with increasing feed intake.

TABLE 6.3 Predicted responses of digestibility, digestible intake, gas emissions and rumen volatile fatty acid (VFA) variables[a] to dry matter intake (DMI) set values of 0.4, 0.6, 0.8, 1.0, 1.2, and 1.6 kg/d for sheep fed white clover and ryegrass forages.

DMI (kg/d)	**0.40**	**0.60**	**0.80**	**1.00**	**1.20**	**1.40**	**1.60**	**P-value**	**SED**
Digestibility (g/kg)									
Dry matter (DDM)	694	697	699	702	704	707	709	0.046	30.96
Organic matter (DOM)	742	743	745	747	749	750	752	0.095	36.16
Neutral detergent fibre (DNDF)	599	603	606	610	613	616	620	0.033	50.06
Digestible intake (kg/d)									
DDM intake (DDMI)	0.30	0.43	0.57	0.70	0.83	0.97	1.10	<0.001	0.029
DOM intake (DOMI)	0.28	0.41	0.54	0.67	0.79	0.92	1.05	<0.001	0.060
DNDF intake (DNDFI)	0.07	0.13	0.19	0.26	0.32	0.38	0.44	<0.001	0.038
Methane (CH_4) emissions									
g CH_4/d	12.4	16.3	20.1	24.0	27.9	31.8	35.6	<0.001	0.415
g CH_4/kg DMI	25.6	24.7	23.8	22.9	22.0	21.1	20.2	<0.001	0.558
g CH_4/kg OMI	31.0	29.7	28.4	27.1	25.8	24.5	23.3	<0.001	0.503
g CH_4/kg DDMI	40.2	38.5	36.8	35.1	33.4	31.7	30.0	<0.001	3.336
g CH_4/kg DOMI	43.2	41.1	38.9	36.8	34.6	32.5	30.3	0.006	1.457
CH_4-E/GEI	0.084	0.081	0.078	0.075	0.071	0.068	0.065	<0.001	0.001
Hydrogen (H_2) emissions									
g H_2/d	0.032	0.040	0.049	0.058	0.066	0.075	0.084	<0.001	0.006
g H_2/kg DMI	0.056	0.055	0.055	0.054	0.053	0.052	0.051	0.686	0.007
Carbon dioxide (CO_2) emissions									
g CO_2/d	626	748	870	992	1113	1235	1357	0.014	24.25
Total VFA (mM)	90.0	89.0	88.0	87.0	86.1	85.1	84.1	0.957	12.08
% of total VFA									
Acetate (A)	68.8	67.8	66.9	65.9	64.9	64.0	63.0	0.401	3.185
Propionate (P)	17.3	18.0	18.7	19.3	20.0	20.7	21.4	0.038	2.385
Butyrate (B)	11.7	11.5	11.3	11.2	11.0	10.8	10.7	0.693	1.445
Ratios									
A:P	4.01	3.83	3.65	3.46	3.28	3.10	2.92	0.046	0.540
A + B/P	4.62	4.42	4.22	4.02	3.82	3.62	3.42	0.041	0.581

[a] Means have been derived from individual data and values present here may not appear compatible

6.3.3 Intake and diet chemical composition

For sheep fed white clover and ryegrass forages, the concentrations (g/kg) and intakes (kg/d) of individual chemical components covered a wide range of values (Table 6.4).

Feed intakes in this study ranged from below ME_m (0.8 x ME_m; 0.44 kg DM/d) to above ME_m (2.6 x ME_m; 1.55 kg DM/d) (Table 6.4). The average DMI was 0.94 kg/d across all four experiments and ranged from 0.44 and 1.55 kg/d, providing good opportunities to identify relationships between feed intake and CH_4 production (g/d) and yield (g/kg DMI). Expression in terms of ME_m adjusts for variation in animal size, on an energy basis. The ME_m requirements for sheep in this study with an average LW of 40-50 kg is about 7 MJ ME/d (Australian Agricultural Council, 1990), which is about 0.6 kg DM/d of good quality white clover or ryegrass forage (11.5 MJ ME/kg DM).

The decision to estimate DMI requirements for maintenance with forages having an ME of 11.5 MJ ME/kg DM was based on published DM digestibilities for white clover (e.g. 84%; Ulyatt and Egan, 1979) and good quality ryegrass pasture (Litherland and Lambert, 2007). The DMI required for maintenance was estimated assuming good quality white clover and ryegrass. Whilst the estimated ME of white clover and ryegrass was about 10.9 MJ ME/kg DM (derived from about 72.3% DMD in Experiments 2 and 4), the lower digestibility of ryegrass fed in Experiment 5 (63.5% DMD) underestimated the ME intakes. So, the true range in ME_m used for analysis was 0.62 to 2.36 x ME_m, rather than 0.69 to 2.64 x ME_m (Table 6.4). This underestimate had a small effect on predictions that were based on intakes expressed as multiples of ME_m, but not on actual data or data expressed on a DMI basis.

There was a two-fold difference in concentrations (g/kg DM) of dietary CP (123 to 264) and NDF (265 to 536). In Experiment 5, the DMD (g/kg) of ryegrass was lower (625 to 648; Appendix 6.1) than values for sheep fed white clover and ryegrass in Experiments 2 and 4 (714 to 727; Appendix 6.1).

TABLE 6.4 Concentrations and intakes of chemical components by sheep fed white clover and perennial ryegrass forages in Experiments 2, 3, 4 and 5. Digestibility data were measured and metabolisable energy (ME) calculated by Near Infrared Reflectance Spectroscopy (NIRS).

Component	Concentration (g/kg DM)[#]		Intake (kg/d)[#]	
	Range	Average	Range	Average
Dry matter[a]	158 – 240	191	0.44 – 1.55	0.94
Organic matter	871 – 911	895	0.40 – 1.39	0.84
Crude protein	123 – 264	179	0.06 – 1.28	0.17
Neutral detergent fibre	265 – 536	412	0.11 – 0.83	0.39
Acid detergent fibre	183 – 268	226	0.09 – 0.42	0.21
Lipid	22 – 38	26.2	0.01 – 0.04	0.03
Lignin	16 – 86	46.4	0.01 – 0.10	0.04
Hot water soluble carbohydrate	98 – 164	117	0.05 – 0.21	0.11
Pectin	7 – 65	27.7	0.01 – 0.08	0.03
Hemicellulose	81 – 268	187	0.04 – 0.42	0.18
Cellulose	97 – 237	180	0.05 – 0.37	0.17
Digestible dry matter	601 – 777	680	0.29 – 1.02	0.67
Digestible organic matter	651 – 812	725	0.28 – 0.97	0.64
Digestible neutral detergent fibre	491 – 706	610	0.11 – 0.54	0.27
Gross energy[b]	17 – 19	18.5		
Dry matter intake x ME_m			0.69 – 2.64	1.52

[#] unless indicated
[a]Dry matter concentration expressed as g/kg wet matter
[b]Gross energy expressed as MJ/kg DM
DM, dry matter; ME_m, metabolisable energy requirements for maintenance

6.3.4 Variables associated with methane production

Data from all four experiments were examined individually for a relationship to CH_4 production (g/d) and regressions were calculated (Table 6.5). Most variation was predicted by OMI (87%) and for every kg increase in daily OMI, daily CH_4 production increased by 20.6 g. Dry matter intake predicted 85% of the variation in CH_4 production, followed by DOMI (76%) and DDMI (72%).

Intakes of individual chemical components (kg/d) all had significant and positive relationships with CH_4 production (g/d), except for pectin. The component intake that predicted the most variation in CH_4 production was acid detergent fibre (ADF; 70%), and this was closely followed by intakes of hot water soluble carbohydrates (HWSC) (65%) and lipid (58%).

The only significant (P=0.055) relationship between CH_4 production (g/d) and concentrations of individual chemical constituents (g/kg DM) was for HWSC, which predicted 3% of the variation in CH_4 production.

Total VFA concentrations predicted 14% of the variation in CH_4 production, with a 1 mM increase in total VFA concentrations associated with a 0.19 g increase in CH_4 production. Digestibility of DM, OM and NDF, as well as individual VFA molar proportions were not related to CH_4 production (Table 6.5).

When intake, digestion and rumen VFAs were analysed by a multiple subsets regression, no combination of variables presented in Table 6.5 could predict more of the variation in CH_4 production than OMI on its own.

6.3.5 Variables associated with methane yield

The relationships between CH_4 yield (g/kg DMI) and intake, digestibility and rumen VFAs were examined by individual regression analysis (Table 6.6). The variation in CH_4 yield from sheep was best predicted by the molar percentage of propionate (60%). A 1% increase in propionate was associated with a decrease of 1.18 g CH_4/kg DMI.

Intakes of DM, OM and all individual constituents of the DM (kg/d) had negative relationships with CH_4 yield. Crude protein intake had the strongest relationship, accounting for 44% of the variation in CH_4 yield (Table 6.6).

Concentrations of chemical constituents in the DM (g/kg DM); except lipid, lignin and HWSC, had significant relationships with CH_4 yield and predicted 3 to 19% of the total variation (Table 6.6). Concentration of CP predicted the greatest variation in CH_4 yield (19%), and this was followed by ADF (13%), NDF (9%), hemicellulose (8%), cellulose (6%) and pectin (3%).

As DM and OM digestibility increased, CH_4 yield declined, predicting 45 and 40%, respectively, of the variation (Table 6.6). Total VFA concentrations were negatively related to CH_4 yield ($P<0.001$), predicting up to 36% of the total variation.

When up to 13 of the most significant variables were included in the multiple subsets regression, the combination of propionate (%), and intakes of HWSC and NDF (kg/d) predicted 84% of the variation in CH_4 yield, where:

CH_4 (g/kg DMI) = -0.65 propionate% – 92.0 HWSC intake + 13.5 NDF intake + 39.3

TABLE 6.5 Methane production (g CH_4/d) and its relationship with intake, digestibility and rumen volatile fatty acid (VFA) variables measured in sheep fed white clover and perennial ryegrass forages over a range of feed intakes[#]. NB based on data from Experiments 2, 3, 4 and 5.

Variable	Methane production (g CH_4/d)			P-value	SED
	Intercept	Slope	Variation predicted (%)		
Intake (kg/d)					
Dry matter intake x ME_m	7.53	9.49	58.5	<0.001	3.87
Dry matter	4.71	18.4	85.2	<0.001	2.31
Organic matter	4.71	20.6	86.5	<0.001	2.22
Crude protein	15.8	41.3	23.6	<0.001	5.25
Neutral detergent fibre	12.5	24.2	53.0	<0.001	4.12
Acid detergent fibre	9.03	60.7	69.6	<0.001	3.31
Lipid	9.56	503	58.1	<0.001	3.89
Lignin	18.9	73.0	11.4	<0.001	5.65
Hot water soluble carbohydrate	7.95	129	65.3	<0.001	3.54
Pectin	21.0	41.1	2.20	0.089	5.94
Hemicellulose	15.5	36.2	37.8	<0.001	4.74
Cellulose	13.8	47.7	46.1	<0.001	4.41
Digestibility (g/kg)					
Dry matter	13.4	0.01	0.40	0.274	4.92
Organic matter	11.6	0.02	0.70	0.236	4.91
Neutral detergent fibre	3.10	0.03	3.20	0.086	4.85
Digestible intake (kg/d)					
Dry matter	6.53	24.6	72.2	<0.001	2.60
Organic matter	6.41	26.1	76.0	<0.001	2.41
Neutral detergent fibre	13.7	34.5	52.5	<0.001	3.40
Total volatile fatty acids (mM)	7.30	0.19	14.1	0.003	4.81
% of total volatile fatty acids					
Acetate (A)	26.9	-0.05	0.00	0.882	5.24
Propionate (P)	14.6	0.47	0.10	0.308	5.19
Butyrate (B)	16.4	0.64	0.00	0.380	5.20
Ratios					
A:P	30.3	-1.91	0.20	0.296	5.19
A + B/P	31.1	-1.86	0.40	0.272	5.18

[#] intakes ranged from 0.44 to 1.55 kg/d

ME_m, metabolisable energy requirements for maintenance.

TABLE 6.6 Methane yield (g CH_4/kg DMI) and its relationship with intake, digestibility and rumen volatile fatty acid (VFA) variables measured in sheep fed white clover and perennial ryegrass forages over a range of feed intakes[#]. NB based on data from Experiments 2, 3, 4 and 5.

Variable	Methane yield (g CH_4/kg DMI)			P-value	SED
	Intercept	Slope	Variation predicted (%)		
Dry matter (g/kg wet matter)	17.8	0.03	17.6	<0.001	2.39
Component concentration (g/kg)					
Organic matter	39.1	-0.02	0.00	0.423	2.64
Crude protein	26.9	-0.02	18.5	<0.001	2.38
Neutral detergent fibre	20.7	0.01	9.40	0.002	2.51
Acid detergent fibre	17.5	0.03	12.5	<0.001	2.46
Lipid	27.2	-0.12	2.90	0.058	2.59
Lignin	24.6	-0.02	1.50	0.129	2.61
Hot water soluble carbohydrate	22.8	0.01	0.00	0.499	2.64
Pectin	24.5	-0.02	3.40	0.044	2.59
Hemicellulose	22.0	0.01	7.50	0.005	2.53
Cellulose	21.7	0.01	6.40	0.009	2.55
Component intake (kg/d)					
Dry matter intake x ME_m	29.2	-3.47	40.5	<0.001	2.03
Dry matter	27.8	-4.12	21.3	<0.001	2.34
Organic matter	27.7	-4.49	20.5	<0.001	2.35
Crude protein	27.6	-24.5	44.4	<0.001	1.96
Neutral detergent fibre	24.6	-1.78	0.20	0.277	2.63
Acid detergent fibre	25.5	-7.32	4.20	0.029	2.58
Lipid	27.4	-142	23.3	<0.001	2.31
Lignin	25.5	-36.0	14.6	<0.001	2.43
Hot water soluble carbohydrate	27.3	-30.9	18.6	<0.001	2.38
Pectin	24.8	-37.5	13.1	<0.001	2.45
Hemicellulose	24.1	-0.81	0.00	0.767	2.65
Cellulose	24.4	-3.07	2.63	0.347	2.63
Digestibility (g/kg)					
Dry matter	48.2	-0.04	45.2	<0.001	1.92
Organic matter	49.2	-0.04	40.1	<0.001	2.01
Neutral detergent fibre	13.6	0.02	2.70	0.105	2.56
Digestible intake (kg/d)					
Dry matter	28.4	-7.13	20.6	<0.001	2.31
Organic matter	28.0	-6.76	17.1	<0.001	2.36
Neutral detergent fibre	22.4	4.31	1.40	0.178	2.58
Total volatile fatty acids (mM)	35.2	-0.14	35.6	<0.001	1.91
% of total volatile fatty acids					
Acetate (A)	-34.9	0.88	54.5	<0.001	1.61
Propionate (P)	46.0	-1.18	60.1	<0.001	1.50
Butyrate (B)	37.2	-1.22	24.1	<0.001	2.07
Ratios					
A:P	6.83	4.73	60.7	<0.001	1.49
A + B/P	5.68	4.37	60.3	<0.001	1.50

[#] intakes ranged from 0.44 to 1.55 kg/d

ME_m, metabolisable energy requirements for maintenance.

6.3.6 Feed intake and methanogen populations

There was no effect of feed intake on rumen methanogen populations for sheep fed ryegrass forages at two intakes of 0.8 x ME_m and 2.5 x ME_m, as indicated by the similar band patterns for treatment groups (i.e. low (L) 1 to L6 vs. high (H) 1 to H6; Figure 6.2).

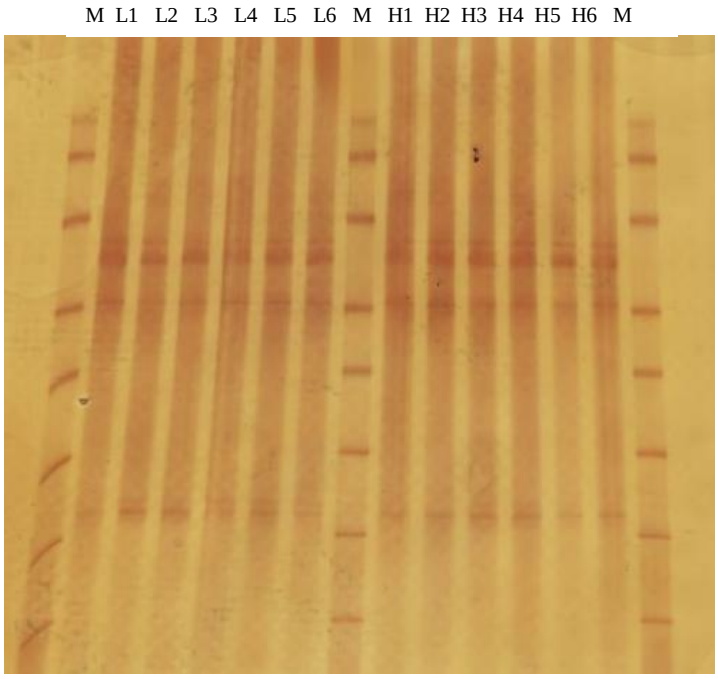


FIGURE 6.2 Denaturing gradient gel electrophoresis (DGGE) fingerprint of ruminal archaea in sheep fed ryegrass forages at low (L; 0.8 x metabolisable energy requirements for maintenance (ME_m)) (L1-L6) and high intakes (H; 2.5 x ME_m) (H1-H6). The outer two and middle lanes were loaded with an external standard (Marker (M) IV, Nippongene).

6.4 DISCUSSION

The main finding from this study confirms the hypothesis that as intakes of white clover and ryegrass forages increased, CH_4 production (g/d) increased and CH_4 yield (g/kg DMI) decreased from sheep. This confirms previous work with fresh forages (Chapter 4, Muetzel *et al.*, 2009; Sun *et al.*, in publication) that increasing DMI decreases CH_4 yield from sheep, and lowers the proportion of GEI lost to CH_4.

Regressing feed chemical constituents and their intakes, and rumen parameters, against CH_4 emissions, provided an insight into factors that were and were not associated with CH_4 production and yield. Organic matter intake accounted for the greatest amount of variation in CH_4 production, whereas propionate accounted for the greatest variation in CH_4 yield, with propionate proportions predicted to increase with increasing DMI. The PCR-DGGE examination of rumen methanogen populations in sheep fed ryegrass forages did not indicate significant differences in methanogen populations at high or low intakes.

6.4.1 Feed intake

The cause of the reduction in CH_4 yield with increasing feed intake has not been clearly defined, although most suggestions are that higher feed intakes result in shorter rumen residence times, with lower digestion, compared to low feed intakes (Ulyatt *et al.*, 1984, Johnson *et al.*, 1993, Dewhurst *et al.*, 2003, Pinares-Patiño *et al.*, 2003b, Pinares-Patiño *et al.*, 2007).

With an increase in feed intake there is more substrate entering the rumen, an increase in rumen digesta load (Ulyatt *et al.*, 1986, Waghorn *et al.*, 1986, Pinares-Patiño *et al.*, 2003c), and more substrate is available for microbial colonisation and H_2 generation (Hegarty *et al.*, 2007b). The mean retention time (MRT) of feed can be influenced by the rumen digesta load, which changes in response to feed intake, and is affected by the physical capacity of the rumen in individual sheep. A short MRT is associated with rapid digestion or outflow from the rumen, and is characteristic of high feed intakes or readily digestible DM. For example, Thornton and Minson (1972) fed sheep lucerne chaff and found that as intakes increased from 607 to 1180 g/d, MRT decreased from 26.7 to 14.5 h, respectively. A short MRT may also be associated with a low CH_4 yield from the diet. A longer MRT is generally associated with low intakes or more fibrous diets, which provide a greater opportunity for digestion and CH_4 production. In this study, sheep fed at low intakes (e.g. 0.40 kg/d), had a similar digestibility, but a higher CH_4 yield, compared to sheep at high intakes (e.g. 1.60 kg/d). Although not measured in this Chapter, differences in CH_4 yield with feed intake could be a consequence of the

interaction between digesta load, residence times and passage rates, and this requires further investigation.

Faster passage rates (i.e. a decreased MRT of digesta in the rumen), characteristic of high feed intakes, are thought to result in a shift in fermentation pathways towards more propionate production and consequently less CH_4 formed per unit of feed eaten (i.e. CH_4 yield) (Dewhurst *et al.*, 2009, Janssen, 2010). When feed intakes were set from 0.40 up to 1.60 kg/d, molar percentages of propionate were predicted to increase as CH_4 yield decreased (Table 6.3). Regression analysis of data from all four experiments found the molar percentage of propionate predicted up to 60% of the variation in CH_4 yield.

Based on evaluations of CH_4 yield in relation to feed intake (e.g. Blaxter and Clapperton, (1965), Johnson *et al.*, (1993), Yan *et al.*, (2000, 2010), Beauchemin and McGinn, (2006), Molano and Clark, (2008), Muetzel *et al.*, (2009), Sauvant and Giger-Reverdin, (2009), Chapter 4), it is established that there is a decrease in CH_4 yield as feed intakes increase. However, this relationship does vary and is less evident for roughages compared to concentrate diets. Blaxter and Clapperton (1965) showed that the reduction in CH_4-E/GEI for each multiple of ME_m increase in intake, was greater for pelleted diets (0.021) compared to roughages (0.008). Increasing intakes of concentrate diets from 1 to 2 x ME_m reduced CH_4-E/GEI by 0.016 in cattle (Johnson and Johnson, 1995) and 0.015 in sheep (Moss *et al.*, 1995). However, when cattle were fed fresh grass or silage diets, the effect of intake on CH_4-E/GEI was less (about 0.008) (Yan *et al.*, 2000, Beauchemin and McGinn, 2006, Yan *et al.*, 2010). Chapter 4 found no effect of ryegrass intakes above ME_m on CH_4 yield from cattle estimated by the SF_6 technique.

Reports for sheep fed pasture-based ryegrass forages at differing intakes have shown variable and inconsistent relationships with CH_4 yield. For example, Molano and Clark (2008) fed lambs and ewes pasture forages from about 0.8 to 2.0 x ME_m, and found no relationship between CH_4 yield and feed intake. In contrast, Muetzel *et al.* (2009) fed sheep pasture forages and reported a decrease in CH_4 yield of 5.3 g/kg DMI (CH_4-E/GEI of 0.016) for every increase in feed intake above ME_m. Sun *et al.* (2011) fed sheep ryegrass forages and found a decrease in CH_4 yield of 25.6 to 21.5 g/kg DMI when intakes increased from 1.3 to 2.2 x ME_m, (CH_4-E/GEI of 0.009). The database

analysis in Chapter 4 found a decrease in CH_4-E/GEI of 0.016 with increasing intakes of ryegrass above ME_m from sheep in respiration chambers.

There is some evidence that high intakes of roughage diets may result in a greater increase in rumen volume (Waghorn *et al.*, 1986) compared to concentrates (Mertens, 1987, Waghorn *et al.*, 2002), and roughages will be affected by forage quality. So, the change in MRT for animals fed roughages (or mature forages) may be less than from concentrate diets, or immature forages. Ulyatt (1969) reported that the MRT of the liquid pool for sheep fed immature ryegrass was only 8 h compared to 12 h for mature ryegrass. This, in combination with substrate suitability for propionate production, may partially explain the different responses in CH_4 yield to increasing feed intake.

Of the 66 species of methanogens found in a variety of anaerobic habitats, seven have been isolated from the rumen (Janssen and Kirs, 2008). PCR-DGGE allows the compositional diversity of the rumen methanogenic community to be visualised by comparing various band patterns and shifts in methanogenic populations. It has been speculated that rumen methanogen diversity can affect CH_4 production (Zhou *et al.*, 2010), but there has been no direct link between changes in methanogen community diversity and CH_4 yield measured from ruminants (Popova *et al.*, 2011). However, knowledge of the compositional diversity of methanogens in the rumen could enable a more targeted manipulation of the rumen system (Leahy *et al.*, 2010). In this study, no differences in PCR-DGGE bands were observed from sheep fed at low or high feed intakes and further investigation of the link between methanogen numbers and their activity on CH_4 emissions is required.

6.4.2 Diet chemical composition

Despite the large range in diet chemical composition achieved by feeding sheep white clover and ryegrass, chemical composition appeared to be of little consequence to CH_4 yield, with most the variation predicted by CP concentration (slope of -0.02, R^2=0.19). Previous attempts to predict variation in CH_4 yield (from unrelated trials) on the basis of diet chemical composition accounted for up to 51% of variation when pasture forages were fed to sheep, but no relationships could be established for cattle (Waghorn and

Woodward, 2006). A more recent evaluation of CH_4 emissions measured in respiration chambers from sheep fed ryegrass forages in unrelated experiments with varying composition found only 20% of the variation in CH_4 yield could be predicted (Chapter 4). Comparisons between white clover and ryegrass forages fed to sheep in three experiments (Chapter 5) showed similar CH_4 yields for both diets (Chapter 5), despite the ryegrass containing 50% more NDF, 80% less pectin, and 40% less CP in the DM, than white clover.

A correlation between CH_4 yield and diet composition was also anticipated because diet composition affects the proportions of VFAs (Bannink and Tamminga, 2005), as well as H_2, CO_2, and microbial growth. Changes in the products of fermentation alter the amount of H_2 formed, so CH_4 formation is likely to vary (Janssen, 2010). Moe and Tyrrell (1979) and Johnson and Johnson (1995) suggested the fermentation of plant cell walls (i.e. NDF) results in a greater CH_4 production compared with non-cell wall components. Ulyatt *et al.* (2002b) and Beauchemin *et al.* (2008) suggested improving diet quality by feeding forages with a lower NDF and higher RFC could also reduce CH_4 emissions. However, based on the results presented here, the variation in CH_4 yield from sheep was poorly related to the chemical composition of the diet. There is no simple relationship between the diet composition and CH_4 yield, but diet composition affects voluntary feed intake (VFI), and intakes appear to be responsible for an appreciable part of the observed variation in CH_4 yields.

6.4.3 Predicting variation in methane emissions

Feed intake predicted the greatest amount of variation in CH_4 production and it is the principle driver of methanogenesis. Mc Court *et al.* (2006) used data from 135 beef steers fed diets of grass silage, grass silage plus concentrates, dried and fresh grass and fodder beet in respiration chambers, to investigate factors influencing CH_4 production. The authors found that DMI on its own predicted up to 62% of the variation in CH_4 production, and adding intake as a multiple of ME_m slightly improved the relationship (64%). Robinson *et al.* (2010) fed sheep lucerne chaff at three different intakes (0.8, 1.24 and 1.6 x ME_m) and found feed intake was strongly correlated with CH_4 production (87%). From this study, OMI on its own predicted up to 87% of the variation in CH_4

production, and incorporation of other individual components did not improve the prediction.

The effects of feed intake on CH_4 yield are more complicated. Regression analysis of CH_4 yields from sheep in this study showed that feed intake only predicted 21% of the variation and suggested a decline of 4.12 g CH_4/kg DMI as feed intakes (of DM) increased. This was equivalent to 3.47 g CH_4/kg DMI per multiple of ME_m intake for a 40-50 kg sheep used here (Table 6.6).

Despite the three-fold range in feed intakes there were no consistent differences in apparent digestibility of white clover or ryegrass in this study. Although DMD predicted up to 45% of CH_4 yield, for every unit increase in DMD (g/kg), only a minor decrease in CH_4 yield of 0.04 g CH_4/kg DMI was a result. An analysis of 118 studies by Johnson and Johnson (1995) found that feed digestibility explained only 5% of the variation in GEI lost as CH_4. This indicates that digestibility is likely to not be a good indicator for CH_4 yield.

Molar percentage of propionate (% of total VFA concentration) predicted the greatest variation in CH_4 yield of 60%. Pinares-Patiño *et al.* (2007) fed non-lactating dairy cows pasture at 90% of VFI and reported that, although CH_4 production was best described by DMI and rumen acetate concentration (mM/L) (88%), CH_4 yield was mainly a function of acetate concentration alone (84%).

According to a review by Moss *et al.* (2000), *in vitro* studies showed strong correlations of molar percentages of propionate with CH_4 production (88%). When Robinson *et al.* (2010) fed sheep lucerne chaff eight times daily at three different intakes, VFA concentrations (mM/L) at various times of the feeding cycle differed in their capacity to predict CH_4 production. Correlations of VFA concentrations with CH_4 production were best 1 h before feeding, and the correlation with propionate concentration (mM/L) was 66%. However, when samples were averaged over 24 h, propionate concentration predicted 26% of the variation in CH_4 production. They concluded that concentration of propionate in the rumen is associated with differences in feed intake and this may explain why it is more closely associated with CH_4 emissions than other VFAs (Robinson *et al.*, 2010).

6.5 CONCLUSION

When feed intakes of sheep fed white clover and ryegrass forages increased three-fold, CH_4 production increased (187%) and yield decreased (21%). The chemical composition of the diet had little effect on CH_4 yield.

The cause of a reduction in CH_4 yield with feed intake has not been clearly defined. Possible factors associated with intake that could affect both CH_4 production and yield include the effect of intake on digesta load, rumen digesta times, rumen volume and passage rates. High intakes have been associated with short a MRT and low CH_4 yield, and these warrant further investigation.

Based on previous work, the relationship between intake and CH_4 yield within diets (i.e. ryegrasses) and between diets (i.e. roughages vs. concentrates) is variable. The ratio of RFC:NDF in roughages compared to concentrates have been implicated as a cause of variation, but poor relationships between diet composition and CH_4 yield do not show simple associations. High intakes of roughage diets can affect rumen volume to a greater extent than concentrates, and physical aspects of digestion may have an important effect on CH_4 emissions.

Faster passage rates and a decreased MRT of digesta in the rumen are believed to shift fermentation pathways towards more propionate production and consequently less CH_4 formed per unit of feed eaten. This was demonstrated by the REML prediction, and molar percentages of propionate may be a good indicator of responses in CH_4 yield brought about by changes in feed intake.

The net effect of high feed intakes will be a reduction of CH_4 emissions associated with increased animal production (reduced emissions intensity) due to effects on CH_4 yield and reductions in the proportion of feed used for ME_m. This is important because ruminants are usually fed to produce human edible food and farmers often need to maximise feed intakes for improved profitability. The lack of significant and meaningful relationships with diet components indicates that parameters of rumen function, particularly that pertaining to rumen residence times and passage rates, require further investigation to understand their effect on CH_4 yields.

APPENDIX 6

APPENDIX 6.1 Intakes, digestibility and gas emissions averaged for each feed intake treatment (based on metabolisable energy requirements for maintenance; ME_m) from sheep fed white clover and perennial ryegrass forages in Experiments 2, 3, 4 and 5.

Experiment	**2**	**3**		**4**	**5**					**P-value**	**SED**	**LSD**
Feed offered (x ME_m)	**1.6**	**0.8**	**2.0**	**1.6**	**0.8**	**1.2**	**1.6**	**2.0**	**2.5**			
Number of animals	16	14	14	8	6	6	6	6	6			
Number of periods	1	1	1	2	1	1	1	1	1			
Intake (kg/d)												
DMI	1.03	0.47	1.18	0.92	0.49	0.76	1.02	1.24	1.51	<0.001	0.019	0.038
OMI	0.91	0.43	1.07	0.83	0.44	0.67	0.90	1.11	1.35	<0.001	0.020	0.040
NDFI	0.37	0.15	0.39	0.34	0.24	0.41	0.54	0.63	0.80	<0.001	0.021	0.042
Digestibility (g/kg)												
DDM	721	N/A	N/A	726	625	634	641	625	648	<0.001	9.180	18.40
DOM	760	N/A	N/A	773	676	681	683	674	693	<0.001	9.320	18.68
DNDF	605	N/A	N/A	603	604	614	622	609	630	0.641	16.010	32.09
Digestible intake (kg/d)												
DDMI	0.74	N/A	N/A	0.67	0.31	0.48	0.65	0.79	0.98	<0.001	0.014	0.030
DOMI	0.69	N/A	N/A	0.64	0.30	0.46	0.61	0.76	0.94	<0.001	0.020	0.040
DNDFI	0.23	N/A	N/A	0.21	0.16	0.25	0.34	0.41	0.50	<0.001	0.011	0.023
Methane emissions												
g CH_4/d	21.4	12.5	26.5	25.1	12.8	19.5	23.2	27.2	31.9	<0.001	0.769	1.529
g CH_4/kg DMI	21.2	26.6	22.6	22.3	26.6	27.0	25.2	25.0	23.9	<0.001	0.775	1.543
g CH_4/kg OMI	23.5	29.2	24.8	30.3	29.5	29.2	25.8	24.3	23.6	<0.001	0.873	1.737
g CH_4/kg DDMI	28.8	N/A	N/A	37.7	42.5	40.6	35.7	34.4	32.3	<0.001	1.000	2.005
g CH_4/kg DOMI	31.0	N/A	N/A	39.2	44.4	42.8	37.8	35.7	34.1	<0.001	1.098	2.201
CH_4-E/GEI	0.063	0.078	0.067	0.086	0.078	0.077	0.068	0.066	0.063	<0.001	0.002	0.005
Hydrogen emissions												
g H_2/d	0.06	0.03	0.06	0.08	0.01	0.02	0.02	0.03	0.03	<0.001	0.011	0.021
g H_2/kg DMI	0.07	0.05	0.05	0.07	0.02	0.03	0.02	0.02	0.02	<0.001	0.012	0.024
Carbon dioxide emissions												
g CO_2/d	949	635	1103	1057	577	718	832	957	1123	<0.001	22.05	43.88

DMI, dry matter intake; OMI, organic matter intake; NDFI, neutral detergent fibre intake; DDM, digestible dry matter; DOM, digestible organic matter; DNDF, digestible neutral detergent fibre; DDMI, digestible dry matter intake; DOMI, digestible organic matter intake; DNDFI, digestible neutral detergent fibre intake; CH_4-E/GEI, methane energy in relation to gross energy intake; H_2, hydrogen; CO_2, carbon dioxide; SED, standard error of differences of means; LSD, least significant difference; N/A, data not available

APPENIDIX 6.2 Measurements of volatile fatty acids (VFAs) averaged for each feeding treatment (based on metabolisable energy requirements for maintenance; ME_m) for sheep fed fresh forages in Experiments 2, 3, 4 and 5. Approximately 10 samples were analysed from each fistulated sheep in Experiment 2, 14 samples from each fistulated sheep in Experiment 4, and 2 samples stomach tubed from each sheep in Experiment 5.

Experiment	**2**	**4**	**5**					**P-value**	**SED**	**LSD**
Feed offered (x ME_m)	**1.6**	**1.6**	**0.8**	**1.2**	**1.6**	**2.0**	**2.5**			
Number of animals	8	8	6	6	6	6	6			
Number of Periods	1	2	1	1	1	1	1			
Number of samples	80	224	12	12	12	12	12			
Total VFA (mM)	89.0	92.4	67.9	72.3	77.4	83.6	78.5	<0.001	3.038	6.112
% of total VFA										
Acetate (A)	64.4	66.0	68.6	68.1	66.2	69.2	67.2	<0.001	0.664	1.336
Propionate (P)	20.2	19.4	16.6	17.8	19.1	17.6	18.7	<0.001	0.574	1.156
Butyrate (B)	11.3	11.6	10.2	10.2	11.1	9.72	10.7	<0.001	0.364	0.733
Valerate	1.14	0.97	0.65	0.69	0.72	0.65	0.68	<0.001	0.049	0.098
Isobutyrate	1.35	0.92	1.00	0.90	0.87	0.78	0.73	<0.001	0.059	0.119
Isovalerate	1.40	0.89	1.10	0.89	0.96	0.78	0.76	<0.001	0.069	0.139
Ratios:										
A:P	3.21	3.49	4.15	3.84	3.49	3.95	3.61	<0.001	0.136	0.274
A + B/P	3.77	4.03	4.77	4.42	4.07	4.51	4.18	<0.001	0.153	0.308

SED, standard error of differences of means; LSD, least significant difference

CHAPTER 7

DIGESTA KINETICS AND METHANE EMISSIONS FROM SHEEP FED WHITE CLOVER (*Trifolium repens*) OR PERENNIAL RYEGRASS (*Lolium perenne*) FORAGES

CHAPTER 7: DIGESTA KINETICS AND METHANE EMISSIONS FROM SHEEP FED WHITE CLOVER (*Trifolium repens*) OR PERENNIAL RYEGRASS (*Lolium perenne*) FORAGES

ABSTRACT

Results from experiments presented in previous chapters have shown methane (CH_4) yields (g/kg dry matter intake; DMI) declined as intakes increased, and although there were few differences between white clover (*Trifolium repens*) and perennial ryegrass (*Lolium perenne*; ryegrass) forages fed here, published data show that diet type affects the extent of the change in yield with intake. Reasons given for the change in CH_4 yield with feed intake are often attributed to the change in residence time of digesta in the rumen, which is likely to be affected by diet type and composition. The main objective of this study was to examine the effect of white clover or ryegrass forages fed at different intakes on digesta kinetics (with or without a 1 L intra-ruminal water balloon) and CH_4 emissions from sheep.

Results from Experiments 4 and 5 were used in this analysis and all CH_4 emissions were measured in respiration chambers for two consecutive days. The objectives of Experiment 4 were to measure and compare CH_4 emissions from sheep fed either white clover or ryegrass forages, and to measure the impact of intra-ruminal water balloons to reduce digesta volume on digesta kinetics. This experiment used 8 sheep; 4 fed white clover and 4 fed ryegrass, and all were fed hourly at 1.6 x metabolisable energy (ME) requirements for maintenance (ME_m). Within dietary treatments, 2 sheep did or did not have a 1 L water filled balloon in their rumen, in a cross-over design. Rumen pool size and outflow rates were estimated, as well as passage of solid and liquid digesta fractions to the faeces. The objective of Experiment 5 was to measure the effect of feed intake on digesta kinetics and CH_4 emissions, as well as digesta passage rates and rumen kinetics. The 30 sheep used in Experiment 5 were fed ryegrass forages at 0.8, 1.2, 1.6, 2.0 and

2.5 x ME_m, twice daily, and there were 6 sheep per intake treatment. Rumen fractional outflow rates (FOR) were estimated primarily from liquid marker dilution (cobalt-EDTA; Co-EDTA) and sampling from the rumen fistulae in Experiment 4 (and from two treatment groups in Experiment 5), but both liquid and solid fraction kinetics were determined from faecal marker analysis (Co-EDTA and chromium-mordanted neutral detergent fibre (Cr-mordanted NDF) for liquids and solids, respectively) in both Experiments.

Placement of a 1 L water balloon in the rumen of sheep ('Balloon') in Experiment 4 did not affect liquid pool size and there was no effect on dry matter (DM) digestibility, CH_4 emissions, rumen pH, concentrations of ammonia (NH_3) and volatile fatty acids (VFAs), or digesta kinetics. Methane yield was similar (22.5 g/kg DMI) for white clover and ryegrass forages, but production of hydrogen (H_2) was higher (P=0.026) in sheep fed white clover compared to ryegrass. There were no differences between diets in DM digestibility (725 g/kg), rumen pH (6.27) or molar percentages of individual VFAs, except for acetate. Total VFA concentration (mM) was higher (P=0.047) in sheep fed white clover (96.9) compared to ryegrass (88.0) and rumen liquid volume was greater (P=0.041) when ryegrass was fed (6.05 vs. 3.96 L). The whole tract liquid mean retention time (MRT) was shorter (P=0.020) for sheep fed ryegrass compared to white clover (10.5 vs. 16.8 h, respectively), but diet did not affect the MRT of solids.

In Experiment 5, a three-fold increase in ryegrass intake (0.50 to 1.51 kg DM/d), reduced CH_4 yield by 11% (P<0.001; from 27.0 to 23.9 g/kg DMI) and increased H_2 production (P=0.026). Digestibility only changed to a minor extent over the range of intakes, but intake did increase rumen VFA concentrations and molar percentage of propionate from 16.6 to 18.7% (P=0.015). Increasing intakes from 0.50 to 1.51 kg DM/d reduced whole tract liquid MRT (P<0.001; from 28.0 to 12.1 h), but there were no changes in solid MRT, which averaged 35.0 h across all intakes.

Increasing feed intake reduced CH_4 yield from sheep fed ryegrass forages, and this was associated with a decrease in the MRT of liquid but not solid digesta. These findings support the hypothesis that a shorter digesta residence time can decrease CH_4 yield. The association between feed intake, CH_4 yield and liquid fraction kinetics, as well as effects on molar percentage of propionate in the rumen digesta, suggests further study of factors regulating outflow and passage of DM in the liquid phase.

7.1 INTRODUCTION

The yield of CH_4 (g/kg DMI) from digestion declines as feed intakes increase above ME_m (Chapter 6; Blaxter and Clapperton 1965; Sauvant and Giger-Reverdin 2009; Yan *et al.* 2010). The rumen digesta pool size can increase in response to increasing feed intakes, but to a limited extent, and Pinares-Patiño *et al.* (2003c) suggested that the decrease in CH_4 yield appears to result from a reduced residence time of digesta in the rumen. Intakes of lignified fibrous roughages will not be as high as diets comprising higher proportions of readily fermentable carbohydrate (RFC), because of bulkiness and extended time required to chew and reduce the particle size of fibre to enable passage from the rumen. As a consequence, responses in digestion and methanogenesis to changing feed intakes will differ according to diet type. Relationships established for concentrate and silage-based feeds may not apply to fresh forages (Table 4.4).

The decision to include a water balloon in some treatments (Table 3.1) was based on the concept that if some rumen capacity was occupied by an inert object, then digesta outflow could be affected (Forbes, 1995). It was important to use a balloon with a fill that would not lower intakes, and based on an estimated rumen digesta pool of 4.5 L, a balloon containing 1 L of water was considered large enough to affect a measurable change in digesta kinetics without lowering intakes.

An extended MRT of feed particles in the rumen is a consequence of either low feed intakes or resistance to particle size reduction (characteristic of lignified forage), and the increased exposure to microbial digestion may affect CH_4 production (Forbes, 1995). Conversely, a short MRT is associated with rapid digestion or outflow from the rumen and is characteristic of high feed intakes and/or readily digestible feed material. Some associations have been developed between forage NDF content and feed intake (Mertens, 1993), and between NDF and methanogenesis (Pinares-Patiño *et al.*, 2003c).

Blaxter and Clapperton (1965) considered the interactions between feed intake and forage quality and effects on CH_4 yield however few analyses have focused on differences between diet types, especially with fresh forages. Multiple regression analyses of the data from respiration chamber measurements with sheep (Chapter 4) showed that chemical composition predicted only a small amount of the variation in CH_4 yield (up to 20%). It also became apparent from Chapter 4 that evaluation of diet

composition and comparisons between diets required feed intakes to be held at a constant value.

The multiple regression analyses of data (Chapters 4 and 6) showed that chemical composition predicted a small amount of variation in CH_4 emissions, but increasing intakes reduced yield (g CH_4/kg DMI) and forage composition can affect intake. Even when there is a strong drive to eat (e.g. during lactation), intakes of diets available *ad libitum* will be limited by the intransience of fibre (associated with lignification, not necessarily NDF concentration), and high water content (Waghorn, 2002), which may influence physical aspects of rumen function and in turn affect methanogenesis.

The measures of chemical constituents used in the evaluation of diet composition upon CH_4 emissions (Chapter 4) did not distinguish between cellulose and hemicellulose, the extent to which fibre was degraded, or its passage following physical reduction through chewing. Chaves *et al.* (2006) showed that ryegrass maturation reduces degradation rates substantially, even though NDF content was similar, and changes in lignin concentration were small (2.5 to 3.0% of DM). Rates and extent of DM degradation will affect particulate DM pools and up to 95% of the microbial biomass in forage-fed ruminants is associated with the particle fractions (Hungate, 1966, Czerkawski, 1986). Hence the quantity, size and surface area of particulate matter in the rumen may indirectly affect methanogen populations (Janssen, 2010) because those not adherent to particles must grow at a higher rate to maintain their presence in the rumen. The liquid passage rate (FOR) is higher than that of particulate DM (Czerkawski, 1986, Owens and Goetsch, 1986) however, interpretation of flows need to consider the quantity of soluble and small particle DM flowing with the liquid fraction.

This chapter examines the effect of feed intake with rumen and whole tract digesta kinetics in relation to CH_4 emissions. It was hypothesised that CH_4 yield would be reduced by an increased FOR or decreased MRT of solid and liquid digesta fractions. Effects were hypothesised to be brought about by increased feed intakes or by reducing rumen digesta volume (through inserting intra-ruminal water filled balloons or feeding white clover which was suggested to degrade more rapidly than ryegrass forages).

7.2 MATERIALS AND METHODS

Measurements of digesta kinetics were made from sheep fed either white clover or ryegrass forages, with and without water-filled balloons in the rumen in Experiment 4, and with a fresh ryegrass diet fed at five different feed intakes in Experiment 5 (Table 7.1). In Experiment 4, during the digestibility period sheep were fed hourly at about 1.6 x ME_m, and in Experiment 5 they were fed twice daily at 0.8, 1.2, 1.6, 2.0 and 2.5 x ME_m. Rumen liquid volume and FOR were measured from marker dilution and sampling from the rumen fistulae in Experiment 4, and MRT by faecal marker analysis in Experiment 5, which used intact animals. Data were evaluated in relation to CH_4 emissions from sheep.

In Experiment 4, the expectation was a lower CH_4 yield from sheep fed white clover, compared to ryegrass. This was based on reports of low yields from sheep fed fresh white clover of 12 to 16 g CH_4/kg DMI (Waghorn *et al.*, 2002, Krause, AgResearch Report), compared to 20 to 24 g CH_4/kg DMI from ryegrass. The experiments were intended initially to compare emissions associated with white clover and ryegrass forages, and the fill of the water balloons was not intended to affect feed intakes, but to increase FOR in sheep. The similar CH_4 yields from both diets (Chapter 5) forced a re-appraisal of objectives to focus on the effects of feed intake. Digesta markers were again used (Experiment 5) to explore relationships between feed intake, CH_4 yield and digesta kinetics. Sheep numbers in Experiment 5 precluded hourly feeding because insufficient belt feeders were available. Principal measurements were DMI, apparent digestibility, solid and liquid digesta kinetics (FOR and MRT), rumen volume, pH, VFA and NH_3 concentrations, as well as emissions of CH_4, H_2 and carbon dioxide (CO_2) (Table 7.1).

A schedule of events for Experiments 4 and 5 are given in Table 7.2. All procedures were reviewed and approved by the AgResearch Palmerston North Animal Ethics Committee.

TABLE 7.1 Overview of experiments used to determine the effects of diet, rumen fill and feed intake on digesta kinetics and methane (CH_4) emissions from sheep fed white clover (WC) or perennial ryegrass (RG) forages.

	Experiment 4	Experiment 5
Date	November to December 2009	April to May 2010
Number of periods	2	1
Number of animals	8 (all with rumen fistulae)	30 intact
Diet	WC & RG	RG
Feed offered (x ME_m)	1.6	0.8, 1.2, 1.6, 2.0 & 2.5
Treatment	Diet ± water balloon[a]	Feed intake
Animals/treatment	4 WC, 4 RG 2 Balloon; 2 Control[a]	6 per feeding treatment
Feeding regime	Hourly	Twice daily
Digesta kinetics	Rumen liquid	Rumen liquid in two groups
	Whole tract liquid and solids	Whole tract liquid and solids
Measurements	DMI, gas emissions (CH_4, H_2 and CO_2), apparent digestibility, rumen samples (VFAs, pH[b] and NH_3[b]),	

ME_m, metabolisable energy requirements for maintenance; DMI, dry matter intake; H_2, hydrogen; CO_2, carbon dioxide; VFAs, volatile fatty acids; NH_3, ammonia
[a]Measurements were made in two periods so data were obtained from all sheep with, and without, an intra-ruminal balloon containing 1 L of water.
[b]Rumen pH and NH_3 were only measured in rumen samples collected from rumen fistulated sheep in Experiment 4.

7.2.1 Animals and diets

Wether sheep aged 1 to 2 years were used for both experiments. The diets fed in Experiment 4 were white clover (*cv.* Kopu II) and ryegrass (*cv.* Quartet) forages, whereas sheep in Experiment 5 were fed ryegrass forages only, with the ryegrass harvested from the same paddock as that used in Experiment 4. Further details of forages and feeding are given in Chapter 3.3.

For Experiment 4, sheep were fed hourly during the digestibility period from overhead feeders which were filled with freshly cut white clover or ryegrass forages at 09:00 and 21:00 h to achieve 'steady-state' feeding for marker measurements. Feeding was twice

daily at 09:00 and 16:00 h during respiration chamber measurements. During Experiment 5, ryegrass forages were fed at 09:00 and 16:00 h for the duration of the experiment as described in Chapter 3.4.

The sheep used in this study have been described in Chapters 5.2.1 and 6.2.1. Briefly, 8 rumen-fistulated wethers were used in Experiment 4, with 4 fed white clover over both periods and 4 fed ryegrass over both periods, all at 1.6 x ME_m. Within each diet 2 animals had a balloon placed in the rumen and filled with 1 L of water (Balloon). This was repeated with the other 2 sheep in the second period. Experiment 5 used 30 intact sheep fed ryegrass forages with 6 sheep allocated to each intake treatment and offered 0.8, 1.2, 1.6, 2.0 and 2.5 x ME_m.

7.2.2 Rumen water balloon treatment

The water balloon treatment involved placement of a deflated balloon into the rumen via the rumen cannula, and a drench gun was used to fill it with 1 L of water (room temperature). The neck of the balloon was knotted and a string tied around the knot. One end of the string was passed through the cannula (enabling the balloon to be located and retrieved), and the bung replaced. The balloons were obtained from a 'novelty' shop and were made of rubber. They were punctured for removal.

7.2.3 Gas measurements

Emissions of CH_4, H_2 and CO_2 were measured using the eight chamber sheep respiration facility of AgResearch Grasslands Research Centre, described in Chapter 3.6. Sheep were placed in individual chambers before 09:00 h and they were opened for feeding and collecting of feed refusals twice daily (09:00 and 16:00 h) and cleaning (09:00 h). Further details are given in Chapter 3.6.

TABLE 7.2 Schedule of events for Experiments 4 and 5. Additional details are provided in Table 5.2.

Experiment 4 (10th November to 22nd December 2009)	
Pre-trial Day -1	8 fistulated sheep fed white clover or ryegrass diets hourly. This trial involved 2 measurement periods; 2 sheep/diet had a intra-ruminal water balloon.
Adaptation (days 1 to 10)	
In vivo digestibility and digesta kinetics	
11	Cr-mordanted fibre (solids marker) given via rumen fistula at 22:00 h.
12	Co-EDTA (liquid marker) given via rumen fistula at 10:00 h.
12 – 18	Apparent digestibility and digesta kinetics determined. Diets fed hourly until day 18, with faecal and rumen sample collection for digestibility and marker measurement (Section 7.2.5).
Gas measurements	
19 – 22	Individual respiration chambers for 48 h.
Water balloon treatment swapped over within treatment groups. Period 2 adaptation commenced 2nd December 2009 with the same routine as above.	
Experiment 5 (12th April to 9th May 2010)	
Pre-trial - 1	30 intact sheep fed ryegrass and randomised into feed intake treatments of 0.8, 1.2, 1.6, 2.0 and 2.5 x ME_m; 6 sheep/feed intake treatment.
Adaptation (days 1 to 11)	
In vivo digestibility and digesta kinetics	
12 – 13	Adaptation of sheep to consumption of 'dummy' solid marker by feeding a 10 g dried grass/molasses mixture for 2 mornings.
14	Solid (Cr-mordanted NDF) and liquid (Co-EDTA) markers administered at 08:30 h and 09:00 h, respectively. Solid markers given via mouth and liquid markers via stomach tubing.
14 – 20	Apparent digestibility and digesta kinetics determined. Diets fed twice daily with feed refusals collected prior to feeding. Faecal and rumen sample collection for digestibility and more frequently for marker measurement (Section 7.2.5).
Gas measurements	
20 – 27	Individual respiration chambers for 48 h.
Liquid kinetics	
28	Co-EDTA given to sheep fed at 1.2 and 2.0 x ME_m and samples were obtained by stomach tube to evaluate this process for determining rumen pool size and FOR. Marker given at 10:00 h and rumen sampled 5 times over 10 h.

ME_m, metabolisable energy requirements for maintenance; Co, cobalt; Cr, chromium; NDF, neutral detergent fibre; FOR, fractional outflow rate

7.2.4 Markers for estimating digesta outflow rates

For both experiments, two markers were used; chromium-mordanted neutral detergent fibre (Cr-mordanted NDF) as the solid marker, and cobalt ethylene diaminetetraacetic acid (Co-EDTA) as the liquid marker. Both markers were prepared according to Uden *et al.* (1980) as detailed in Chapter 3.8.

Digesta kinetics were estimated during the digestibility period in both experiments. Manipulations with fistulated sheep fed white clover in Experiment 4 were made difficult by excessive foaming of rumen contents, so all sheep (including those fed ryegrass) were given a 5 ml drench of sunflower oil before marker administration. Before markers were given, rumen and faecal samples were taken from each sheep to determine background concentrations of Cr and Co.

Details of marker administration are given in Chapter 3.8. In both experiments, sheep were given 55 ml of 23.4 mM Co-EDTA solution on day one of the digestibility period (approximately 76 mg of Co per sheep). In Experiment 4, Co-EDTA was administered into the rumen via the cannulae at approximately 10:00 h, whereas in Experiment 5 Co-EDTA solution was given by stomach tube between 08:30 and 09:30 h (Table 7.2). A similar procedure was used for 12 sheep (fed at 1.2 and 2.0 x ME_m) at the end of Experiment 5 to evaluate sampling by stomach tube for estimating rumen liquid pool size and FOR.

Sheep in Experiment 4 were each given 5 g Cr-mordanted NDF directly via the rumen cannulae the night before (22:00 h) day 1 of the digestibility period. The dosage provided 165 mg and 190 mg Cr for sheep fed white clover and ryegrass forages, respectively (Table 7.3). Intact sheep in Experiment 5 were trained to consume a dried grass molasses mixture before feeding of actual Cr-mordanted NDF. For two mornings during the acclimatisation period, 10 g of dried grass, 4 ml molasses and 1 teaspoon of rock salt were mixed and offered to each animal. Sheep had around 5 min to consume the dried grass before morning feeding. Each sheep was fed 10 g Cr-mordanted NDF at 08:30 h on the first day of the digestibility period, containing 370 mg Cr (Table 7.3).

TABLE 7.3 Dosing of Cr-mordanted NDF to sheep in Experiments 4 and 5.

	Experiment 4		Experiment 5
	White clover	Perennial ryegrass	Perennial ryegrass
Number of sheep	4	4	30
Number of periods	2	2	1
Marker dosing method	Rumen cannulae	Rumen cannulae	Oral
Cr concentration (g/kg NDF)	33	38	37
Cr-mordanted NDF given (g)	5	5	10
Cr given (mg)	165	190	370

Cr, chromium; NDF, neutral detergent fibre

7.2.5 Sample collection, processing and laboratory analyses

Details for sample collection, processing and analysis of feed offered, refused, faeces and rumen fluid are given in Chapter 3 and a brief overview has been given in Chapter 5.2.3.

Faeces were collected and weighed from each sheep during the 7-d digestibility period, using either collection bags or trays placed under the animals (Chapter 3.5). In Experiment 4, faecal sampling for estimating whole tract solids MRT, required collection at: 10, 16, 22, 28, 34, 40, 48, 52, 58, 70, 82, 94, 106, 118 and 130 h after Cr-mordanted NDF were given. Analyses of samples also included Co, used for rumen liquid measurements. In Experiment 5, faecal samples were taken at: 6, 9, 12, 15, 18, 22, 26, 30, 35, 41, 48, 72, 96 and 120 h after eating the Cr-mordanted NDF.

Faecal collection times differed between experiments because the behaviour of Co and Cr markers was expected to differ, and sampling was intended to capture a range of concentrations for measuring solid and liquid kinetics in Experiment 5. Analyses of kinetics required measurements of concentrations before and after peak values, and there was a need to limit analytical costs. Approximately 5 g sub samples were taken from each faecal sample for Cr and Co analysis by inductively coupled plasma optical emissions spectrometry (ICP-OES).

Rumen fluid collection (Chapter 3.7) during Experiment 4 resulted in 14 rumen samples per animal over 2 d, during the digestibility period at: -1.0, 0.5, 1.0, 1.5, 2.0, 3.0, 4.0, 5.0, 6.0, 7.0, 8.0, 10.0, 12.0, and 22 h after Co-EDTA dosing. Samples were used to

determine marker concentrations for the calculation of rumen liquid FOR, and for pH, VFA and NH_3 analyses. In Experiment 5, rumen fluid was collected 1 h before and 1 h after feed had been offered to all sheep during the digestibility period for VFA analysis. At the end of Experiment 5, Co-EDTA was given to sheep fed 1.2 and 2.0 x ME_m, and rumen fluid samples were obtained by stomach tube at 1, 2, 4, 6 and 8 h post dosing to determine marker concentrations.

Rumen samples were frozen for later analysis. After thawing and centrifugation, approximately 5 ml of supernatant was obtained for measurement of Co concentration by ICP-OES. An additional aliquot of supernatant was frozen and thawed for VFA and NH_3 analyses (Chapter 3.9).

7.2.6 Marker calculations

Two sets of equations have been used to interpret digesta kinetics, one based on faecal marker analysis (whole tract passage and MRTs) and the other based on the rumen liquid pool (rumen liquid pool size and FOR). It is important to realise that measurements in Experiment 4 were made from sheep fed hourly, to approximate steady state (ideal) conditions, but in Experiment 5 the twice daily feeding would alter rumen volume and this could affect the estimation of rumen size made from sheep in two feed intake treatments.

7.2.6.1 Passage rate of solids and liquids (chromium and cobalt faecal excretion)

Faecal Cr and Co concentrations (less background concentrations) were fitted to a multi-compartmental model, based on Dhanoa *et al.* (1985), to determine whole tract solid and liquid kinetics. Details of the model and its equation (Eq.) are in Chapter 3.8.

7.2.6.2 Passage rate of fluids (cobalt disappearance from the rumen)

The liquid dilution rate (*k*) of Co-EDTA in the rumen was calculated from the decrease in Co concentration over time, described by Eq. 5 in Chapter 3.8.3.1, and log transformation enables *k* to be estimated by linear regression (Eq. 6) (López, 2008).

Rumen liquid volume (L) was estimated from the Co dose and concentration at zero time (Eq. 7, Chapter 3.8.3.1) (Bartocci *et al.*, 1997).

The outflow of liquid from the rumen (ml/h) was calculated by multiplying the ruminal liquid pool size by the dilution rate (*k*) (Eq. 8, Chapter 3.8.3.1).

7.2.7 Statistical analysis

Data were analysed by ANOVA for each experiment independently. Data from Experiment 4 evaluated effects of diet and rumen fill (balloon) in relation to a range of parameters, and similar relationships were developed for Experiment 5, but only the most relevant effects are presented here (Tables 7.5 to 7.8) with other associations in Appendix 7. The parameters used in the evaluations include:

- intake: DMI; organic matter intake, OMI; NDF intake, NDFI;
- digestibility: digestible DM, DDM; digestible OM, DOM; digestible NDF, DNDF;
- digestible intake: DM, DDMI; OM, DOMI; NDF, DNDFI;
- emissions of CH_4: g CH_4/d; g CH_4/kg DMI; g CH_4/kg OMI; g CH_4/kg DDMI; g CH_4/kg DOMI; and CH_4 energy (CH_4-E) in relation to gross energy intake (GEI), CH_4-E/GEI;
- emissions of H_2: g H_2/d; g H_2/kg DMI;
- emissions of CO_2: g CO_2/d;
- rumen parameters: pH; NH_3; total VFAs; individual VFA molar proportions;
- digesta kinetics: FOR (*k*) and MRT of solids and liquid whole tract digesta fractions based on faecal analyses; rumen liquid volume, outflow rate and rumen MRT.

In Experiment 4, treatment effects were determined by ANOVA (Payne *et al.*, 2010). The blocked effects were animal and period within animal, because there were two measurement periods but the same animals were used in each. The Balloon and Control treatments were compared between Periods 1 and 2 within sheep, as each sheep was measured with and without a balloon. Both the fixed and random statistical models for

analysis of data from Experiment 4 are detailed in Chapter 5.2.4. Forage chemical composition within each experiment was expressed as single sample values (Table 7.4).

A similar analysis was applied to Experiment 5, but the 'Treatment' term in the fixed model was feed intake group: 0.8, 1.2, 1.6, 2.0 and 2.5 x ME_m, and there was no random effect (only one measurement period). Rumen pH and NH_3 were not measured.

Results of the individual experimental analyses are expressed in Tables 7.5 to 7.8 as means ± standard error of the difference of the mean (SED) and p-values. Additional data are given in Appendices 7.1 and 7.2. Data are derived from individual animals and means presented within tables will not always appear compatible.

The equation used to fit the multi-compartmental model were solved using SAS (2005; SAS Inst. Inc, Cary, NC, USA). A least squares fit of the data was obtained by an iterative adjustment of parameter values (Berman *et al.*, 1962). Statistical analysis of digesta kinetics data were based on values obtained from the multi-compartmental model, but individual data that failed to converge were excluded. Excluded data included four sheep where faecal Co data failed to converge, and one sheep where faecal Cr data did not converge, all from Experiment 4. All data converged for samples analysed from sheep in Experiment 5.

7.3 RESULTS

7.3.1 Chemical composition of diets offered

The composition of diets fed in Experiments 4 and 5 are summarised in Table 7.4. The ryegrass fed in Experiment 5 had a higher NDF concentration (g/kg DM) relative to that in Experiment 4 (554 vs. 466) and the value was much lower for white clover (296). The ratio of readily fermentable carbohydrate (RFC) to NDF was about twice as high in white clover, compared to ryegrass (0.59 vs. 0.29 and 0.21) and the crude protein (CP) concentration (g/kg DM) in white clover was about twice that of ryegrass (220 vs. 117 and 102) (Table 7.4).

TABLE 7.4 Chemical composition of white clover and perennial ryegrass forages offered to sheep in Experiments 4 and 5 during digestibility.

Chemical composition (g/kg DM)	Experiment 4		Experiment 5
	White clover[a]	Perennial ryegrass[a]	Perennial ryegrass[a]
Gross energy (MJ/kg DM)	18.9	17.8	18.6
Dry matter (g/kg wet matter)	160	184	240
Organic matter	903	898	888
Crude protein	220	117	102
Lipid	23.4	27.1	26.5
Hot water soluble carbohydrate	106	126	106
Pectin	62.8	7.30	8.15
RFC[b]	169	133	114
Neutral detergent fibre	296	466	554
Acid detergent fibre	203	237	283
Cellulose	134	219	256
Lignin	68.6	18.1	32.6
RFC: NDF	0.57	0.29	0.21

All analyses were by wet chemistry.
RFC, readily fermentable carbohydrate; NDF, neutral detergent fibre
[a]*n* = 1, with 6 samples per forage pooled to make one sample for analysis
[b]RFC = hot water soluble carbohydrate + pectin

7.3.3 Intakes, digestibility, gas emissions and rumen parameters

Data from Experiment 4, which investigated the effect of diet and rumen fill (Control or Balloon) on digestibility, gas emissions and rumen parameters in sheep, are summarised in Table 7.5. Corresponding results for the effect of feed intake measured from sheep in Experiment 5 are presented in Table 7.6, and additional data are located in Appendices 7.1 and 7.2.

7.3.2.1 Diet treatment (Experiment 4)

Sheep fed either white clover or ryegrass forages had numerically similar DM intakes (0.91 and 0.93 kg/d; Table 7.5), and a similar DM digestibility (726 g/kg) (Table 7.5). Diet did not affect CH_4 emissions from sheep fed white clover or ryegrass forages, with an average CH_4 production of 25.1 g/d and yield of 22.5 g/kg DMI. Sheep fed white clover had greater emissions of both CO_2 (P=0.032) and H_2 (P=0.026), but H_2 was less than 0.5% of CH_4 emissions (g/d). There was no difference in rumen pH (6.27)

between diets, but both NH_3 and total VFA concentrations were higher for sheep fed white clover (18.6 and 96.9 mM, respectively) compared to ryegrass (5.50 and 88.0 mM, respectively) (Table 7.5). Molar percentage of acetate was lower ($P<0.01$) in sheep fed white clover compared to ryegrass.

7.3.2.2 Rumen fill treatment (Experiment 4)

The presence of a 1 L intra-ruminal water balloon in sheep had no effect on intake, digestibility, gas emissions or rumen parameters (Table 7.5). In retrospect, it appeared that a larger balloon may have been preferable.

7.3.2.3 Feed intake treatment (Experiment 5)

Feed intakes of sheep in Experiment 5 had a three-fold range (0.49 to 1.51 kg/d). There were small differences in DMD between intake treatments, but this had no relationship with DMI (Table 7.6). With increasing feed intake there was a corresponding increase ($P<0.001$) in CH_4 production (13.1 to 31.9 g/d) and decrease ($P<0.001$) of 11% in CH_4 yield (27.0 to 23.9 g/kg DMI). Feed intake had no effect on H_2 yield (0.02 g/kg DMI), but production of CO_2 (g/d) increased nearly two-fold ($P<0.001$) with high intakes compared to low intakes. Total VFA concentrations averaged from pre and post feeding samples, increased from 67.9 to 83.6 mM with feed intakes of 0.49 to 1.26 kg/d, but at 1.51 kg DMI/d, concentrations averaged 78.5 mM. Molar percentages of acetate, propionate and butyrate were all affected by feed intake, but there were no consistent patterns (Table 7.6).

TABLE 7.5 Intakes, digestibility, gas emissions and rumen measurements[a] from sheep fed either white clover or perennial ryegrass forages at 1.6 x metabolisable energy requirements for maintenance (ME_m), with and without an intra-ruminal water balloon in Experiment 4.

	Diet				**Rumen fill**			
	White clover	Perennial ryegrass	P-value	SED	Balloon	Control	P-value	SED
Number of animals	4	4			4	4		
Number of periods	2	2			2	2		
DMI (kg/d)	0.91	0.93	0.026	0.006	0.92	0.91	0.613	0.011
DDM (g/kg DM)	725	727	0.915	15.40	729	723	0.829	27.10
Methane emissions								
g CH_4/d	25.7	24.5	0.338	1.070	24.9	25.3	0.543	0.610
g CH_4/kg DMI	22.5	22.0	0.563	0.857	22.2	22.3	0.858	0.581
Other gas emissions								
g H_2/d	0.11	0.05	0.026	0.020	0.08	0.08	0.886	0.022
g H_2/kg DMI	0.09	0.04	0.026	0.018	0.07	0.07	0.879	0.020
g CO_2/d	1077	1037	0.032	14.30	1048	1066	0.519	25.30
Rumen pH	6.31	6.23	0.439	0.095	6.34	6.19	0.444	0.178
NH_3 (mM)	18.6	5.50	<0.001	1.800	11.7	12.3	0.846	2.930
Total VFA (mM)	96.9	88.0	0.047	3.550	92.2	92.7	0.951	7.290
% of total VFA								
Acetate	64.7	67.3	0.009	0.682	66.2	65.8	0.647	0.846
Propionate	19.4	19.4	0.987	0.863	19.4	19.4	0.970	0.853
Butyrate	11.9	11.3	0.404	0.579	11.5	11.7	0.765	0.935

DMI, dry matter intake; DDM, digestible dry matter; CH_4, methane H_2, hydrogen; CO_2, carbon dioxide; NH_3, ammonia; VFA, volatile fatty acid; SED, standard error of the difference of the mean

[a] Means have been derived from individual data and values presented here may not appear compatible

TABLE 7.6 Intakes, digestibility, gas emissions and rumen volatile fatty acids (VFAs)[a] from sheep fed perennial ryegrass forages at five feed intakes ranging from 0.8 to 2.5 x metabolisable energy requirements for maintenance (ME_m) in Experiment 5.

	Feed offered as a multiple of ME_m					P-value	SED
	0.8	**1.2**	**1.6**	**2.0**	**2.5**		
Number of animals	6	6	6	6	6		
Number of periods	1	1	1	1	1		
DMI (kg/d)	0.49	0.76	1.02	1.26	1.51	<0.001	0.016
DDM (g/kg DM)	625	634	641	625	648	0.021	7.600
Methane emissions							
g CH_4/d	13.1	19.5	23.2	27.1	31.9	<0.001	0.831
g CH_4/kg DMI	27.0	27.0	25.2	25.3	23.9	<0.001	0.709
Other gas emissions							
g H_2/d	0.01	0.02	0.02	0.02	0.03	0.026	0.006
g H_2/kg DMI	0.02	0.03	0.02	0.02	0.02	0.880	0.007
g CO_2/d	557	718	832	935	1123	<0.001	23.10
Total VFA (mM)	67.9	72.3	77.4	83.6	78.5	0.004	3.380
% of total VFA							
Acetate	68.6	68.1	66.2	69.2	67.2	0.005	0.767
Propionate	16.6	17.8	19.1	17.6	18.7	0.015	0.700
Butyrate	10.2	10.2	11.1	9.72	10.7	0.015	0.370

DMI, dry matter intake; DDM, digestible dry matter; CH_4, methane; H_2, hydrogen; CO_2, carbon dioxide; SED, standard error of the difference of the mean

[a] Means have been derived from individual data and values presented here may not appear compatible

7.3.4 Digesta kinetics

Estimates of digesta passage were for whole tract digestion (based on faecal marker analysis), and rumen liquid kinetics (based on rumen liquid analysis), which included rumen volume, as well as outflow as ml/h, or as a fraction of the liquid pool. Predictions of digesta solids and liquid MRT, and passage rates (*k*) derived from the multi-compartmental model analysis, were analysed to determine if there were differences associated with diet or rumen fill in Experiment 4 (Table 7.7) or feed intake in Experiment 5 (Table 7.8). Rumen digesta kinetic estimations include liquid passage rate (e.g. *k*) and MRT as well as rumen liquid pool size (based on Co marker concentrations and rumen sampling). In each experiment, faecal analyses were undertaken and Cr concentrations were used to predict the digesta kinetics of solids,

whereas faecal Co concentrations were used for the liquid fraction. Details of faecal Cr and Co concentrations following marker administration in Experiments 4 and 5 have been presented in Appendices 7.3 and 7.4, respectively, and rumen Co concentrations from Experiment 4 and two treatments in Experiment 5 have been given in Appendix 7.5.

7.3.3.1 Diet, rumen fill and digesta kinetics (Experiment 4)

Whole tract MRT of solids were not significantly different for sheep fed white clover and ryegrass forages, averaging 26 h, with a FOR (*k*) of 5%/h (Table 7.7). Faecal Co analysis suggested the MRT of liquid was longer (P=0.027) for white clover (16.6 h) compared to ryegrass (10.5 h), and the MRT of the digesta liquid fractions were much lower than that of DM for both diets.

Measurements of rumen liquid FOR showed that there were no differences between diets, averaging 14.5%/h, with a MRT of 7.2 h (Table 7.7). However, rumen liquid volume was smaller (P=0.041) when white clover was fed (3.96 L), compared to ryegrass (6.05 L), so liquid outflow was less (555 ml/h) from the white clover diet compared to ryegrass (838 ml/h), fed at the same feed intake (Table 7.7).

The placement of a 1 L water balloon in the rumen of sheep did not affect whole tract digestion kinetics (Table 7.7) or rumen volume. Hence, there were no differences in rumen liquid volume (averaging 5.0 L) or turnover of liquid (averaging 14%/h) (Table 7.7).

TABLE 7.7 Effect of diet and intra-ruminal water filled balloons on whole tract passage of solid (chromium marker) and liquid (cobalt marker) fractions and rumen liquid kinetics. Whole tract calculations are based on faecal analyses using the multi-compartmental model of Dhanoa *et al.* (1985).

	Diet				**Rumen fill**			
	White clover	Perennial ryegrass	P-value	SED	Balloon	Control	P-value	SED
No. of animals	4	4			4	4		
No. of periods	2	2			2	2		
Whole tract solids digestion kinetics								
k (%/h)	4.25	5.66	0.337	0.010	4.28	5.63	0.243	0.007
MRT (h)	27.7	24.8	0.766	9.290	30.0	22.6	0.162	4.520
Whole tract liquid kinetics								
k (%/h)	6.12	9.89	0.020	0.011	7.26	8.75	0.202	0.009
MRT (h)	16.8	10.5	0.027	2.030	15.2	12.2	0.266	2.180
Rumen liquid digesta kinetics								
k (%/h)	14.5	14.4	0.976	0.022	14.0	14.9	0.404	0.010
MRT (h)	7.11	7.30	0.854	0.973	7.34	7.06	0.583	0.480
Volume (L)	3.96	6.05	0.041	0.809	4.86	5.15	0.689	0.694
Outflow (ml/h)	555	838	0.018	88.10	666	728	0.518	90.40

No., number; *k*, fractional outflow rate; MRT, mean retention time (1 x 100/*k*); SED, standard error of the difference of the mean

7.3.3.2 Feed intake and digesta kinetics (Experiment 5)

Measurement of whole tract digestion kinetics in sheep fed ryegrass forages twice daily over a range of feed intakes (0.49 to 1.51 kg DM/d) did not show any effects of intake on MRT of solids (averaging 35 h) or FOR (averaging 3.02%/h) (Table 7.8). However, as feed intakes increased, there was a 125% increase ($P<0.001$) in liquid FOR (3.72 to 8.38%/h), and a decrease ($P<0.001$) in liquid MRT from 28 to 12 h (Table 7.8).

The exploratory trial to determine whether rumen liquid kinetics could be determined using a stomach tube to both administer Co-EDTA marker and sample contents in sheep suggested a 46% faster liquid outflow rate (*k*), and 35% shorter MRT for sheep fed at 2.0 vs. 1.2 x ME_m (9.7 vs. 14.9 h, respectively), but these differences were not statistically significant (Table 7.7). The analysis showed that sheep fed at 2.0 x ME_m had a 25% greater ($P=0.009$) rumen liquid volume compared to those fed at lower

intakes (Table 7.7), and there was nearly a two-fold increase (P=0.003) in rumen liquid outflow between the two groups (1029 vs. 526 ml/h).

TABLE 7.8 Effect of feed intake on whole tract passage of solid (chromium marker) and liquid (cobalt marker) fractions and rumen liquid kinetics. Whole tract calculations are based on faecal analyses using the multi-compartmental model of Dhanoa *et al*. (1985).

	Feed offered as a multiple of ME_m					P-value	SED
	0.8	1.2	1.6	2.0	2.5		
No. of animals	6	6	6	6	6		
No. of periods	1	1	1	1	1		
Whole tract solids digestion kinetics							
k (%/h)	3.08	3.01	2.88	2.99	3.13	0.980	0.004
MRT (h)	34.6	35.5	35.0	35.9	34.0	0.997	5.410
Whole tract liquid kinetics							
k (%/h)	3.72	4.47	6.57	7.14	8.38	<0.001	0.009
MRT (h)	28.0	24.2	16.5	14.7	12.1	<0.001	3.061
Rumen liquid digestion kinetics							
k (%/h)	N/A	7.60	N/A	11.1	N/A	0.104	0.019
MRT (h)	N/A	14.9	N/A	9.68	N/A	0.068	2.568
Volume (L)	N/A	7.09	N/A	9.48	N/A	0.009	0.733
Outflow (ml/h)	N/A	526	N/A	1029	N/A	0.003	129.4

No., number; ME_m, metabolisable energy requirements for maintenance; *k*, fractional outflow rate; MRT, mean retention time (1 x 100/*k*); N/A, not available; SED, standard error of the difference of the mean

7.4 DISCUSSION

This study tested the hypothesis that increasing rumen FOR will result in a decrease in CH_4 yield from sheep fed fresh forages. Measurements of rumen kinetics were complemented by whole tract digesta flow, and the three-fold increase in feed intake (0.49 to 1.51 kg/d), and associated increase in liquid FOR, did reduce CH_4 yield. However, neither the presence of a 1 L water balloon in the rumen, or diet (white clover vs. ryegrass) had significant effects. The yield of CH_4 was higher when less digestible

ryegrass was fed at similar intakes. Figure 7.1 and Table 7.9 summarise the effects of diet and feed intake on CH_4 emissions and digesta kinetics.

FIGURE 7.1. Diagram of digestion parameters, and trends associated with (a) white clover versus perennial ryegrass (ryegrass) forages (b) increasing feed intakes of ryegrass forage. CH_4, methane; H_2, hydrogen; VFA, volatile fatty acid; FOR, fractional outflow rate.

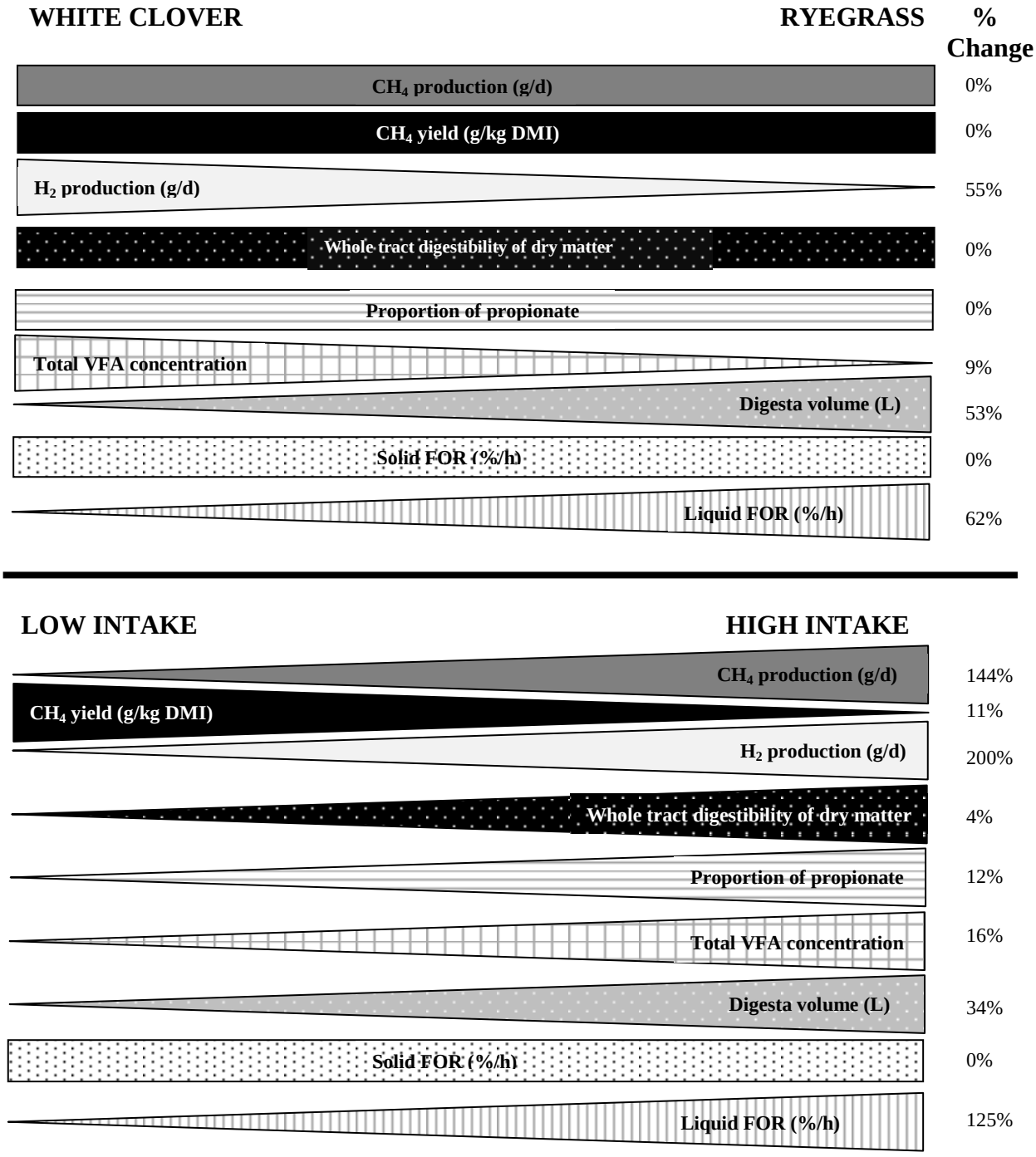


TABLE 7.9 Effect of feed intake on whole tract and rumen digesta kinetics, based on sheep fed either white clover or ryegrass forages in Experiments 4 and 5.

Diet	Feed offered x ME_m	Dry matter digestibility (%)	Whole tract				Rumen			
			Solids		Liquid		Liquid			
			MRT (h)	*k* (%/h)	MRT (h)	*k* (%/h)	MRT (h)	*k* (%/h)	Volume (L)	Outflow (ml/h)
Ryegrass (Expt. 4)	1.60	72.7	24.8	5.66	10.5	9.89	7.30	14.4	6.05	838
White clover (Expt. 4)	1.60	72.5	27.7	4.25	16.8	6.12	7.10	14.5	3.96	555
Ryegrass (Expt. 5)	0.80	62.5	34.6	3.08	28.0	3.72				
Ryegrass (Expt. 5)	1.20	63.4	35.5	3.01	24.2	4.47	14.9	7.6	7.14[a]	526
Ryegrass (Expt. 5)	1.60	64.1	35.0	2.88	16.5	6.57				
Ryegrass (Expt. 5)	2.00	62.3	35.9	2.99	14.7	7.14	9.68	11.1	9.48[a]	1029
Ryegrass (Expt. 5)	2.50	64.8	34.0	3.13	12.1	8.38				

[a] values are possibly over-estimated because marker was diluted through both increased pool size (during eating) as well as normal outflow post-marker administration

ME_m, metabolisable energy requirements for maintenance; Expt., experiment; MRT, mean retention time; *k*, fractional outflow rate

7.4.1 Gas emissions and digesta kinetics with feed intake

Sheep with high intakes of ryegrass forages had an 11% lower CH_4 yield than sheep fed low intakes, but the whole tract MRT of solids was not affected by intake. The increase in feed intake increased the FOR of the liquid fraction by 125%, and the H_2 production measured from the breath was higher, compared to animals fed at a lower intake (Figure 7.1). The hypothesis was partly proven because the FOR of liquid but not solids from the rumen was associated with a change in CH_4 yield. The methanogens in the rumen are essential for maintaining a low H_2 partial pressure, and H_2 produced during fermentation is used as an energy source by methanogenic archaea which produce CH_4 (Wolin *et al.*, 1997). The presence of respired H_2 indicates a high partial pressure, and presumably a high concentration of dissolved H_2 (Janssen, 2010), which would favour fermentation pathways that produced less H_2 per unit of feed fermented (e.g. propionate) and lower CH_4 yield. Propionate formation results in a net utilisation of H_2, with less available for CH_4 formation. A rumen environment resulting in a high H_2 concentration (e.g. white clover diets, or a high intake of ryegrass; Figure 7.1) is expected to yield high VFA concentrations and a higher molar proportion of propionate, compared to a slower fermentation or lower intake.

The growth rate of methanogens in the rumen and H_2 concentrations are dynamically linked through both methanogen kinetics and thermodynamics of rumen fermentation. Janssen (2010) postulated that a high passage rate would decrease CH_4 yield in response to H_2 concentrations, which in turn would affect the fermentation of feed.

Pinares-Patiño *et al*. (2003c) reported a significant relationship between CH_4 production (g/d) and both OMI and rumen OM pool size. They suggested little change in MRT of the solid (OM) fraction with increasing intakes. Similar whole tract MRT (and *k*) values for sheep fed ryegrass forages at a range of intakes (Experiment 5) supports the concept of increasing the rumen solids pool size in response to increasing intakes, especially as digestibility did not change in relation to intake (Table 7.9). Although the total amount of solids flowing through the gastro-intestinal tract (GIT) increased with intake, the exposure to digestion was similar for all intakes, which is in marked contrast to the digesta liquid fraction, where the FOR increased from 3.7 to 8.4%/h with increasing intake. The faster liquid outflow rate supports the concept of reduced CH_4 yields as reviewed by Janssen (2010), and the three-fold increase in DMI was associated

with a reduction in CH_4 yield from 27.0 to 23.9 g/kg DMI. In addition, molar percentages of propionate were higher in sheep with high feed intakes compared to low intakes (18.7% vs. 16.6%, respectively).

When feed intake increases there is more substrate entering the rumen, an increase in rumen digesta load (Ulyatt *et al.*, 1986, Waghorn *et al.*, 1986, Pinares-Patiño *et al.*, 2003c), and more substrate is available for microbial colonisation. However, the dynamics of particle size distribution and forage cell rupture is likely to differ with feed intakes. High intakes result in less time is spent chewing per unit of DMI, which will affect the rate and extent of cell contents release, particulate surface area, and rate of digestion (Ulyatt *et al.*, 1984). Although findings presented here show liquid outflow appears more strongly related to CH_4 yield than digestion of solids, questions remain about the amount of 'solid' passing with the liquid fraction. Flow rates of solid and liquid fractions vary with diet and feed intake. The liquid FOR from the rumen is considerably higher than that of DM (Egan and Doyle, 1984, Ulyatt *et al.*, 1984), even though very small particles flow at a similar rate to water (Ulyatt *et al.*, 1986). Waghorn *et al*. (1986) showed that 30 to 50% of rumen DM in sheep fed a lucerne chaff diet was small enough to pass a sieve with a 0.25 mm aperture and the proportion of DM in this fraction appeared to be higher in animals given fresh, compared to dry, feeds (Ulyatt *et al.*, 1986). Some of the DM able to pass a 0.25 mm sieve will pass out of the rumen with the liquid fraction, and future experiments could measure the association of solid fraction markers with particle size fractions in faeces. In Experiments 4 and 5 the Cr marker was associated with NDF, and this would have been more representative of cell wall than total DM.

The rate of outflow (*k*) may have a greater effect on methanogenesis than the extent of digestion, because outflow could affect both the activity and populations of methanogens and other microflora. An understanding of the location of methanogens in the rumen, the outflow of liquid and solid fractions from the rumen, and the efficacy of markers; i.e. what DM is labelled by 'solid phase' markers, may also affect the interpretation of data. Morgavi *et al.* (2010) showed methanogens are found in the rumen liquid and on protozoa, and are associated with the rumen epithelium, but most are associated with particulate DM fractions. Rumen microbes that do not adhere to particles must grow at a faster rate to maintain themselves in the rumen, because the

liquid outflow is more rapid than solids, and if the growth rate is not sufficient the species would disappear (Janssen, 2010). Van Soest *et al.* (1988) predicted an increased FOR of digesta in the rumen would increase microbial efficiency because of the decrease in microbial maintenance requirements relative to growth, resulting in an increased production of microbial cells and VFAs.

Changes in digesta parameters associated with an increase in feed intake have been reported in deer. Deer have seasonal cycles in voluntary feed intake (VFI) which are associated with their seasonal cycle of growth, with marked increases in the summer compared to the winter (Domingue *et al.*, 1991). In deer, the 35% increase in VFI during summer resulted in a 51% increase in rumen digesta load, with no affect on solids MRT or fibre digestibility, when compared to winter (Domingue *et al.*, 1991). This variation in rumen digesta kinetics corresponds with effects of feed intake in the sheep measured here. Observations of liquid FOR and CH_4 emissions in cattle (Okine *et al.*, 1989) also support measurements made here. They added inert material to the rumen to reduce the volume by 8.5 L and this increased solid and liquid outflow rates by 63% and 43%, respectively, with a 29% decrease in CH_4 production.

Stanier and Davies (1981) used a continuous fermentation system and showed a change in liquid FOR affected CH_4 production, with an effect on net fermentation, in particular, total VFA production and yield of microbial matter. The difference in CH_4 emissions between individuals fed high and low intakes in this study may have affected fermentation in the rumen, but similar apparent digestibilities suggested any changes had been compensated by digestion in the hindgut.

Future opportunities to prove the relationship between rumen liquid FOR and methanogenesis could utilise intra-ruminal infusions of either artificial saliva (4 L/d) or artificial saliva containing 4% or 8% polyethylene glycol (PEG). This has been shown to increased liquid FOR (%/h) from 0.060 (control sheep) to 0.109 to 0.117 and 0.140, respectively (Harrison *et al.*, 1975 and 1976). Infusion of water was shown to not affect liquid FOR, and the addition of PEG to saliva was to increase the osmotic pressure of the rumen liquor without increasing its ionic strength.

7.4.2 Methane emissions and digesta kinetics with diet

There was no difference in CH_4 yield, whole tract or rumen solids digesta kinetics for animals fed white clover or ryegrass forages in Experiment 4. The only effect of diet was a lower rumen volume, a lower rumen outflow (but similar FOR), and a longer whole tract liquid MRT in sheep fed white clover, compared to ryegrass with a similar digestibility. The two-fold higher NDF concentration in ryegrass, compared to white clover, and the need for rumination to reduce particle size of NDF (33 vs. 13 min/100 g, respectively; Moseley and Dellow, 1985), is likely to have increased saliva production, which in turn would contribute to a larger rumen pool size and be partly responsible for the increase in liquid FOR of ryegrass compared with white clover. Different patterns of rumen liquid FOR with grass and legume forages have also been reported by Waghorn *et al.* (1989), who fed cows freshly cut ryegrass and lucerne (*Medicago sativa*) forages. They found rumen liquid outflow after feeding was 10.2 L/h for ryegrass diets, and 6.0 L/h for lucerne.

The lack of difference in solid FOR or DDM between white clover and ryegrass diets was unexpected, because white clover is often associated with faster rates of particle breakdown and passage out of the rumen, higher digestibility and degradation, and a faster reduction in size of particulate DM in the rumen compared to grasses (Ulyatt, 1969, Moseley, 1981, Moseley and Jones, 1984, Beever and Thorp, 1996). It appears that the lignification of ryegrass fed in Experiment 4 was insufficient to affect retention time in the GIT (and probably the rumen) at intakes of 1.6 x ME_m, and the difference between feeds in RFC:NDF ratios were not an important influence on methanogenesis.

Janssen (2010) suggested the high fibre content of ryegrass would decrease solids passage rate and result in lower rumen H_2 concentrations, favouring the formation of H_2 and result in higher molar proportions of acetate and butyrate, less propionate, and a higher CH_4 yield, compared to white clover. However, the comparison in Experiment 4 resulted in similar solid FOR and digestibility for both diets, and the only difference was the higher rumen liquid outflow for ryegrass (Figure 7.1).

Ryegrass forage is affected by lignification and tensile strength (Evans, 1964), and this usually results in a high number of chews and a low rate of eating compared to legumes (Ulyatt *et al.*, 1986). So, differences in the physical structure of the white clover (not

necessarily indicated by chemical composition *per se*) are likely to result in a rapid degradation and a low proportion of large particle DM in the rumen. It is important to realise that forages such as white clover have a low percentage of cell walls, relative to grasses, and irrespective of the dynamics and efficacy of chewing, less cell wall material will be available in the particulate fraction for microbial (including methanogen) adhesion. Rumen particle size and liquid dynamics are complicated and perhaps best understood through simulation modelling (Baldwin *et al.*, 1977).

If white clover is rapidly degraded in the rumen, pathways would favour a rapid fermentation, a decrease in H_2, and formation of propionate. In this study, sheep fed white clover had higher total VFA concentrations (96.9 vs. 88.0 mM for ryegrass), suggesting a rapid fermentation, and more H_2 appeared in the respired gas (0.11 vs. 0.05 g H_2/d, respectively), but molar proportions of propionate and CH_4 yields were similar (Figure 7.1). One explanation for similar values, despite differences in liquid digesta kinetics is that a lesser proportion of digestion occurred in the rumen, with more post-ruminally when white clover was fed, and this could improve the energy capture relative to ryegrass (Ulyatt and MacRae, 1974). Whole tract digesta kinetics did not discriminate between ruminal and intestinal processes and this could be an important aspect of future research.

7.4.3 Digestibility

The absence of consistent effects of feed intake on digestibility was unexpected because digestibility of forages with a high fibre concentration are assumed to diminish as intakes increase (Hungate, 1975), sometimes in association with a decrease in rumen solids MRT (Woods *et al.*, 1999). However, effects of intake on digestibility has been more pronounced for rations containing high proportions of concentrate as opposed to forage diets (Andersen *et al.*, 1959). Dellow *et al*. (unpublished) found no effect of intake (from 0.50 to 1.50 kg DM/d) of either fresh white clover, immature or mature ryegrass fed to sheep on digestibility of DM, OM, or NDF for either of the diets. Data from Experiment 5 were similar, and the three-fold increase in intakes resulted in similar whole tract solids MRT and FOR, suggesting an increase in rumen digesta volume (Table 7.9).

Blaxter and Clapperton (1965) showed an increased digestibility reduced CH_4 yield when intakes were considerably greater than ME_m, and Johnson *et al.* (1994) found a very weak relationship (R^2=0.05) between diet digestibility and CH_4-E lost as a proportion of GEI. The absence of a relationship between CH_4 yield, feed intake and DM digestibility of sheep fed ryegrass forages have also been reported by Sun *et al.* (in publication) with sheep fed at 1.3 vs. 2.2 x ME_m, and Molano and Clark (2008) who fed sheep at 0.75 and 2.0 x ME_m. Hence, it appears that digestion of ryegrass forages differs from those based on grains and silages, where increasing intakes both lower digestibility and reduce CH_4 yield. With ryegrass forages, CH_4 yield is reduced, but this is a function of digesta kinetics, rather than whole tract digestibility.

7.4.4 Methane emissions and variation

Variation in CH_4 yield was observed between experiments. Sheep fed ryegrass at 1.6 x ME_m in Experiment 4 had a lower CH_4 yield compared to sheep fed ryegrass at a similar feed intake in Experiment 5 (22.0 vs. 25.2 g/kg DMI, respectively). The ryegrass fed in Experiment 5 was a lower quality compared to that fed in Experiment 4; characterised by a lower RFC:NDF ratio, higher lignin concentration and lower digestibility (Tables 7.4 to 7.6). Chaves *et al.* (2002) has shown that the principle consequences of increased ryegrass maturity were slower degradation rates, and it is likely that the ryegrass fed to sheep in Experiment 5 was more resistant to particle breakdown in the rumen, compared to that fed in Experiment 4. This was reflected by longer whole tract solids and liquid MRT, and slower passage rates for sheep fed ryegrass at 1.6 x ME_m in Experiment 5, compared to sheep fed ryegrass at the same intake in Experiment 4 (Table 7.9). This supports the observation of Pinares-Patiño *et al.* (2003c); that MRT and proportion of NDF in the diet are main factors responsible for variation between sheep in CH_4 yields.

7.4.5 Technique for estimating rumen liquid digesta kinetics

In this study, two techniques were used for estimating rumen liquid digesta kinetics: administration of liquid marker and sampling directly from the rumen via cannulae (Experiment 4); and stomach tubing to administer both the liquid marker and sample

rumen contents (Experiment 5). Although rumen liquid pool sizes appeared to be over-estimated in Experiment 5, compared to Experiment 4, this was possibly due to the differences in feeding regimes, which altered the volume and flow of digesta (Table 7.9). Sheep in Experiment 4 were fed hourly to achieve 'steady state' conditions, and it is likely that the twice daily feeding in Experiment 5, when markers were administered during eating, may have resulted in an increased rumen pool size and caused a rapid dilution of the marker, overestimating rumen liquid FOR as well.

Despite problems associated with twice daily feeding, the use of stomach tubing (Experiment 5) to estimate rumen liquid kinetics appeared to be successful. Sensible data were acquired, and sheep fed at higher intakes (2.0 x ME_m) had a faster liquid FOR, shorter MRT and a greater rumen volume than sheep fed at lower intakes (1.2 x ME_m) (Table 7.9). These data correspond with measurements of whole tract liquid flow, and suggest rumen liquid digesta kinetics can be estimated from intact sheep using stomach tubing, but future consideration needs to be given to the timing of marker administration relative to feeding. Use of frequent feeding to achieve a 'steady state' remains the method of choice for estimating digesta kinetics.

7.5 CONCLUSION

When sheep increased their intakes of ryegrass forages, CH_4 yields declined, and this was associated with an increase in whole tract liquid passage rates. The measurements undertaken here have not demonstrated a causative association between CH_4 yield and liquid passage, but the increased intakes were not related to a change in either digestibility or MRT of the solids fraction of digesta. Furthermore, increased intakes (and whole tract liquid FOR) resulted in higher H_2 emissions, greater concentrations of VFAs and molar proportions of propionate, all of which have been linked by thermodynamics affecting methanogen activity to a reduction in CH_4 yield. It is suggested that the higher FOR (shorter MRT) of the liquid fraction of digesta can reduce methanogenesis when forages are fed, especially at high intakes. Future work may investigate the consequences of increasing liquid FOR (i.e. infusions of artificial saliva containing PEG) whilst maintaining intakes, on the end products of fermentation and methanogenesis.

Data from the research presented here, suggest that the rate of liquid flow through the digestive tract will have a greater effect on methanogenesis than either the chemical composition of forages or the extent of digestion. It is assumed that the effects of intake, liquid flow and CH_4 yield are associated primarily with rumen activity, because this is the site of most methanogenesis and about 60% of digestion, but intestinal contributions to these relationships should not be discounted (e.g. hindgut fermentation). A few measurements of rumen liquid kinetics have been presented here, and more need to be conducted to define the effects of intakes on liquid outflow kinetics, especially in relation to the activity and populations of methanogens and other microflora.

It is suggested that a high liquid FOR could reduce the amount of digestion taking place in the rumen and affect methanogenesis. This is based on the nutrient DM flowing with the liquid fraction, comprising dissolved (soluble) DM and small particles, less than 0.25 mm in size. Dry matter passage in the liquid fraction may be greater in animals fed at high intakes compared to low intakes, but also may account for the lack of difference in CH_4 emissions when diets differing in chemical composition are fed. Although sheep fed ryegrass forages had a higher liquid FOR than sheep fed white clover, the physical structure and low NDF content of white clover is likely to affect a rapid degradation, resulting in a large proportion of soluble matter (less than 0.25 mm) able to pass out of the rumen. Further work is needed to determine the association of the marker with particle size fractions and degradation rates, which affect digestion and methanogenesis.

An important benefit of high feed intakes is a reduction in emissions intensity (Ei) due to a decrease in CH_4 yield, a reduction in the proportion of feed used for ME_m and high levels of animal production.

APPENDIX 7

APPENDIX 7.1 Intakes, digestibility, gas emissions and rumen measurements[a] from sheep fed either white clover or perennial ryegrass forages at 1.6 x metabolisable energy requirements for maintenance (ME_m), with and without an intra-ruminal water balloon in Experiment 4.

	Diet				**Rumen fill**			
	White clover	Perennial ryegrass	P-value	SED	Balloon	Control	P-value	SED
Number of animals	4	4			4	4		
Number of periods	2	2			2	2		
Intake (kg/d)								
OMI	0.82	0.84	0.008	0.005	0.83	0.83	0.659	0.010
NDFI	0.25	0.43	<0.001	0.002	0.34	0.34	0.787	0.009
Digestibility (g/kg)								
DOM	795	751	0.011	12.18	773	774	0.965	18.54
DNDF	532	674	<0.001	20.80	610	596	0.795	50.40
Methane emissions								
g CH_4/kg OMI	31.3	29.3	0.149	1.199	30.0	30.6	0.536	0.971
g CH_4/kg DDMI	39.0	36.5	0.045	1.005	37.1	38.4	0.404	1.363
g CH_4/kg DOMI	39.4	39.0	0.765	1.069	38.8	39.6	0.464	1.069
CH_4-E/GEI	0.087	0.085	0.548	0.003	0.085	0.087	0.555	0.003
% of total VFA								
Valerate	1.13	0.81	0.001	0.057	0.94	1.01	0.134	0.043
Isobutyrate	1.25	0.60	<0.001	0.049	0.93	0.91	0.840	0.101
Isovalerate	1.32	0.46	<0.001	0.050	0.89	0.89	0.978	0.127
Ratios								
A:P	3.37	3.49	0.533	0.175	3.45	3.40	0.765	0.156
A + B/P	3.99	4.08	0.713	0.218	4.05	4.01	0.855	0.208

OMI, organic matter intake; NDFI, neutral detergent fibre intake; DOM, digestible organic matter; DNDF, digestible neutral detergent fibre; CH_4, methane; DDMI, digestible dry matter intake; DOMI, digestible organic matter intake; CH_4-E/GEI, methane energy as a proportion of gross energy intake; VFA, volatile fatty acid; A:P, acetate to propionate ratio; A + B/P, acetate + butyrate/propionate; SED, standard error of the difference of the mean

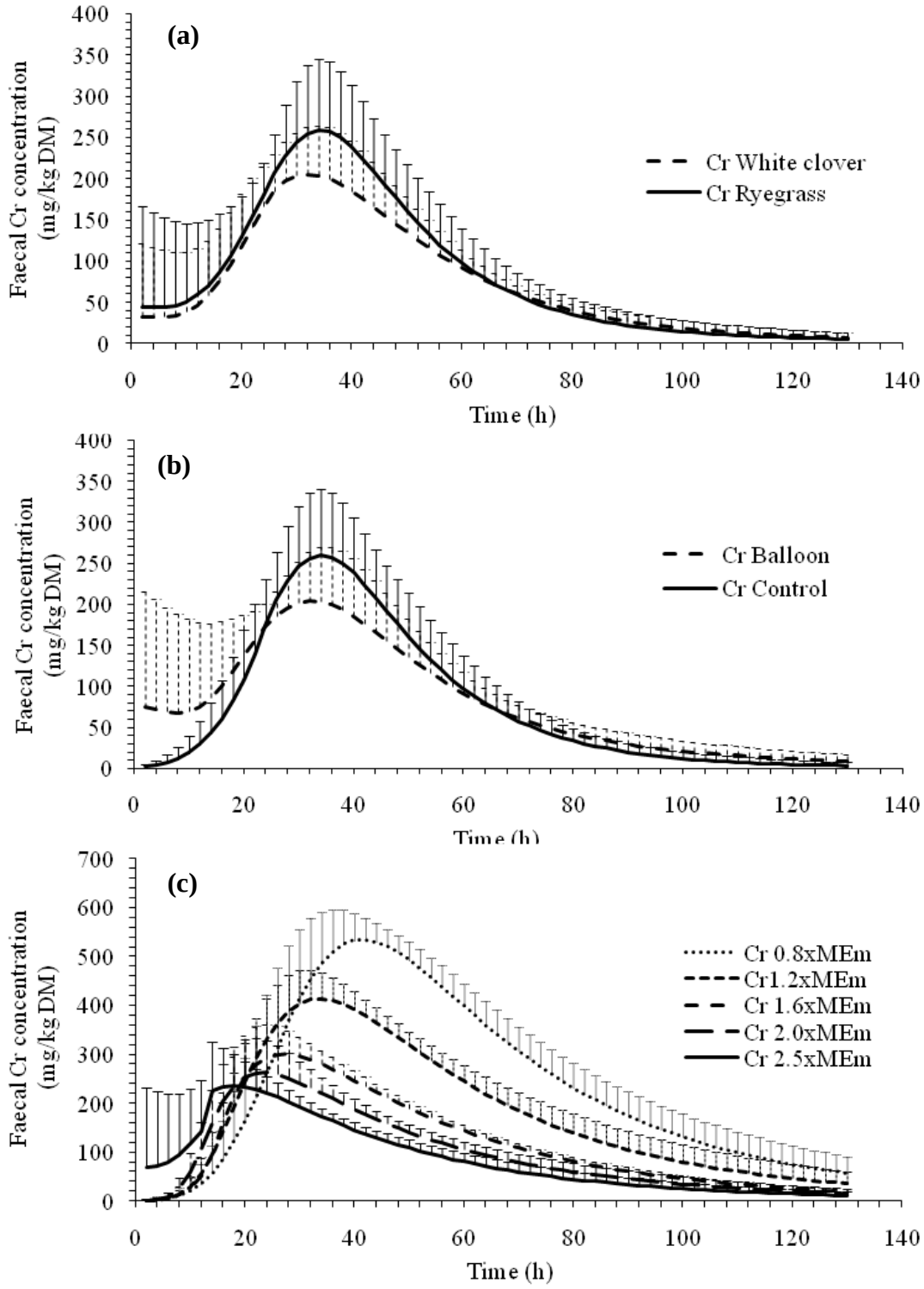
[a] Means have been derived from individual data and values presented here may not appear compatible

APPENDIX 7.2 Intakes, digestibility, gas emissions and rumen volatile fatty acids (VFAs)[a] from sheep fed perennial ryegrass forages at five feed intakes ranging from 0.8 to 2.5 x metabolisable energy requirements for maintenance (ME_m) in Experiment 5.

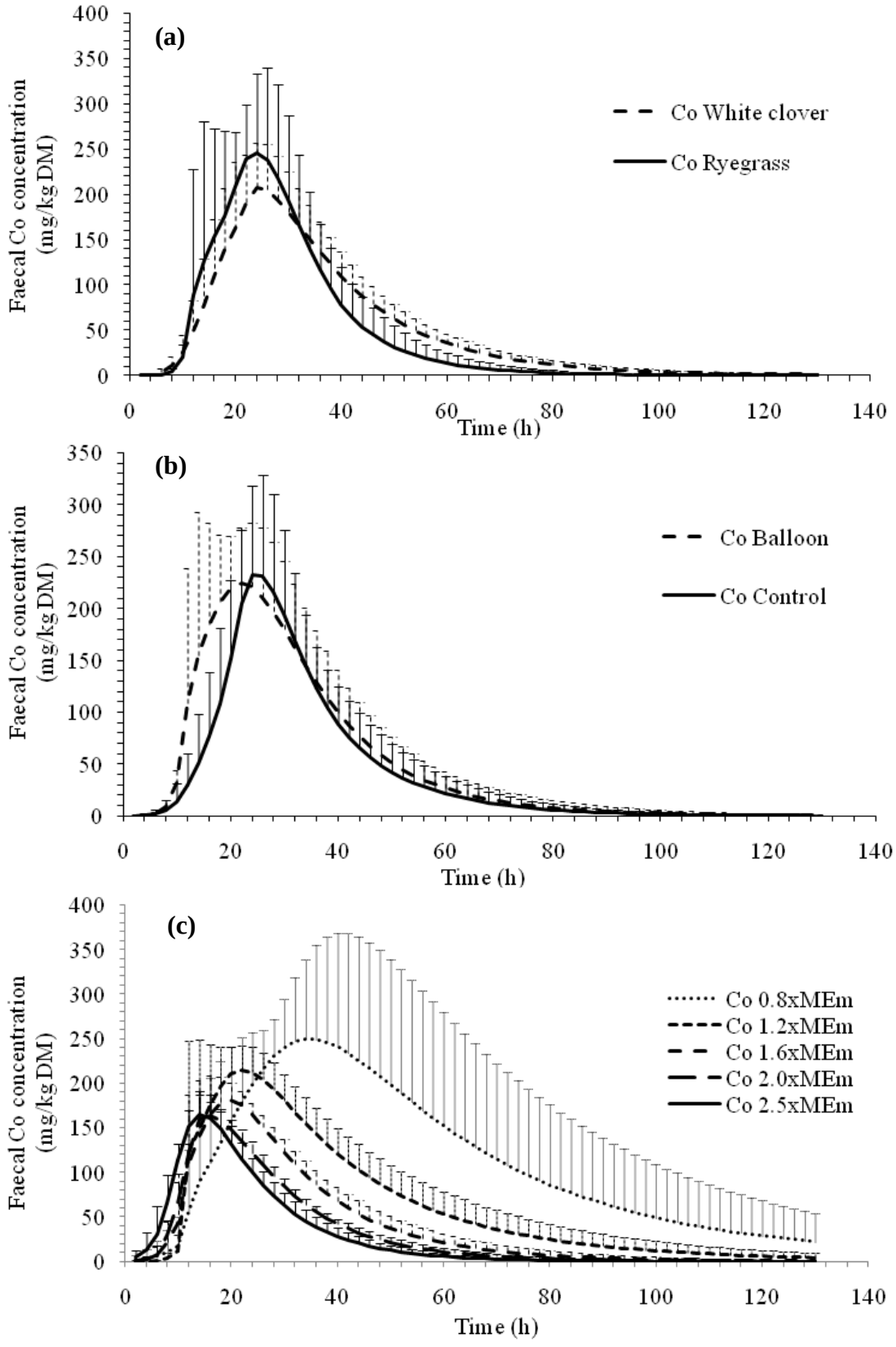
	Feed offered as a multiple of ME_m					P-value	SED
	0.8	**1.2**	**1.6**	**2.0**	**2.5**		
Number of animals	6	6	6	6	6		
Number of periods	1	1	1	1	1		
Intake (kg/d)							
OMI	0.44	0.67	0.90	1.13	1.35	<0.001	0.013
NDFI	0.27	0.41	0.54	0.68	0.80	<0.001	0.009
Digestibility (g/kg)							
DOM	676	681	683	674	693	0.088	6.900
DNDF	604	614	622	609	630	0.106	9.920
Methane emissions							
g CH_4/kg OMI	30.0	29.2	25.8	24.1	23.6	<0.001	0.835
g CH_4/kg DDMI	42.5	40.6	35.7	34.5	32.7	<0.001	1.223
g CH_4/kg DOMI	44.4	42.8	37.8	35.7	34.1	<0.001	1.244
CH_4-E/GEI	0.079	0.077	0.068	0.064	0.063	<0.001	0.002
% of total VFA							
Valerate	0.65	0.69	0.72	0.65	0.68	0.268	0.037
Isobutyrate	1.00	0.90	0.87	0.79	0.73	0.009	0.071
Isovalerate	1.10	0.89	0.96	0.78	0.76	0.002	0.080
Ratios							
A:P	4.15	3.84	3.49	3.95	3.61	0.008	0.179
A + B/P	4.77	4.42	4.10	4.51	4.18	0.014	0.198

OMI, organic matter intake; NDFI, neutral detergent fibre intake; DOM, digestible organic matter; DNDF, digestible neutral detergent fibre; CH_4, methane; DDMI, digestible dry matter intake; DOMI, digestible organic matter intake; CH_4-E/GEI, methane energy as a proportion of gross energy intake; A:P, acetate to propionate ratio; A + B/P, acetate + butyrate/propionate; SED, standard error of the difference of the mean

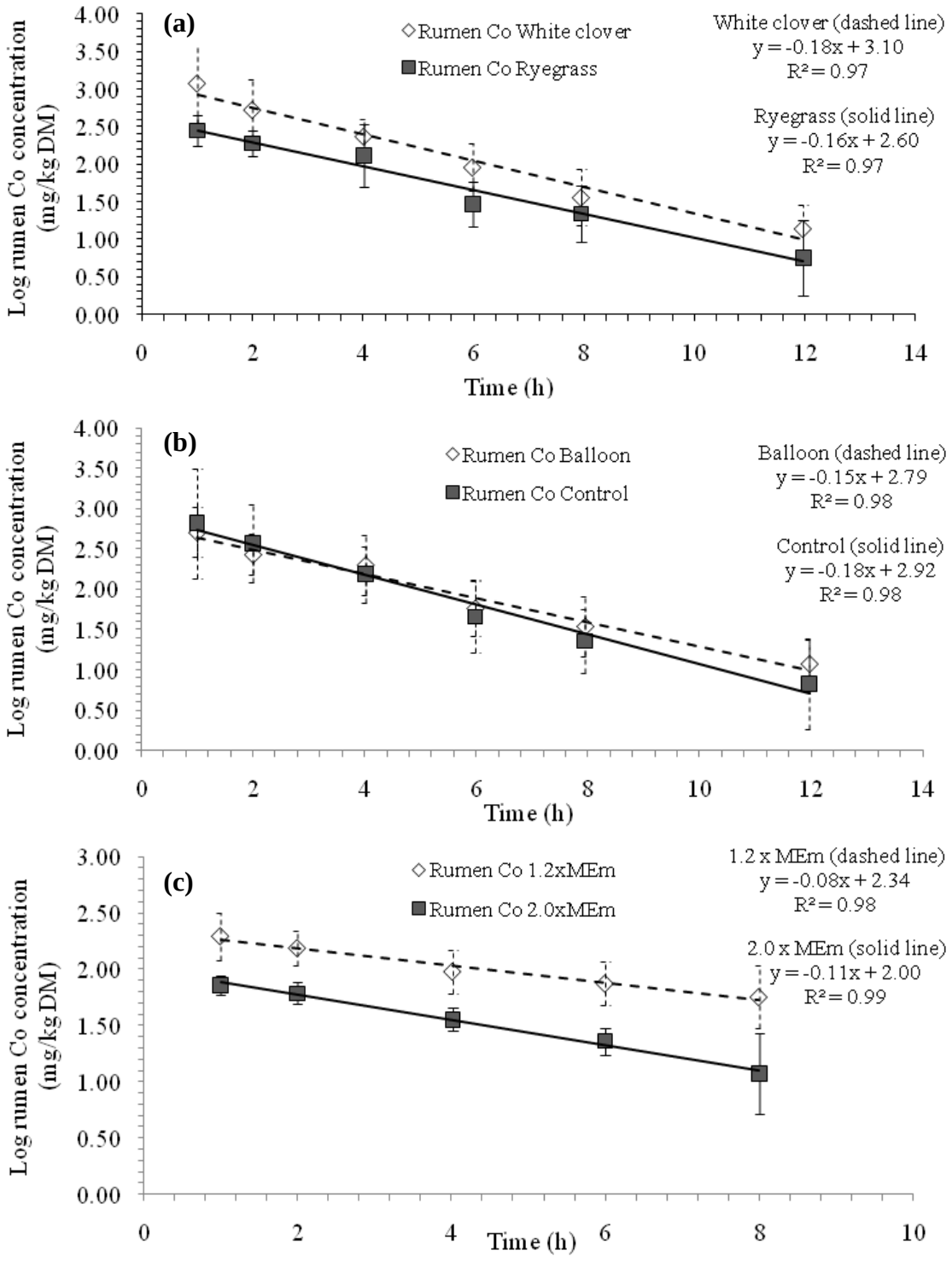
[a] Means have been derived from individual data and values presented here may not appear compatible



APPENDIX 7.3 Predicted faecal chromium (Cr) concentrations over time, used to determine whole tract digesta kinetics of solids from sheep in comparisons of (a) white clover vs. ryegrass, (b) rumen fill affected by insertion of a balloon and (c) ryegrass intakes. Vertical lines are SD from 6 to 8 measurements at each time.



APPENDIX 7.4 Predicted faecal chromium (Co) concentrations over time, used to determine whole tract digesta kinetics of liquids from sheep in comparisons of (a) white clover vs. ryegrass, (b) rumen fill affected by insertion of a balloon and (c) ryegrass intakes. Vertical lines are SD from 6 to 8 measurements at each time.



APPENDIX 7.5 Predicted rumen cobalt (Co) concentrations over time from sheep used to determine pool size and outflow for treatments comparing (a) white clover vs. ryegrass, (b) rumen fill (presence or absence of a 1L water filled balloon), in Experiment 4, and (c) evaluating stomach tubing in intact sheep fed at two feed intakes in Experiment 5. Vertical lines are SD from 6 to 8 measurements at each time.

CHAPTER 8

GENERAL DISCUSSION

CHAPTER 8: GENERAL DISCUSSION

8.1 GENERAL DISCUSSION

This study initially focused on white clover (*Trifolium repens*) and perennial ryegrass (*Lolium perenne*; ryegrass) forages because they are the dominate species grazed by ruminants in New Zealand. Based on previous work, it was initially hypothesised that white clover forages would result in lower methane (CH_4) yields (g/kg dry matter intake (DMI)) when fed to sheep than ryegrass. However, early measurements from experiments conducted for this thesis showed there was little difference in CH_4 emissions from sheep fed the two forages. The focus of the thesis was changed to better understand the variation in CH_4 emissions from ruminants fed either white clover or ryegrass forages. It was hypothesised that variation in CH_4 emissions from ruminants could be partly predicted by diet chemical composition, rumen function and feed intake. The research presented in this thesis has addressed these hypotheses and a general discussion of the outcomes is given here.

8.1.1 The importance of ruminants fed fresh forages and methane emissions

Livestock farmers are unlikely to adopt strategies to mitigate greenhouse gas (GHG) emissions if production or profitability is reduced. The objective of research to reduce emissions from ruminants must take into account their importance for food production. By 2050, the human population is predicted to reach 9 billion and the demand for livestock products is expected to double (United Nations, 2009). The concept of feeding fresh forages to ruminants is important, because forages are able to sustain livestock, and ruminants should not compete directly with humans for the same food source (Garnett, 2009). The ability of grazing livestock to turn human inedible products into human edible products may become increasingly important in terms of global food security, because there is 3.4 billion ha of grazing land and only 1.5 billion ha of cropping land worldwide (FAOSTAT, 2010). Previous reports of CH_4 yields have shown substantial variation in emissions between diets, with lower values reported from

legumes than grasses. This variation suggested a practical potential avenue for mitigating CH_4 emissions from ruminants, particularly as the New Zealand grazing system tends to have fewer inputs, lower costs and fewer options for GHG mitigation than intensive animal production industries (Eckard *et al.*, 2010).

8.1.2 Measurements and techniques

This study used respiration chambers for measuring CH_4 emissions, which have the advantage of direct and accurate gas measurements, and enable accurate measurement of feed intakes. However, respiration chambers also confine animals and alter their behaviour, compared to their 'normal' production environment (McAllister *et al.*, 2011), and this is especially true for pastoral grazing. The sulphur hexafluoride (SF_6) tracer technique is less intrusive for CH_4 determinations and has the advantage in that it can be used to estimate emissions from both grazing and confined animals. However, recent evaluations have challenged the precision of the SF_6 technique for estimating CH_4 emissions (Vlaming *et al.*, 2005, McGinn *et al.*, 2006, Vlaming *et al.*, 2007, Pinares-Patiño and Clark, 2008, Pinares-Patiño *et al.*, 2011a), and intakes of animals grazing mixed pastures cannot be measured accurately. Chapter 4 was a CH_4 database analysis which compared SF_6 and respiration chamber data from sheep fed measured amounts of fresh ryegrass from several unrelated experiments. Although mean CH_4 yields were not affected by technique, direct measurements derived from respiration chambers had less variation and were more precise than those from SF_6 tracer technique (23.1 ± 2.90 versus 23.4 ± 5.70 g CH_4/kg DMI, respectively), suggesting the SF_6 technique contributed to the variation in CH_4 emissions.

The comparisons undertaken in Chapter 4 resulted in all CH_4 measurements for the animal trials conducted in this study being made using respiration chambers. This was because the focus was to understand the relationships between diets and the mechanisms responsible for changes in CH_4 production and yield. The dynamic nature of CH_4 emissions, especially variation associated with feeding can provide additional information to assist with interpretation of the causes of variation, and this is not available from daily CH_4 totals determined by the SF_6 technique.

8.1.3 Diet chemical composition and methane emissions

An assessment of diet chemical composition on CH_4 emissions was warranted because forage composition and quality affect feed intake and determine the feeding value of the forage. Feeding value determines the potential for production and profitability for farmers. When this thesis was started, most published CH_4 measurements were based on dried and ensiled diets fed to sheep or cattle. The objective of Chapter 4 was to relate CH_4 emissions from individual animals with measured intakes of ryegrass forages to chemical composition. Expectations of causative relationships between CH_4 yield and diet composition were based on previous work (Waghorn *et al.*, 2002, Krause, AgResearch Report), where estimates of CH_4 yield by the SF_6 technique were lower from fresh legumes and a herb (lotus major, *Lotus pedunculatus*; lucerne, *Medicago sativa*; sulla, *Hedysarum coronarium*; white clover, and chicory, *Cichorium intybus*) compared to fresh ryegrass. It was hypothesised that the variation in CH_4 emissions with diet type could be attributed to differences in diet chemical composition, which affects the end products of fermentation and CH_4 production.

The database evaluated in Chapter 4 included measurements using ryegrass forages with a wide range in chemical composition (g/kg DM) e.g. 396 - 626 neutral detergent fibre (NDF); 71 - 398 non-fibre carbohydrate (NFC), and CH_4 yields (10 - 38 g/kg DMI from SF_6 determinations and 15 - 32 g/kg DMI from respiration chamber measurements). However, regression analyses of variation in CH_4 yield from sheep showed ryegrass chemical composition accounted for <2% of the variation in CH_4 yield determined by SF_6, and only 20% of variation based on data from respiration chambers. Only 13% of the variation in CH_4 yield was predicted by the composition of ryegrass fed to cattle. Variation in CH_4 yield was best predicted by DMI, which accounted for up to 36% of the variation.

Analyses based on measurements undertaken in three separate experiments summarised in Chapter 5, showed similar CH_4 yields for sheep consuming white clover and ryegrass forages (22.6 g/kg DMI), despite ryegrass containing 50% more NDF, 80% less pectin, and 40% less crude protein (CP) in the DM, than white clover.

When values from previous work with alternative fresh forages (Waghorn *et al.*, 2002, Krause, AgResearch Report) to ryegrass were adjusted for the reduction in CH_4

associated with intakes above metabolisable energy requirements for maintenance (ME_m) (Chapter 5), differences between forages were less apparent, and they appeared to be in part due to the higher intakes of white clover, compared to ryegrass forages.

The overall conclusion, based on all analyses, suggested chemical composition of DM in ryegrass forages had little effect on CH_4 yield from sheep, and there are no simple relationships between chemical components of fresh forages and CH_4 yield. Other factors affecting digestion or retention time, such as the degree of lignification of plant cell walls or presence of secondary compounds (e.g. condensed tannins), may have greater effects on CH_4 emissions than diet chemical composition.

8.1.4 Feed intake, digestibility and methane emissions

Published analyses with animals fed conserved feeds (e.g. Blaxter and Clapperton, (1965), Johnson *et al.,* (1993), Beauchemin and McGinn, (2006), Yan *et al.,* (2010)) show an inverse relationship between CH_4 yield and feed intake. However, the relationship between feed intake and CH_4 yield is poorly understood, and at the commencement of this thesis there was a lack of data investigating the effects of fresh forage intake on CH_4 yield. It was hypothesised that increasing intakes of white clover and ryegrass forages would decrease CH_4 yield, and that whole tract digestibility, fermentation end products and methanogen community composition would account for the effects of feed intake on CH_4 emissions from sheep.

Some analyses undertaken in this thesis have expressed intakes in terms of ME_m because this takes into account variations in animal size (although all sheep used here had a similar live weight) and diet quality. Expression of CH_4 emissions in relation to ME_m also enabled comparisons with published data presented in this form. When intakes of white clover and ryegrass forages were similar, so were CH_4 yields, whole tract digestibility and molar percentages of most volatile fatty acids (VFA) (Chapter 5).

In Chapters 4 and 6, the average decline was 3.19 and 3.47 g CH_4/kg DMI, respectively, for each multiple of ME_m increase in feed intake, above ME_m, for sheep fed white clover and ryegrass forages, which is similar to reports for cattle fed grain/silage diets (Yan *et al.*, 2010).

In Chapter 6, as intakes of white clover and ryegrass increased four-fold (0.40 to 1.60 kg DM/d), CH_4 yield declined by 21%, and there was no change to whole tract apparent DM digestibility or methanogen community composition. However, as intakes increased, molar percentage of propionate increased from 17.3 to 21.4% and this was able to predict 60% of the variation in CH_4 yield. Increasing propionate production will utilise hydrogen (H_2), so that less will be available for methanogenesis. A reduction in CH_4 yield from sheep fed increasing intakes of ryegrass forage, independent of apparent digestibility, has also been shown by Molano and Clark (2008) and Sun *et al*. (in publication). Hence the reduction in CH_4 yield in response to increased intakes can be independent of total tract digestion.

The reduced CH_4 yield in animals with high intakes and high levels of productivity will lower the emissions/unit production. High production also 'dilutes' CH_4 emissions associated with feed requirements for maintenance, and even though the relationship between intake and CH_4 yields were similar for white clover and ryegrass forages, high quality diets will maximise both intakes and production and emissions/product.

Although the hypothesis; that increasing intakes of white clover and ryegrass forages would decrease CH_4 yield, was supported, these analyses did not explain the bases of the relationship with intake. The importance of fermentation end products (particularly propionate) were highlighted in Chapter 6 and it was postulated that an understanding of the association between feed intake and rumen fill, fermentation and digesta kinetics could give a better understanding of CH_4 emissions.

8.1.5 Digesta kinetics and methane emissions

It was hypothesised that the decrease in CH_4 yield with increasing feed intakes was attributed to lower mean retention times (MRT) of liquid and solid fractions within the gastro-intestinal tract (GIT). The physical attributes of feeds and digestive processes associated with the quantity of feed eaten can affect changes in the rumen and whole tract residence time, and the estimates of whole tract digestion kinetics in Chapter 7 suggested liquid, rather than solid fractions were associated with the decrease in CH_4 yield. When feed intakes increased from 0.50 to 1.51 kg DM/d, there was an 11%

reduction in CH_4 yield, and a 125% increase in whole tract liquid fractional outflow rate (FOR; 3.72 to 8.28%/h) (Chapter 7). Whole tract MRT of the digesta solid fraction was not affected by intake but rumen liquid pool size appeared to increase with intake, and was larger when sheep were fed ryegrass than white clover.

Increasing the flow of liquids relative to solids can increase the efficiency of microbial protein synthesis and microbial protein outflow from the rumen (Grovum, 1984), and the liquid flux carries small particulate and dissolved (soluble) DM from the rumen. If more soluble (plant cell contents) DM was flushed from the rumen to the intestines for endogenous enzymatic digestion, less would be available for fermentation and CH_4 production in the rumen. However, a reduced digestion in the rumen was not supported by the higher concentrations of VFA in rumen contents (Chapters 6 and 7) of sheep having high intakes.

Increased molar percentages of propionate in rumen digesta of sheep fed at high intakes (Chapters 6 and 7) supports the relationships between rumen outflow rates and methanogenesis proposed by Janssen (2010). One consequence of a high rumen outflow rate would be an increase in H_2 concentrations which would favour fermentation pathways that lowered H_2 production and increased propionate production, with an overall decrease in CH_4 yield (Figure 7.1). Future research should focus on the inter-relationships between rumen pool size, liquid and solid FOR, digestion (including VFA production and particle kinetics) and methanogenesis.

8.1.6 Practical application of findings to lower methane emissions

White clover and ryegrass are the two main forage species that dominate the New Zealand pastoral grazing system. Although ryegrass accounts for 80 to 90% of intakes under many situations (Waghorn and Clark, 2004), white clover is responsible for nitrogen (N) fixation (Wilkins, 2008) when urea is not applied in excess. White clover generally has a high feeding value, whereas ryegrass must be intensively managed to maintain a high feeding value. Both are relatively inexpensive as a feed resource and their robust root system provides resilience in a variable climate, prevents soil erosion, reduces nutrient leaching, and replenishes soil organic matter (Janzen *et al.*, 1998).

Despite similar CH_4 yields from sheep fed white clover and ryegrass forages, the high intakes and growth rates expected from lambs fed white clover (Ulyatt, 1981) would result in a lower emissions intensity (Ei, emissions per unit of animal product) than would be achieved from ryegrass. This is because a higher proportion of intake (and CH_4 emissions) will be directed toward growth (Ulyatt, 1981, Waghorn *et al.*, 2007), and lambs will reach slaughter weight at a younger age with less feed required (reduced Ei).

The digestibility of white clover is often higher than that fed here (73%; Chapter 5), with values of 84% reported by Ulyatt and Egan (1979) but digestibility of ryegrass can be quite variable. Lignification and senescence affects the feeding value of ryegrass, causing reductions in both intake and digestibility, and intakes of forages tend to decrease as digestibility decreases (Freer and Jones, 1984, Hegarty *et al.*, 2010). Although Johnson and Johnson (1995) found that the digestibility of the diet explained less than 5% of the variation in CH_4 yield, lower digestibility has major implications for intake and animal production, and farmers must manage pastures to maximise ruminant production.

This study was not intended to promote white clover as a preferred forage for pastoral farming, and the lack of a difference with ryegrass enables farming objectives to focus on animal production (e.g. per ha) and Ei. This is important because yields of white clover grown as a monoculture are less than ryegrass (Moot *et al.*, 2007), and white clover would not have been a viable option for farmers, even if CH_4 yields had been less than ryegrass.

It is essential that estimates of GHG emissions from farming systems have well defined assumptions, so that meaningful and honest evaluations of mitigation strategies can be made. Although GHG emissions from livestock are closely related to ruminant numbers, factors that impact on intake and productivity over an entire life cycle have the greatest effect on food production and Ei (O'Mara, 2011). The largest gains for production with reduced emissions will be associated with good animal management practice (i.e. appropriate feeding, genetics and reproductive performance), which will improve profitability and lower Ei (Waghorn and Hegarty, 2011). For example, Hegarty *et al.* (2010) reported the three most effective management strategies for

reducing CH_4 production per lamb sold were to increase the culling age of ewes (a 6.4% reduction by retaining for 6 not 5 years); increase conception rates of ewes (a 7.8% reduction per 10% unit increase), and the lambing of ewes as hoggets rather than 1 year later (a 11.7% reduction). Beukes *et al.* (2011) developed a whole farm model of a pasture-based New Zealand dairy farm to evaluate a range of GHG mitigation strategies. In order to increase milk production by 10 to 15% and decrease net GHG emissions by 15 to 20%, a combination of strategies had to be employed. These included improved reproductive performances, increased genetic merit, lower stocking rates and longer lactations, as well as the use of stand-off feeding pads, and reducing N fertiliser use. Systems modelling, in association with improved measurements and understanding of factors affecting GHG emissions, need to be evaluated on-farm to demonstrate their benefits for production, profitability and the environment. The measurements and relationships between feeding and methanogenesis developed from research undertaken for this thesis will contribute to these objectives.

8.2 CONCLUSIONS

- The initial focus of this thesis was to explore the reasons for an apparently lower CH_4 yield from sheep fed white clover compared to ryegrass. However, respiration chambers measurements showed CH_4 yields from sheep fed white clover and ryegrass was similar over a range of feed intakes.
- There was less variation in CH_4 emissions from sheep fed ryegrass forages in respiration chamber than estimates using the SF_6 technique.
- There was little direct association between chemical components of fresh forages and CH_4 yield from sheep and cattle.
- A clear relationship between DMI of sheep fed fresh forages and CH_4 yield has been established, with a decline of about 3.3 g CH_4/kg DMI for each multiple of ME_m increase in intake, above ME_m.
- The decline in CH_4 yield with increasing intakes was associated with a higher whole tract liquid, but not solids, FOR. The 125% increase in digesta liquid FOR and higher proportions of propionate in the rumen VFA, associated with a three-fold

increase in feed intake, supports theoretical drivers of methanogenesis associated with rumen digestion kinetics.

- A reduction in CH_4 yield associated with high feed intakes will achieve high levels of production and achieve substantial reductions in Ei.

8.3 RECOMMENDATIONS FOR FUTURE RESEARCH

Basic science is needed to improve the understanding of rumen ecology and animal factors that affect CH_4 emissions from ruminants. In particular, how components of rumen function, such as volume, passage rate and degradation kinetics interact with the composition of fresh forages to affect CH_4 emissions.

There are differences between animals in CH_4 yield, even when fed the same diet at the same intake, and this variability is poorly understood. Selection of divergent animals for CH_4 yield may provide a good model for measuring the impact of rumen function on methanogenesis.

Further work is required to understand the drivers of liquid FOR and effects on methanogenesis. This could include measurements of CH_4 emissions from animals in respiration chambers whilst receiving intra-ruminal infusions of artificial saliva. More information is also required about passage of 'solids' DM with the liquid fraction from the rumen and through the digestive tract, to better interpret the effects of intake on FOR. The dynamics of forage cell rupture and particle size distribution are likely to differ with feed intakes and diet quality, and could have important consequences for methanogenesis and hindgut fermentation. Future experiments could examine the association of 'solids fraction' markers with particle size fractions in the faeces.

Polymerase chain reaction-denaturing gradient gel electrophoresis (PCR-DGGE) examination of rumen methanogen community composition did not show relationships to intakes of fresh ryegrass. However, theoretical associations between methanogen growth rates and rumen outflow rates, suggests the activity and populations of methanogens and other microflora require further investigation.

ANNEX A

INTRA-RUMINAL INFUSION OF ACID OR BICARBONATE ON METHANE EMISSIONS FROM SHEEP

ANNEX A: INTRA-RUMINAL INFUSION OF ACID OR BICARBONATE ON METHANE EMISSIONS FROM SHEEP

A.1 INTRODUCTION

One factor that may account for differences in methane (CH_4) yield of individual animals is rumen pH, which is affected by diet, saliva production, ruminal fermentation patterns as well as digesta passage rates and mean retention times (MRT) (McCaughey *et al.*, 1999, O'Mara *et al.*, 2008). The optimum rumen pH for methanogens is between about 6.0 and 6.4 (Jarvis *et al.*, 2000, Rea *et al.*, 2007) and growth rates will be lower when the pH is outside of this range (Janssen, 2010). Inducing a change in rumen pH has decreased CH_4 emissions (Burrin and Britton, 1986, Van Kessel and Russell, 1996, Lana *et al.*, 1998), with methanogens losing their ability to utilise hydrogen (H_2) below a pH of 5.5, giving rise to free H_2 in the gas phase (Van Kessel and Russell, 1996).

This pilot trial was an attempt to gain insights on the effect of rumen pH on CH_4 emissions from sheep fed perennial ryegrass (*Lolium perenne*; ryegrass) forages whilst in respiration chambers. The objective was to change rumen pH by imposing a hydrogen ion (H^+) load in the rumen using intra-ruminal infusions of acid, and comparing effects on methanogenesis with infusions of sodium bicarbonate (bicarbonate) and water. The concentrations and infusion rates of the solutions were exploratory and based on similar measurements with cattle by Williams *et al.* (2004). It was hypothesised that a low rumen pH (in response to acid infusion) would reduce CH_4 yield from sheep compared higher yields due to an elevated pH (in response to bicarbonate infusion).

A.2 MATERIALS AND METHODS

This pilot trial was conducted with 4 rumen-fistulated sheep fed fresh ryegrass and given a continuous intra-ruminal infusion of either acid (hydrochloric, HCl/acetic

mixture), or bicarbonate or water. Sheep were placed in respiration chambers for measuring CH_4 as well as dry matter intake (DMI), ruminal pH, concentrations of ammonia (NH_3) and volatile fatty acids (VFAs). All procedures were reviewed and approved by the AgResearch Animal Ethics Committee in Palmerston North, New Zealand. Table A.1 illustrates the timetable of events.

TABLE A.1 Timetable of events for a pilot trial with sheep fed ryegrass forages to evaluate effects of an intra-ruminal infusion with either acid, water or sodium bicarbonate, on methanogenesis.

Pilot Trial (8^{th} to 12^{th} June 2009)	
Day 1 – 28	Four rumen-fistulated sheep had been fed ryegrass in either pens or metabolism crates.
29	Two sheep were given an intra-ruminal infusion of dilute acid for 24 h. Rumen samples collected and pH monitored.
30	Sheep were placed in individual respiration chambers for 24 h whilst acid infusion was maintained. Feed refusals and rumen samples collected.
31	Animals stayed in respiration chambers for a further 24 h and infused with water from 09:00 h. Feed refusals and rumen samples collected.
32	Infusion solution changed at 09:00 h from water to sodium bicarbonate and a further 24 h of measurements undertaken. Feed refusals and rumen samples collected.
33	Sheep released to pasture at 09:00 h.

This timetable was repeated with the remaining two rumen-fistulated sheep. The only difference was a 24 h acid infusion in respiration chambers was followed by a 24 h bicarbonate infusion (i.e. no 24 h water infusion occurred).

A.2.1 Animals, feeding and gas measurements

Four sheep with rumen-fistulae (30 mm (o.d.) cannulae; Beruc Equipment Ltd., Benoni, South Africa) were used for this pilot trial. They were two year old wethers and had an average live weight (LW) ± SD of 45.8 ± 3.24 kg.

The sheep were fed ryegrass (*cv.* Quartet) forages which were grown near Palmerston North, New Zealand, and harvested daily using a sickle bar mower (1995 S.E.P, San

Martino in Rio, Italy). Cut ryegrass was either fed on the afternoon of delivery or stored at 4ºC overnight prior to feeding. Ryegrass was offered at 1.6 x metabolisable energy (ME) requirements for maintenance (ME_m) in equal portions at 09:00 and 16:00 h. Water was available *ad libitum*. More specific details for sample collection, processing and laboratory analyses of feed offered and refused are given in Chapter 3.

Measurements of CH_4 emissions were by placing each sheep into a cattle respiration chamber, but other aspects of measurements are those detailed in Chapter 3.5. Sheep were placed in individual chambers before 09:00 h and the chambers were opened for feeding and collection of feed refusals, rumen sampling and checking infusions twice daily (09:00 and 16:00 h).

A.2.2 Acid and sodium bicarbonate solutions

Calculation of the quantity of acid to be infused was based on projected VFA production, with an intention of increasing H^+ by 10%, by acid infusion. If dry matter (DM) digestibility is 70%, and 65% of digestion takes place in the rumen, then, based on an intake of 1 kg DM/day, approximately 455 g DM will be digested in the rumen with about 66% released as VFAs (i.e. 300 g; Waghorn *et al.,* 2007). The moles (M) of H^+ associated with 300 g of VFAs were calculated (Table A.2) to be about 4.5 M from an intake of 1 kg DM.

TABLE A.2 Calculation of hydrogen ion production from rumen digestion in a sheep, adjusted to an intake of 1 kg forage dry matter.

	Acetate	Propionate	Butyrate	Total
Molar %[1]	65	22	13	
Molecular weight (Mwt)[1]	60	74	88	
(Molar % x Mwt)/100	390	163	114	667
Adjusted to 300 g	175	74	51	300
Hydrogen Moles in 300 g	2.92	1.00	0.58	4.5

[1]Sourced from Waghorn *et al*. (2007).

If an infusion of acid were to increase H^+ by 10%, approximately 0.45 M of acid would need to be infused over 24 h. The acid solution consisted of 0.22 M HCl (37%; 11.7 M) and 0.22 M acetic acid (100%, 17.4 M). This 0.44 M acid solution was made up to 2.16 L with water and infused into the rumen at a rate of 2.16 L/day.

Bicarbonate has properties that will increase ruminal outflow (Okeke *et al.*, 1983, Giduck *et al.*, 1988) and 0.52 M of bicarbonate was infused at a rate of about 1% of DMI. Hence, 0.52 M bicarbonate was made up to 2.16 L of water and infused over 24 h.

Day one of the pilot trial determined the effect of acid infusion on rumen pH, taking care not to reduce pH below 5.6. An infusion of 0.44 M acid/24 h into the rumen was undertaken with two sheep fed ryegrass, and rumen pH was measured hourly for 10 h and then every 5 h. The decline in rumen pH was not excessive and the two sheep were then moved to respiration chambers where CH_4 emissions were measured whilst acid infusion was maintained for 24 h. This was followed by a 24 h continuous infusion of water (2.16 L/24h), and a final 24 h infusion of bicarbonate (0.52 M/24 h).

The infusion process described above was repeated with another two fistulated sheep fed fresh ryegrass in the respiration chambers, but only acid and bicarbonate were infused over this two day measurement period.

A.3 RESULTS

There was no effect of infusion treatment on feed intakes (average DMI of 0.90 kg/d) (Table A.3), but the first two sheep that were placed in the respiration chambers stopped eating for a short time towards the end of the acid infusion treatment. They resumed eating after about 6 h of water infusion and daily intakes were similar during water and bicarbonate treatments. The drop in feed intake was reflected in CH_4 production, which decreased during the water infusion, but was similar during acid and bicarbonate infusions (Table A.3).

There was a significant effect of infusion treatment on rumen pH ($P<0.001$), with a low value (5.60) during the pre-chamber acid infusion period, but whilst in respiration

chambers, rumen pH averaged 6.14 during the acid infusion, compared to 6.77 during the bicarbonate treatment (Table A.3). Acid infusion increased ($P<0.001$) NH_3 concentrations, especially when pH was low before entering the chamber (34.1 mM). Values were similar for the acid and bicarbonate infusion treatments when CH_4 was measured. Evaluation of VFAs were complicated because acetic acid was included in the infusion mixture, but the concentrations of isobutyrate and isovalerate were much lower during the acid infusion, than at other times.

TABLE A.3 Effect of acid, water and sodium bicarbonate infusion on intake, methane (CH_4), and rumen parameters from sheep fed perennial ryegrass forages.

Parameter	Acid Pre chamber period	Respiration chamber period			P-value	SED	LSD
		Acid	Water	Sodium bicarbonate			
Number of animals	2	4	2	4			
DMI (kg/d)	0.93	0.86	0.93	0.89	0.937	0.17	
Methane emissions							
g CH_4/d	N/A	19.2	13.7	20.5	0.083	2.572	
g CH_4/kg DMI	N/A	23.1	14.8	23.4	0.128	3.961	
Rumen pH	5.60	6.14	6.73	6.77	<0.001	0.254	0.51
Ammonia (mM)	34.1	21.8	15.2	16.9	<0.001	5.681	11.5
Total VFA (mM)	86.3	72.5	61.3	70.6	<0.001	7.033	14.2
% of total VFA:							
Acetate (A)	69.1	73.8	70.4	68.1	0.010	2.081	4.19
Propionate (P)	19.1	16.6	17.5	19.1	0.041	1.312	2.64
Butyrate (B)	9.46	7.38	8.69	9.32	0.019	0.920	1.87
Valerate	0.87	0.67	0.77	0.88	0.043	0.100	0.21
Isobutyrate	0.67	0.82	1.37	1.31	<0.001	0.145	0.28
Isovalerate	0.66	0.78	1.28	1.28	<0.001	0.162	0.33
Ratios							
A:P	3.78	4.51	4.05	3.61	0.167	0.511	1.03
A + B/P	4.28	4.95	4.55	4.10	0.213	0.511	1.03

DMI, dry matter intake; VFA, volatile fatty acid, N/A, not available

A.4 DISCUSSION

Infusion of acid and bicarbonate altered rumen pH, but the average values (6.14 and 6.77) were within or similar to optimal values reported for methanogens by (Jarvis *et al.*, 2000, Rea *et al.*, 2007) and there were no effects on CH_4 emissions. The main effect of acid infusion was on NH_3 concentrations, which were markedly elevated on the

day before the first two sheep were put in the respiration chamber. Of greater significance was the depression in isobutyrate and isovalerate concentrations when acid was infused, suggesting a reduction in catabolism of some amino acids, but not others. Erfle *et al.* (1982) also reported a decrease in percentages of isobutyrate and isovalerate when pH was decreased *in vitro*.

The effects of high concentrations of cereal grains in ruminant diets to reduce both rumen pH and CH_4 emissions, is well known (Van Kessel and Russell, 1996). Other studies have examined rumen pH on CH_4 emissions *in vivo* by comparing ruminants fed either a forage diet to induce a high ruminal pH, or a concentrate diet to cause a low ruminal pH (Burrin and Britton, 1986, Van Kessel and Russell, 1996, Lana *et al.*, 1998). This pilot study attempted to induce changes in rumen pH in sheep fed a forage diet, but was largely unsuccessful, and manipulation of pH through acid infusion is difficult.

The acid infusate used here comprised equal molar proportions of acetic acid and HCl, because HCl alone resulted in an unstable pH. However, an infusion containing acetate precluded a useful interpretation of the effects of pH on the major VFAs.

A.5 CONCLUSION

This pilot study showed it was possible to manipulate rumen pH by infusion, but the quantities of both acids and bicarbonate used here did not affect major changes in pH and had no effect on methanogenesis. We suggest caution in future attempts to replicate this pilot study, especially with acid infusion, because excess acid will stop the animal eating and the reduction in salivary inflow to the rumen will exacerbate acidosis. A more detailed experiment, with continuous rumen pH monitoring, would be required to change rumen pH sufficiently to affect a change in CH_4 emissions.

CHAPTER 9

BIBLIOGRAPHY

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