METHANE EMISSION FROM FORAGE-FED SHEEP, A STUDY OF VARIATION BETWEEN ANIMALS

A thesis presented in partial fulfillment of the requirements for the degree of Doctor of Philosophy in Animal Science in the Institute of Veterinary, Animal and Biomedical Sciences, College of Sciences, Massey University, Palmerston North, New Zealand

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DECLARATION

The studies presented in this thesis were completed by the author whilst a postgraduate student in the Institute of Veterinary, Animal and Biomedical Sciences, College of Sciences, Massey University, Palmerston North, New Zealand. This is all my own work and the views presented are mine alone. Any assistance received is acknowledged in the thesis. All references cited are included in the bibliography.

I certify that the substance of the thesis has not been already submitted for any degree and is not being currently submitted for any other degree. I certify that to the best of my knowledge any help received in preparing this thesis, and all sources used, have been acknowledged in this thesis.

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ABSTRACT

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Rumen methanogenesis represents a loss of between 2 to 15% of the energy intake by the animal, and methane (CH_4) has a role in the global warming phenomenon. Thus, any reduction of ruminant CH_4 emission would have both environmental and nutritional benefits. The development of cost-effective strategies to mitigate ruminant CH_4 without causing a negative impact on animal production, especially for systems based on forages, is a major challenge. Large between-sheep (within a breed) variations in CH_4 emission, under controlled and grazing conditions, have been described in the literature (Chapter 1). This thesis studied the nature and causes of the between-sheep variation in CH_4 emission, with the objective of using it as a tool to reduce CH_4 emission. The sulphur hexafluoride (SF_6) tracer technique was used to measure CH_4 emission throughout this study, therefore three trials were conducted with penned sheep in order to evaluate this technique against the standard respiration chamber (Chapter 2). Poor ventilation in the building and prolonged *in rumen* deployment of the SF_6 permeation tubes were identified as reasons for poor agreement between the techniques in the initial trials. However, when these problems had been overcome, good agreement (r=0.79, p=0.02) between the techniques was found, with the tracer CH_4 values being 10% lower than the chamber values. The tracer technique was then used to screen grazing sheep for their rates of CH_4 emission, and three groups of sheep (8, 10 and 8 animals), each comprising sheep with low or high CH_4 emissions, were selected, and their CH_4 emissions were monitored during 12, 12 and 5 months, respectively (Chapter 3). This study showed that sheep did not maintain their rankings with respect to CH_4 emission when they were brought from pasture to restricted indoor feeding conditions. However, they did maintain their rankings under generous grazing conditions, although the persistence of rankings weakened with time. A detailed study of rumen digestion (Chapter 4) was carried out with sheep fed indoors on lucerne hay at a restricted level (1.2 maintenance). This study revealed that particulate fractional outflow rate from the rumen (particulate FOR, % h^{-1}) explained a large proportion (R^2=0.57) of the between-animal variation in CH_4 emission (%GEI). In addition to the negative relationship to CH_4 emission (%GEI), particulate FOR was negatively correlated with rumen fill (g) (r=-0.69, p=0.03) and with digestibility of cellulose (r=-0.65, p=0.04). Based on the latter results, a simple field index for screening grazing sheep for rumen particulate FOR or rumen volume, based on changes in liveweight, was tested (Experiment 1, Chapter 5). LW change during short-term grazing, following overnight fasting, was strongly correlated with maximum

rumen fill (determined at the end of evening grazing). This index was used to screen sheep and to select 10 sheep with 'small' rumen volumes and 10 sheep with 'large' rumen volumes. However, this index was not repeatable in subsequent measurements (Experiment 2, Chapter 5). In a later study (Chapter 6), sheep (6 animals) and alpaca (6 animals), two animal species with known differences in forestomach particulate FOR (lower in alpaca), were successively fed *ad libitum* on three different forages: (1) indoors on chaffed lucerne hay, (2) grazed on ryegrass/white clover pasture (RG/WC), and (3) grazed on *Lotus corniculatus* pasture (Lotus). In general, the quality of diets selected by the sheep, their voluntary feed intakes (per kg metabolic liveweight) and their CH_4 emissions (g d^{-1}) were higher than those of alpaca, but their CH_4 emissions per unit of intake (%GEI) were lower than those of alpaca. On lucerne hay, the digestibility of cell walls was higher and the urinary energy loss lower in alpaca than in sheep. The sheep produced much less CH_4 (%GEI) while grazing on Lotus than on the other feeds, whereas alpaca grazing Lotus showed low values for apparent digestion of cell walls.

In conclusion, this thesis showed that particulate FOR, an animal-related factor, was a major contributor to the between-sheep variation in CH_4 emission (%GEI) and particulate FOR was also suggested to be the underlying mechanism by which alpaca and sheep differed in their rates of CH_4 emission (%GEI). Sheep ranked initially low or high for CH_4 emission rates (%GEI) persisted in their rankings only under generous grazing conditions and this persistence weakened with time. Changes in diet selection or particulate FOR in response to the seasonal changes in pasture quality and composition may have been the reasons for the weakened persistence in CH_4 emission. Future developments in techniques which can measure between-sheep differences in particulate FOR or rumen volume with large numbers of animals under grazing will be useful in the assessment of the benefits of these animal factors for CH_4 mitigation. The depressing effect of *Lotus corniculatus* on CH_4 emissions by sheep shown in this study represents another benefit of condensed tannin-containing temperate legumes for sustainable pastoral production systems.

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LIST OF ABBREVIATIONS

A/P	acetate/propionate
ADF	acid detergent fibre
ADFI	acid detergent fibre intake
ADL	acid detergent lignin
AMRT	apparent mean retention time, h
ATP	adenosine triphosphate
C_2H_6	ethane
CFC	chlorofluorocarbons
CH_4	methane
CO_2	carbon dioxide
CP	crude protein
Cr-EDTA	chromium ethylenediaminetetra acetic acid
CT	condensed tannins
d	day
DE	digestible energy
DEI	digestible energy intake
DM	dry matter
DMD	dry matter digestibility
DMI	dry matter intake
DNDFI	digestible neutral detergent fibre intake
DOMR	digestible OM fermented in the rumen
ED	apparent digestibility of energy
F/G	overnight fasting and subsequent grazing
F:C	forage:concentrate
FOR	fractional outflow rate, %/h
g	gram
GC	gas chromatograph
GEI	gross energy intake
Gg	gigagram, 10^9 g
GWP	global warming potential
H_2	hydrogen
I	infusion rate
IR	intake rate
IRGraz	intake rate during grazing

kg	kilogram
$kg^{0.75}$	metabolic liveweight, $LW^{0.75}$
Lotus	*Lotus corniculatus* pasture
LW	liveweight
LWG	liveweight gain
M/D	metabolisability of diet (MJ/kg DM)
MaxFill	maximum rumen fill
ME	metabolisable energy
MEI	metabolisable energy intake
MJ	mega Joule
ml	milliliter
MRT	mean retention time, h
N	nitrogen
n	number of observations
N_2O	nitrous oxide
NDF	neutral detergent fibre
NDFI	neutral detergent fibre intake
NH_3	ammonia
NIR	near-infrared reflectance
O	oxygen
O_3	ozone
OM	organic matter
OMD	organic matter digestibility
OMI	organic matter intake
PD	purine derivatives
ppbv	parts per billion by volume
pptv	parts per trillion by volume
PR	permeation rate
Q	quantity
RCRFast	rumen clearance rate during fasting
RG/WC	perennial ryegrass/white clover pasture
S	sheep subgroups
SAC	South American camelids
SC	soluble carbohydrates
SF_6	sulphur hexafluoride

SR	specific radioactivity
STP	standard temperature and pressure
T	trials
Tg	teragram, 10^{12} g
TMR	total mixed ration
VFA	volatile fatty acid
VFI	voluntary feed intake
VR	ventilation rate
Wm^{-2}	watts per square meter
Y_{ATP}	ATP-derived yield of cell mass
ΔLwFast	change in LW during overnight fasting
ΔLwGraz	change in LW during short-term grazing
$[CH_4]$	CH_4 concentration
$[SF_6]$	SF_6 concentration
'calorimetric'	CH_4 measured in respiration chambers
'tracer'	CH_4 measured by SF_6 tracer technique

INTRODUCTION

A unique property of ruminants is their ability to convert cellulose, hemicellulose and non-protein nitrogen to useful animal products. Plant cell wall polysaccharides can only be utilized in animal metabolism after their fermentation by the microbial communities found in the digestive tract. Feed is firstly exposed to microbial digestion in the reticulo-rumen (hereafter called the rumen or forestomach), then hydrolytic digestion by the animals' enzymes takes place in the abomasum and small intestine. In the lower gastro-intestinal tract, undigested feed and endogenous substances are again submitted to bacterial attack and digestion (Van Nevel and Demeyer, 1996).

Fermentation in the rumen is considered an anaerobic oxidation of feed organic compounds (Russell and Strobel, 1993). Primary digestive microorganisms, including bacteria, protozoa and fungi, hydrolyse plant cell-wall polymers, starch and proteins, producing sugars and aminoacids, which are in turn fermented to volatile fatty acids (VFAs), hydrogen and carbon dioxide (CO_2) by both primary and secondary digestive microorganisms (McAllister et al., 1996). As a last step in the process, methanogens reduce CO_2 to methane (CH_4) with H_2 as energy source. Thus, CH_4 formation acts as an electron sink into which the hydrogen from all ruminal microorganisms drains, allowing a higher yield of adenosine triphosphate (ATP) and therefore increasing the efficiency of the system (McAllister et al., 1996), at the same time avoiding the formation of lactate and ethanol (Miller, 1995).

Methane production, however represents a loss of between 2 to 15% of the gross energy in the feed (Johnson et al. 1991). During the 1960s and 1970s, considerable research on production and inhibition of rumen methanogenesis was carried out with the aim of increasing feed efficiency (Czerkwaski 1969; Demeyer and Van Nevel, 1975). During the last few years, however, a renewed interest in inhibition of ruminant CH_4 production has been observed, because CH_4 plays a role in the global warming phenomenon and the destruction of the ozone layer (Crutzen et al. 1986; Johnson et al. 1991).

As a greenhouse gas CH_4 is second in global importance to carbon dioxide. Its atmospheric burden, currently ~4850 Tg (teragrams, 10^{12} g), has increased 2.5 fold in the last two centuries due to human activity, and continues to increase at a rate of 35-40 Tg yr^{-1} (IPCC, 1996). Of the globally aggregated source of ~535 Tg yr^{-1} (IPCC, 1996), enteric (i.e. gut) fermentation accounts for 85 Tg yr^{-1} (Houghton, 1997), farmed ruminants being responsible for about 95% of this (Crutzen et al., 1986; Johnson et al., 1991). In New Zealand, about 88% (1.435 Tg yr^{-1}) of the anthropogenic (released by human activities) CH_4 emission is attributed to livestock (UNFCCC, 1999), which on a per capita basis is ten times higher than the global average (Ministry for the Environment, 1997).

Compared to the other greenhouse gases, CH_4 is an excellent candidate to reduce global warming in the near term (Chynoweth, 1996). Because of its much shorter lifetime in the atmosphere (about 12 years compared with 100-200 years for CO_2), only a relatively small reduction in the anthropogenic emission of CH_4, about 8%, would be required to stabilize its concentration at the current level (Houghton, 1997). Enteric CH_4 emission is one of the few global sources of CH_4 that can be reduced relatively simply (Leng, 1993). It is more easy to manipulate than, for instance, CH_4 produced from marshes or in rice production (Leng, 1993). Furthermore, CH_4 reduction strategies from livestock will directly benefit the farmers by improved animal productivity.

With the exception of improved feeding management, the current technologies to control CH_4 emission from ruminants are seen with pessimism (Van Nevel and Demeyer, 1996; Johnson et al., 1996). In ruminant production systems based on forages, however the improvement of feeding might not only be unaffordable, but it may undermine the unique ability of ruminants, which is the utilisation of fibrous feed resources. Therefore, the development of cost-effective strategies to mitigate CH_4 production in ruminants, without causing a negative impact on ruminant production, continues to be a major challenge for ruminant nutritionists (McAllister et al., 1996).

Between-sheep (within a breed) variability in CH_4 emission has long been recognised from standardised *in vivo* (Blaxter and Clapperton, 1965) and *in vitro* (Demeyer and Van Nevel, 1975) studies, and recently confirmed under grazing conditions (Lassey et al., 1997). If such variability is persistent in the long-term and the animal trait(s) that

account for such variation is (are) inherited, breeding of animals for low CH_4 production might be viable (Gibbs et al., 1989). In this respect, the sulphur hexafluoride (SF_6) tracer technique for CH_4 measurement (Johnson et al., 1994) has been claimed (Johnson et al., 1998; Lassey and Ulyatt, 1999; Ulyatt et al., 1999) to be reliable for measurements on individual animals both under field and controlled indoor conditions, thus allowing treatment comparisons to be made. This thesis studies the nature and causes of the between-animal variation in CH_4 emission from forage-fed sheep with the objective of using it as a tool to reduce CH_4 production. Alpaca, a species with different anatomo-physiological characteristics, was also compared to sheep in one experiment.

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Chapter 1

Review of literature

1.1 INTRODUCTION

This review of the published literature deals in the first instance with the global and national (New Zealand) importance of CH_4 as a greenhouse gas and its source from ruminant livestock. In the second instance, methanogenesis as an integrated part of enteric fermentation in ruminants is described. Thirdly, the current and future technologies for control of CH_4 emission from ruminants are reviewed. Lastly, the techniques for the measurement of CH_4 emission from individual animals are described.

1.2 METHANE EMISSION FROM RUMINANTS AND GLOBAL WARMING

1.2.1 Global warming, greenhouse gases and New Zealand's share

The phrase 'global warming' has become familiar to many people as one of the important environmental issues of our day. Global warming is simply the proposal that increased ***greenhouse gases*** cause the Earth's temperature to rise globally (Houghton, 1997). According to Houghton (1997), there are three facts supporting the belief that the Earth's temperature is rising as a consequence of human influence: (1) The global average surface air temperature between 1860 and 1995 has increased between 0.3 and 0.6 ^{o}C. (2) Over the last hundred years the sea level has risen by between 10 and 25 cm (Houghton, 1997). The later is attributed to the thermal expansion of ocean water and to the retreat of glaciers. (3) Close agreement is found between the observed warming of the Earth surface (over the last century) and the predictions of climate models allowing for greenhouse gases and sulphate aerosols (cooling effect).

The United Nations Convention on Climate Change (IPCC, 1996) defined 'climate change' as: "a change of climate which is attributed directly or indirectly to human activity that alters the composition of the global atmosphere and which is in addition to natural climate variability observed over comparable time periods". Although some scholars believe that the recorded change in global climate is in the range of natural variability, the Intergovernmental Panel on Climate Change (IPCC, 1996) has suggested that there is a discernible human influence on global climate.

The ***greenhouse effect***, the cause of global warming (Houghton, 1997), can be understood by considering the *solar radiation,* which warms the Earth's surface and

the *thermal radiation* from the Earth and the atmosphere, which is re-radiated out to space. On average these two streams must balance. If the balance is disturbed (e.g. by increased atmospheric CO_2) it can be restored by an increase in the Earth's surface temperature. The gases nitrogen and oxygen which make up the bulk of the atmosphere (78 and 21%, respectively), neither absorb nor emit thermal radiation. However, the water vapour, carbon dioxide and other minor gases present in the atmosphere in much smaller quantities (***greenhouse gases***) absorb some of the thermal, shortwave, radiation leaving the Earth surface, acting as a partial blanket for this radiation and thus warming the atmosphere. This blanketing is known as the 'natural' greenhouse effect, because all the atmospheric gases (except chlorofluorcarbons – CFCs) were there long before human beings came on the scene. In contrast, the 'enhanced' greenhouse effect, the added effect caused by the gases present in the atmosphere due to human activities is the cause of major concern (IPPC, 1996). Any increase in the concentration of greenhouse gases in the atmosphere will reduce the efficiency with which the Earth cools to space.

The most important of the greenhouse gases is water vapour, but its concentration in the atmosphere is not changing directly because of human activities. The important greenhouse gases which are directly influenced by human activities are carbon dioxide (CO_2), methane (CH_4), nitrous oxide (N_2O), the chlorofluorocarbons (CFCs) and ozone (O_3) (Houghton, 1997). Because CFCs destroy some O_3, the greenhouse effect of the CFCs is partially compensated by the reduced greenhouse effect of atmospheric O_3. If we ignored the effects of CFCs and of changes in ozone, about 70, 24 and 6% of the enhanced greenhouse effect would be attributed to the increase in CO_2, CH_4 and N_2O, respectively (Houghton, 1997).

Table 1.1 presents the change in atmospheric concentrations (attributed to human activities), lifetime, *radiative forcing*[1] and *global warming potential*[2] (GWP) of the major (CO_2, CH_4, N_2O) and other minor (CCl_3F, a chlorofluorocarbon-CFC; CHClF,

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[1] *Radiative Forcing*, a simple measure of the importance of a potential climate change mechanism, is the perturbation to the energy balance of the Earth-atmosphere system (in watts per square metre, Wm^{-2}) (IPCC, 1996).

[2] *Global Warming Potential* (GWP), defined as the cumulative radiative forcing between the present and some chosen time horizon caused by a unit mass of gas emitted now, expressed relative to that for CO_2 (IPCC, 1996).

a hydrochlorofluorocarbon-HCFC; SF_6, a perfluorinated) greenhouse gases. The atmospheric concentrations of the major greenhouse gases, *inter alia* CO_2, CH_4 and N_2O have grown significantly since pre-industrial times (~1750 AD) by about 30%, 145% and 15%, respectively. The growth of other minor greenhouse gases (Table 1.1) has shown drastic changes during the last decade. For example, hydrochlorofluorocarbons are being used as substitute for chlorofluorocarbons.

Table 1.1 Atmospheric concentrations and current annual growth rates, lifetimes, radiative forcing and global warming potentials (GWP) of the major greenhouse gases and other groups' representative gases (from: IPPC, 1996).

	CO_2	CH_4	N_2O	CCl_3F	$CHClF_2$	SF_6
Concentration (*ppbv*[1]):						
Pre-industrial	278000	700	275	0	0	0
Year 1992	356000	1714	311	0.268	0.100	0.032
Current annual growth	1600	8	0.8	0.000[2]	0.005[2]	0.0002
Lifetime (years)	50-200	12	120	50	12	3200
Radiative Forcing (Wm^{-2})	1.56	0.47	0.14	0.06	0.02	0.002
GWP:						
20 years	1	56	280	4900	4000	16300
100 years	1	21	310	3800	1500	23900
500 years	1	6.5	170	1400	510	34900

[1] *ppbv*, parts per billion by volume

[2] Gases with rapid changing growth rates over the past decade.

Increases in greenhouse gas concentrations have led to a positive *radiative forcing* of climate. The direct radiative forcing of the long-lived greenhouse gases (2.45 Wm^{-2}) is due primarily to increases in the concentrations of CO_2, CH_4 and N_2O. The direct radiative forcing due to the CFCs and hydrochlorofluorocarbons (HCFCs) combined is 0.25 Wm^{-2}. However, the net radiative forcing of CFCs and HCFCs is reduced by about 0.1 Wm^{-2} because they have caused stratospheric ozone depletion which gives rise to a negative radiative forcing.

Different greenhouse gases vary in their ability to trap heat. The GWP index depicts these differences using CO_2 as a benchmark for comparison. Factors, which affect the GWP of a gas, include its chemical make-up and the length of time it can remain intact in the atmosphere (Ministry for the Environment, 1997). Thus, although CO_2 is

the most notorious of the greenhouse gases it is actually very weak in comparison to the others (see Table 1.1). The most potent greenhouse gas is sulfur hexafluoride (SF_6) with a GWP nearly 35,000 times (at 500 years horizon) greater than CO_2.

However, the GWP alone is not the full picture because greenhouse gases vary in their abundance in the atmosphere (Table 1.1). CO_2, despite its relatively low warming potential, has the greatest total impact on atmospheric temperatures, and it is said to have the highest warming 'commitment' (Ministry for the Environment, 1997). The global warming commitment of a gas is simply its GWP multiplied by its abundance.

According to the Intergovernmental Panel on Climate Change (IPCC, 1996), the "best estimate" model-projected rise in global average temperature due to increase in greenhouse gases and aerosols (cooling effect) from 1990 to 2100 is 2 °C. For the same period, sea level is predicted to rise in about 50 cm (IPCC, 1996). Reduction in the human-made emissions of greenhouse gases is urged to stabilise their atmospheric concentrations. The most important of the greenhouse gases that result from human activities is CO_2. It is expected that CO_2 concentration in the atmosphere will increase to about 2.5 times its pre-industrial value by the year 2100. This burden will be little affected even when the emission of this gas is stabilized at its current levels. The time taken for atmospheric CO_2 to adjust to changes in sources or sinks is between 50-200 years. On the other hand, CH_4, the second most important greenhouse gas has a shorter lifetime (12 years) than CO_2 and therefore is an excellent candidate to be stabilised; a reduction of only 8% in the anthropogenic emission would be required to stabilise its atmospheric concentration (Houghton, 1997).

For the year 1993, the New Zealand's estimated emissions of CO_2, CH_4 and N_2O were 27.3, 1.605 and 0.037 Tg, respectively (Ministry for the Environment, 1997). These emissions accounted only for 0.1, 0.6 and 1.2% of the global emissions of CO_2, CH_4 and N_2O, respectively. Nevertheless, because New Zealand's population (3.5 million people) represented only 0.06% of the world's population, on per capita basis New Zealand's emissions of CO_2, CH_4 and N_2O were respectively 2.2, 9.7 and 18.6 times the global average (Ministry for the Environment, 1997).

1.2.2 Methane emission from the world and New Zealand's ruminants

The current global CH_4 emission is estimated to be 535 Tg/year. From this, about 375 Tg (70%) has anthropogenic sources (IPCC, 1996). About 80 to 85 Tg/year CH_4 emission is attributed to enteric fermentation (Houghton, 1997; Crutzen et al., 1986; Johnson and Ward, 1996). Between 89 and 93.6% of CH_4 emission from enteric fermentation is attributed to domestic ruminants of which cattle and buffalo are the most important (Crutzen et al., 1986). CH_4 generated in the rumen as a percentage of feed energy consumed varies from 2% to 12% (Johnson et al., 1993). With highly digestible diets, CH_4 emission is at the low end of the range (3-4%), but diets of this type can produce very high CH_4 yields (10-12%) when fed at about maintenance requirements. For forage and mixed diets, CH_4 yield is around 6.5%.

Johnson and Ward (1996) quoted an estimate of 13.84 Tg/year for CH_4 emission from livestock manure, however, Houghton (1997) quoted a much higher amount for this source (25 Tg/year). The majority of the world's ruminants are free ranging and under these circumstances CH_4 emission from manure was found to be less than 1% of the potential emission (Williams et al., 1993 and Lodman et al., 1993, cited by Johnson et al., 1993).

Unlike other countries where CO_2 assumes a major role as a greenhouse gas, in New Zealand CH_4 is of first importance (UNFCCC, 1999). In fact in a 100 year horizon GWP basis, emissions of CO_2 and CH_4 for the year 1990 accounted for 35.9 and 48.3%, respectively of the total greenhouse emissions. Corresponding values for the year 1996 were 40.4 and 44.3% for CO_2 and CH_4, respectively.

Table 1.2 shows the anthropogenic sources of CH_4 emission in New Zealand for the years 1990 to 1996 (UNFCCC, 1999). Agriculture is the dominant source of CH_4 emissions. In 1996, agriculture accounted for about 90% of the total national CH_4 emission, followed by waste disposal (8%). Ruminant livestock accounts for about 98% of the agricultural emissions (Ministry for the Environment, 1997). Table 1.2 shows that agricultural source of CH_4 emission declined (4%) from 1990 to 1996, which was attributed to a significant decline in sheep and cattle numbers in that period (UNFCCC, 1999).

Table 1.2 Anthropogenic CH_4 emission (Gg = gigagrams, 10^9 g) in New Zealand, 1990-1996 (Source: UNFCCC, 1999).

Sources:	1990	1991	1992	1993	1994	1995	1996
Agriculture	1492	1458	1435	1433	1439	1437	1431
Waste disposal	141	143	138	135	128	120	114
Energy generation	35.41	32.63	32.84	32.80	34.08	38.24	42.08
Land-use change & forestry	3.78	3.09	3.13	3.97	4.74	5.12	5.66
Industrial processes	0.12	0.12	0.10	0.12	0.12	0.12	0.10
TOTAL	**1673**	**1637**	**1609**	**1605**	**1607**	**1601**	**1593**

Lassey et al. (1992) produced the first comprehensive estimate of CH_4 emission from New Zealand's ruminants at 1.24 ± 0.19 Tg/year for 1988. Ulyatt et al. (1992) based their estimation of 1.50 Tg/year for 1990 on a mathematical model and a more detailed scrutiny of the seasonal fluctuation of ruminant population. Both estimates agreed in that about 58, 18 and 21% of the New Zealand's ruminants emissions were accounted from sheep, dairy cattle and beef cattle, respectively.

1.3 METHANOGENESIS; AN INTEGRATED PART IN THE DIGESTION PROCESS

1.3.1 Feed digestion in the rumen, methanogenesis the last step

1.3.1.1 The process of microbial fermentation

Over 200 species of bacteria, 100 species of protozoa and 12 species of fungi inhabiting the rumen have already been described (Theodorou and France, 1993) and new species are likely to be isolated. According to Armstrong (1993) and McAllister and Cheng (1996) each ml of rumen fluid contains 10^9-10^{10} bacteria, 10^5-10^6 protozoa and 10^3-10^4 fungi. Thus, because of their numerical predominance and metabolic diversity, the ruminal bacteria are believed to be responsible for most of the feed digestion in the rumen.

The absence of oxygen and the production of reducing agents (e.g. sulphide) in the rumen creates a highly reduced environment (E_h = -250 to -450 mV) that is suitable for the growth of strictly anaerobic microbia (Russell and Strobel, 1993). The energy associated with the phosphoanhydride bonds of adenosine triphosphate (ATP), or similar nucleoside triphosphates, is the major energy source for microbial cell metabolism (Van Houtert, 1993). However, because oxygen is not available as an

electron acceptor, ruminal bacteria (Russell and Strobel, 1993) employ anaerobic oxydation (an incomplete oxidation) closely coupled to reduction reactions. Fermentation is the process of energy yielding oxidation-reduction reactions in which both the initial electron donor(s) and final electron acceptor(s) are organic compounds (Prins, 1977).

The consortium of ruminal microbes ferment carbohydrates and other minor organic feed components (protein and lipids) to acetate, carbon dioxide and ammonia, with concomitant production of reduced end products, mainly CH_4, propionate and butyrate as a result of electron and proton transfer reactions (Van Nevel and Demeyer, 1996). During the fermentation process, energy is conserved in the form of ATP and subsequently utilised for the maintenance (heat production) and growth (cell synthesis) of the microbial population (Moss, 1994; France and Siddons, 1993). Obviously, heat is also produced during the fermentation process (Russell and Strobel, 1993).

Dietary carbohydrates, the main fermentation substrates, are degraded to their constituent hexoses and pentoses before being fermented to volatile fatty acids (VFA) via pyruvate (Figure 1.1). Pentoses are converted to hexose and triose phosphate by the transketolase and transaldolase reactions of the pentose cycle so that the majority of dietary carbohydrate metabolism proceeds via hexose, which is metabolized to pyruvate almost exclusively by the Embden-Meyerhof glycolytic pathway (Baldwin et al., 1963). Acetyl CoA is an intermediate in the formation of both acetate and butyrate, whilst propionate formation occurs mainly via succinate, although an alternative pathway involving acrylate is also operative.

In the oxidation of sugars to pyruvate, electrons, which can be referred to as liberated hydrogen, are taken up by the intracellular electron carrier coenzyme NAD^+, which is reduced to NADH (Immig, 1996). NADH has to be reoxidized to NAD^+ to continue the fermentation of sugars (Figure 1.1). In the rumen the different species of bacteria, protozoa and fungi evolved different strategies to reoxidize NADH. Some species have pathways where the hydrogen from NADH is used to form butyrate from pyruvate. Others use pyruvate and NADH to synthesize propionate. Almost all rumen species form acetate in a non-hydrogen consuming pathway via acetyl CoA with a net release of H_2 and CO_2 (Figure 1.1). Despite these different hydrogen sink reactions a

considerable amount of NADH has to be reoxidized by reactions different from those involved in propionate or butyrate formation. Under low hydrogen partial pressure in the rumen NADH is reoxidized to NAD^+ and H_2 (Immig, 1996). This metabolic H_2 provides another sink for the disposal of reducing power (electrons) generated during the oxidation of sugars and amino acids (Wolin, 1975). Since molecular hydrogen acts as a feedback inhibitor in the fermentation process, it has to be removed. Methanogens use H_2 to reduce CO_2 to CH_4. In this pathway 4 mol of H_2 are used to produce one mol of CH_4 (Immig, 1996). If CH_4 is formed via formate, 3 mol H_2 are used (Figure 1.1). The overall reactions can be summarized as:

$$\text{Hexose} + 2NAD^+ + 2ADP \rightarrow 2\ \text{Pyruvate} + 2[H] + 2NADH + 2ATP \quad (1)$$

$$2\ \text{Pyruvate} + 2H_2O + 2ADP \rightarrow 2\ \text{Acetate} + 2CO_2 + 2H_2 + 2ATP \quad (2)$$

$$2\ \text{Pyruvate} + 4[H] + 4NADH + 2ADP \rightarrow 2\ \text{Propionate} + 2H_2O + 4NAD^+ + 2ATP \quad (3)$$

$$2\ \text{Pyruvate} + 2[H] + 2NADH + ADP \rightarrow \text{Butyrate} + 2H_2 + 2CO_2 + 2NAD^+ + ATP \quad (4)$$

$$CO_2 + 4H_2 + ADP \rightarrow \text{Methane} + 2H_2O + ATP \quad (5)$$

$$\text{Formic acid} + 3H_2 \rightarrow \text{Methane} + 2H_2O \quad (6)$$

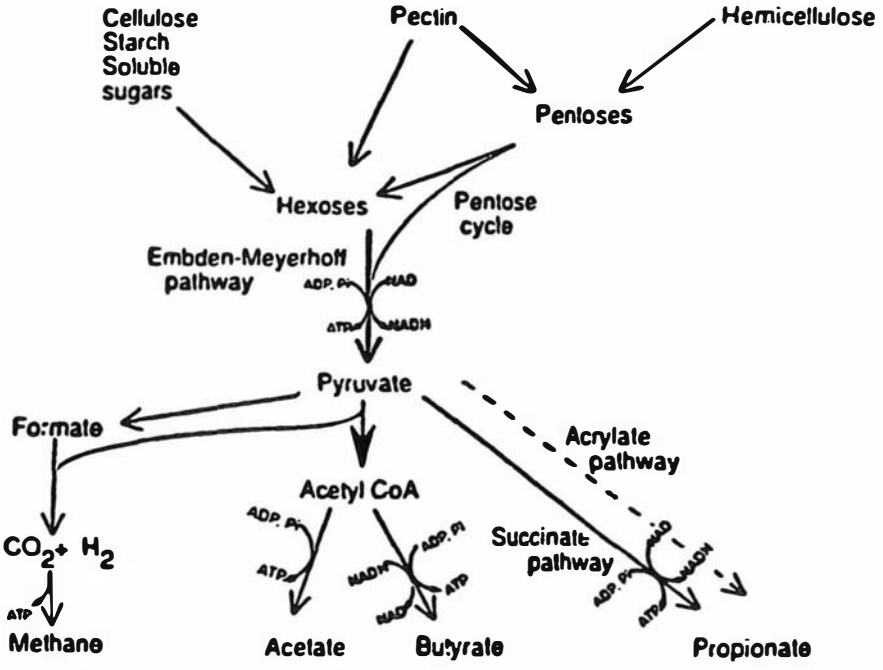

Figure 1.1 A schematic representation of the major patways of carbohydrate metabolism in the rumen (adapted from France and Siddons, 1993 and Immig, 1996).

Apart from acetate, propionate and butyrate, lesser amounts of other VFAs such as valerate, caproate, isobutyrate, isovalerate, 2-methylbutyrate and traces of various higher acids are produced as end products of carbohydrate fermentation (France and Siddons, 1993). Depending upon diet composition, proteins and lipids can also contribute to VFA production in the rumen. Only glycerol and galactose arising from lipid hydrolysis are fermented. On the other hand, when diets with high contents of rumen-degradable protein (RDP) are fed, the amino acids resulting from hydrolysis are deaminated before conversion to VFAs such as isobutyric, isovaleric and 2-methylbutyric (Cotta and Hespell, 1986).

The majority of VFAs produced in the rumen pass through the rumen wall into the circulatory system and are oxidized in the liver, supplying a major part of the energy needs of the animal (Moss, 1994). VFAs may be utilised directly by the animal as building blocks for synthesis of cell material. Fermentation is also coupled to microbial growth and the microbial cell protein synthesised forms the major source of protein for the animal. The gases produced are waste products and are lost from the animal through absorbtion, respiration and eructation (Van Houtert, 1993).

1.3.1.2 Stoichiometry of VFA production

Fermentation of hexose to the various VFAs is determined to a large extent by conditions favouring or diminishing the activities of particular groups of ruminal microorganisms. The availability and nature of the substrate, the presence of electron donors and acceptors and microbial interactions are some of the many factors, which influence the pathways of conversion of pyruvate to VFAs (Sutherland, 1977).

Beever (1993) summarized estimations of fermentation yields of carbohydrate from three contrasting dietary conditions:

High forage: $1\ CHO \rightarrow 1.34\ C_2 + 0.45\ C_3 + 0.11\ C_4 + 0.61\ CH_4 + 4.62\ ATP$

High concentrate: $1\ CHO \rightarrow 0.90\ C_2 + 0.70\ C_3 + 0.20\ C_4 + 0.38\ CH_4 + 4.38\ ATP$

High molasses: $1\ CHO \rightarrow 0.94\ C_2 + 0.40\ C_3 + 0.33\ C_4 + 0.54\ CH_4 + 4.54\ ATP$

where :

CHO = mixed dietary carbohydrate, C_2 = acetate, C_3 = propionate, C_4 = butyrate and CH_4 = methane, and ATP, all in moles.

From this, the total VFA yields of 1.90, 1.80 and 1.67 mol VFA per mol of carbohydrate can be computed, suggesting that an acetate-inducing fermentation (forage) is most efficient with respect to both VFA and ATP yield, but produces a high CH_4 yield (resulting from greater net H_2). When the yields of total VFA were computed in energy terms, the high-forage and the high-molasses fermentations give values of 2.109 and 2.163 MJ, equivalent to 73 and 75% of the original CHO energy (2.882 MJ/mol), or 85 and 87% of CHO if the energy content of ATP is taken into account. However, with the high cereal (concentrate) diet, VFA energy was 80% of CHO energy and inclusion of ATP energy gave an overall efficiency of 92%.

1.3.1.3 Microbial growth and fermentation

As a consequence of the microbial degradation of the various carbohydrate components within the rumen, the immediate end-products are primarily hexoses and pentoses. Subsequently these are used by the microbial population either to supply essential carbon skeletons for the synthesis of microbial biomass, or to provide ATP for both microbial maintenance and growth requirements (Beever, 1993). Degraded hexose is a significant substrate in the synthesis of nucleic acids and lipid by the microbial population (Black et al., 1980/1981) and a significant proportion of microbial protein is synthesized from ammonia (Maeng and Baldwin, 1976) with hexose as the preferred source of the essential carbon skeleton. Microbial polysaccharide is almost certainly derived from degraded hexose (Beever, 1993). Each of these synthetic reactions requires ATP, which will be derived from the fermentation of degraded hexose (Beever, 1993).

According to Preston and Leng (1987) the major factors that affect microbial cell synthesis in the rumen are the availability in rumen fluid of cell precursors (e.g. glucose, nucleic acids, aminoacids, peptides, ammonia and minerals), the maintenance requirements of the microbes, the turnover of microbial cells and the destruction of bacteria by predatory protozoa. A continous supply of fermentable carbohydrates to maintain both fermentation and the supply of precursors for cell growth is paramount to efficient use of ATP. The rate of fermentation must be synchronised to the rate of uptake and therefore availability of microbial nurients (Preston and Leng, 1987).

The efficiency of ATP generation and cell growth also depends on the substrates that provide the 'building blocks' of the microorganisms. If the microbial cell components are synthesised from the glucose (e.g. from cellulose), then growth is highly efficient. If, however, the end-products of fermentation (i.e. VFAs) are used, the synthesis of microbial cells is much lower per unit of organic matter fermented. Estimates of microbial cell yields in terms of carbohydrates fermented, indicate that it is the intermediates in the breakdown of glucose in the rumen that are used to synthesise microbial cells (Preston and Leng, 1987).

It has been estimated (Baldwin et al., 1970) that the synthesis of 100 g microbial biomass comprising 0.60 protein, 0.08 nucleic acid, 0.10 polysaccharide, 0.10 lipid and 0.12 inorganic material, will require 0.662 mol hexose and 3.914 mol ATP. Then, assuming a fractional microbial turnover rate of 4.0 d^{-1}, the synthesis of 100 g microbial biomass will require a viable microbial mass within the rumen of 25 g, which, on the basis of a proposed maintenance cost of 1.63 mmol ATP g^{-1} microbial biomass h^{-1} (Issacsson et al., 1975, cited by Beever, 1993), will add a further ATP requirement of 0.98 mol d^{-1}, thus given a total requirement of 4.89 mol ATP d^{-1}.

Given that on average, 1 mole of fermented hexose yields 4.5 mol ATP, it can be calculated that the total microbial requirement of hexose to support the synthesis of 100 g biomass is 1.749 mol (i.e. 0.662 + 4.89/4.5). This indicates that the partition of degraded hexose between direct incorporation into microbial biomass (0.662) and fermentation (1.087) to provide ATP is approximately 0.6:1, i.e. 62% of degraded hexose will be fermented (Beever, 1993). Thus in a dairy cow consuming 18 kg DM of a high energy diet, the potential yield of degradable hexose within the rumen may approach 8 kg d^{-1}, equivalent to 49 mol d^{-1}. In theory this should be capable of sustaining a synthesis of 2800 g microbial biomass d^{-1} (1680 g microbial protein), if no other nutrients are limiting (Beever, 1993).

Factors determining the efficiency of microbial growth are of great importance for the nutrient economy of the host ruminant. A proposed relationship between microbial growth efficiency and the proportion of fermentable organic matter entering microbial cells, VFA, CH_4 and heat is shown in Figure 1.2 (Van Houtert, 1993). Energy losses as heat in the rumen result from the conversion of carbohydrate to VFAs and microbial cells. According to this model, as the efficiency of cell synthesis increases

there is a decrease in CH_4 production and heat of fermentation. Thus any manipulation which increases microbial cell yield may lead to an increased availability of metabolisable energy. However because microbes are 79-95% digestible in the intestines, the extra energy losses in the feces may remove any energetic advantage of such manipulation. Nevertheless, the protein/energy ratio in absorbed nutrients would be increased markedly (Preston and Leng, 1987).

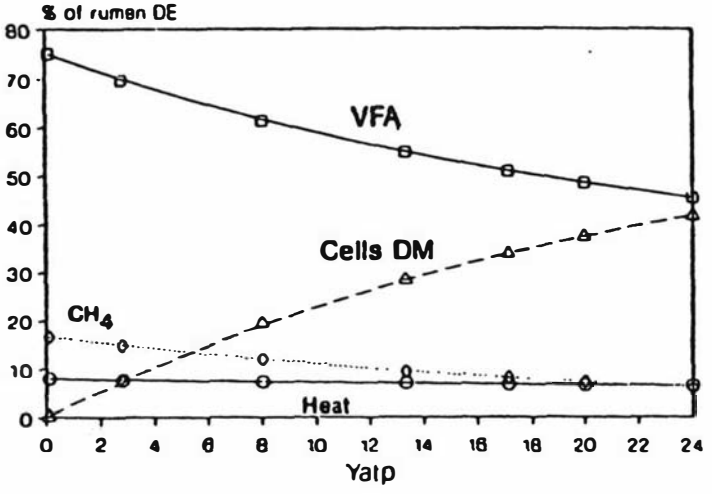

Figure 1.2 The relationship between Y_{ATP} and the proportion of rumen digestible energy (DE) entering microbial cells, VFA, CH_4 and heat (from Leng, 1982 by Van Houtert, 1993).

Assuming a daily intake of 49 moles of potentially fermentable hexose by a dairy cow, Beever (1993) calculated that the change of the diet from high-forage to high-concentrate resulted in reduction in daily CH_4 production from 17 to 10.6 moles. On the other hand, changing the partitioning of hexose utilization towards microbial synthesis at the expense of fermentation from 0.4:1 to 1.2:1 caused a reduction in CH_4 production from 17.9 to 11.4 moles/day.

Because of methanogenesis, more acetate is produced, and consequently, the ATP yield during fermentation is increased, leading to a higher microbial growth efficiency (Wolin and Miller, 1988). However, assuming that approximately 5 g of cells are produced per mole of CH_4 formed, in a cow producing 200 l of CH_4 per day, methanogens would contribute only about 1% of the total microbial matter leaving the rumen (Van Nevel and Demeyer, 1995). Furthermore, from *in vitro* inhibition of methanogenesis, it has been speculated (Van Nevel and Demeyer, 1995) that the loss of ATP yield caused by lower acetate and CH_4 productions is compensated by an increased ATP yield from propionate formation.

1.3.2 Methanogens and rumen methanogenesis

1.3.2.1 Evolution of methanogenesis and taxonomy of methanogens

1.3.2.1.1 Evolution of methanogenesis

It has been assumed (Miller and Wolin, 1986, cited by Hackstein et al. 1996) that the composition of the microbial biota (including methanogens) living in the gut of animals is crucially related to the feeding habits. Recently, from extended screenings for CH_4 emissions by vertebrates (253 species) and arthropods (153 species), Hackstein and co-workers (Hackstein et al., 1996; Hackstein and Van Allen, 1996) have suggested that the presence of methanogens in the digestive tract is under evolutionary rather than dietary control.

Thus, competence for methanogens is a primitive-shared character, which seems to be prerequisite for the evolution of anatomic specializations of the digestive tract (Hackstein, 1997). Accordingly, in "methanogenic" branches of the evolutionary tree, a variety of differentiations of the large intestine evolved and, in some cases, differentiations of the foregut. In contrast, the lack of competence for methanogens in chiropterans/insectivores and carnivores apparently has precluded the evolution of specialised fermenting differentiations of the digestive tract. The correlation between intestinal methanogens and the presence of fermenting differentiations might be indicative of the long history of the symbiosis between methanogens and vertebrates and suggests a high specificity of the associations (Hackstein and Van Allen 1996).

1.3.2.1.2 Major characteristics and taxonomy of methanogens

The methanogens together with the halobacteria and the thermoacidophiles were grouped into the Archaea urkingdom (Jones, 1991), separated from the urkingdoms of "normal" bacteria (prokaryotes) and of eukaryotes (plants, animals, fungi and protozoa). The name "urkingdom" was coined to refer to a taxonomic grouping higher than the kingdom (such as Planta, Animalia). From 16S rRNA studies it has been suggested that the three urkingdoms are distinct cell lines, which have evolved along different pathways early in the evolution of life on Earth (Boone, 1993; Miller, 1995).

Methanogens possess unique cofactors (e.g., coenzyme M, HS-HTP, F420) and lipids (Miller, 1995). The cell envelopes of methanogens can contain pseudomurein, protein, glycoprotein or heteropolysaccharides. The characteristic peptidoglycan polymer of the cell walls of Bacteria is absent from methanogens (Miller, 1995).

Methanogens not only derive energy from the production of CH_4 but can also derive their cellular carbon from single carbon precursors. The methanogens have a modified reversed citric acid cycle (Moss, 1993). The synthesis by methanogens of all amino acids except isoleucine appears to be the same as in typical bacteria. Ammonia ions are the major source of N for these organisms, which have growth rates and cell yields well below values obtained for many other bacteria (Demeyer and Van Nevel, 1975).

Common physiological characteristics of methanogens are their requirement of anaerobiosis and environments with redox potentials below –300 mV (Stewart and Bryant 1988, cited by McAllister et al., 1996), their simple nutritional requirements, and most importantly, the use of CH_4 formation as the sole energy-generating mechanism (Miller, 1995).

Hydrogenotrophic methanogens have evolved a unique metabolic route that enables them to conserve energy from the reduction of CO_2 to CH_4 with hydrogen as the energy source. The availability of hydrogen poses its specific demands to the methanogenic metabolism. Keltjens and Vogels (1996) have reported a putative sensor system in which factor F_{390} plays a central role. In this way the metabolic status of the cells is elegantly sensed by the F_{390} concentrations. The compound could specifically be detected under hydrogen-limited conditions. Coordinated expression of the genes might provide the methanogens with a means to adapt to varying hydrogen concentrations (Keltjens and Vogels 1996).

Hydrogen, formate, acetate, methanol and mono-, di- and tri-methylamine can all serve as substrates for methanogenesis (Wolin and Miller, 1988). Complete anaerobic bioconversion ecosystems (e.g. waste digesters), where organic matter is retained for weeks or months, convert all organic carbon to CO_2 and CH_4 (Hobson and Wallace, 1982, cited by McAllister et al., 1996). In contrast, the 1-2 d turnover of organic matter in the rumen is too rapid for complete anaerobic bioconversion of carbon to carbon dioxide and CH_4.

According to Jones (1991), the phylogenetic data currently support the taxonomic organization of methanogenic Archaea into three major groups (orders), comprising six families and nineteen genera. From sixty-six species of methanogens, isolated (to date) from a variety of anaerobic habitats (Mackie et al. 1992), only five species (corresponding to two orders, see below) are isolates from the rumen, and only two, *Methanobrevibacter ruminantium* and *Methanosarcina* sp, have been found in the rumen at populations greater than $1x10^6$ ml^{-1} (McAllister et al., 1996). Nevertheless, methanogens constitute a fundamental component of the rumen microbiota, becoming established very early in the life of the ruminant (Morvan et al., 1994).

The few rumen methanogen species classify in the following way:

Order: Methanobacteriales
- Fam. Methanobacteriaceae
  - *Methanobacterium formicicum*
  - *Methanobrevibacter ruminantium*

Order: Methanomicrobiales
- Fam. Methanomicrobiaceae
  - *Methanomicrobium mobile*
- Fam. Methanoplanaceae
  - *Methanosarcina barkeri*
  - *Methanosarcina mazei*

A recent SSU ribosomal RNA analysis on ruminal flora (Lin et al., 1997) suggested that family Methanobacteriaceae are dominant in cattle and goat rumen, whereas Methanomicrobiales are dominant in sheep.

The cofactor Coenzyme M is found only in methanogens and is required for the terminal reaction of methanogenesis (DiMarco et al., 1990). Coenzyme M-requiring and non-requiring strains of *Methanobrevibacter* spp. were isolated from bovine rumen contents (Miller, 1995). In this respect, Lin et al. (1997) and Baker (1999) have suggested that there may be greater diversity of methanogens in the rumen.

1.3.2.2 The $H_2 + CO_2$ pathway of methane production

A very restricted number of substrates are used for methanogenesis. They are H_2-CO_2, formate, acetate, methanol, and mono-, di-, and tri-methylamine (Miller, 1995). With H_2 and CO_2 as substrates, CH_4 is formed by the reduction of CO_2, whereas with formate, it is converted first to H_2 and CO_2 with subsequent reduction of CO_2 to CH_4. The $H_2 + CO_2$ pathway (Figure 1.3) of CH_4 formation is the most predominant in the

rumen (Mah et al., 1977; Miller, 1995). Methanogens convert CO_2 to CH_4 through four reductive intermediates: formyl, methenyl, methylenyl (or methylene) and methyl. These compounds are not present as free intermediates, and it was suggested that the one-carbon unit is attached to a series of carriers during sequential reduction (Barker, 1956, cited by McAllister et al., 1996). To date, six coenzymes are recognized as participants in the sequential reduction of CO_2 to CH_4 (Figure 1.3). One of these coenzymes, F_{420}, a flavin analog, functions as a two electron acceptor in H_2 oxidation and plays a major role in the transfer of electrons during CH_4 synthesis (Miller, 1995).

The electron transport phosphorylation is responsible for ATP synthesis in methanogens (Deppenmeier et al., 1996). The reduction of methyl-coenzyme M and 5, 10-methylenyl-H_4MPT are exergonic reactions, being possible sources of energy for ATP synthesis (Ferry, 1992, cited by McAllister et al. 1996). Four mols of H_2 are used per each mol of CH_4 produced in the H_2 + CO_2 pathway (Czerkawski, 1986). Then, assuming that about -37 kJ of energy is needed to produce one mol of ATP, a minimum of 3 mols ATP per mol of CH_4 would be evolved from the reduction of CO_2 by H_2 (Moss, 1993).

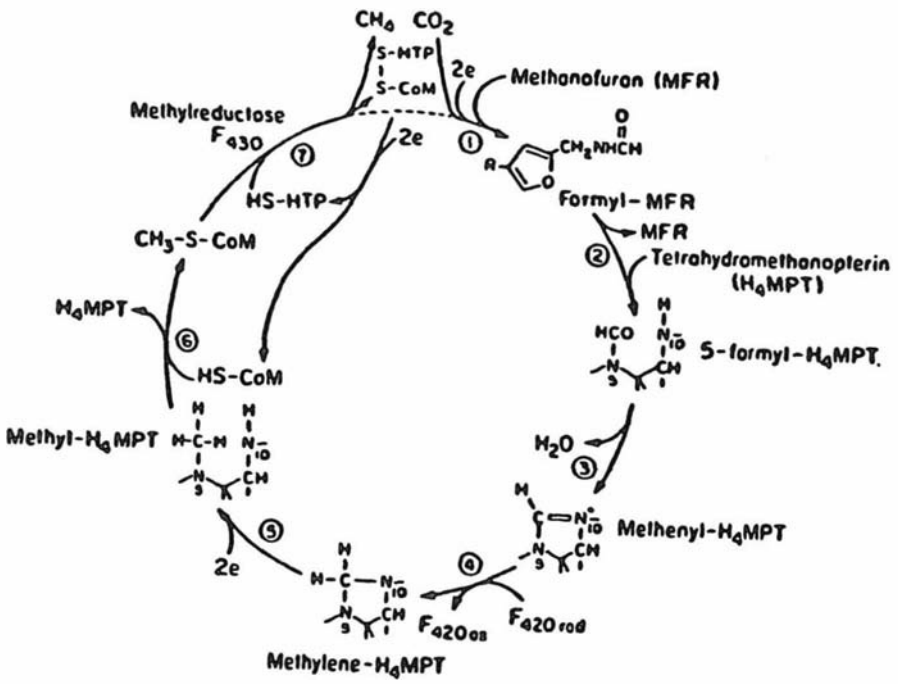

Figure 1.3 Pathway for the reduction of CO_2 to CH_4 (from Rouviere and Wolfe, 1988 by Miller, 1995).

1.3.2.3 The inter-species H_2 transfer and role of H_2 in methanogenesis control

1.3.2.3.1 Inter-species hydrogen transfer

According to Hegarty and Gerdes (1999) hydrogen for CH_4 synthesis occurs in three key states in the rumen, these being hydrogen gas (H_2), reduced cofactors (NADH and NADPH), and as free protons (H^+). Hydrogen in all its forms is a central metabolite in rumen fermentation and for this reason it has been referred to as the "currency" of rumen fermentation (Czerkawski, 1986).

During the fermentation, H_2 is formed in large amounts, but it does not accumulate because it is immediately used by the methanogens. A significant portion of the H_2 arises from the enzymatic oxidation of NADH, formed during glycolysis, to NAD^+ and H_2 (Hegarty and Gerdes, 1999). This occurs only if the concentration of H_2 is kept below one kPa. Methanogens maintain the required partial pressure of H_2 when they form CH_4. The collaboration between fermenting species and H_2-using methanogens is called 'interspecies H_2 transfer' (Iannotti et al., 1973).

Although methanogens do not produce fibrolytic enzymes, co-cultures of methanogens with cellulolytic bacteria (Beaudette, 1994, cited by McAllister et al., 1996) and fungi (Joblin et al., 1990) have shown improved cell wall digestion in comparison with monocultures of cellulolytic organisms. Methanogens enhance the energetic and digestion efficiency of other microorganisms by preventing the accumulation of reduced nucleotides (e.g. NADH) through interspecies hydrogen transfer (McAllister et al., 1996). Acetate production by the fermentative species increases because NADH is oxidised to H_2 instead of being used to form ethanol and lactate (Miller, 1995).

As a consequence of interspecies H_2 transfer, the products formed by the fermentative organisms in the absence of H_2 utilization differ from those produced when H_2 is used to form CH_4. For example, *Ruminococcus albus*, a major rumen cellulolytic species, produces ethanol, acetate, H_2, and CO_2 in monoculture where H_2 accumulates. NADH is re-oxidized by the enzymes used to produce ethanol from acetyl-CoA (Miller, 1995). When co-cultured with a methanogen, ethanol formation by *R. albus* ceases or is vastly diminished. *R. albus* oxidizes pyruvate to acetyl-CoA, H_2, and CO_2 (Miller 1995). The organism also produces H_2 by oxidizing NADH to NAD and H_2.

Hydrogen does not inhibit the oxidation of pyruvate to H_2, but it inhibits the formation of H_2 from NADH (Glass et al., 1977). The maintenance of a low partial pressure of H_2 by methanogens promotes the transfer of electrons to coenzyme F420, increases the production of H_2, acetate, and ATP by *R. albus*, and eliminates its production of ethanol.

Ruminococcus flavefaciens, another cellulolytic species, reoxidizes NADH by reducing oxalacetate through a series of reactions to produce succinate, but when this organism is co-cultured with a methanogen, succinate production is dramatically reduced and the flow of carbon is diverted from succinate to acetate formation (Latham and Wolin, 1977). Similarly, *Selenomonas ruminantium* ferments carbohydrates to acetate, propionate, and CO_2, but when it is grown with a methanogen, propionate formation is significantly decreased and acetate formation increased (Chen and Wolin, 1977).

Interspecies hydrogen transfer is not restricted to cellulolytic bacteria. Bauchop and Mountfort (1981) have reported that lactate and ethanol are the major fermentation products of anaerobic cellulolytic phycomycetes, but when co-cultured with methanogens, the production of these major products decreases while acetate increases.

Direct physical associations to facilitate syntrophy exist in the rumen such as those between rumen protozoa and endo- and ecto-symbiotic methanogens (Finlay et al., 1994; Newbold et al., 1995). Boone et al. (1989) showed that methanogens benefited directly from hydrogen released from hydrogen-producing bacteria if they were within 10 mm of the bacteria. Methanogens also benefit from the increasing H_2 concentration in the bulk solution. In fact, a change from low to high p_{H_2} increased ATP yield (per mole of CH_4 produced) from 0.33 to 2 moles (Keltjens and Vogels, 1996).

Because of the interspecies transfer of hydrogen, the end products of rumen metabolism are almost exclusively CO_2, CH_4, and VFAs. Although large quantities of H_2 are liberated during fermentation (~ 800 l/day in a cow), the partial pressure of H_2 in the rumen is kept low by methanogens (Wolin and Miller, 1988; Hungate, 1966). Thus methanogens enhance the activity of the fermentation process by

removing hydrogen and reducing the feedback inhibition. Thus, CH_4 formation may be looked upon as an energy sink into which the hydrogen from all rumen organisms drains (Hungate, 1963 cited by Demeyer and Van Nevel, 1975).

1.3.2.3.2 Role of H_2 in control of rumen methanogenesis

Because the rate of rumen CH_4 production is directly proportional to the concentration of dissolved H_2 (Czerkawski et al., 1972), considerable opportunities may exist to reduce methanogenesis by depriving rumen methanogens of H_2. Hydrogen gas, arising primarily from reduction of protons by the hydrogenases associated with the ferredoxin oxidoreductase enzyme systems in rumen microbes, is the central metabolite in rumen fermentation (Hegarty and Gerdes, 1999).

The availability of hydrogen to rumen methanogens may be altered by reducing H_2 release, by promoting oxidation of NAD(P)H in reactions that do not transfer electrons to hydrogenase, and by promoting non-methanogenic reactions that utilise H_2 (Hegarty, 1999b). Decreasing the H_2 liberation may be achieved by either reducing the total flow of organic matter through the fermentation pathways or by shifting the balance from acetate (and H_2) production to propionate and butyrate yielding fermentations, which are net consumers of protons.

The rate of carbon flow through the glycolytic pathway controls substrate pressure and ratios of $NADH/NAD^+$ and ATP/ADP, thereby regulating the partitioning of carbon between acetate, propionate and butyrate production (Sutherland, 1977). Rate of feed intake may therefore be expected to alter VFA balance, with slow intake leading to a higher molar proportion of acetate and greater phosphoroclastic H_2, but in practice, the rate of feed consumption does not normally affect VFA proportions (Hegarty, 1999b). Changing the form of carbohydrate does affect the acetate/propionate (A/P) ratio and therefore H_2 production (Beever, 1993).

The formation of propionate requires hydrogen. Hence, production of CH_4 and propionate both compete for hydrogen with other reducing reactions such as production of butyrate and synthesis of some amino acids (Van Houtert, 1993). Propionate production may be enhanced by modifiers of fermentation such as monensin by affecting growth of important acetate producers and causing high

intracellular NADH/NAD^+ ratio, and thus favouring propionate production reoxidising NADH while limiting the H_2 yielding acetate pathway (Hegarty, 1999b).

These and other alternatives for rumen fermentation control are discussed in section 1.4 of this chapter.

1.3.3 Fermentation in the hindgut and methanogenesis

Hindgut digestion may become important in ruminants when substrate degradability in the rumen decreases, i.e., after feeding diets which have been ground or pelleted, diets containing high amounts of starch or fat, after defaunation or after feeding high amounts of resistent starch (Immig, 1996). Depending on the diet characteristics and the type of animal, between 3 and 14% of starch and between 17 and 35% of fiber ingested can become available for fermentation in the large intestine (caecum and colon) (Immig, 1996).

From CH_4 dynamics studies with sheep fed lucerne hay, Murray et al. (1976) found that production of CH_4 in the rumen accounted for 87% of the total production and 95% of it was excreted by eructation. Of the CH_4 produced in the lower digestive tract (13% of total), 89% was excreted through the lungs and 11% through the anus. No transfer of CH_4 between rumen and hindgut pools was observed. More recently, Torrent and Johnson (1994), using a rumen emptying technique in sheep fed either cracked corn plus pelleted lucerne or dried distillers grain, found a similar proportional contribution from the hindgut (11.5-12.5%), whereas Hofmeyr et al. (1984) reported a much lower contribution (6.7%) from the hindgut.

The VFAs fermentation pattern of caecal contents revealed (Immig, 1996) 77% acetate, 19% propionate and 4% butyrate, with a higher acetate concentration and a lower butyrate concentration than in the rumen fluid. Assuming that the carbohydrates degraded in the intestines are fermented to pyruvate via the glycolytic pathway, theoretically between 12 and 17% of the total VFAs production and between 6 and 14% of the daily CH_4 production would be produced in the hindgut (Immig, 1996).

Combining data for VFAs production on a lucerne-based diet (Ulyatt et al., 1975) and data for CH_4 production on the same type of diet (Murray et al., 1976), Immig (1996) calculated a rate of production of 0.72 and 0.59 mmol CH_4 per mmol VFA for

the rumen and caecum, respectively. These values are in line with the low production of CH_4 observed in the gut of termites, rodents and even humans (Cummings and Macfarlane, 1991).

In vitro experiments carried out by De Graeve and Demeyer (1988, cited by Immig 1996) and Váradyová (2000) using rumen and caecum contents obtained from sheep and cattle, showed not only the obvious lower CH_4 production from the hindgut than from the rumen contents, but also that the hydrogen recovery for the caecum contents was substantially lower. Therefore, it is likely that an alternative hydrogen sink such as reductive acetogenesis occurs in the caecum.

1.4 CONTROL OF METHANOGENESIS IN THE RUMEN

1.4.1 Interventions by ration manipulation

1.4.1.1 Level of feeding, forage to concentrate ratio and type of concentrate

Early studies by Blaxter and Clapperton (1965) reported that the yield of CH_4 per unit of intake of good quality diets decreased with increasing level of feeding, but with poor quality diets, increase in feeding level had little effect on CH_4 yield. The different effects of level of intake between concentrate and roughage diets on CH_4 yield may be attributed to the fact that the rate of passage through the rumen is much less affected by level of feeding with roughage diets than with concentrate or mixed diets (Owens and Goetsch, 1986).

Blaxter and Clapperton (1965) also observed that at maintenance level of feeding, increases in apparent digestibility resulted in higher CH_4 yield. More recently, Johnson and Johnson (1995) have suggested that high-grain diets (>90% concentrate) fed at near *ad libitum* intake levels may reduce CH_4 losses to 2-3% of gross energy intake, but as diet digestibility increases, variability in CH_4 loss also increases (Johnson and Johnson, 1995).

Studies carried out by Czerkawski (1969), Holter and Young (1992) and Orskov et al. (1968) have shown that the rate of fermentation is also important; i.e. substrates with slow fermentation rate (e.g. structural carbohydrates) yield more CH_4 (per unit substrate fermented) than those which are rapidly fermentable. In the study of Orskov et al. (1968), cows on the ration containing 0.25 forage to concentrate ratio produced

only half the quantity of CH_4 produced by cows fed 100% forage. High grain diets are associated with increased passage rate, lower ruminal pH, and decreases in protozoa and methanogens numbers (Mathison et al., 1998; Johnson et al., 1996).

Lower CH_4 production and a decreased A/P have been observed in ruminants offered *ad libitum* access to diets rich in starch (Orskov et al., 1968) or infused with glucose (Demeyer and Van Nevel, 1975). This shift in rumen fermentation pattern has been attributed to an increase in the rate of ruminal fermentation, which favours the production of propionate over CH_4 (Demeyer and Van Nevel, 1975). Some starch-fermenting ruminal bacteria produce propionate, but starch feeding can also cause a decrease in ruminal pH and a marked shift in the numbers of other ruminal bacteria (Mackie et al., 1978; Orskov, 1994).

Moss et al. (1995) investigated the effects of four proportions of grass silage (1.00, 0.75, 0.50, 0.25) in diets including barley, on CH_4 production at two feeding levels. They found that at the maintenance level of feeding, a decreasing forage:concentrate (F:C) ratio tended to increase the volume of CH_4 produced per animal per day and also slightly increased the CH_4 generated per unit of digested OM. In contrast, at a higher level of feeding (1.5 x maintenance), there was a quadratic effect of decreasing the F:C ratio on CH_4 production, which was attributed to an associative effect of barley and silage. From studies involving sheep fed at a level equivalent to 90% *ad libitum* intake of 100% forage diet, on diets containing different proportions of forage (1.00, 0.83, 0.66 and 0.50), Dynes et al. (1997) found that reductions in CH_4 production and A/P ratio were more evident only at 50% forage diet.

Chaudhry et al. (1998) fed sheep at two levels (1.6 and 3.2 % LW) with isoenergetic and isonitrogenous diets containing high or low soluble carbohydrate (SC) contents. CH_4 emission (g/kg DMI) for the high SC diet decreased by 25% (21.5 vs. 16.2) with increase in feeding level, whereas the corresponding decrease for the low SC diet was only 6% (18.8 vs 17.7). The higher CH_4 yield observed for the high SC diet fed at the lower level agrees with the findings from other studies (Blaxter and Clapperton, 1965; Bonhomme, 1990; Moss and Givens, 1993, cited by Islam and Begum, 1997). Under these feeding conditions (concentrate feeding at maintenance or concentrate inclusion in forage based diets), the ciliate population increased (Bonhomme, 1990; Moss, 1994; Faichney et al., 1997).

Zervas et al. (1999) studied the rumen fermentation pattern in lactating ewes fed diets consisting of 0.50 kg grass hay and 0.75 kg either fibrous (HRFC) or starchy (HRSC) concentrates (high F:C ratio), or 0.25 kg hay and 1.0 kg either fibrous (LRFC) or starchy (LRSC) concentrates (low F:C ratio). All diets provided similar intakes of energy and crude protein. A linear increase in total VFA concentration, but a decrease in A/P ratio, pH and protozoa counts from HRFC→ HRSC→ LRFC→ LRSC were observed. The highest (for HRFC) and lowest (for LRSC) values of A/P ratio, pH and protozoa ($x10^5$/ml) were 3.36 and 1.68; 6.04 and 5.71; 13.16 and 9.75, respectively. Increases in milk yield and milk protein content were maximum with the LRSC diet. Although CH_4 was not measured, it was expected that it followed the same trend as A/P ratio.

Van Kessel and Russell (1996) have shown that ruminal methanogens are sensitive to even modest decreases in pH. They fed cows on either timothy hay or corn and soybean meal. With the forage diet, ruminal pH remained more or less constant (6.7-6.9) and the VFA concentration was 103 mM/l with A/P ratio of 4.2. On the other hand, with the concentrate diet, the rumen pH decreased dramatically from 6.1 (prior to feeding) to 5.4 within 3 h after feeding and the VFA concentration and A/P ratio were 157 mM/l and 1.8, respectively. *In vitro*, ruminal fluid taken from the forage-fed cow converted CO_2 and H_2 to CH_4, and CH_4 production increased 8-fold when H_2 was added, but no CH_4 was produced at pH values less than 6.0. In contrast, ruminal fluid from the concentrate-fed cow did not produce CH_4 even when H_2 was added. However, when the pH of the ruminal fluid from the concentrate-fed cow was adjusted to pH 7.0, CH_4 was produced at a rate similar to that of the forage-fed cow.

In another *in vitro* study, Russell (1998) found that over a pH range of 6.5 to 5.3, CH_4 production was positively correlated (R^2=0.80) with A/P ratio, with the relation being both pH- and substrate-dependent. About 25% of the decrease in A/P ratio was explained by the effect of pH alone. A recent *in vivo* study (Lana et al., 1998) showed a high positive relationship (R^2=0.80) between rumen pH (range 5.7 to 6.8) and A/P ratio. In turn, the *in vivo* ruminal pH correlated positively (R^2=0.50) with the *in vitro* methanogenesis.

Lower pH resulting from concentrated feeding is often associated with deleterious effects on fibre digestion, feed intake and animal health (liver damage, and acidosis). Orskov (1994) found that feeding concentrates more frequently or mixing them completely with roughage (TMR) reduces the adverse effects of concentrates in rumen pH. However, it is not known how these practices affect rumen methanogenesis.

1.4.1.2 Forage species and type of forages

Properties of the forage that decrease the rate of digestion, or prolong the time of retention in the rumen may increase the amount of CH_4 produced per unit of forage digested (McAllister et al. 1996). Mambrini and Peyraud (1994) reported that total mean retention time of fresh perennial ryegrass fed *ad libitum* to dairy cows tended to increase (43 vs 49 h) with forage maturity (28 vs 49 days growth). In turn, Sundstøl (1981) reported that CH_4 yield tended to increase with the maturity of the forage.

McCaughey et al. (1999) measured CH_4 emission from lactating beef cows, while grazing on either alfalfa-dominant pasture or bromegrass. CH_4 yield (as a % GEI) of alfalfa grazed cows was 25% less (7.1 vs 9.5) than those on bromegrass, reflecting the better quality of the alfalfa.

CH_4 production from forage is higher when it is dried than when it is ensiled (Sundstøl, 1981). Accordingly, it has been suggested that high propionate fermentation is not so apparent in silage-based diets as on hay-based diets when concentrates are included (Thomas and Chamberlain, 1982).

Secondary compounds that are found in some forage species may affect rumen methanogenesis. An *in vitro* study (McMahon et al., 1999) found that CH_4 production declined as the proportion of sainfoin:alfalfa increased; whereas the A/P ratio was unchanged. Sainfoin (*Onobrychis viciifolia*) contains condensed tannins (CT, 10-11% DM). *Lotus* is another plant genera containing CT. *Lotus pedunculatus* and *Lotus corniculatus* are promising forage species for temperate climates. Recently, CH_4 measurements in sheep fed either fresh ryegrass, lucerne or *Lotus pedunculatus* (Waghorn, 1996) have revealed lower rates of CH_4 emission (%GEI) for lotus (3.9%) than for the other two feeds (6.2 and 5.9% for ryegrass and lucerne, respectively).

1.4.1.3 Physical treatment of forages

Grinding and pelleting of forages alters the utilization of the energy through higher fecal losses, lower urine and CH_4 losses and marked reduction in heat losses (Blaxter and Graham, 1956). The reduction in CH_4 loss was associated with a marked reduction of cell wall digestibility, an increase in the proportion of propionate and in the level of intake, and a reduction in the proportion of the ration digested in the rumen (Blaxter and Graham, 1956).

Altering the physical form of a forage diet by grinding and pelleting leads to a faster rate of passage of undigested food particles from the rumen, which is the major factor contributing to the reduced digestion of energy in the rumen (Thomson, 1972). Hironaka et al. (1996) reported that pelleting of lucerne hay reduced CH_4 yield (g/kg DMI) and DM digestibility by about 10% (relative to values for chopped hay) and no effect of feeding level of the pelleted hay upon CH_4 yield was observed.

1.4.1.4 Chemical treatment of forages

The chemical treatments of straw and other agricultural residues with sodium hydroxide, ammonia, urea (Perdok et al., 1988; Givens and Moss, 1995) and lime (Gandi et al., 1997; Sahoo et al., 2000) can improve the feeding value of these feed resources. Moss et al. (1994) showed that NaOH treatment of cereal straw decreased CH_4 emission (% GEI) from 6.0 (untreated) to 5.6 (treated); whereas the corresponding CH_4 emission values found by Wanapat et al. (1985) were 10.1 and 8.4 when the treated diet was supplemented with a small amount of fish meal. Meeske et al. (1993) also showed a lower A/P ratio for NaOH treated wheat straw, which suggested lower CH_4 production. Contrary to these findings, Birkelo et al. (1986) found no differences in CH_4 production between cattle fed on non-treated or anhydrous NH_3 - treated wheat straw.

Treatments with formaldehyde or bacterial inoculants have been reported to increase the digestibility of silages. McLellen and McGinn (1983) showed a slight but non-significant reduction in CH_4 energy loss from grass silage treated with a formaldehyde-acid mixture. However, since the daily liveweight gains of lambs fed the treated silage increased, CH_4 production per unit of product was 9% less on the treated compared with the untreated silage. Martin et al. (1994) reported that the

metabolism of lactic acid from silage by the rumen microbes was a major determinant of rumen fermentation pattern, and it was positively related to the molar proportion of rumen propionate.

1.4.2 Interventions by the use of additives with feed

1.4.2.1 Halogenated methane analogues and other compounds

Bauchop (1967) discovered that halogenated CH_4 analogues, chloroform, carbon tetrachloride and methylene chloride are potent CH_4 inhibitors. Further research (e.g. Van Nevel et al., 1969; Van Nevel and Demeyer, 1992; McCrabb et al., 1997) showed that halogenated compounds (e.g. chloral hydrate, methylene bromide, di-and trichloroacetamide, nitrapyrin, bromochloromethane), also have methane-inhibitory effects. It was suggested that halogenated methane analogues react with corrinoid compounds thus preventing the cobamide methyl transfer leading to CH_4 formation (Wood et al. 1968, cited by Van Nevel and Demeyer, 1995).

2-Bromoethanesulfonic acid (BES), a bromite analogue of coenzyme-M reductase in methanogens (Balch and Wolfe, 1979) has been shown to specifically inhibit *in vitro* growth of methanogens (Sparling and Danields, 1987; Sauer and Teather, 1987). However, *in vivo* studies found adaptation by the methanogens after 3 days (Van Nevel and Demeyer, 1995). Recently, Dong et al. (1999) reported that BES depressed *in vitro* CH_4 production by 51%, without affecting fiber digestion.

1.4.2.2 Ionophores

Most studies on changes in ruminal fermentation that are associated with ionophore feeding have fed monensin or lasalocid (Van Nevel and Demeyer, 1996). Ionophores effect a 5 to 6% improvement in feed efficiency and commonly they shift the fermentation pattern to a higher propionate production and reduced methane production (Johnson et al., 1996). *In vitro* experiments have shown that the effect of ionophores on methanogenesis is indirect, through inhibition of microrganisms which decompose formate to CO_2 and H_2 (Van Nevel and Demeyer, 1997). Ionophores interfere with Gram-positive bacteria, while Gram-negatives are less sensitive (Van Nevel and Demeyer, 1996). The effect of ionophores seems to be due to their interference with transport of ions across biological membranes and disruption of

transmembrane ion gradients (Russell, 1997). As a consequence, there is a shift in the microbial population towards less sensitive or resistent species producing propionate.

Monensin, an ionophore used widely in beef fattening, inhibits CH_4 production *in vivo* by an average of about 25% (Wedegaertner and Johnson, 1983; Van Nevel and Demeyer, 1995). About 55% of the reduction in CH_4 production by steers could be accounted for by their depressed feed intake (anorectic effect), leaving 45% due to specific effects of the monensin on rumen fermentation (O'Kelly and Spiers, 1992). McCaughey et al. (1997) have reported no effects of slow-release capsules containing monensin on CH_4 production by grazing steers.

Some long-term *in vivo* trials have shown that inhibition of methanogenesis by monensin and lasalocid did not persist for more than two weeks (Rumpler et al., 1986; Johnson et al., 1991), but others (Mbanzamihigo et al., 1996; Rogers et al., 1997) have reported no adaptation by the rumen microbia.

1.4.2.3 Organic and inorganic subtances as alternative H_2 acceptors

It has been hypothesised (Martin, 1998; López et al., 1999b) that organic acids (aspartate, fumarate and malate) act as electron sinks for hydrogen. These acids, when added to *in vitro* mixed ruminal microorganism fermentations, decreased CH_4 production, but increased propionate concentration and pH (Callaway and Martin, 1996; Asanuma et al., 1999; Carro et al., 1999; López et al., 1999b). Furthermore, an additive effect was observed with incubations containing monensin and organic acids (Callaway and Martin, 1996). On the other hand, *in vivo* effects of organic acids in high concentrate feeding conditions are conflicting, although DL-malate tended to alleviate sub-clinical acidosis and animal performance (Martin et al., 1999).

Martin (1998) suggested that forages rich in organic acids might serve as vehicles for providing malate to ruminants. This author observed that malate concentration was higher in alfalfa (up to 4.5% DM) than in grasses.

Reduction of sulfate and nitrate are thermodynamically more favourable than reduction of CO_2 to CH_4 (Hegarty, 1999b; Weimer, 1998). On a molar basis, all 3 processes consume an equal quantity of H_2, therefore, decreasing CH_4 emission from sheep by 50% (0.75 mol/day) would require consumption of 0.75 mol sulfate or 0.75

mol nitrate. In practice however, because of toxicity effects, these reductions are undesirable (Weimer, 1998).

1.4.2.4 Lipids

For many years it has been known that dietary lipids act as inhibitors of CH_4 production in the rumen (Czerkawski et al., 1966; Demeyer et al., 1969). Lipids often lead to a decrease in the extent of fibre degradation, but at the same time, the VFA pattern is shifted towards an increase in propionic acid and a decrease in acetic and butyric acids (Doreau and Chilliard, 1997; Lewis et al., 1999).

Inhibition of CH_4 production increases with the degree of fatty acid unsaturation (Dong et al., 1997). Van Nevel and Demeyer (1988) have indicated that in order to interfere with methanogenesis, fatty acids must have a free carboxylic group, but because the reduction in CH_4 production induced by unsaturated fatty acids was not directly proportional to the number of double bonds introduced, factors other than the consumption of hydrogen via biohydrogenation have been suggested (Van Nevel and Demeyer, 1988; Dong et al., 1997).

A direct toxic effect of unsaturated fatty acids on methanogens has been reported *in vitro* (Demeyer et al., 1969; Henderson, 1973; Maczulac et al., 1981). Reduced protozoa counts, and even complete defaunation were observed with both polyunsaturated oils (Newbold and Chamberlain, 1988) and fats rich in medium chain fatty acids (Newbold and Chamberlain, 1988; Machmüller et al., 1998). Methanogens are attached (or symbiotic) to ciliates (Finlay et al., 1994; Ushida and Jouany, 1996), then the loss of this habitat after feeding lipids can explain the inhibited methanogenesis (Van Nevel and Demeyer, 1995). However, Broudiscou et al., (1990) showed that fermentation and CH_4 production in the rumens of normal and protozoa-free sheep were affected similarly by soybean fatty acids.

Certain plant oils have been described as CH_4 reducing feed ingredients for ruminants (Van Nevel and Demeyer, 1996; Dong et al., 1997). Relatively low proportions of coconut oil (3 and 6%) were more effective *in vitro* inhibitors of CH_4 production than whole crushed oilseeds (Machmüller et al., 1998). A recent *in vivo* study (Machmüller and Kreuzer, 1999) found that diets containing 3.5 and 7% coconut oil reduced protozoa counts by 88 and 97% and CH_4 production by 28 and 73%, respectively,

compared with the unsupplemented diet. However, since the forage:concentrate ratio for the diet with 3.5% coconut oil supplementation was 3 times higher than for the 7% level and substitution of concentrate for hay occurred, a net reduction of 51% in CH_4 production resulted from the 7% coconut oil supplementation.

1.4.3 Biotechnological interventions

1.4.3.1 Microbial feed additives

Microbial feed additives (probiotics) used in ruminant feeding are live cells and growth medium of *Saccharomyces cereviseae* (SC) and fermentation extracts of *Aspergillus oryzae* (AO). AO improves productivity by increasing the degradability of forages and enhancing the flow of microbial protein from the rumen, both of which in turn seem to be caused by increased bacterial numbers and activity within the rumen (Fondevila et al., 1990). On the other hand, addition of SC to the feed has been associated with a shift in the molar proportions of ruminal VFA, increased nutrient digestibility, reduced ruminal NH_3 concentration, and a shift in bacterial populations (Putnam et al., 1997). Garcia et al. (2000) observed *in vivo* that SC addition decreased the protozoa count by 42%, but the A/P ratio remained unchanged.

Van Nevel and Demeyer (1995) have reviewed the effect of probiotics on methanogenesis and concluded that their effects on methanogenesis are still unconvincing. In two experiments (Martin and Nisbet, 1990), SC and AO stimulated CH_4 production (50-60%) in incubations without substrate, while no effect at all was seen when starch, Trypticase or bermuda grass were incubated. On the other hand, when 250 mg AO was added daily to Rusitec (Frumholtz et al., 1989), it decreased the percentage of CH_4 in the headspace gas by 50%; the A/P ratio remained unchanged, but the production of butyrate and valerate was increased in line with stoichiometry. The fact that protozoal numbers were reduced by 45% suggested that AO indirectly decreased CH_4 production by affecting the balance of the microbial population. When positive responses did occur, they were only short term effects (Mutsvangwa et al., 1992).

Finally, when combinations of monensin and AO were studied (Newbold et al., 1993) in Rusitec, monensin generally suppressed the effects of AO. Similar observations were reported for SC and monensin in vivo (Garcia et al., 2000).

1.4.3.2 Defaunation

Ciliate protozoa are important components of the rumen ecosystem (in sheep, 10^5 ciliates/ml) and more than 50% of the ruminal biomass is accounted by ciliate protozoa (Jouany, 1978, cited by Ushida et al., 1997). Although the presence of protozoa in the rumen is not essential for the host, it is now certain that they play an important role in overall rumen digestion (De Smet et al., 1992).

Rumen methanogens use hydrogen and formate as the source of reduction power, and CO_2 as a major electron acceptor. Methanogens do not produce these compounds directly, but depend upon the supply of these compounds by hydrolytic and fermentative microorganisms (Ushida et al., 1997). Among the hydrolytic microorganisms, ciliate protozoa are the most potent hydrogen producers (5 nmol/day/cell). Thus, the observed attachment or juxtaposition of methanogens to ciliates and even methanogens living symbiotically inside the protozoa cell (Imai and Ogimoto, 1978; Finlay, et al., 1994; Ushida et al., 1997) confirm a mechanism to make a more efficient hydrogen transfer from ciliates to methanogens.

A daily production potential of 50 l of hydrogen and 50 moles of formate by the rumen ciliate population in a normal sheep rumen has been estimated (Ushida et al., 1997). Then, the potential CH_4 production from these electron donors may explain a large part of the source of ruminal CH_4.

According to Ushida et al. (1997) rumen methanogens may be distributed into four fractions in terms of association with ciliate protozoa: an attached population, an engulfed population, an endosymbiotic population and a free-living but surrounded population. It has been suggested that these populations are specific in their association to the host (Ushida et al., 1997). Finlay et al. (1994) estimated that 37% of the rumen CH_4 production is due to endosymbiont methanogens. In contrast, Newbold et al. (1995) estimated that endo- and ecto-symbiotic methanogens are responsible only for 25% of the rumen CH_4 production.

Ushida et al. (1997) found that protozoa taken just before feeding can produce between 0.5 and 1.6 nmol of CH_4 per cell per day. Therefore, in a sheep with 5 litres of liquid volume and 10^5 ciliates/ml, about 18 litres of CH_4 can be attributed to the ciliate population, which would represent about 50% of the total CH_4 production in

the rumen. This may however be an underestimation, since the apparent CH_4 emission from ciliates 1 hr after feeding was twice as high as the pre-feeding level (Ushida et al., 1997).

Defaunation markedly increases the total bacterial number, but significantly reduces the number of methanogens (Takenaka and Itabashi, 1995; Mathieu et al., 1996). The reduction in number of methanogens after defaunation may be due to the loss of preferable colonization sites for them (Ushida et al., 1997). However, studies reviewed by Hegarty (1999a) reported only a weak correlation between methanogenesis and methanogen numbers.

The effect of defaunation upon CH_4 production is diet-dependent (Kreuzer et al., 1986; Van Nevel and Demeyer, 1996; Ushida et al., 1997; Hegarty, 1999a). When concentrate diets are given, defaunation reduces CH_4 emission by 30-45%, but on forage diets, the reduction in CH_4 production is insignificant. De Smet et al. (1992) have found that with a roughage diet (hay), defaunation increased the volume of digesta and decreased the fractional outflow rate; while the opposite effects were observed on a pelleted concentrate diet. On the other hand, although a small decrease in the ruminal digestibility of organic matter most frequently occurs in the rumen due to defaunation, this is partially compensated by an increase in digestion in the lower tract (Hegarty, 1999a).

Defaunation on a large practical scale is seriously limited by the lack of an appropriate method (Van Nevel and Demeyer, 1995). Recently, two separate studies (Odenyo et al., 1997; Newbold et al., 1997) have screened multipurpose tropical trees for their effects on rumen protozoa. In general no or mild anti-protozoal effects were reported, except for *Acacia saligna* (Odenyo et al. 1997) and *Sesbania sesban* (Newbold et al., 1997). In the latter case, saponins, rather than tannins were found to have the anti-protozoal activity. Nevertheless, the anti-protozoal activities of these compounds were only of short-term effectiveness and therefore they might be useful only in animals with no previous dietary exposure (Teferedegne, et al., 1999).

Ushida et al. (1997) have suggested that CH_4 production in the rumen may be influenced by generic composition of the ciliate protozoa. Thus control of fauna composition, rather than total elimination of ciliate protozoa, may have the potential

to control methanogenesis without significant reduction of OM digestion in the rumen.

1.4.3.3 Introduction of reductive acetogenesis in the rumen

The management of hydrogen produced by microbes during forage digestion is the key to controlling ruminant CH_4 emissions (Joblin, 1999). An alternative pathway for H_2-deposition under anaerobic conditions is the H_2-oxidizing, and CO_2-reducing acetate production by acetogenic bacteria (Immig, 1996; Van Nevel and Demeyer, 1996). If acetogenesis can be established in the rumen, H_2 would be diverted away from methanogens for production of acetate, a nutrient, which would result in increased animal performance as well as decreased CH_4 emission (Joblin, 1999).

Similar to methanogenesis, 4 mol of H_2 are consumed in the formation of a mol of acetic acid (Van Nevel and Demeyer, 1996):

$$2\ CO_2 + 4\ H_2 \rightarrow \text{Acetic acid} + 2\ H_2O$$

Eubacterium limosum was the first ruminal acetogen isolated from the rumen of sheep fed on molasses-based diet (Genthner et al., 1981) and then about 9 years later another acetogen, *Acetitomaculum ruminis* was isolated from cattle's rumen (Greening and Leedle, 1989). Significant populations of acetogenic bacteria belonging to about 10 species now have been found in the rumen of cattle, sheep, deer, and bison (Joblin, 1999).

Although all of these acetogens have been shown to be hydrogenotrophs *in vitro*, it is not known if they act similarly *in vivo* (Joblin, 1999). Acetogenesis occurring in the rumen has been suggested (Faichney et al., 1999) from low recovery of hydrogen (55-73% less than the expected value) under unstable rumen conditions. Similarly, Váradyová (2000) reported that *in vitro* fermentation of fibrous materials by sheep's hindgut inocula produced less CH_4 and lower hydrogen recovery compared to inocula from the rumen, suggesting the occurrence of acetogenesis in the hindgut.

Nollet et al. (1998) suggested that acetogens and methanogens are not strictly competitive, in that acetogenesis requires a higher H_2 partial pressure (H_2 threshold) than does methanogenesis. Therefore, a strategy such as a selective inhibition of

methanogenesis in order to allow H_2 to accumulate, and then stimulation of the establishment of reductive acetogenesis (Hegarty, 1999b; Joblin, 1999; López et al., 1999a) seems promising for controlling CH_4 emission from the rumen. Currently, technology exists to raise the H_2 pressure in the rumen (e.g. by defaunation). In addition, archaeal viruses and bacteriocins acting against methanogens and protozoa might be developed in the future (Klieve and Hegarty, 1999).

1.4.3.4 Reducing animal's maintenance energy requirements

Animal species differ in their maintenance energy requirements. For example in northern Australia, part of the reason why *Bos indicus* cattle are more productive than *Bos taurus* is that they have lower maintenance requirements and so they cope better with periods of nutritional stress (Frisch and Vercoe, 1977). However, even within breeds, genetic variation in maintenance energy requirements has been reported (Archer et al., 1999), suggesting that potential exists to reduce maintenance energy requirements by selection.

Biotechnology interventions by using synthetic anabolic steroids or α2-adrenoceptor agonists to reduce basal metabolism by targeting ion pumping and protein turnover, the two metabolic processes that are the main contributors of the cost of basal metabolism have been suggested (Hunter et al., 1993; Derno et al., 1998).

1.4.4 Production system interventions

Methane emissions are approximately constant per unit of diet; thus mitigation or amelioration of CH_4 emissions from livestock is more effectively approached by strategies to reduce feed input per unit of product output (Johnson et al., 1996). This approach also encourages adoption because it is usually also economically advantageous. Enhancing ruminant productivity generally requires simultaneous improvements in nutrition, genetics, health and animal welfare.

When animal productivity is increased the absolute amount of CH_4 per animal will increase, but the CH_4 yield per unit of animal product will decrease. The benefits of increasing animal productivity on CH_4 emission mitigation will result from the 'dilution' effect of the 'maintenance' CH_4 production over the CH_4 associated with productive functions (Kurihara et al., 1999).

1.4.4.1 Feeding intensity

According to a simulation study, Kirchgeβner et al. (1995) estimated that the 'maintenance' CH_4 emission for a 600 kg dairy cow accounts for about 200 g/day, which is more than a half of the total daily CH_4 released from an average lactating dairy cow. It was estimated that because of the 'dilution effect' of the 'maintenance' CH_4, an asymptotic decline of CH_4 emission per unit of product (milk) will result from an increasing milk yield. Thus, an increase in milk yield, for example from 4000 to 6000 kg milk/cow/year will account for 25% less CH_4 emitted per kg milk.

A simulation study carried out by McCrabb and Hunter (1999) reported that finishing of cattle for 2-5 months on a grain based feedlot was associated with a 34-54% reduction in lifetime CH_4 production per kg saleable beef yield. Walker et al. (1997) confirmed that steers fed at high intensity reached slaughter weight 90 days earlier and consequently they produced less CH_4 (by 12 kg) during the whole period than the control steers. Accordingly, a study carried out by Kurihara et al. (1999) involving *Bos indicus* cattle fed on three feeds ranging from a low quality tropical forage to a high-grain feedlot feed reported a negative liveweight gain (LWG) on the low quality forage, whereas LWG along the other two feeds were positive. When LWG was positive, CH_4 emission per kg LWG declined in a curvilinear fashion with increasing LWG (the 'dilution effect'). CH_4 yield (per kg LWG) of cattle fed on medium quality forage (Rhodes grass) was 4 fold higher than of cattle fed on feedlot feed.

Hunter et al. (1993) estimated that on a whole herd basis, the metabolisable energy contained in carcasses sold from a herd in Northern Australia represented only 2-3% of the metabolizable energy required to produce them. Thus, for some extensive ruminant production systems working below their potential, reduction in maintenance energy requirements on a herd basis would have a large impact on the efficiency of animal production and therefore reduction in CH_4 emission. For example, in a recent study of a particular herd in Ukrania, Martinez et al. (1995, cited by Johnson et al., 1996) found that in spite of higher genetic potential, the productivity figures of the herd were 2200 kg milk/cow/year and 300 g LWG/day. Simulation of three fold increase in productivity per head, while holding total milk and meat production constant, indicated a 33 % reduction in CH_4 emission in the system, attributable to the decreased numbers of slow growing and replacement animals. Total numbers of cattle

decreased by 49%, but each was fed better, grew faster, and produced more milk. For an individual cow, CH_4 yield (g/kg milk) was halved (from 29 to 14) when the baseline productivity was increased by 3 fold.

1.4.4.2 Strategic supplementation of ruminants fed on poor quality forages

The vast majority of ruminants in developing countries are supported on by-products of agriculture, or graze forages of relatively poor nutritional value. These systems are unlikely to change in the foreseeable future (Devendra, 1989). Therefore, there is probably no place for pure intensification arguments to reduce CH_4 emission from these systems. Instead, system-wide strategies of development, based on full and efficient exploitation of the existing animal and feed resources, are suggested (Ehui et al., 1998).

Srinivas and Gupta (1997) and studies reviewed by Leng (1993) have suggested that in cattle on poor quality forage, microbial efficiency in the rumen is low due to the deficiency of essential microbial nutrients and CH_4 emission may represent up to 15-18% DEI. However, when strategic supplementation matched the requirements of the rumen microbia and balanced the absorbed nutrients to the animal's requirements, production of CH_4 (g/kg LWG) declined in a curvilinear manner with increasing metabolisability (M/D) of the improved diet. For example, addition of a molasses-urea block and a small amount of bypass protein (350 g/day) supplementation on crop residues resulted in 6 fold reduction in CH_4 emission per unit of animal product (from 1200 to 200 g/kg LWG). Huque and Chowdhury (1997) arrived at a similar conclusion.

Addition of small amounts of fresh green herbage to straw-based diets is a common practice among villagers in developing countries. Leng (1990) has suggested that it may have a number of beneficial effects, including supply of vitamins and essential minerals, ammonia and amino acids. Furthermore, it appears that fresh herbage provides a highly colonized fibre source to "seed" bacteria onto the less-digestible straw fibre (i.e. colonization from fibre to fibre). Microbial protein synthesis was more efficient when steers fed grass silage of poor quality were supplemented with fresh grass (McKee et al., 1996).

1.4.4.3 Matching ruminant species to agro-ecological niches

It is well recognized that different breeds and species of ruminants differ in their adaptation to the environment and the feed resources (Kennedy, 1995; Chilliard et al., 1995; Orskov, 1989). Usually, animals select feeds or parts of feeds appropriate to the morphology and capacity of their stomach and the retention time they can tolerate. For example, grazers usually have a longer retention time in the gut than browsers such as goats or deer. Yet camelids, with their different forestomach structure, have a long retention time for fibrous residues (Engelhardt et al., 1986). A recent study (Odenyo et al., 1999) has reported that even within browsers there are differences in their abilities to utilise tannin-rich fodders.

Environmental adaptation of species for more productive farming practices has received great interest from scientists. An animal species is considered as being adapted to a particular environment if they can maintain and reproduce themselves over the long term (Kennedy, 1995). To do this it requires physiological, structural and behavioural adaptations to counter environmental stresses and fluctuating feed resources. For example, superior digestion in riverine and swamp buffaloes than in cattle, and differences in microbial activity and numbers not attributable to nutrient supply have been detected in some experiments (Wanapat, 1989). In the same way, the superior digestive efficiency of some breeds and even ecotypes in a particular environments and systems are well documented (Orskov, 1989; Chilliard, et al., 1995). Prolonged retention in the rumen of fibrous feeds to extract the maximal amount of energy would appear to be the strategy adopted by ruminants when feed is scarce (Kennedy, 1995; Orskov, 1989).

Terada et al. (1985) compared metabolizability of the same diets by cattle, sheep and goats. They found that CH_4 energy loss (% GE) of cattle was higher than that of sheep or goats, but not when expressed as a percentage of DE. Species differences in energy digestibility and metabolizability increased as dietary crude protein contents decreased; which were attributed to large differences in fecal energy losses with relative small differences in urinary and CH_4 energy losses. Similarly, comparative feeding studies with sheep and red deer (Simpson et al., 1978) showed that losses of dietary energy in feces, urine and CH_4 were similar in both species, but red deer had much higher heat production than sheep. On the other hand, it has been shown (San

Martin, 1991; Warmington et al., 1989) that South American camelids are better suited than sheep to utilise poor forages, which may be attributed to longer rumen retention time and therefore better digestion (San Martin, 1991).

1.4.4.4 Environment control

Working with sheep Graham et al. (1959) and Kennedy and Milligan (1978) reported that CH_4 production decreases by about 20-30% with cold temperature exposure and cold adaptation. In contrast, recently Von Keyserlingk and Mathison (1993) found that CH_4 production was 25% greater in sheep housed at 4.7°C than in those housed at 21°C, but when expressed as a percentage of DE, 14% more CH_4 was still produced. Sheep in the cold environment ate 8% more DM. In the study of Kennedy and Milligan (1978), a 30% reduction in CH_4 production in the rumen in cold-adapted sheep was associated not only with a 54 and 68% increase of liquid and particulate passage rates, respectively, but also a decrease in the A/P ratio.

Energy losses from lactating cows in CH_4 and urine, and heat production, expressed as MJ/kg DMI, increased as environmental temperature was raised from 18 °C to 28 °C, whereas, milk energy yield and energy retention (MJ/kg DMI) decreased (Terada and Muroaka, 1994)

1.4.4.5 Using the between- and within-species variation in methanogenesis

Daily CH_4 production (g/day) is significantly different between ruminant species and types of production. Dairy cows emission varies between 200 and 400 g (Tyrrell et al., 1988; Kirchgeβner et al., 1991; Holter and Young, 1992; Saama and Mao 1993), whereas in beef cattle CH_4 emission is found in the range from 70 to 200 g (Thornton and Owens, 1981; Wedegaertner and Johnson, 1983; Johnson et al., 1991; Reynolds et al., 1991). Daily CH_4 production by sheep and goats is found in the range between 10 and 30 g (Sawyer et al. 1974; Rowe et al. 1985; Aguilera and Prieto, 1991; Arieli, 1994; Crovetto et al. 1994). However, on average, the CH_4 emission per unit of feed intake (~20-22 g/kg DMI) does not differ too much between ruminant species (Kurihara et al., 1997; Mathison et al., 1998), although, the differences may become larger at extremes of feed quality and level of intake.

Based on a systematic screening (faecal samples) of a large number of species for CH_4 emission, Hackstein et al. (1996) have challenged the conventional belief (Hungate, 1966) that both vegetarian feeding habits and the presence of intestinal "fermentative" differentiations of the host are the necessary pre-requisites for the presence of symbiotic methanogens (see section 1.3.2.1.1 in this chapter). According to these authors (Hackstein et al., 1996; Hackstein, 1997) the phylogenetic position of the host is more important for methanogenesis than the feeding behaviour or the presence of elaborated fermenting devices of the digestive tract. However, because the rumen as well as the caecum evolved exclusively in methanogenic taxa, it has been suggested (Hackstein, 1997) that non-methanogenic hosts possessing such structures lost their intestinal methanogens secondarily during their evolution from methanogenic ancestors. Thus, the character " CH_4 production" obeys Dollo's law, i.e. methanogenesis does not reappear in those branches of the phylogenetic tree that are characterized by a previous loss of this trait (Hackstein, 1997).

Based on the fact that mutant hosts which lack significant numbers of intestinal methanogens exist in various methanogenic local populations of primates and humans, Hackstein (1997) has suggested that the ability to host methanogens primarily depends on a heritable character of the hosts (segregating as an autosomal dominant Mendelian factor) and not only on the availability of suitable intestinal redox-potentials and pH values. In this respect, in methanogenic hosts the period from birth to weaning would provide the basis for the persistence of methanogens in the gut (Hackstein, 1997).

If the hypothesis of Hackstein and co-workers that 'methanogenesis is likely under genetic control and not under dietary control' was true, reduction of CH_4 emissions by selecting non- or low-producers would be feasible. The existence of between-sheep variation in CH_4 emission has been reported both from controlled (Blaxter and Clapperton, 1965; Demeyer and Van Nevel, 1975) and grazing conditions (Lassey et al., 1997). Nevertheless, in order for animal breeding to be effective, the relevant animal characteristics and the mechanisms via which the characteristics influence methanogenesis by microbes in the rumen, must be identified (Gibbs et al., 1989).

1.5 TECHNIQUES FOR MEASUREMENT OF METHANE PRODUCTION ON INDIVIDUAL ANIMALS

1.5.1 Enclosure techniques

1.5.1.1 Total enclosure of animal

The technique of open-circuit indirect respiration calorimetry was first devised by Pettenkoffer and Voit in 1875 (McLean and Tobin, 1987) and since then, it has been widely used with all animal species to determine the expenditure of energy, and the partition of dietary energy. In ruminants, CH_4 production is measured in conjunction with O_2 consumption and CO_2 production.

The basic open-circuit indirect calorimeter consists of an airtight chamber with an air conditioning system, a gas meter (outflow rate at standard temperature and pressure, STP) and a system to analyse representative samples of the gas entering and leaving the chamber for O_2, CO_2 and CH_4 (Miller and Koes, 1988).

The minimum space within the chamber is recommended be 150 l/ $BW^{0.75}$ (IAEA, 1992). Chambers must be sufficiently rigid to tolerate normal behaviour of animals. All joints between panels, doors, windows, etc. must be made to a high standard, minimizing air-leaks by using appropriate seals and paints. The chamber must have a small door at the animal's head end in order to permit feeding, and plexiglass windows must be fitted in both sides in order to avoid claustrophobia. Environmental conditions (temperature, relative humidity) within the chamber are controlled by an air conditioning system.

Inlet air must be supplied from an outside source, otherwise the background concentration of gases will rise and be highly variable (IAEA, 1992). A sustained slightly negative pressure with respect to atmospheric pressure must be maintained inside the chamber by continuous removal of air by a vacuum pump. Flow rate within the chamber must be set to give about 1.1% in CO_2 concentration in the air leaving the chamber.

The accurate measurement of air flow through the chamber is critical. A dry gas meter is commonly used, with corrections to dry volume at STP. Once the outgoing air leaves the gas meter, its concentrations of CH_4, CO_2 and O_2 are measured. For this

purpose, a small but constant aliquot of the exhaust gas can be collected over the entire period of measurement and stored in a spirometer. All moisture in air samples is removed by passage through a desiccant before entering the O_2, CO_2 and CH_4 analysers. Oxygen concentration can be measured using a paramagnetic analyzer; CO_2 and CH_4 concentrations are determined using infrared analyzers. Analyzers must be calibrated and checked against gas mixtures of known composition.

At all parts in the system up to and including the air outflow meter, it is essential that the pressure is maintained below that of the atmosphere, so that any air leakage is inwards. Thereafter, once the volume has been measured, it is necessary to achieve a slight positive pressure in order to ensure that any leakage will be outwards, so that CH_4 concentration remains unchanged by any possible leaks (IAEA, 1992; Young et al. 1975).

The reliability of the entire calorimetry system must be tested by releasing CH_4 from cylinders into the chamber at measured rates (Birkelo et al., 1986). Similar tests can be carried out for CO_2 recovery (Miller and Koes, 1988), but absolute ethanol (1 mol) burning is a simple test for recoveries of CO_2 and O_2 (Wedegaertner and Johnson, 1983; Birkelo et al. 1986). Gas recovery rates between 97 and 103% indicate good reliability.

Finally, before actual determination of CH_4 production, the animal must be accustomed to the chamber by placing it in the chamber for 6 to 8 h on several occasions before the measurement period.

Although the major advantage of whole-animal calorimeters is that they measure the total digestive tract CH_4 output, they are fixed or of limited mobility, requiring a large building to house them and therefore tend to be very costly. In addition, the data is only directly relevant to the conditions in the chamber, with restrained animals.

1.5.1.2 Masks and hoods

The use of face masks and head stalls (hoods) provides a robust and direct measurement of CH_4 production. The method relies on collecting a representative sample of expired (and eructated) gas and then estimating the gas concentrations (IAEA, 1992). The animal inspires from, and expires to, a stream of air drawn across

its face (McLean and Tobin, 1987). Animals must be properly trained to wear masks or to be kept in hoods.

Masks must be light, but strong for attachment of the sampling tube and to secure it to the animal's head by elastic straps (IAEA, 1992). Fibreglass is an ideal material, but fitting and sealing is improved by strip foam rubber in the edges. The inlet and gas sampling vents need to be at least 50 mm diameter to allow for a free flow of gas. Flexible tubes allow the animal to move. Negative pressure in the sampling line is maintained, so leaks are inwards.

Since masks prevent animals from eating, they are appropriate only for short-term CH_4 measurements, ideally under steady state conditions or at a standardised time after feeding (IAEA, 1992). The prolonged use of masks may cause discomfort and distress to the animals and consequently can affect the gas measurements. Therefore, the use of hoods is preferred to masks (Takahashi et al. 1999).

Hoods should be designed to provide sufficient feeding space and enough room for the animal to move its head in an unrestricted way. However, dead space must be minimized to enable the equipment to respond quickly to changes in rate of gaseous exchange and animal metabolism (Takahashi et al. 1999). Hoods must have a clear window or door at the front to facilitate feeding and watering. A nylon curtain or a sleeve with a hole for the animal's neck and a draw string to minimise gas leakage replaces the rear panel of the hood (IAEA, 1992). The length of the sleeve should allow the animal to stand up and lie down.

In general, the basics for gas collection, gas flow measurement, gas lines, pumps and gas analysis are similar to those in total enclosure calorimeters, irrespective of whether hoods or masks are used (IAEA, 1992). In fact, Young et al. (1975) developed a versatile respiratory pattern analyzer, which includes chambers, masks and hoods.

1.5.2 Tracer techniques

1.5.2.1 Isotopic methods

CH_4 production can be estimated by isotopic dilution using ^3H or ^{14}C-labelled CH_4. The accuracy of isotope-labelled CH_4 depends on its efficient mixing with the pool which is to be measured, in this case CH_4 in the rumen (IAEA, 1992). The basis of the technique is that if a known quantity of radioactively labelled CH_4 is infused into the ventral rumen in such a way as to mix with the CH_4 in solution in the rumen fluid, after equilibration, the specific radioactivity (SR) of CH_4 in the gas phase would indicate the rate of production of CH_4 in this organ (Murray et al., 1976). If simultaneously, the whole digestive tract CH_4 production (as assessed by total collection) is measured, the rate of production in the lower digestive tract can be estimated by difference.

Murray et al. (1976) measured rumen and lower digestive tract CH_4 kinetics in sheep. For this purpose, intra-ruminal and intra-caecal infusions of ^{14}C- and ^3H-labelled CH_4 in solution were made, whereas respired (and eructed) gas ('head' gas) was collected (between feedings) by the mask method. Flatus gas was collected using similar procedures as by the mask method. A sub-sample of the 'head' and 'flatus' gas were drawn from the main stream for analysis of radioactivity by an ion chamber, and CH_4 by infrared analiser.

The rate of CH_4 production in the rumen (M_R, g/min) was calculated from the infusion rate (I, μCi/min) and the mean SR of CH_4 in rumen gases (SR_R, μCi/g):

$$M_R = I/SR_R$$

Similarly, the total CH_4 collected from the 'head' (M_H, g/min) was calculated from the infusion rate (I) and the plateau SR of CH_4 at the mask (SR_H):

$$M_H = I/SR_H$$

Given that the CH_4 produced in the lower digestive tract (M_L, g/min) is partly excreted in flatus (M_{LF}) and partly through the lungs and breath (M_{LH}), and that $M_{LH} = M_H - M_R$, then $M_L = M_H - M_R + M_{LF}$.

The proportion of CH_4 produced in the rumen that is absorbed and excreted through the lungs was estimated by measuring the radioactivity excreted at the 'head', when rumen gas was allowed to escape through an open cannula.

It is also possible to measure CH_4 production from a single dose injection of radioactive tracer (France et al., 1993). In this case, mathematical models can be used to estimate methanogenesis in various compartments of the rumen.

CH_4 is highly diffusible (IAEA, 1992). Thus, strict precautions must be taken during the preparation of the radioactive solution, handling, infusion and sampling. Animals with leak-proof rumen cannulas are required. The technique requires all-metal infusion pumps and lines. Establishment of steady state rumen conditions (hourly feeding) helps to minimise variations over the time.

1.5.2.2 Non-isotopic methods

1.5.2.2.1 The SF_6 bolus technique

Development of the sulphur hexafluoride (SF_6) tracer technique (Johnson et al., 1994) was based on the knowledge that about 95% of the total digestive tract CH_4 production is excreted in the exhaled gases at the animal's mouth and nostrils (Murray et al., 1976, see section 1.3.3 in this chapter). The technique was validated against the calorimetry chamber technique using penned cattle (Johnson et al., 1994), and since then it has been widely used for CH_4 measurement in grazing sheep (Lassey and Ulyatt, 1999; Judd et al., 1999; Leuning et al., 1999), dairy cattle (Johnson et al., 1998; Lassey and Ulyatt, 1999), beef cattle (McCaughey et al., 1997; Johnson et al., 1998; McCaughey et al., 1999) and water buffalo (Turnbull et al., 2000).

The technique involves inserting a 'permeation tube' that emits the gas sulfur hexafluoride (SF_6) at a low but steady rate into the rumen of every participating animal. The technique is based in the fact that at least 95% of the CH_4 produced within the digestive tract is exhaled (chiefly by eructation) at the mouth and nose (Murray et al., 1976). Thus, assuming that both SF_6 and CH_4 have similar emission pathways and dilution rates (Johnson et al., 1994), the CH_4 emission rate (Q_{CH_4}, both eructed and expired) is assessed as:

$$Q_{CH_4} = Q_{SF_6} \times [CH_4]/[SF_6]$$

where $[CH_4]$ and $[SF_6]$ denote the concentrations of the two gases in the exhaled air in excess of background concentrations and Q_{SF_6} is the known rate of SF_6 permeation from the particular tube.

The calibrated tracer source, the collection of gas and its subsequent analysis are the major components of the SF_6 tracer technique (Lassey et al., 1997). A permeation tube (brass, ~ 32-33 g), with a permeable Teflon™ membrane at one end and containing the SF_6 gas is prepared, calibrated and inserted about 7 days before the measurements are to begin. The rate of SF_6 release from each permeation tube, initially charged at 500-800 mg SF_6 (ultra-pure), is determined by weekly weighing in laboratory for at least 2 months to establish a steady permeation rate, while maintained at rumen temperature (39 °C). The typical range of permeation rates for tubes are 0.7-1.7 and 1.8-3.5 mg SF_6 d^{-1}, for sheep and cattle, respectively (Lassey and Ulyatt, 1999).

The air exhaled by the animals is unobtrusively sampled from around the nostrils, without the need to determine sampling efficiency (the $[CH_4]/[SF_6]$ ratio is unaffected). Samples are drawn into a pre-evacuated yoke-shaped pvc canister via a capillary-based connection, mounted on a harness (Lassey et al., 1997). The capillary tubing system controls the sample delivery rate at about the half of yoke volumes (1.7 l for sheep and 2.5 l for cows) during about 24 h sampling duration. A similar system is used to collect background air samples.

At the end of sampling, yokes are checked for pressure and then over-pressured (diluted) with pure nitrogen to ~ 1.5 atm, and a corresponding dilution factor calculated. The gas chromatography is used to analyse CH_4 and SF_6 gases, using flame ionisation detection and electron capture detection, respectively. Final concentrations of CH_4 and SF_6 gases (after dilution) are typically found in the range 7-70 ppmv and 100-600 pptv, respectively in sheep yokes (Lassey et al., 1997).

Moate et al. (1997) argued that the use of SF_6 as a tracer for CH_4 measurement is ironic, because SF_6 is itself a long-lived greenhouse gas, for which the entire source of emission is anthropogenic (Victor and MacDonald, 1999). The atmospheric lifetime ('turnover time') of CH_4 and SF_6 are 12 and 3,200 years, respectively, whereas the

GWP (100 years time horizon) of CH_4 and SF_6 are 21 and 23,900, respectively (IPCC, 1996). Nevertheless, the amount of SF_6 released from permeation tubes used in the SF_6 tracer technique is only in the range of 0.7 to 2 mg per day.

1.5.2.2.2 The C_2H_6 infusion technique

An alternative tracer technique for CH_4 production measurement in grazing ruminants, using ethane (C_2H_6), was recently proposed by Moate et al. (1997). They have argued that since C_2H_6 is closest to CH_4 in the alkane homologous series and therefore has similar physical and chemical properties to CH_4, C_2H_6 should be an ideal tracer of CH_4.

In the experiment reported by Moate et al. (1997), dairy cows were allowed to graze for 2 h and then confined for the measurement of the gas kinetics of the rumen headspace. Cows were administered local anaesthetic in the left side *paralumbar fossa*. A sample of rumen headspace gas (c. 10 ml) was withdrawn into a gas-tight glass syringe using a stainless steel needle (16 g x 150 mm). C_2H_6 (100 ml) was then quickly injected through the needle into the rumen headspace. Aditional samples (6-10) of rumen headspace gas were collected at timed intervals over the subsequent 20 min period. The gas samples were analysed for C_2H_6 and for the principal rumen gases by gas chromatography using Helium as a carrier gas. The C_2H_6 data were analysed as a one-compartment model, which calculated directly the rumen headspace volume and the fractional clearance rate (FCR) of the tracer. The total rate of entry (ROE) of gas into the rumen headspace was calculated as the product of the headspace volume and the FCR. The ROE into the rumen headspace of an individual gas was calculated as the product of its proportional composition in the rumen headspace and the total ROE of rumen gas.

Moate et al. (1997) claimed that the C_2H_6 dosing technique is a simple way to estimate CH_4 production by grazing ruminants. However, it must be noted that it was invasive and cows were not grazing when gases were measured. Furthermore, the 100 ml injection of C_2H_6 represented about 3% of rumen headspace volume, which was not a trace amount (Shipley and Clarke, 1972). Finally, this technique only accounted for the instantaneous measurement of CH_4 production, thus, applying only to steady-

state rumen conditions. Perhaps in the future the C_2H_6 tracer may be developed in a technique similar to that of the current SF_6 technique.

1.5.3 Indirect methods

1.5.3.1 From VFA production

The relative rates of formation of acetate (A), propionate (P) and butyrate (B) largely determine the amount of excess metabolic H_2 available in the rumen which is ultimately converted to CH_4 by the methanogenic archaea (Czerkawski, 1969; Wolin, 1960; Whitelaw et al., 1984). Thus, assuming that in the absence of other H_2 donors and acceptors in the rumen, the rumen hydrogen balance can be calculated from the molar amounts of A, P, B and CH_4 (M) produced during the fermentation process (Demeyer and Van Nevel, 1975), the balance can be represented as:

2H released = 2A + P + 4B = 4M + 2P + 2B = 2H accepted

Alternatively, the eventual production of hydrogen (2H), lactate (L) and valerate (V), and consumption of oxygen (O) can be incorporated:

2A + P + 4B + L +3V = 4M + 2P + 2B + 4V + 2H + L + 2O

The values for CH_4 obtained in these stoichiometric calculations are maximum values since they make the assumption that all dietary hexose is fermented to VFA and that all 'excess' metabolic H_2 produced is utilised in the reduction of CO_2 to CH_4 (Whitelaw et al., 1984). In practice, however, the hydrogen recovery (2H accepted/2H released) from the stoichiometric balance is often in the range of 78-96% (Demeyer et al., 1989). Reasons for this include the presence in the rumen of hydrogen acceptors not related to carbohydrate metabolism such as nitrate, sulphate, unsaturated fatty acids, phytol, alkaloids and formononetin (Demeyer and Van Nevel, 1975) and also incorporation of H_2 into bacterial and protozoal proteins (Whitelaw et al., 1984). On the other hand, VFA in the rumen may result from non-carbohydrate substrates.

The above problems have been recently demonstrated by Faichney et al. (1999), working with defaunated (isolated) and re-faunated lambs. They found that the 2H recoveries for defaunated animals were 55-73% less than expected. Thus, an

unidentified 2H sink (probably reductive acetogenesis) was probably responsible for the low recovery.

1.5.3.2 From feed characteristics

Based on statistical analysis of more than 2,500 calorimetric CH_4 measurements made in sheep and cattle, Blaxter and Clapperton (1965) proposed the following CH_4 prediction equation:

$$CH_4 \text{ (kcal/100 kcal GEI)} = 1.30 + 0.112D - L(2.37 - 0.05\ D)$$

where D is digestibility of feed gross energy (GE) and L is the ratio of GEI to maintenance energy requirements. Johnson and Johnson (1995) indicated that for most feeds, this equation gave prediction values in the range of 6 to 10% (kcal CH_4/100 kcal GEI), whereas the actual values ranged from 2 to 11%.

Moe and Tyrrell (1979) derived an equation based on 404 CH_4 measurements (in respiration chambers) made in dairy cows fed high quality rations. It incorporates three carbohydrate fractions:

$$CH_4 \text{ (MJ/day)} = 3.406 + 0.510S + 1.736H + 2.648C$$

where S, H, C are soluble residue, hemicellulose and cellulose, respectively in kg fed/day. Wilkerson et al. (1994) examined seven published equations for predicting CH_4 production from dairy cows and concluded that the Moe and Tyrrell (1979) equation provided the highest performance and lowest errors.

Kirchgeβner et al. (1994) proposed a prediction equation based on the intake of crude nutrients as variables:

$$CH_4 \text{ (g/day)} = 63 + 79CF + 10*NFE + 26\ CP - 212EE$$

where CF, NFE, CP and EE are the intakes of crude fibre, N-free extract, crude protein and ether extract, respectively in kg/day. The validation of this equation (Kirchgeβner et al., 1995) reported high accuracy (R^2 = 92%) over a wide range of CH_4 releases from lactating dairy cattle, non-lactating and non-pregnant cows and beef cattle.

Recently, Pelchen and Peters (1998) reviewed 1137 observations of CH_4 from sheep. These authors computed regression equations to predict CH_4 emissions from ration descriptive variables subdivided in various class intervals (for example, various levels of feeding). Data sub-division showed benefits in the accuracy of CH_4 estimations. On the other hand, equations including the variables crude fibre and N-free extract yielded higher R^2 than those including NDF and ADF.

Limitations of the empirical regression equations to predict CH_4 emission in all circumstances have been recognised by Johnson and Johnson (1995). Dynamic and mechanistic models (Baldwin et al., 1987; Dijkstra et al., 1992) have the potential to overcome these. A study carried out by Benchaar et al. (1998) compared the prediction accuracies of two regression equations (Blaxter and Clapperton, 1965 and Moe and Terryll, 1979) and two mechanistic models (Baldwin et al., 1987 and Dijkstra et al., 1992) by challenging diet data for dairy cows selected from 13 publications. They found better prediction of CH_4 production with mechanistic models (R^2=0.71) than with regression models (R^2=0.42, for Moe and Terryll, 1979).

1.6 CONCLUSIONS AND NEEDS FOR RESEARCH

1. CO_2, CH_4 and N_2O are, in this order, the most important greenhouse gases. CH_4 concentration has increased at faster rates (2.5 times) compared to CO_2 since the pre-industrial period, and is currently increasing at 35-40 Tg y^{-1}. Because of its shorter lifetime in the atmosphere (12 years), CH_4 is an excellent candidate for mitigation purposes. A reduction of only 8% in emission rate is needed to stabilise its atmospheric concentration at current levels

2. New Zealand's share in the global emissions of CO_2, CH_4 and N_2O is only 0.1, 0.6 and 1.2%, respectively. Nevertheless, on *per capita* basis New Zealand's emissions of CO_2, CH_4 and N_2O are respectively 2.2, 9.7 and 18.6 times the global average. Contrary to the rest of the world, where CO_2 emission is the most important, in New Zealand, the most important greenhouse gas is CH_4. About 88% of the New Zealand's anthropogenic CH_4 emission is derived from enteric fermentation in grazing ruminants. Efforts to mitigate CH_4 production will contribute to enhance the country's global image.

3. In ruminant production systems, CH_4 production is an integrated part of the feed digestion process. By reducing CO_2 with H_2 as the only energy source, methanogens keep the partial pressure of hydrogen in the rumen very low, which has an important effect on the overall fermentation end-products and efficiency. Nevertheless, methanogenesis represents between 2 and 15% loss of the energy intake. Ruminants fed on forage diets produce CH_4 at higher rates than ruminants on concentrate-based diets.

4. Mitigation of CH_4 emission from ruminants would have both nutritional and environmental benefits. However, most of the current technologies available for inhibition or reduction of methane emission using feed additives are seen with pessimism because of their adverse effects on the ruminal and animal system, and their lack of effectiveness in systems based on forages. In these systems, interventions at the dietary level by ration manipulation or by management interventions to minimise CH_4 yield per unit of animal product appear more practical. Nevertheless, interventions requiring high inputs of supplementary feeding could compromise the unique ability of ruminants, which is the conversion of fibrous feed resources into high quality food.

5. Until recently, CH_4 production was generally measured by the standardised calorimetry chamber technique or estimated by indirect methods. The development of the SF_6 tracer technique has been claimed to be relatively inexpensive for reliable measurements of CH_4 emission under field conditions. Thus, this technique offers great potential for the accurate assessment of CH_4 mitigation effects by increasing the size of the sample. Nevertheless, the use of this technique in sheep has not been evaluated yet.

6. Since variations in CH_4 emission between individual animals, within a breed, have been observed, animal breeding for low-CH_4 production may be feasible provided that the trait is inherited and is not negatively correlated with other desirable characteristics, such as efficiency of digestion. For this purpose, the relevant animal characteristic(s) associated with the variation need(s) to be identified.

7. If the between-animal variation in CH_4 emission has a genetic basis, it must be persistent with time and different feeding conditions. This hypothesis needs to be tested.

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Chapter 2

Evaluation of the sulphur hexafluoride (SF_6) tracer technique for the measurement of methane emission from sheep

2.1 ABSTRACT

Two groups (group 1: 8 animals and group 2: 10 animals) of chryptorchid Romney sheep were involved in a study of the long-term persistence of methane (CH_4) emission. Three trials were carried out both with sheep group 1 (T_{11}, T_{12} and T_{13}) and sheep group 2 (T_{21}, T_{22} and T_{23}). Indoors parallel CH_4 emission measurements by the sulphur hexafluoride (SF_6) tracer technique ('tracer') and by the calorimetry chamber technique ('calorimetric') were carried out in T_{13} (group 1) and trials T_{22} and T_{23} (group 2) in order to evaluate the SF_6 tracer technique for CH_4 measurement. In T_{13}, sheep were fed perennial ryegrass (*Lolium perenne*) pasture, whereas chaffed lucerne hay was fed in T_{22} and T_{23}. Feeding level was restricted to 1.2 times the maintenance requirements in all trials, distributed in two (T_{13} and T_{22}) or 12 (T_{23}) meals per day.

The 'tracer' CH_4 measurements were carried out for 4 (T_{13}), 2 (T_{22}) and 5 (T_{23}) days while sheep were kept in digestibility crates placed 2-3 m from each other within a building (T_{13} and T_{22}) or covered yard (T_{23}). Following the 'tracer' CH_4 measurements, sheep were transferred to open-circuit calorimetry chambers where, after 3 days acclimatization, CH_4 was measured for 3 (T_{13}), 7 (T_{22}) and 3 (T_{23}) days. Simultaneous with 'calorimetric' CH_4 measurements, the chamber gases were also collected into Tedlar bags (T_{13}) or PVC containers-'yokes' (T_{22}) for CH_4 and SF_6 analysis by gas chromatography and subsequent CH_4 emission calculations ('modified-tracer' procedures) using either the 'known' ('mod.-tracer, known PR') or the *in chamber* 'measured' ('mod.-tracer, measured PR') permeation rates (PR) of SF_6.

Except in T_{23}, both the 'tracer' (in digestibility crates) and the 'modified-tracer' (in calorimetry chambers) CH_4 emission values were much higher and more variable (within sheep) than the 'calorimetric' values. The limited ventilation in the building, and the subsequent unrepresentative sampling efficiencies of CH_4 and SF_6, and possible problems during handling and storage of gas contained in Tedlar bags were suggested to be responsible for the discrepancies observed in T_{13}. The discrepancies observed in T_{22} were attributed to the depressed *in rumen* PR of SF_6 resulting from the long period of deployment of permeation tubes. In the final trial, T_{23}, the 'tracer' (in crates) and the 'calorimetric' CH_4 emission values agreed very well. It is concluded that provided that pre-insertion and post-recovery PR of tubes are closely monitored and tubes are deployed for short-time, the SF_6 tracer technique is very effective for measuring CH_4 emission by individual sheep. Finally, because little is known about the *in situ* (*in rumen*) permeation behaviour of tubes, studies addressing the effects of time and rumen environment are needed.

2.2 INTRODUCTION

Measurements of methane (CH_4) emission from livestock have generally relied upon the indirect calorimetry technique. While this technique is both accurate and reliable, the expense and need for animal training limit the numbers of animals that can be measured (Johnson et al., 1998). In addition, the extent to which chamber results can be extrapolated to free ranging animals in which diet, behaviour and environment are different from those in chambers has been questioned and has stimulated the development of measurement techniques suitable for grazing animals (O'Kelly and Spiers, 1992; Johnson et al., 1994; Harper et al., 1999; Leuning et al., 1999).

Studies with sheep (Murray et al., 1976; Torrent and Johnson, 1994; Immig, 1996) have established that the rumen accounts for about 87-88% of the total digestive tract CH_4 production. About 95% of the rumen CH_4 is excreted by eructation, whereas 89% of the lower gut CH_4 production (12-13% of the total) is excreted through the lungs (Murray et al., 1976). Thus, about 95% of the total tract CH_4 production is exhaled through the mouth and nostrils. Based on this knowledge, Johnson et al. (1994) developed the sulphur hexafluoride (SF_6) tracer technique for CH_4 measurement, which has been used with penned and grazing sheep and cattle (Lassey et al., 1997; Lassey and Ulyatt, 1999; Judd et al., 1999; Leuning et al., 1999; Johnson et al., 1998; McCaughey et al., 1997; McCaughey et al., 1999).

Johnson et al., (1994) showed a good agreement between the SF_6 tracer technique and calorimetry chamber CH_4 measurements in cattle, but to date no evaluation of this technique has been carried out with sheep. Although it is not known whether CH_4 production dynamics differ between sheep and cattle, it is known that digestion efficiency and digesta kinetics differ between these species, especially when they are fed on forages (De Boer et al., 1984; Colucci et al., 1984). The present study therefore sought to compare sheep CH_4 emissions measured by the SF_6 tracer technique and the standard respiration calorimetry chamber technique.

2.3 MATERIALS AND METHODS

2.3.1 Experimental design and animals

Two groups (1 and 2) of chryptorchid Romney sheep, which were involved in a study of the long-term persistence in CH_4 emission, were each monitored in three trials (group 1: trials T_{11}, T_{12} and T_{13}; group 2: trials T_{21}, T_{22} and T_{23}), and this persistence study is the subject of Chapter 3 of this thesis. However, trials T_{13} (group 1) and T_{22} and T_{23} (group 2) also measured CH_4 emissions in parallel with both the sulphur hexafluoride (SF_6) tracer technique (Johnson et al., 1994) and the calorimeter chamber method, and the present chapter is concerned with these parallel measurements.

The trials (T_{13}, T_{22} and T_{23}) (Table 2.1) were carried out indoors at AgResearch Grasslands and the Animal Physiology Unit, Massey University, Palmerston North, NZ during January-February 1997 (T_{13}), October-November 1997 (T_{22}) and May-June 1998 (T_{23}). Before the start of experiments and between experiments, sheep were maintained at grazing on ryegrass/white clover pasture. In T_{13} (group 1) sheep were 17-months old; whereas in T_{22} and T_{23} sheep belonging to group 2 were 15 and 22 months old, respectively (Table 2.1). Sheep from group 2 were prepared with rumen cannulae (65 mm internal diameter) six months prior to the commencement of T_{23}. Plastisol washers were fitted around the cannulae to minimize loss of gases through the rumen fistula.

In all experiments sheep were managed in pairs in order to synchronize the availability of the two respiration chambers. Sheep were brought into covered yards and put in digestibility crates. After acclimatization, a period of feed digestibility measurement took place (Figure 2.1). During this period CH_4 production was measured by the SF_6 technique for 4, 2 and 5 days in T_{13}, T_{22} and T_{33}, respectively (Figure 2.1). At the end of this period, sheep were transferred to calorimetry chambers for 3 days of acclimatization followed by 3 (T_{13}), 7 (T_{22}) and 3 (T_{23}) days of CH_4 measurement. In T_{13}, tracer CH_4 measurement was carried out 2 days before and 2 days after the calorimetry period (Figure 2.1).

2.3.2 Feeds and feeding

Once removed from grazing, sheep were placed in digestibility crates. In all the trials, the feeding level during the measurement periods (both in crates and chambers) was restricted to about 1.2 times the maintenance energy requirements (Table 2.1). In trials T_{13} and T_{22}, the sheep were fed twice daily, both in digestibility crates and chambers; whereas in T_{23} feeding frequency in digestibility crates was 12 times a day, but it was reduced to 2 times daily when the sheep were moved into the calorimetry chambers. The daily feed allowance was distributed evenly between the feedings. Manual feeding (twice a day) took place at 08:00 and 16:00 h. Feed on offer and refusals were recorded daily and samples were taken for daily DM determinations (100 °C, 48 h).

The feed during T_{13} was fresh cut pasture. Pasture was cut early in the morning (about 07:00) and, after mixing, fed fresh at the morning feeding, while the afternoon allowance was kept in a cold room. The pasture used in this trial (T_{13}) was predominantly composed of perennial ryegrass (>80% DM basis) with minor contributions of annual meadow grass, white clover and chicory. Variation in pasture quality with progress during the experiment was minimized by using a cutting sequence to give similar periods of growth for the daily supply of pasture.

Chaffed (~5 cm) lucerne hay from one uniform batch was fed in T_{22} and T_{23}. Within these trials (T_{22} and T_{23}), the quality of the feed offered was controlled by thorough mixing of the total experimental requirements prior to the experiment and weighing the daily allowance for individual animals into plastic bags.

Drinking water was made available *ad libitum* both in the digestibility crates and calorimetry chambers.

Table 2.1: Animal and experimental conditions during the trials (T_{13}, T_{22} and T_{23}).

	T_{13}	T_{22}	T_{23}
	Sheep Group 1	**Sheep Group 2**	
Date	Jan.-Feb. '97	Oct.-Nov. '97	May-June '98
Sheep:			
Number	8	10	10
Age (months)	17	14	22
Liveweight (kg)	57.3	40.4	46.9
Feed and Feeding:			
Feed	cut pasture	lucerne hay	lucerne hay
M/D (MJ/kg DM)	10.6	8.3	8.4
Level of feeding (x maint.)	1.2	1.2	1.2
Feedings per day	2	2	12[1]
CH_4 measurements (days):			
by calorimetry chambers	3	7	3
by SF_6 in digest. crates	4[2]	2	5
by mod. SF_6 in chambers[3]	3	2-5	nil
Permeation tubes (SF_6 technique):			
Insertion dates	14/03/96[4] & 9/01/97[4]	15/03/97	20/04/98
Retrieve date	30/04/97	17/04/98	11/06/98
Days *in situ*[5]	380 &110	397	51

[1] Feeding frequency: 12 times a day in digestibility crates, but twice a day in calorimeter chambers.

[2] Two days before and two days after calorimeter chamber measurements.

[3] Gas chromatography (GC) analysis (under SF_6 technique's routine) of chamber's inflow and outflow air, sub-sampled into gas-proof Tedlar bags (in T_{13}) or evacuated PVC containers 'yokes' (in T_{22}).

[4] In T_{13} each sheep (group 1) had two permeation tubes: 1 presumably expired (inserted 11/01/96) and 1 active (inserted on 9/01/97). The two sets were recovered (30/04/97) after 380 and 110 days *in situ*.

[5] Days *in situ* (in sheep) deployment of permeation tubes.

SHEEP GROUP 1:

T_{13}	Location	digestibility crates												calorimetry chambers						digestib. crates	
	Days	1	2	3	4	5	6	7	8	9	10	**11T**	**12T**	13	14	15	16	17	18	**19T**	**20T**
	Activities	acclimatisation										FI&FD,		acclimatisation			FI&FD, calorimetry CH_4			FI&FD	

SHEEP GROUP 2:

T_{22}	Location	digestibility crates												calorimetry chambers									
	Day	1	2	3	...	20	21	22	**23T**	24	25	26	**27T**	28	29	30	31	32	33	34	35	36	37	38
	Activities	acclimatisation						feed intake and digestibility							acclimatisation			feed intake, calorimetry CH_4						

T_{23}	Location	digestibility crates																			calorimetry chambers					
	Days	1	2	..	21	22	**23T**	24	25	**26T**	**27T**	28	29	30	31	32	**33T**	34	35	**36T**	37	38	39	40	41	42	43
	Activities	acclimatisation				feed intake and digestibility						FI		digestion dynamics								acclimatisation			FI, calorimetry CH_4		

Abbreviations: FI, feed intake; FI&FD, feed intake and digestibility; **11T, 12T, 19T, 20T** in T_{13}, **23T, 27T** in T_{22} and **23T, 26T, 27T, 33T, 33T** in T_{23} indicate the days on which CH_4 was measured by the tracer technique (called 'tracer CH_4').

Figure 2.1 Experimental design for the trials, showing the location, days and main activities involved. Digestion dynamics (T_{23}) is described in Chapter 4.

2.3.3 The CH_4 measurements

2.3.3.1 By open-circuit respiration chambers ('calorimetric')

Two calorimeters (1 and 2) were used in this study (Holmes, 1973). Temperature in the chambers was maintained at 14-16 °C. An infrared gas analyser (Servomex, UK) was used to measure the CH_4 concentration on aliquot representative samples (c. 7000 ml) collected, in spirometers sealed with liquid paraffin, at constant rate (during about 22 h) from the inflow and outflow airstreams.

The two calorimeters differed in construction, with chamber 1 being older than chamber 2. For this reason, except in T_{13}, chamber 1 was used only for acclimatisations prior to CH_4 measurements in chamber 2.

A gravimetric method was used to test the recovery of CH_4 in the chambers. Pure CH_4 was released at about 25 ml/min into each chamber for about 2.5 hours and the cylinder was weighed at the start and the end of the release periods to determine the weight of CH_4 released. The mean (±s.d.) CH_4 recovery for chambers 1 and 2 were 88.8±7.3 and 95.2±5.0%, respectively.

CH_4 was measured by the calorimetric technique (hereafter called 'calorimetric' CH_4 emission) for 3, 7 and 3 days in T_{13}, T_{22} and T_{23}, respectively (Figure 2.1).

On some days during T_{13} and T_{22}, after the daily CH_4 analysis by the calorimeter chamber routine was finished, the remainder of the aliquot samples from the chamber's outflow and inflow gases, which had been collected in the spirometers, were drawn into gas-proof Tedlar bags or PVC containers ('yokes') for gas analysis under the routine of the SF_6 tracer technique (Table 2.1), as described in the section 2.3.3.3 of this chapter

2.3.3.2 By the SF_6 tracer technique ('tracer')

The calibrated SF_6 tracer source, the gas collection system and the subsequent analysis of samples are the three major components of the SF_6 tracer technique (Johnson et al., 1994). The application of the SF_6 technique for methane measurement in sheep has been described by Lassey et al. (1997). Very briefly, the technique involved the slow release of an inert tracer, sulphur hexafluoride (SF_6) from a 'permeation tube' dosed into the rumen. A sample of air exhaled by each sheep was

drawn continuously (controlled by capillary tubing) from near the mouth and nostrils over ~24 h into a lightweight pre-evacuated PVC container (yoke) suspended on the digestibility crates. Gas chromatography (GC) was used to analyse the concentrations of CH_4 and SF_6 gases, using flame ionisation detection and electron capture detection, respectively.

The daily CH_4 emission (g/day) (hereafter called 'tracer' CH_4 emission) was calculated using the ratio of CH_4 to SF_6 concentrations (above the background) in the sample, in conjunction with the 'known' permeation rate (PR) of SF_6 ('known' PR, g/day), as shown by *Equation* 2.1 (Johnson et al., 1994).

'Tracer' CH_4, g/day = 'known' PR x $[CH_4]/[SF_6]$ ***Eq.* 2.1**

CH_4 measurements by the SF_6 technique ('tracer' CH_4) were carried out while animals were kept in digestibility crates placed 2-3 m from each other within a building. The building (Animal Physiology Unit, Massey University) used in T_{13} and T_{22} was naturally well ventilated during the working hours (07:00-18:00 h) by two doors at each end of the building (one large and one small). Night ventilation in T_{13} was, however, restricted to two small windows; whereas in T_{22}, the small door was kept open during the nights. In T_{23}, the sheep were kept in a well-ventilated covered yard (AgResearch Grasslands). The 'tracer' CH_4 measurements were carried out for 4, 2 and 5 days in T_{13}, T_{22} and T_{23}, respectively (Table 2.1).

Table 2.1 shows the dates of insertion and retrieval of permeation tubes deployed in each trial. In T_{13} (sheep group 1) two permeation tubes were present in each animal: one was assumed to have expired ('old', inserted on 14/03/96) and one was active ('new', inserted on 9/01/97). To confirm that the 'old' tubes had effectively expired (predicted *Use-by-date*[1]: June-November 1996) a 24-h collection of exhaled gases (the SF_6 technique) was carried out on the 8th January 1997 (i.e. one day before inserting the 'new' set of tubes) while sheep were grazed, but the samples were not analysed until later, together with those from T_{13}. Based on the fact that in our laboratory, the mean $[CH_4]/[SF_6]$ ratio in samples is found about $57x10^3$ (Ulyatt et al., 1999), the $[CH_4]/[SF_6]$ ratios found in samples taken on the 8th January 1998 (Table

[1] *Use-by-date*: estimated date at which the SF_6 load in the permeation tube falls to 150 mg, with the assumption of linear mass loss (K. Lassey, personal communication).

2.9, Appendices) suggest that the 'old' tubes were still permeating at low but unknown rates, far below their 'known' permeation rates. Samples from sheep deploying the tubes 321 and 344 were effectively dead (zero SF_6 concentration).

2.3.3.3 By application of the SF_6 technique's principles on gases collected from calorimetry chambers ('modified-tracer')

In T_{13} and T_{22}, while sheep were in calorimetry chambers, samples of the chamber's inflow and outflow air gases were drawn into Tedlar bags (T_{13}) or evacuated yokes (T_{22}), and CH_4 and SF_6 concentrations were analysed by gas chromatography (GC), under the SF_6 technique's routine. The concentrations of the gas SF_6 in these samples were used to calculate the amount of this gas released in the chamber ('measured' PR of SF_6, g/day) from a particular tube located in the rumen of a particular sheep, using the following equation:

$$\text{'Measured' PR of } SF_6\text{, g/day} = \frac{([\text{out } SF_6] - [\text{in } SF_6]) \times VR \times 146}{22.4} \qquad \textit{Eq. 2.2}$$

where [out SF_6] and [in SF_6] are the concentration of SF_6 in the outflow and inflow air, respectively; VR is the standardized ventilation rate (l/day) in the chamber, 146 is the molecular weight (g) of SF_6 and 22.4 is the molar volume (l) of gas.

Then, the SF_6 technique principles were applied to calculate the CH_4 emission from sheep in calorimetry chambers (hereafter called 'modified-tracer' CH_4 emission) based on the concentrations of CH_4 and SF_6 gases in the chamber's inflow and outflow air samples (determined by gas chromatography) in conjunction with either the 'known' or the 'measured' PRs of SF_6 from permeation tubes.

To calculate the CH_4 emission (g/day) based on the concentrations of gases in the chamber together with the 'known' PR of SF_6 (hereafter called 'modified-tracer, known PR' CH_4 emission), the following equation was used:

$$\text{'Mod.-tracer, known PR' } CH_4\text{, g/day} = \text{'known' PR x } \left(\frac{[\text{out } CH_4] - [\text{in } CH_4]}{[\text{out } SF_6] - [\text{in } SF_6]} \right) \qquad \textit{Eq. 2.3}$$

where the 'known' PR is the 'known' PR of SF_6 (g/day) from a particular tube and [out] and [in] denote concentrations of the particular gas (CH_4 or SF_6) in the chambers' outflow and inflow air samples, respectively.

Alternatively, to calculate the CH_4 emission (g/day) based on the concentrations of gases in the chamber together with the 'measured' PR of SF_6 (hereafter called 'modified-tracer, measured PR' CH_4 emission), the following equation was used:

$$\text{'Mod.-tracer, measured PR' } CH_4\text{, g/day} = \text{'measured' PR} \times \left(\frac{[\text{out } CH_4] - [\text{in } CH_4]}{[\text{out } SF_6] - [\text{in } SF_6]} \right) \qquad \textbf{\textit{Eq.} 2.4}$$

where 'measured' PR is the 'measured' PR of SF_6 (g/day) (*Eq.* 2.2) from a particular tube and [out] and [in] are the gases concentration as in *Eq.* 2.3.

Finally, once permeation tubes were retrieved from the rumen of the animals (at slaughter or by surgery), their weight loss was monitored (for various months) in the laboratory at 39 °C. Thus, a 'post-recovery' PR was determined. The 'post-recovery' PR was not used to re-calculate the CH_4 emission, but it was compared with the 'known' and the 'measured' PRs.

Given that the 'in situ' PR, i.e. the PR while the permeation tube was deployed in sheep, can not be measured directly, it was assumed that the pre-insertion 'known' PR corresponds to the 'in situ' PR. However, if the recovery of SF_6 in the calorimetry chamber was about 100%, the 'measured' PR would be a good estimator of the 'in situ' PR.

Table 2.2 summarizes the concepts and keywords used in this chapter regarding the CH_4 measurements. Hereafter, only the keywords will be used.

Table 2.2 Definition of keywords used in the explanation of methodology of CH_4 measurements.

KEYWORDS	DEFINITION
Tubes' permeation rates (PR):	
'known'	After filling with SF_6, permeation tubes were weighed in laboratory (39 °C) for a minimum of 2 months. The 'known' PR was determined by fitting linear regression to the tube weighings. Only those with high linearity (R^2>0.99) were deployed in CH_4 measurement participating animals.
'measured'	Determined from SF_6 concentrations (gas chromatography analysis) in the chamber's outflow and inflow air samples and the rate of ventilation of the chamber (***Eq.* 2.2**). An estimation of the amount of SF_6 released from a particular tube located in a particular sheep, while it was in calorimetry chamber.
'in situ'	The actual (*in rumen*) permeation rate. If the recovery of SF_6 in the calorimetry chamber was about 100%, the 'measured' PR will be a good estimator of the 'in situ' PR.
'post-recovery'	Tubes recovered from the animal at the end of trials were weighed for more than two months. 'Post-recovery' PR was determined from linear regression fitting to the post-recovery weighings in laboratory (39 °C).
CH_4 emission measurements:	
'calorimetric'	CH_4 emission measured by the standard calorimetry chamber routine.
'tracer'	CH_4 emission measured in digestibility crates by the SF_6 tracer technique in digestibility crates. CH_4 emission determined by ***Eq.* 2.1**, i.e. using the 'known' PR of SF_6 of permeation tubes.
'modified-tracer, known PR'	CH_4 emission determined by ***Eq.* 2.3**, using SF_6 and CH_4 concentrations in calorimetry chamber's outflow and inflow gases and the 'known' PR of SF_6 of permeation tubes.
'modified-tracer, measured PR'	CH_4 emission determined by ***Eq.* 2.4**, using SF_6 and CH_4 concentrations in calorimetry chamber's outflow and inflow gases and the 'measured' PR of SF_6 of permeation tubes.

2.3.4 Data calculation and analysis

For each trial, the mean values and the coefficient of variation (CV) of CH_4 emission (g/kg dry matter intake, DMI and g/animal/d) measured by the 'tracer' technique (*Eq.* 2.1) and by the 'calorimetric' technique were calculated. The two CH_4 measurement techniques were compared by the paired t-test (Cody and Smith, 1991). In addition, the mean CH_4 emission values were subject to correlation analysis. Similar statistical analyses were carried out to compare the daily CH_4 emission (g/animal) measured by the 'calorimetric' technique and those measured by the 'modified-tracer' procedures

(*Eq*s. 2.3 and 2.4). For this, only the 'calorimetric' CH_4 emission values corresponding to the days when the 'modified-tracer' procedures took place were computed.

The 'measured' PR (*Eq*. 2.2) and the 'post-recovery' PR of SF_6 from the permeation tubes used in each trial were expressed as a percent of the 'known' PR and paired t-tests were carried out to compare mean values. Coefficients of correlation between these permeation values were also calculated.

2.4 RESULTS

2.4.1 'Tracer' versus 'calorimetric' CH_4 emission values

In general, the CH_4 emission rates measured by the 'tracer' and by the 'calorimetric' techniques differed from each other (Table 2.3). For trials T_{13} and T_{22}, the 'tracer' technique produced significantly greater CH_4 emissions, both per animal (g animal^{-1}) and per unit of intake (g kg^{-1} DMI) than the 'calorimetric' technique; whereas the opposite was observed in T_{23}. In T_{23}, the two techniques differed significantly from each other only for the CH_4 emission per unit of intake, and not for CH_4 per sheep. In all the trials, the 'calorimetric' technique produced more consistent CH_4 emission values, with less variation between-days within sheep (CV) than the 'tracer' technique (Table 2.3).

Table 2.4 shows that in trials T_{13} and T_{22}, the CH_4 emission measurements by the 'tracer' and the 'calorimetric' techniques were moderately correlated, but not significantly ($p>0.05$) for daily CH_4 emission expressed per animal. However, in T_{23}, a high (r=0.93) and significant ($p<0.001$) relationship between the two techniques for daily CH_4 emission expressed per animal was observed. For CH_4 emission expressed per unit of intake (g kg^{-1} DMI), the techniques were also highly and significantly correlated (r=0.79; $p=0.02$) in T_{23}, but not in T_{13} or T_{22}.

Table 2.3 Mean (± standard error) and coefficient of variation (CV[1]) of daily CH_4 emission (g animal^{-1} and g kg^{-1} DMI) as measured by the SF_6 tracer technique ('tracer', in digestibility crates) and by the calorimetry chamber ('calorimetric') and test of the difference of their means. (n=total number of observations).

CH_4 Emission	'Tracer' Technique		'Calorimetric' Technique		'Tracer'-'Calorimetric' [2]
	mean±s.e.	CV (%)	mean±s.e.	CV (%)	mean±s.e.
T_{13}	*n=29*		*n=24*		*n=8*
g animal^{-1}	32.2±1.8	14.2	25.0±0.7	8.8	7.4±3.0 $_{\bullet}$
g kg^{-1} DMI	26.5±1.4	14.9	19.8±0.5	5.2	7.7±2.6 $_{\bullet}$
T_{22}	*n=20*		*n=77*		*n=10*
g animal^{-1}	24.7±1.6	18.4	17.8±0.3	6.7	6.2±1.8 $_{\bullet\bullet}$
g kg^{-1} DMI	21.3±1.2	18.6	15.8±0.2	5.8	4.9±1.6 $_{\bullet\bullet}$
T_{23}	*n=40*		*n=24* [3]		*n=8* [3]
g animal^{-1}	18.8±0.4	7.8	19.5±0.6	4.3	-0.7±0.5 $_{ns}$
g kg^{-1} DMI	17.1±0.3	7.4	19.3±0.4	4.7	-2.2±0.4 $_{\bullet\bullet\bullet}$

[1] CV (%), calculated by dividing the root mean square error by the mean value.

[2] Paired *t*-test of Ho: 'tracer'-'calorimetric'=0. Subscripts indicate that within particular trial, the difference of means is significantly ($_{\bullet\bullet\bullet}$, $p<0.001$; $_{\bullet\bullet}$, $p<0.01$; $_{\bullet}$, $p<0.05$) or not (ns, $p>0.05$) different from zero.

[3] In T_{23}, 'calorimetric' CH_4 measurements were computed only for 8 sheep (feed intake by 2 sheep drastically dropped while in the chambers).

Table 2.4 Correlation coefficients[1] between mean[2] CH_4 emissions (g animal^{-1} or g kg^{-1} DMI) measured by the SF_6 tracer technique ('tracer'), the calorimetry chamber technique ('calorimetric') and by the 'modified-tracer' procedures[3] ('mod.-tracer, known PR' and 'mod.-tracer, measured PR') (blank cells, not determined[4]).

	'Tracer' (g animal^{-1})	'Calorimetric' (g kg^{-1} DMI)	'Calorimetric' (g animal^{-1})	'Mod.-tracer, known PR' (g animal^{-1})
			T_{13}	
'Tracer' (g kg^{-1} DMI)		0.50 *0.20*		
'Calorimetric' (g animal^{-1})	0.48 *0.22*		1.00 *0.00*	
'Mod.-tracer, known PR' (g animal^{-1})	0.91 *0.001*		0.49 *0.22*	1.00 *0.00*
'Mod.-tracer, measured PR' (g animal^{-1})			0.89 *0.003*	0.71 *0.05*
			T_{22}	
'Tracer' (g kg^{-1} DMI)		-0.12 *0.72*		
'Calorimetric' (g animal^{-1})	0.53 *0.09*		1.00 *0.00*	
'Mod.-tracer, known PR' (g animal^{-1})	0.88 *0.001*		0.44 *0.18*	1.00 *0.00*
'Mod.-tracer, measured PR' (g animal^{-1})			0.64 *0.03*	0.58 *0.06*
			T_{23}	
'Tracer' (g kg^{-1} DMI)		0.79 *0.02*		
'Calorimetric' (g animal^{-1})	0.93 *0.001*		1.00 *0.00*	

[1] *in italics*, probability values for H_0: p=0.

[2] Sheep's mean emission values (n=number of sheep): T_{13}=8; T_{22}=10; T_{23}=8.

[3] See Table 2.2 for description of key words.

[4] Other possible correlations not determined because lack of relevance. For example 'tracer' (g kg^{-1} DMI) vs 'calorimetric' (g animal^{-1}) is less relevant than the relationship between these techniques per CH_4 expressed for similar units. For the same reason, the simultaneous (in chamber) CH_4 emissions (g animal^{-1}) measured by the 'mod.-tracer, known PR', 'mod.-tracer, measured PR' and 'calorimetric' techniques were not compared to those determined by the 'tracer' technique (in digestibility crates). The 'mod.-tracer, known PR' and the 'tracer' CH_4 values (g/animal/d) were compared because both values were calculated with the 'known' PR.(see Table 2.2).

2.4.2 'Calorimetric' versus 'modified-tracer' ('mod.-tracer, known PR' and 'mod.-tracer, measured PR') CH_4 emission values

Table 2.5 presents the daily CH_4 emission values (g animal^{-1}) determined by the *in chamber* 'modified-tracer' procedures ('mod.-tracer, known PR' and 'mod.-tracer, measured PR', see Table 2.2 for definitions) and by the calorimetry chamber ('calorimetric') technique. In T_{13}, the CH_4 emission values determined by the 'mod.-tracer, known PR' procedure were much higher than those determined by the 'mod.-tracer, measured PR' procedure (+ 26.3 g) and by the 'calorimetric' technique (+ 20.6 g). The 'calorimetric' value was 5.7 g higher than the value obtained by the 'mod.-tracer, measured PR' procedure. This pattern of differences in CH_4 emission between measurement procedures was repeated in T_{22}, but the differences were smaller. In fact, in this trial (T_{22}) the CH_4 emission determined by the 'mod.-tracer, measured PR' procedure was not different ($p>0.05$) from the 'calorimetric' value.

In agreement with the previous section (2.4.1) the variation between-days (within sheep) in CH_4 emission was larger with the tracer-based measurements ('mod.-tracer, known PR' and 'mod.-tracer, measured PR') than with the calorimetry technique (see CV in Table 2.5).

Table 2.4 summarises the correlation analysis between the CH_4 emission values (g animal^{-1}) determined by the 'mod.-tracer, known PR', 'mod.-tracer, measured PR' and 'calorimetric' procedures. The CH_4 emissions determined by 'mod.-tracer, known PR' and 'mod.-tracer, measured PR' correlated well in both T_{13} (r=0.71, p=0.05) and T_{22} (r=0.58, p=0.06). In turn, in both T_{13} and T_{22}, the 'mod.-tracer, measured PR' values were more closely correlated to the 'calorimetric' values than were the 'mod.-tracer, known PR' values (r=0.89, p=0.003 vs r=0.49, p=0.22 in T_{13} and r=0.64, p=0.03 vs r=0.44, p=0.18 in T_{22}). Furthermore, in both T_{13} and T_{22}, very high and significant correlations were observed between the SF_6 ('tracer') and the 'mod.-tracer, known PR' CH_4 emission (g animal^{-1}) values (r=0.91, p=0.001 in T_{13}; r=0.88, p=0.001in T_{22}) (Table 2.4).

Table 2.5 Mean (± standard error) and coefficient of variation (CV[1]) of CH_4 emission (g animal^{-1}) determined by the 'modified-tracer' procedures[2] ('mod.-tracer, known PR' and 'mod.-tracer, measured PR') and by the calorimetry chamber technique ('calorimetric'), and tests of difference of their mean values. (n= total number of observations).

Trials	'Mod.-tracer, known PR' (1)		'Mod.-tracer, measured PR' (2)		'Calorimetric' (3)		Means Difference Tests[3] (1) - (3)	Means Difference Tests[3] (2) - (3)
	mean±s.e.	CV (%)	mean±s.e.	CV (%)	mean±s.e.	CV (%)	mean±s.e.	mean±s.e.
T_{13}	46.0±3.9 *n=20*	22.5	19.7±1.5 *n=20*	23.3	25.4±0.7 *n=20*	7.6	20.6±5.3 •• *n=8*	-5.7±1.2 •• *n=8*
T_{22}	27.0±1.1 *n=28*	20.1	19.5±0.6 *n=28*	13.8	18.6±0.4 *n=28*	5.5	9.5±1.1 ••• *n=10*	1.3±0.6 ns *n=10*

[1] CV (%), calculated by dividing the root mean square error by the mean value.

[2] See Table 2.2 for description of key words.

[3] Paired *t*-test of Ho: Difference of means=0. Subscripts indicate that within particular trial, the difference of means is significantly (•••, $p<0.001$; ••, $p<0.01$; •, $p<0.05$) or not (ns, $p>0.05$) different from zero.

2.4.3 Permeation rates (PR) of SF_6 from permeation tubes: relationships between the 'known' (pre-insertion), *in chamber* 'measured' and the 'post-recovery' PR

As shown in Table 2.6, both in T_{13} and T_{22}, there were significant differences ($p<0.001$) between the 'known' and 'measured' PR of the permeation tubes. In T_{13}, the 'measured' PR were only 48% of the 'known' PR; whereas in T_{22}, for different set of tubes, the corresponding value was 80%.

Table 2.6 also shows that for trials T_{13} and T_{23} the 'post-recovery' PR did not differ ($p>0.05$) from the 'known' PR, whereas for T_{22} the 'post-recovery' PR was 22.2% lower ($p<0.001$) than the 'known' PR.

The comparison between the 'measured' and the 'post-recovery' PR was possible only for T_{13} and T_{22} (Table 2.6). In T_{13} the 'measured' PR value was 54% less ($p<0.001$) than the 'post-recovery' value, whereas in T_{22} these PRs did not differ ($p>0.05$).

The correlation analysis between the PR (Table 2.7) revealed that 'known' and 'measured' PR were highly correlated ($r=0.93$, $p<0.0001$) in T_{22}, but not in T_{13}. In T_{13}, the 'post-recovery' PR correlated well both to the 'known' ($r=0.68$, $p=0.06$) and 'measured' ($r=0.66$, $p=0.07$) PRs. The corresponding correlations between these PRs were higher and more consistent in T_{22} ($r=0.97$, $p=0.0001$ for 'post-recovery' vs 'known' and $r=0.90$, $p=0.0009$ for 'post-recovery' vs 'measured').

In T_{23}, an almost perfect correlation ($r=0.98$, $p<0.0001$) between the 'post-recovery' and the 'known' PRs was observed. This finding is illustrated in Figure 2.2, where the 'known' (pre-insertion) and the 'post-recovery' PRs of tube 344 deployed in sheep No. 2 typically represent the permeation behaviour of the set of tubes used in T_{23}, i.e. there was very little change in PR between the pre-insertion and the post-recovery stages.

Table 2.6 Mean (± standard error) values of the 'known', the *in chamber* 'measured' and the 'post-recovery' permeation rates (PR) of SF_6 from permeation tubes[1] used in each trial and tests of difference between their means. All values (except column **(2)-(3)**) expressed as percent of the 'known' PR (100%). (n=number of permeation tubes).

Trials	n	'Known' PR (1)	'Measured' PR (2)	'Post-recovery' PR (3)	Means Difference Test[2] (2) – (1)	(3) – (1)	(2) – (3)[3]
T_{13}	8	100	48.4±4.1	104.2±5.6	-51.7±4.1 ***	4.2±5.6 ns	-53.9±2.2 ***
T_{22}	10[4]	100	79.6±3.4	77.8±3.1	-20.4±3.4 ***	-22.2±3.1 ***	5.5±4.2 ns
T_{23}	10	100	nd[5]	99.1±1.0	nd	-0.9±1.0 ns	nd

[1] Independent set of tubes deployed at each trial.

[2] Paired *t*-test Ho: Difference of means=0. Subscripts indicate that within a trial, the difference of means is significantly (***, $p<0.001$) or not (ns, $p>0.05$) different from zero.

[3] **(2) – (3)**: The 'measured' PR value **(2)** expressed as a percent of the 'post-recovery' PR value (100%) **(3)**.

[4] In T_{22}, n=8 for 'post-recovery' PR. Two permeation tubes were beyond their *expire date* at the time when they were recovered.

[5] nd: not determined.

Table 2.7 Correlation coefficients[1] between the 'known', the *in chamber* 'measured' and the 'post-recovery' permeation rates (PRs) of SF_6 from permeation tubes deployed in trials[2]. Cells in blank, not determined.

	'Known'	'Measured'	'Post-recovery'
		T_{13}	
'Known'	1	0.18 *0.68*	0.68 *0.06*
'Measured'		1	0.66 *0.07*
		T_{22}	
'Known'	1	0.93 *0.0001*	0.97 *0.0001*
'Measured'	1	1	0.90 *0.0009*
		T_{23}	
'Known'	1		0.98 *0.0001*

[1] *in italics*, probability values for H_0: p=0.

[2] Mean permeation rates (n=number of permeation tubes): T_{13}=8; T_{22}=10; T_{23}=10. In T_{23}, n=8 for 'post-recovery' PR, two permeation tubes were beyond their *expire date* when they were recovered.

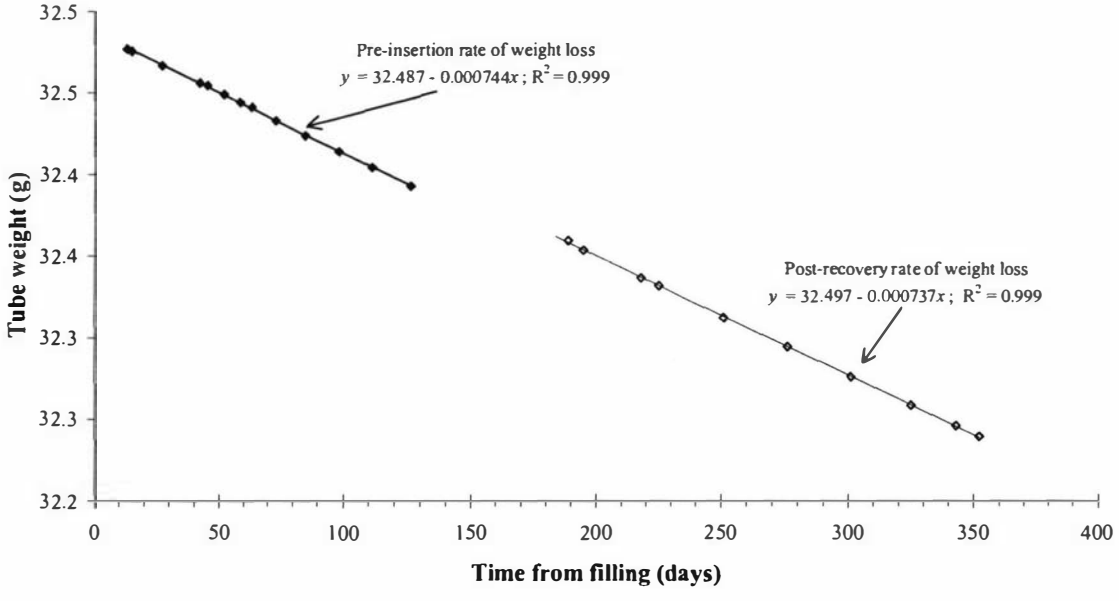

Figure 2.2 Pre-insertion ('known') and 'post-recovery' rates of SF_6 permeation from Tube 344 deployed in Sheep No. 2 during trial T_{23}. The daily 'post-recovery' PR was only 0.007 mg less than the pre-insertion PR (0.733 vs 0.744 mg/day).

2.5 DISCUSSION

Based on the principles of the SF_6 tracer technique (Johnson et al., 1994) and the dynamics of CH_4 production in ruminants (Murray et al., 1976), slightly smaller (by ~5%) CH_4 emission rates are expected for the 'tracer' technique than those measured by the 'calorimetric' technique. However, the results of this study (Table 2.3) showed that in T_{13} and T_{22}, respectively the 'tracer' emission values were 1.3 and 1.4 times greater than the 'calorimetric' values. In contrast, in T_{23}, in which performance of the permeation tubes was almost perfect, the emission values (per animal) recorded by the two techniques were not statistically different from each other.

The observed values were compared to the predicted values calculated by the equation of Blaxter and Clapperton (1965) and the results are shown in Table 2.8. This Table indicates that for T_{13} and T_{22}, the 'tracer' technique CH_4 emission values (% gross energy intake, %GEI) are closer to the predicted values than are the 'calorimetric' values, which apparently casts doubt on the reliability of the standard 'calorimetric' values. Nevertheless, the 'tracer' CH_4 emissions observed in T_{13} and T_{22} can not be assumed to be correct. Pelchen and Peters (1998) have recently found that for 1137 CH_4 emission observations in sheep, the Blaxter and Clapperton (1965) equation had a very poor predictive value (R^2=0.19), while, Murray et al. (1975, cited by Johnson at. al., 1994) found that this equation overestimates the CH_4 output by 10-30%. Secondly, the 'tracer' CH_4 values observed in T_{13} and T_{22} were associated with large coefficients of variation (Table 2.3), whereas the 'calorimetric' values were more repeatable, with CV (5-8%) similar to those usually observed (e.g. Blaxter and Clapperton, 1965).

As shown in Table 2.5 the CH_4 emission measurements by the 'modified-tracer' procedures ('mod.-tracer, known PR' and 'mod.-tracer, measured PR') also produced larger values than the 'calorimetric' technique. The only exception was that in T_{22}, the 'mod.-tracer, measured PR' technique and the 'calorimetric technique, both produced similar values. The 'modified-tracer' procedures produced much larger CVs than the 'calorimetric' technique.

Table 2.8 Expected (from Blaxter and Clapperton, 1965) and measured CH_4 emission values (% gross energy intake, %GEI).

TRIALS	Feed energy digestibility (%)	CH_4 energy (% GEI)		
		Predicted by equation of Blaxter & Clapperton (1965)[1]	Measured (this study)	
			by 'tracer'	by 'calorimetric'
T_{13}	70.3	7.8	7.8	6.0
T_{22}	53.8	6.9	6.5	4.6
T_{23}	56.7	7.1	5.2	5.4

[1] CH_4 (% GEI) = 1.3 + 0.112D – L (2.37 – 0.05D), where D=digestibility of energy (%) and L=level of feeding (times maintenance).

According to the principles of the SF_6 tracer technique (Johnson et al., 1994), the CH_4 emission measurement is only dependent upon the permeation rate (PR) of SF_6 and the $[CH_4]/[SF_6]$ ratio in the gas samples taken. In the present study, the above principles apply both to the 'tracer' technique (*Eq.* 2.1) used in digestibility crates, and also to the 'modified-tracer' procedures ('mod.-tracer, known PR', *Eq.* 2.3 and 'mod.-tracer, measured PR', *Eq.* 2.4) used in calorimetry chambers. Thus, in spite of the lack of agreement between the 'tracer' (in digestibility crates) and 'calorimetric' CH_4 values in T_{13} and T_{22} (Table 2.3), the following outcomes (all for CH_4 expressed as g/animal/d) would be expected: (1) Similar and highly correlated 'tracer' vs 'mod.-tracer, known PR' values, (2) Similar and highly correlated 'calorimetric' vs 'mod.-tracer, known PR' values and (3) At least high correlation between 'mod.-tracer, known PR' vs 'mod.-tracer, measured PR' and 'mod.-tracer, measured PR' vs 'calorimetric' values, even if recovery of gases in the chamber was not accurate. The results showed that for T_{13}, the 'tracer' and the 'mod.-tracer, known PR' values were dissimilar (Tables 2.3 and 2.5), but highly correlated (Table 2.4); 'mod.-tracer, known PR' and 'calorimetric' values were neither similar (Table 2.5) nor correlated (Table 2.4); whereas, both 'mod.-tracer, known PR' vs 'mod.-tracer, measured PR' and 'mod.-tracer, measured PR' vs 'calorimetric' correlated well (Table 2.4). Similar outcomes were found for T_{22}, but the magnitude of the dissimilarities were smaller and the correlations were smaller than those for T_{13}.

To explain the above outcomes (for T_{13} and T_{22}), three hypotheses were formulated. First, when sheep were in digestibility crates, the sampling efficiency for CH_4 was higher than for SF_6, thus the ratio $[CH_4]/[SF_6]$ in samples did not accurately represent the exhaled air. Second, the *in situ* PR of SF_6 from permeation tubes were smaller than the 'known' PR, but similar to the *in chamber* 'measured' PR. Third, the *in situ* PR of SF_6 was similar to the 'known' PR, but greater than the 'measured' PR. Table 2.6 shows that, both for T_{13} and T_{22}, the 'measured' PR of tubes were smaller than the 'known' PR, but whereas for T_{13} the 'measured' and 'known' PR did not correlate to each other (r=0.18, p=0.68), they did for T_{22} (r=0.93, p=0.0001) (see Table 2.7). On the other hand, for T_{22}, the 'post-recovery' PR were highly correlated both to the 'known' and the 'measured' PR (Table 2.7), whereas for T_{13} these correlations were not so strong. Thus, it can be suggested that the outcomes from T_{13} fit within a combination of the first and third hypotheses, whereas those from T_{22} fit within the second hypothesis.

In T_{13}, the $[CH_4]/[SF_6]$ ratio in the 'breath' samples was $240x10^3 \pm 270x10^3$ (s.d.), which is high compared to that ($57x10^3$) usually found under field conditions (Ulyatt et al., 1999). The molecule of SF_6 is about 9 times heavier than that of CH_4. Thus, the limited night-time ventilation of the building used for T_{13} when samples were taken in digestibility crates, might have caused poor mixing of gases and therefore a non-representative sampling of excreted gases. Recycling of the gases into the animal might have also occurred.

In T_{13} the 'known' and the 'post-recovery' PRs were similar (Table 2.6) and were closely correlated with each other (Table 2.7), therefore the lack of correlation between the 'measured' and the 'known' PRs observed in this trial suggests that the 'measured' PR values were incorrect. In this trial (T_{13}), the 'measured' PR and consequently the 'mod.-tracer, measured PR' CH_4 emission values were derived from the analysis of gases collected into and stored in Tedlar bags instead of 'yokes'. Some problems (e.g. contamination, gas losses) during the handling and storage of gases contained in Tedlar bags might have occurred. The SF_6 tracer technique as used by Lassey et al., (1997) involves the analysis of gases collected in evacuated yokes and after dilution with nitrogen (Lassey et al., 1997). A similar procedure might have had errors with samples contained in bags.

There was no clear evidence that the 'old' set of tubes existing in T_{13} (Table 2.1) at an apparent expired *use-by-date* stage, could have contributed to the discrepancy in CH_4 emission values between the techniques. Breath samples taken on 08/01/97 (a day before the insertion of the 'new' set of tubes) revealed that two tubes (321 and 344) were effectively dead, whereas the others were permeating at unknown rates, apparently below their 'known, PRs (in our laboratory, the mean ratio of $[CH_4]/[SF_6]$ in samples is found to be about $57x10^3$) (Table 2.9, Appendices). In addition, the 'post-recovery' measurements of weight loss of these tubes (Table 2.9 and Figure 2.3, Appendices) revealed that on average they were permeating inconsistently (R^2=0.57) at about 3.3% of their 'known' PR and that this pattern was probably the same in T_{13} (100 days earlier). In addition, the 'calorimetric' and 'tracer' CH_4 values in T_{13} for the two sheep (No. 116 and 125) with effectively expired 'old' tubes and the 'post-recovery' PRs (% of 'known' PR) of the 'new' tubes (Table 2.9, Appendices) do not differ from the general patterns of discrepancies already described.

The SF_6 technique (Johnson et al., 1994) assumes that SF_6 PR from permeation tubes is constant. However, this study and other observations (Ulyatt et al., 1999; K.R. Lassey, personal communication) have shown that PR declines with time of deployment of tubes in the rumen, and it has been suggested that a quadratic function would explain better the PR (K.R. Lassey, personal communication). It is unknown whether this change in PR is an effect of the rumen environment, time or both. T_{22} took place after about 250 days of insertion of the permeation tubes and the 'post-recovery' PRs were derived 150 days after the end of this trial. Yet the rate of depression in PR appeared to be homogeneous for all the tubes (high correlation between the 'known' and 'post-recovery' PRs, see Table 2.7).

In conclusion, except in T_{23}, both the SF_6 tracer technique ('tracer' in digestibility crates) and the *in chamber* 'modified-tracer' techniques ('mod.-tracer, known PR' and 'mod.-tracer, measured PR') CH_4 emission values were much higher and less consistent (larger coefficients of variation) than the values determined by the calorimetry chamber technique ('calorimetric'). Although no clear conclusions can be made, it is suggested that in T_{13} the major factor responsible for the discrepancies between the techniques was the limited ventilation in the building (when CH_4 was measured in digestibility crates) and probably errors or contamination during the

handling and storage of the chamber's gases collected and stored in Tedlar bags. On the other hand, a depressed SF_6 permeation rate associated with an increased time of deployment appears to be the reason for the discrepancies observed in T_{22}. However, when the problems identified in T_{13} and T_{22} were overcome, by improved ventilation in the building and the short-term deployment (50 days) of permeation tubes in T_{23}, a good agreement between the techniques for CH_4 measurement was found.

Finally, provided that pre-insertion and post-recovery PRs of tubes are closely monitored and tubes are not deployed for too long the SF_6 tracer technique provides an effective method to measure CH_4 emission rates by individual sheep, confirming the earlier validation tests of the technique with cattle (Johnson et al, 1994; Johnson et al., 1998). The effects of time and rumen environment upon the *in situ* permeation behaviour of tubes require further study.

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2.7 APPENDICES

Table 2.9 Features of the 'old' permeation tubes existing in sheep at 08/01/97 (a day before the 'new' tubes' insertion) when one-day exhaled gases were sampled at grazing for SF_6 and CH_4 concentrations measurement, and the CH_4 emission (g/kg DMI) in trial T_{13} by sheep No. 116 and 125, which 'old' tubes were dead (i.e. zero SF_6 at 08/01/97).

SHEEP No.	'OLD' PERMEATION TUBES: INSERTED 14/03/96 and RETRIEVED 30/4/97							CH_4 (g kg^{-1} DMI) in trial T_{13}	
	Permeation Tube Features			[gases] on samples 08/01/97			'Post-recovery' PR (%)[2]	by 'calorimetry'	by 'tracer' ('new' tube only)
	Tube No.	'known' PR	Use-by-date	$[SF_6]^1$	$[CH_4]^1$	$[CH_4]/[SF_6]$ (10^3)	(May '97)		
106	335	1.80	11/11/96	664	46	69	6.6		
116	321	2.50	20/7/96	0	34		2.4	22.6	24.0
117	314	2.69	07/6/96	361	33	91	1.5		
125	344	2.50	16/8/96	0	76		2.0	19.6	31.3
131	347	2.05	11/11/96	804	75	93	8.1		
135	326	2.88	18/6/96	72	106	1472	1.4		
145	303	2.86	20/7/96	66	71	1076	6.9		
148	342	2.71	24/6/96	46	30	434	4.4		

[1] Concentrations (above the background) of SF_6 (*pptv*, parts per trillion by volume) and CH_4 (*ppmv*, parts per million by volume) on one-day samples, the SF_6 technique.

[2] Permeation rate of 'old' tubes recovered from sheep (30/04/97) and monitored by two months. Values expressed as % of the 'known' PR.

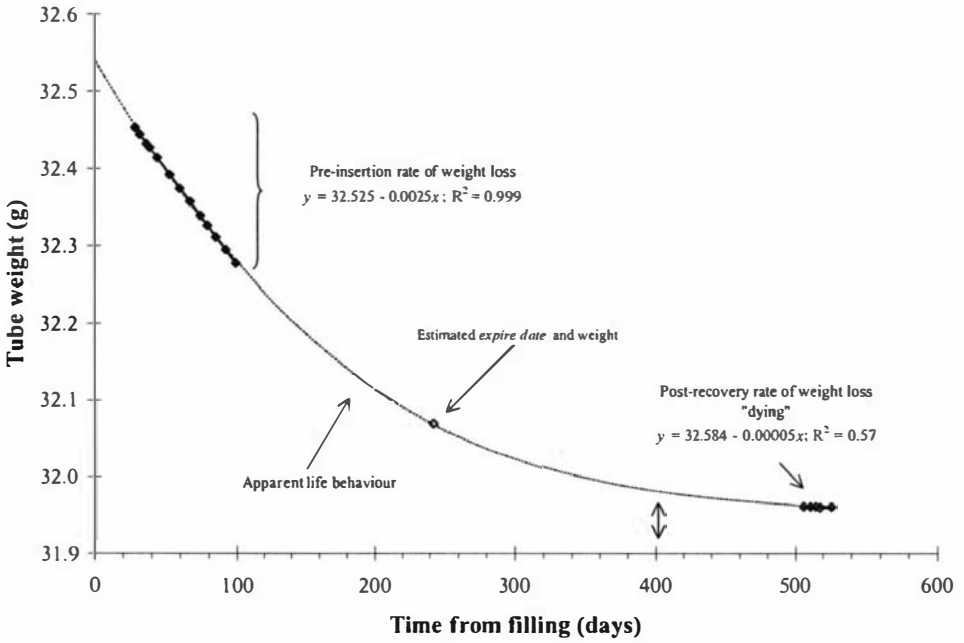

Figure 2.3 Pre-insertion ('known'), 'post-recovery' and apparent whole life permeation rates of Tube 344, existing in sheep 125 at an apparently dead stage in T_{13}. ↕ indicates T_{13}.

Chapter 3

Persistence of the between-sheep variation in methane emission

3.1 ABSTRACT

This study explored the medium- to long-term persistence of between-sheep variation in methane (CH_4) emission involving three experimental groups (1, 2 and 3) of cryptorchid Romney sheep. These sheep were selected on the basis of measurements of initial CH_4 emission rates made at grazing (on perennial ryegrass and white clover pasture) using the sulphur hexafluoride (SF_6) tracer technique. Group 1, comprising 4 low and 4 high CH_4 emitters, was selected in March-April 1996 (group 1: trial 1, $\mathbf{T_{11}}$) from a series of CH_4 emission measurements involving a flock of 50 sheep, and two subsequent indoor trials were carried out in August-September 1996 (group1: trial 2, $\mathbf{T_{12}}$) and January-February 1997 (group1: trial 3, $\mathbf{T_{13}}$). Group 2, comprising 5 low and 5 high CH_4 emitters, was selected in June 1997 (group 2, trial 1, $\mathbf{T_{21}}$) from a series of CH_4 emission measurements involving 92 sheep, and two subsequent indoor measurements were carried out in October-November 1997 (group 2: trial 2, $\mathbf{T_{22}}$) and May-June 1998 (group 2: trial 3, $\mathbf{T_{23}}$) on the same group of sheep. Group 3, comprising 3 low and 4 high CH_4 emitters were selected in October 1999 (group 3: trial 1, $\mathbf{T_{31}}$), based on CH_4 emission measurements over 3 days in 20 animals. Three subsequent grazing CH_4 measurements were carried out in this group (3) in November 1999 (group 3: trial 2, $\mathbf{T_{32}}$), January 2000 (Group 3: trial 3, $\mathbf{T_{33}}$) and February 2000 (group 3: trial 4, $\mathbf{T_{34}}$), using the tracer technique over 3 days.

In all the trials which involved grazing, a fresh area of pasture was offered daily and a generous pasture allowance was offered in order to achieve maximum feed intakes. During all the indoor trials (T_{12}, T_{13}, T_{22} and T_{23}), sheep were kept in digestibility crates. Lucerne pellets and fresh cut pasture were fed in T_{12} and T_{13}, respectively; whereas lucerne hay from one batch was fed in T_{22} and T_{23}. During the indoor trials, following an acclimatisation period, feeding was restricted to 1.2 times the maintenance requirements, and feed digestibility was measured. At the end of the digestibility period, sheep were transferred to calorimeter chambers, where, after 3 days of acclimatization, CH_4 production was measured for 7 (T_{12} and T_{22}) or 3 (T_{13}) days by the calorimetric method. In T_{23} (sheep group 2) CH_4 production was measured for 5 days by the tracer technique in digestibility crates.

The CH_4 emission data were analysed independently for each group of sheep using split-plot analysis of variance. In groups 1 and 2, CH_4 emission subgroups (S, low and high) and trials (T) significantly ($p<0.05$) interacted for CH_4 emission (expressed as g d^{-1}, %GEI and %DEI). In contrast, in group 3, no SxT interaction was observed, and both the low and high CH_4 emission subgroups consistently maintained their initial rankings throughout the subsequent trials. The correlation analysis of rank order for CH_4 emission rates showed very poor

between-trials relationships for both groups 1 and 2; whereas strong between-trials correlation coefficients were observed for sheep in the group 3, although this was weaker in the last trial (T_{34}). Consequently, from the three groups of sheep involved in this study, initial CH_4 emission rates persisted only in group 3. The lack of persistence in CH_4 emission by sheep in groups 1 and 2 might be a consequence of the changing feeding conditions between-trials. In group 3, the acetate/propionate ratio in rumen fluid was positively correlated with CH_4 emission rates, which suggests that low and high emitters differed in rumen fermentation pattern. It is suggested that feeding conditions, which maximize feed intake (e.g. generous allowance of good quality pasture under grazing) favors the expression of between-sheep differences in CH_4 emission (per unit of intake), and under these circumstances sheep persist better in their CH_4 emission rates, but persistence still diminishes with time.

3.2 INTRODUCTION

Rumen methanogenesis results in the loss of up to 12% of gross energy intake (Johnson et al., 1993). Methane (CH_4), a potent greenhouse gas, is estimated to contribute about 24% of the overall global warming potential, second only to carbon dioxide (CO_2) (Houghton, 1997), and its atmospheric concentration has increased over recent years at the rate of about 0.9% per year (Crutzen, 1995). New Zealand's pastoral farming contributes about 88% of the national CH_4 emission (UNFCCC, 1999), which on a per capita basis is ten times greater the global average (Ministry for the Environment, 1997), a consequence of high ruminant livestock and low human populations.

Compared to the other sources of CH_4 emission, ruminant CH_4 can be manipulated relatively simply (Leng, 1993). With appropriate policies, the current and potential future technologies and management practices could reduce CH_4 emissions per unit of animal product by 25 to 75% (Gibbs et al., 1989; Leng, 1993; Mosier et al., 1998). However, with the exception of improved feeding management, the current technologies to control CH_4 emission from ruminants are seen with pessimism (Van Nevel and Demeyer, 1996; Johnson et al., 1996). In addition, for ruminant production systems based on forages, the necessary improvement of feeding might not only be unaffordable, but it may undermine the role of ruminants, which is to utilise low-cost fibrous feed resources. Therefore, the development of cost-effective strategies to mitigate CH_4 production in ruminants, without causing a negative impact on ruminant

production, continues to be a major challenge for ruminant nutritionists and microbiologists (McAllister et al., 1996).

Between-sheep variation in CH_4 emission has long been recognised from standardised measurements *in vivo* (Blaxter and Clapperton, 1965) and *in vitro* (Demeyer and Van Nevel, 1975), and recently confirmed under grazing conditions (Lassey et al., 1997; Ulyatt et al., 1999). The latter authors reported that about 85% of the variation in daily CH_4 emission from sheep grazing temperate pastures was due to variation between-animals. If such between-animal variability is persistent in the long-term, and the animal trait(s) that account for such variation is (are) inherited, breeding of animals for low CH_4 production might be viable (Gibbs et al., 1989).

The present study was planned to assess the medium- to long-term persistence of CH_4 emission by sheep selected initially for low and high CH_4 emission rates.

3.3 MATERIALS AND METHODS

3.3.1 Experimental design and animals

Methane emissions from three groups of sheep (1, 2 and 3), each composed of low and high CH_4 emitters, were independently monitored for 3, 3 and 4 periods (hereafter called trials, T), respectively (Table 3.1) at AgResearch Grasslands and the Animal Physiology Unit, Massey University, Palmerston North, NZ. The interval of time between the first and the last trial was 12, 12 and 4 months for groups 1, 2 and 3, respectively.

The initial selection of animals for the low and high CH_4 emission subgroups (S) and the subsequent trials were as follows:

Group 1

During March and April 1996, CH_4 emissions by 50 sheep were measured, using the sulphur hexafluoride (SF_6) tracer technique (hereafter named 'tracer technique') (Johnson et al., 1994), for 5 days, while grazing perennial ryegrass (*Lolium perenne*)/white clover (*Trifolium repens*) pasture (hereafter named 'RG/WC pasture'). For this purpose, three subflocks (**a**,**b**,**c**) of similar size were processed on three successive weeks (Figure 3.1). Two "control sheep" selected from subflock '**a**', on the basis of consistency of CH_4 emission and temperament, were also monitored during

the weekends and at the time when CH_4 emissions from subflocks '**b**' and '**c**' were measured. A total of 258 sheep-days of CH_4 emission measurements were obtained. Based on these measurements (group 1: trial 1, $\mathbf{T_{11}}$), eight sheep with highly contrasting (4 low and 4 high, $p<0.001$) daily CH_4 emissions (g kg^{-1} dry matter intake, DMI) were selected. Two subsequent indoor trials involving the 8 selected animals were carried out in August-September 1996 (group 1: trial 2, $\mathbf{T_{12}}$) and January-February 1997 (group 1: trial 3, $\mathbf{T_{13}}$).

Group 2

In March 1997, CH_4 emission rates by 92 sheep were measured for one day, using the tracer technique, while grazing on RG/WC pasture. Twenty animals with contrasting (low or high) methane emissions (g kg^{-1} DMI) were selected. From this initial selection group, a final selection of 10 animals (5 low and 5 high CH_4 emitters) was carried out in June 1997, based on tracer CH_4 measurements over 4 days, while sheep were grazing on RG/WC pasture (group 2: trial 1, $\mathbf{T_{21}}$). Two subsequent indoor trials were carried out in October-November 1997 (group 2: trial 2, $\mathbf{T_{22}}$) and May-June 1998 (group 2: trial 3, $\mathbf{T_{23}}$) on these 10 sheep. At the end of T_{22} the sheep were cannulated at the rumen for rumen digestion studies, which are the subject of Chapter 4 of this thesis.

Group 3

In October 1999, twenty sheep were selected on the basis of uniform liveweight (45 kg LW) from a flock of 200. On this group of 20 sheep, CH_4 emission rates by each sheep were measured by the tracer technique for 3 days, while the sheep were grazing on RG/WC pasture. Based on these measurements (group 3: trial 1, $\mathbf{T_{31}}$), eight sheep (4 low and 4 high CH_4 emitters) with contrasting CH_4 emission rates (g kg^{-1} DMI) were selected. Three subsequent CH_4 emission measurements, using the tracer technique, were carried out on the selected sheep in November 1999 (group 3: trial 2, $\mathbf{T_{32}}$), January 2000 (group 3: trial 3, $\mathbf{T_{33}}$) and February 2000 (group 3: trial 4, $\mathbf{T_{34}}$), all at grazing conditions on RG/WC pasture. Because, at the end of T_{31}, one sheep ranked as low emitter had chronic footroot, the final sheep numbers in this group (3) were: 3 low and 4 high emitters.

Figure 3.1 shows the experimental design for each trial with each group of sheep. At the start of each trial, either at grazing or indoors, sheep were acclimatised to the experimental and management conditions before measurements began. This included adaptation to the faeces and gas collection harnesses, and to the feed and feeding management conditions. Since sheep were maintained at grazing when not in trials, acclimatisation for trials involving grazing was set at 5 days. During indoor trials, sheep were managed in pairs (1 low and 1 high CH_4 emitter) because two calorimeter chambers were available. For indoor trials, acclimatisation was set at 10 days when cut pasture was fed (T_{13}) and 21 days when lucerne hay was fed (T_{22} and T_{23}). Additional acclimatisation (3 days) was also allowed in the calorimeter chambers (Figure 3.1).

In all the trials with grazing sheep and in T_{23} (indoors), CH_4 emission was measured by the tracer technique, whereas in the other trials (T_{12} and T_{13}, sheep group 1 and T_{22}, sheep group 2) CH_4 emission was measured by the calorimeter chamber. When CH_4 emission was measured by calorimeter chamber, it followed the feed intake and digestibility measurement period (Figure 3.1). In T_{23} (indoors), CH_4 emission was measured by the tracer technique in digestibility crates, simultaneously with other animal measurements (Figure 3.1).

All the experimental sheep were cryptorchid Romney. In the initial trials, sheep in the group 1 (T_{11}), 2 (T_{21}) and 3 (T_{31}) were 8, 11 and 15 months old, respectively (Table 3.1). Sheep were weighed at the beginning and end of each trial.

Table 3.1 Animal and feeding conditions and the number of days and techniques involved in the methane measurements in each trial (T) with the three groups of sheep (1, 2 and 3).

Conditions	Trials			
Group 1:	T_{11}	T_{12}	T_{13}	
Date	Mar.-Apr. '96	Aug.-Sep. '96	Jan.-Feb. '97	
System	grazing	indoors	indoors	
Sheep (n=8)				
Age (months)	8	13	18	
LW (kg)[1]	37.5±2.8	41.3±2.0	57.3±3.6	
Feeding				
Feed	RG/WC pasture[2]	Lucerne pellets	cut pasture[3]	
Frequency of feeding	*ad libitum*	twice a day	twice a day	
Methane measurement				
Number of days	5 (17 on 2 sheep)	7	3	
Technique[4]	tracer	cal. chamber	cal. chamber	
Group 2:	T_{21}	T_{22}	T_{23}	
Date	June '97	Oct.-Nov. '97	May-June '98	
System	grazing	indoors	indoors	
Sheep (n=10)				
Age (months)	11	15	22	
LW (kg)[1]	40.4±4.0	40.4±4.2	46.9±4.8	
Feeding				
Feed	RG/WC pasture[2]	Lucerne hay	Lucerne hay	
Frequency of feeding	*ad libitum*	twice a day	2-hourly	
Methane measurement				
Number of days	4	7	5	
Technique[4]	tracer	cal. chamber	tracer	
Group 3:	T_{31}	T_{32}	T_{33}	T_{34}
Date	Oct. '99	Nov. '99	Jan. '00	Feb. '00
System	grazing	grazing	grazing	grazing
Sheep (n=7)				
Age (months)	15	16	18	19
LW (kg)[1]	44.6 ± 2.9	47.9 ± 4.0	52.4 ± 4.2	52.5 ± 4.7
Feeding				
Feed	RG/WC pasture[2]	RG/WC pasture[2]	RG/WC pasture[2]	RG/WC pasture[2]
Frequency of feeding	*ad libitum*	*ad libitum*	*ad libitum*	*ad libitum*
Methane measurement				
Number of days	3	3	3	3
Technique[4]	tracer	tracer	tracer	tracer

[1] Standard deviation (± kg).

[2] Perennial ryegrass/white clover pasture.

[3] Perennial ryegrass with minor contributions (<20% DM basis) of white clover, meadow grass and chicory.

[4] Methane emission measured by the SF_6 (tracer) technique or by the calorimetry chamber.

GROUP 1:

T_{11}	Location	controlled grazing																		
	Days	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20
	Activities	acclimatisation					subflock '**a**': feed intake, tracer CH_4					subflock '**b**': feed intake, tracer CH_4					subflock '**c**': feed intake, tracer CH_4				

T_{12}	Location	digestibility crates																	calorimetry chambers							
	Days	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27
	Activities	acclimatisation										feed intake and digestibility							acclimatisation			feed intake, calorimeter CH_4					

T_{13}	Location	digestibility crates												calorimetry chambers						digestib. crates						
	Days	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20					
	Activities	acclimatisation										FI&FD		acclimatisation			FI&FD, calorimetry CH_4			FI&FD						

GROUP 2:

T_{21}	Location	controlled grazing							
	Days	1	2	3	4	5	6	7	8	9
	Activities	acclimatisation					feed intake, tracer CH_4			

T_{22}	Location	digestibility crates													calorimetry chambers											
	Day	1	2	3	...	20	21	22	23	24	25	26	27	28	29	30	31	32	33	34	35	36	37	38		
	Activities	acclimatisation						feed intake and digestibility							acclimatisation			feed intake, calorimetry CH_4								

T_{23}	Location	digestibility crates																								
	Days	1	2	3	...	19	20	21	22	**23T**	24	25	**26T**	**27T**	28	29	30	31	32	**33T**	34	35	**36T**	37		
	Activities	acclimatisation							feed intake and digestibility								FI, digestion dynamics									

GROUP 3:

T_{31}	Location	controlled grazing								
to	Days	1	2	3	4	5	6	7	8	9
T_{34}	Activities	acclimatisation					feed intake, tracer CH_4			A/P in rumen liqour

Abbreviations: FI, feed intake; FI&FD, feed intake and digestibility; A/P, acetate/propionate ratio; **23T**, **26T**, **27T**, **33T**, **36T** show the days in T_{23} on which CH_4 was measured by tracer.

Figure 3.1 Experimental design for the trials (T) with the three groups of sheep (1, 2 and 3), showing the location, days and main activities involved.

3.3.2 Feeds and feeding

During grazing trials, sheep were offered a fresh area of pasture each day. The daily area was controlled by electric fences to give about 10-12% of their body weight as total herbage dry matter (DM) offered, in order to maximise intake (Hodgson, 1990).

In all the indoor trials sheep were placed in digestibility crates and offered an *ad libitum* feeding level during the acclimatisation period. Then, feeding level during the measurement period, both in digestibility crates and calorimeter chambers, was restricted to about 1.2 times the maintenance energy requirements (Geenty and Rattray, 1987). Feed was offered (Table 3.1) twice a day (08:00 and 16:00 h), except in T_{23} when the sheep were fed every 2 hours by overhead mechanical feeders.

Fresh drinking water was made available *ad libitum* both at the grazing and indoor conditions.

Sheep in group 1 were fed lucerne pellets in T_{12}, and fresh cut pasture (zero grazed) in T_{13}. The pasture used in this trial (T_{13}) was predominantly composed of perennial ryegrass (> 80% in DM basis) with minor contributions of meadow grass, white clover and chicory. Variation in pasture quality with progress during the experiment was minimized by a cutting sequence to give similar periods of regrowth for the daily supply of pasture. Pasture was cut early in the morning (about 07:00 h) and, after mixing, fed fresh at the morning feeding, while the afternoon allowance was kept in a cold room.

Chaffed (~ 5 cm) lucerne hay from one batch was fed to sheep group 2 in trials T_{22} and T_{23}. Within each of these trials (T_{22} and T_{23}), the quality of the feed offered was controlled by thorough mixing of the total experimental requirements and weighing the daily allowance for individual sheep.

3.3.3 The methane measurements

3.3.3.1 The SF_6 tracer technique

The application of the sulphur hexafluoride (SF_6) tracer technique (Johnson et al., 1994) for methane measurement in grazing sheep has been described by Lassey et al. (1997) and this protocol was followed in the present study when CH_4 emission rates were measured with grazing sheep (trials T_{11}, T_{21}, T_{31}, T_{32}, T_{33} and T_{34}).

When the tracer technique was applied to sheep kept in metabolic crates (T_{23}), the gas collection PVC containers (yokes) were suspended above the rear end of the digestibility crates and the sample conduction tube was closely attached to the animal's back in order to prevent damage by chewing. During measurements, sheep were placed 2-3 m from each other within a naturally well-ventilated covered yard.

The number of days of CH_4 emission measurements by the SF_6 tracer technique were 5 (17 on 2 sheep), 4, 5, 3, 3, 3 and 3 in T_{11}, T_{21}, T_{23}, T_{31}, T_{32}, T_{33} and T_{34}, respectively (Table 3.1).

3.3.3.2 The open-circuit respiration chambers

The two calorimeter chambers (1 and 2) used in this study were described by Holmes (1973). Temperature in the chambers was maintained at 14-16 °C. An infrared gas analyser (Servomex, UK) was used to measure the CH_4 concentration on aliquot samples collected at constant rate for about 22 h from the inflow and outflow airstreams.

A gravimetric test of CH_4 recovery in the chambers was carried out. This test revealed recovery rates of 88.8±7.3 and 95.2±5.0% in chamber 1 and 2, respectively. During T_{22}, all the CH_4 measurements were carried out in chamber 2, after sheep had acclimatised in chamber 1.

The calorimetric chamber CH_4 measurements followed the feed intake and digestibility measurements in digestibility crates. In all the trials, except in T_{13}, only feed intake and CH_4 emission were measured when sheep were in calorimetric chambers (Figure 3.1).

CH_4 emission measurements by the calorimetric chamber were carried for 7, 3 and 7 days in T_{12}, T_{13} and T_{22}, respectively (Table 3.1).

The periods on which CH_4 emissions were measured are shown in Figure 3.1.

3.3.4 Feed intake, feed digestibility and laboratory analysis

In the grazing trials dry matter intakes (DMI) by individual sheep were estimated from the *in vitro* pasture's dry matter (DM) digestibility (DMD) determined by the near-infrared reflectance technique (NIR), in conjunction with the twice-daily

collection of total faeces output using a harness and canvas bag. Faeces were collected for 5, 4, 3, 3, 3 and 3 days in T_{11}, T_{21}, T_{31}, T_{32}, T_{33} and T_{34}, respectively (Figure 3.1). Collection of faeces was delayed 1 d relative to breath collection. Faeces from each animal was weighed, pooled within each day and sampled for DM determination (100 °C, 48 h).

Samples of herbage were collected each morning before grazing by hand-cutting at the height to which the sheep had grazed on the previous day and imitating the selective grazing of sward components. Herbage samples were dried (60 °C, 72 h), ground to pass through a 1- mm sieve, pooled for the whole period of CH_4 measurements and analysed for chemical composition and digestibility (NIR), and for gross energy content using an adiabatic bomb calorimeter (Gallenkamp Autobomb; Loughborough, Leies, UK). The digestible energy content in the herbage (DE, MJ/kg DM) was estimated using the equation DE=19.66DMD – 0.70 (Minson, 1990).

During the indoor trials, daily feed consumption and apparent *in vivo* feed digestibility were determined for each sheep, during a 7-d period (Figure 3.1). The amounts of feed offered and refused, and faeces excreted were recorded daily and sub-samples were taken for daily DM determinations (100 °C, 48 h). Other daily sub-samples (10%) of feed on offer, refusals and faeces were stored frozen (-20 °C) and later pooled for each individual animal. Chemical analyses were carried out on pooled, freeze dried and ground (1mm sieve) samples. Energy content in feed on offer, refusals and faeces were determined using the bomb calorimeter. Thus, both DM and energy daily intake and their apparent digestibilities were determined.

At the end of the last day of breath collection in all the trials involving sheep group 3, rumen contents were sampled by stomach tube within 1 h after sheep were removed from grazing (08:00 h). About 15-20 ml of rumen contents were taken and it took between 1-5 min per animal. Rumen contents were squeezed through 1 layer of cheese cloth and sampled. Samples were processed (acidified, deproteinized and centrifuged) and later analysed for volatile fatty acids (VFAs) concentrations as described by Domingue et al. (1991). Because in some cases the rumen contents samples were contaminated with saliva, only the molar proportions of acetic acid to propionic acid (A/P) was computed. Within each trial, correlation analysis between

A/P ratio and CH_4 emission values (corresponding to the last day of measurement) were carried out.

3.3.5 Statistical analyses

Data from groups 1, 2 and 3 of sheep were independently processed and analysed. CH_4 emission was expressed both on an absolute basis (g d^{-1}) and as CH_4 energy as a percentage of the gross energy intake (%GEI) or digestible energy intake (%DEI).

In the first instance, within each particular trial and ignoring sheep emission subgroups, the proportion of the total variation in CH_4 emission (g d^{-1} and %GEI) attributable to the between-animal variation were determined. For this purpose analysis of variance using the GLM procedure of SAS (SAS, 1987) was carried out considering sheep as a class. In the SAS output R^2 represents the proportion of variation attributable to the variable (class) fitted in the model.

Secondly, for each experimental group, the persistence of sheep rankings for CH_4 emission was assessed from: (1) a split-plot analysis of variance, and (2) a between-trials rank order correlation analysis.

In the split plot analysis of variance (Gill, 1986), the daily CH_4 emissions (g d^{-1}, %GEI and %DEI), were analysed using the GLM procedure of SAS (SAS, 1987). Effects of CH_4 emission subgroups (S, low or high) were tested using the animal (A) within emission subgroups [A(S)] component as the error term; whereas the effects of trials (T) and SxT were tested using TxA(S) as the error term. The PDIFF option in SAS (SAS, 1987) was used to test the differences between least squared means. If the SxT interaction from the analysis of variance of CH_4 emission data was statistically significant, it implied that sheep subgroups (S) were not persistent in CH_4 emission even when the S main effects were significant. The influence of LW or metabolic liveweight ($kg^{0.75}$) on CH_4 emission was assessed by including it (as a covariate) in the model of analysis of variance.

For the correlation analysis, the mean CH_4 emission values for individual sheep were ranked within each trial using the rank procedure of SAS (SAS, 1987).

The split plot analysis of variance, with similar sources of variation to that for analysis of CH_4 emissions, was also carried out for data concerning liveweight (LW), feed

intake, apparent feed digestibility and A/P ratio (sheep group 3 only). For sheep group 3, a correlation analysis between the A/P ratio and the CH_4 emission was also carried out. For this purpose, only the CH_4 emissions corresponding to the last day of measurements were considered, because rumen contents were sampled on this day.

3.4 RESULTS

3.4.1 Feed quality, feed intake and sheep liveweight

Table 3.2 shows the chemical composition (g kg^{-1} DM) of the diet in each trial. Pasture diets, both grazed and cut, were of much better nutritive value (higher crude protein and soluble carbohydrates, but lower ADF contents) than the lucerne pellets or hay.

Table 3.2 Chemical composition (g kg^{-1} DM) of sheep's diet during each trial as determined by near-infrared reflectance (NIR).

TRIALS	FEED	Feed Components[1] (g kg^{-1} DM)					
		CP	SC	NDF	ADF	Lipid	Ash
Sheep Group 1							
T_{11}	grazed pasture	249	75	444	245	42	104
T_{12}	lucerne pellets	177	51	390	335	33	110
T_{13}	cut pasture	209	nd[2]	nd	nd	nd	nd
Sheep Group 2							
T_{21}	grazed pasture	247	123	341	199	36	109
T_{22}	lucerne hay[3]	nd	nd	nd	nd	nd	nd
T_{23}	lucerne hay	191	27	401	354	18	114
Sheep Group 3							
T_{31}	grazed pasture	242	118	365	206	47	108
T_{32}	grazed pasture	221	104	413	236	42	108
T_{33}	grazed pasture	225	113	375	216	47	107
T_{34}	grazed pasture	293	87	397	210	47	110

[1] CP, crude protein; SC, soluble carbohydrates; NDF, neutral detergent fibre; ADF, acid detergent fibre.
[2] nd, not determined.
[3] Chemical composition not determined. Lucerne hay used in T_{22} and T_{23} were from the same batch.

Table 3.3 shows the mean (± s.e.) liveweight (LW, kg $head^{-1}$), daily gross energy intake (MJ GEI) per kg metabolic liveweight ($kg^{-0.75}$) (MJ GEI $kg^{-0.75}$) and the apparent digestibility of energy (ED%) for each CH_4 emission subgroup of sheep and trial. In general, the LW of sheep in all the experimental groups (1,2 and 3) increased from the first to the last trial (Table 3.3). There were no differences in LW between the low and high CH_4 emission subgroups of sheep in group 1, whereas the differences were significant in groups 2 and 3 (Table 3.3). In contrast, CH_4 emission subgroups of sheep from groups 2 and 3 differed significantly from each other in all the trials (Table 3.3). In all the trials, the low CH_4 emitters from both group 2 and 3 were significantly heavier than the high CH_4 emitters (by 5.3, 5.2 and 4.2 kg in group 2 and by 1.5, 3.1, 4.0 and 5.5 kg in group 3; Table 3.3).

The daily intake of gross energy (GEI) per kg metabolic liveweight (MJ GEI $kg^{-0.75}$) differed between trials for all the three experimental groups (Table 3.3). As expected intakes under grazing conditions were significantly larger than under controlled indoor feeding conditions. Nevertheless, intake by group 2 differed even at controlled feeding conditions ($T_{22}>T_{23}$). In the grazing trials involving group 3, feed intake at T_{33} was significantly ($p<0.05$) lower than at the other trials (Table 3.3). No differences in feed intake were found between CH_4 emission subgroups, except in T_{21} (Table 3.3).

The chemical compositions of the diets (Table 3.2) were reflected in their apparent digestibilities of energy (ED, %) (Table 3.3), i.e. grazed or cut pasture had higher digestibility values than lucerne pellets or hay. For group 1, the digestibility of feed was much higher ($p<0.05$) in T_{11} (pasture) than in T_{12} (lucerne pellets) and T_{13} (cut pasture). For group 2, the digestibility of the feed used in T_{21} (cut pasture) was much higher than for the lucerne hay (T_{22} and T_{23}). Even though the lucerne hay used in T_{22} and T_{23} were from the same batch, digestibility differed significantly between the two trials ($p<0.05$). In all the indoor trials, the *in vivo* apparent digestibilities of energy did not differ between CH_4 emission subgroups (Table 3.3). Finally, for group 3, the *in vitro* pasture energy digestibility was high in all the trials, especially in T_{31} and T_{34}.

Table 3.3 Mean[1] (± s.e.) liveweight (LW, kg head^{-1}), daily feed gross energy intake (MJ GE kg$^{-0.75}$) and apparent digestibility of energy (%) for CH_4 emission subgroups (low and high) and trials (T) within each of the three groups of sheep. (n=number of observations[2]).

Variables	Trials[1]			
Group 1:	T_{11}	T_{12}	T_{13}	
LW (kg head^{-1})	n=8	n=8	n=8	
Low	37.1 ± 1.1 ^{a}	41.8 ± 1.1 ^{b}	57.3 ± 1.6 ^{c}	
High	37.8 ± 1.1 ^{a}	40.8 ± 1.1 ^{a}	57.4 ± 1.6 ^{b}	
Probability[3]	*0.64*	*0.52*	*0.96*	
Feed Int. (MJ GE kg$^{-0.75}$)	n=67	n=56	n=24	
Low	1.72 ± 0.04 ^{a}	1.21 ± 0.04 ^{b}	1.11 ± 0.05 ^{b}	
High	1.64 ± 0.04 ^{a}	1.21 ± 0.04 ^{b}	1.09 ± 0.05 ^{b}	
Probability[3]	*0.13*	*0.96*	*0.82*	
Apparent Dig. of Energy (%)	n=3	n=8	n=8	
Low	73.7 ± 0.6 ^{a}	58.1 ± 0.6 ^{b}	70.0 ± 0.8 ^{c}	
High	74.1 ± 0.6 ^{a}	58.9 ± 0.6 ^{b}	70.6 ± 0.8 ^{c}	
Probability[3]	*0.65*	*0.31*	*0.61*	
Group 2:	T_{21}	T_{22}	T_{23}	
LW (kg head^{-1})	n=10	n=10	n=10	
Low	43.0 ± 1.1 ^{a}	43.0 ± 1.0 ^{a}	49.0 ± 1.0 ^{b}	
High	37.7 ± 1.1 ^{a}	37.8 ± 1.0 ^{a}	44.8 ± 1.0 ^{b}	
Probability[3]	*0.004*	*0.004*	*0.009*	
Feed Int. (MJ GE kg$^{-0.75}$)	n=40	n=70	n=50	
Low	1.67 ± 0.04 ^{a}	1.31 ± 0.03 ^{b}	1.12 ± 0.04 ^{c}	
High	1.55 ± 0.04 ^{a}	1.33 ± 0.03 ^{b}	1.11 ± 0.04 ^{c}	
Probability[3]	*0.04*	*0.70*	*0.86*	
Apparent Dig. of Energy (%)[4]	n=1	n=10	n=10	
Low	77.8 ^{a}	53.9 ± 0.5 ^{b}	56.1 ± 0.6 ^{c}	
High	77.8 ^{a}	53.6 ± 0.5 ^{b}	57.3 ± 0.6 ^{c}	
Probability[3]	--	*0.69*	*0.17*	
Group 3:	T_{31}	T_{32}	T_{33}	T_{34}
LW (kg head^{-1})	n=7	n=7	n=7	n=7
Low	45.5 ± 0.5 ^{a}	49.7 ± 0.5 ^{b}	54.7 ± 0.5 ^{c}	55.7 ± 0.5 ^{c}
High	44.0 ± 0.4 ^{a}	46.6 ± 0.4 ^{b}	50.7 ± 0.4 ^{c}	50.2 ± 0.4 ^{c}
Probability[3]	*0.025*	*0.0001*	*0.0001*	*0.0001*
Feed Int. (MJ GE kg$^{-0.75}$)	n=21	n=21	n=21	n=21
Low	2.39 ± 0.09 ^{a}	1.95 ± 0.09 ^{b}	2.29 ± 0.09 ^{a}	2.36 ± 0.09 ^{a}
High	2.21 ± 0.08 ^{a}	1.83 ± 0.08 ^{b}	2.15 ± 0.08 ^{a}	2.39 ± 0.08 ^{a}
Probability[3]	*0.15*	*0.30*	*0.25*	*0.81*
Apparent Dig. of Energy (%)[4]	80.7	75.2	77.5	81.0

[1] Rows with different letter in their superscripts indicate that trials are significantly different (p<0.05) for the particular variable.

[2] Number of observations: number of replicated measurements within each animal x number of animals.

[3] Probabilty value for Ho: Low subgroup=High subgroup, within a particular trial.

[4] Digestibilities in T_{21} (group 2) and in all the trials with group 3, were determined on within-trial pooled samples.

3.4.2 Between-sheep variation in CH_4 emission

Table 3.4 shows, for each individual trial, the proportion (%) of the total variation in CH_4 emission attributable to the between-sheep variation. In general, except in T_{21} (group 2) and T_{33} (group 3), the between-sheep variations in CH_4 emission were high (above 75%).

Table 3.4 Between-sheep variation (% of the total variation) in CH_4 emission (g d^{-1} and % GEI).

CH_4	Group 1			Group 2			Group 3			
	T_{11}	T_{12}	T_{13}	T_{21}	T_{22}	T_{23}	T_{31}	T_{32}	T_{33}	T_{34}
g d^{-1}	92	94	74	56	80	76	97	48	40	82
% GEI	96	92	88	35	78	62	94	78	45	88

3.4.3 CH_4 emission and pattern of persistence

The effects of LW or metabolic liveweight ($kg^{0.75}$) (covariate) in the model of analysis of variance of CH_4 emission data were not significant ($p>0.45$), and the data were not adjusted for differences in LW or $kg^{0.75}$.

In both group 1 and group 2, CH_4 emission subgroups (S) and trials (T) interacted ($p<0.05$) in their effects upon all the CH_4 emission variables (g d^{-1}, %GEI and %DEI). In contrast, in group 3, no SxT interaction ($p>0.23$) was found for any CH_4 emission variable. In this group (group 3), the CH_4 emission per unit of feed intake (%GEI and %DEI), but not per day (g d^{-1}), was significantly influenced by both the S ($p<0.01$) and the T ($p<0.001$) effects. Thus, only the sheep in group 3 maintained their respective rankings for CH_4 emission rates (low or high) throughout the trials.

The SxT interaction effects for groups 1 and 2 are presented in Tables 3.5 and 3.6, respectively; whereas Table 3.7 shows the SxT effects (not statistically significant) for group 3. Figure 3.2 shows the mean CH_4 emissions (%GEI) for low and high CH_4 emission subgroups during the trials with the three groups of sheep.

Table 3.5 (group 1) and Table 3.6 (group 2) show a clear SxT interaction for all the three CH_4 emission variables. These interactions are present because while the differences between emission subgroups (S) were highly significant in the initial trials ($p<0.01$), there were no significant differences between them in the subsequent trials (Figure 3.2 a,b).

For both emision subgroups (S) from groups 1 (Table 3.5) and 2 (Table 3.6), the absolute amounts of CH_4 emission (g d^{-1}) were higher at grazing than in indoor controlled conditions. The high CH_4 emission (g d^{-1}) observed in T_{13}, when pasture was fed was associated with the heavier LW of sheep (Table 3.3). CH_4 emissions per unit of intake (%GEI and %DEI) however were smaller at grazing than with restricted indoor feedings.

Table 3.5 Sheep Group 1: Mean (± s.e.) daily CH_4 emission (g d^{-1}, % GEI and % DEI) for emission subgroups (low and high) and trials (T_{11}, T_{12}, T_{13}). (n=143 observations; on average= number of replications within sheep within trial x 8 sheep x 3 trials).

Methane Emission	Sheep Subgroups[1] (S)	Trials (T)		
		T_{11}	T_{12}	T_{13}
g d^{-1}:				
	Low	16.1 ± 0.9 [a]	17.7 ± 0.9 [a]	25.7 ± 1.3 [b]
	High	20.9 ± 1.0 [a]	17.0 ● 0.9 [b]	24.3 ± 1.3 [a]
	Probability[2]	*0.003*	*0.60*	*0.45*
% GEI				
	Low	3.5 ● 0.2 [a]	4.9 ± 0.2 [b]	6.1 ± 0.3 [c]
	High	4.6 ± 0.2 [a]	4.8 ± 0.2 [a]	5.9 ± 0.3 [b]
	Probability[2]	*0.005*	*0.81*	*0.63*
% DEI				
	Low	4.7 ± 0.4 [a]	8.4 ± 0.4 [b]	8.8 ± 0.5 [b]
	High	6.2 ± 0.4 [a]	8.2 ± 0.4 [b]	8.4 ± 0.5 [b]
	Probability[2]	*0.01*	*0.64*	*0.57*

[1] Means, within a row, with the same letter in their superscript are not significantly different ($p>0.05$).

[2] Probabilty value for Ho: Low subgroup=High subgroup, within a particular trial.

Table 3.6 Sheep Group 2: Mean (± s.e.) daily CH_4 emission (g d^{-1}, % GEI and % DEI) for emission subgroups (low and high) and trials (T_{11}, T_{12}, T_{13}). (n=160 observations; on average = number of replications within sheep within trial x 10 sheep x 3 trials).

Methane Emission	Sheep Subgroups[1] (S)	Trials (T)		
		T_{21}	T_{22}	T_{23}
g d^{-1}:				
	Low	23.1 ± 1.3 [a]	18.3 ± 1.0 [b]	19.5 ± 1.2 [b]
	High	25.2 ± 1.3 [a]	17.0 ± 1.0 [b]	17.9 ± 1.2 [b]
	Probability[2]	*0.27*	*0.36*	*0.37*
% GEI				
	Low	4.6 ± 0.2 [a]	4.6 ± 0.2 [ab]	5.2 ± 0.2 [b]
	High	6.0 ± 0.2 [a]	4.6 ± 0.2 [b]	5.2 ± 0.2 [b]
	Probability[2]	*0.001*	*0.93*	*0.84*
% DEI				
	Low	5.9 ± 0.4 [a]	8.5 ± 0.3 [b]	9.3 ± 0.3 [b]
	High	7.7 ± 0.4 [a]	8.6 ± 0.3 [ab]	9.0 ± 0.3 [b]
	Probability[2]	*0.003*	*0.83*	*0.48*

[1] Means, within a row, with the same letter in their superscript are not significantly different (p>0.05).
[2] Probabilty value for Ho: Low subgroup=High subgroup, within a particular trial.

No SxT interaction effect upon CH_4 emission occurred in group 3 (Figure 3.2c), although the CH_4 emissions by both subgroups of sheep were higher in T_{32} than in the other trials (Table 3.7). Low and high CH_4 emitters maintained their initial rankings throughout all the trials, although the differences between them were not significantly different in T_{33} for all the CH_4 emission variables (Table 3.7).

Overall, the CH_4 emission by sheep in all the three groups (Tables 3.5, 3.6 and 3.7) ranged from 16 to 37 g d^{-1} at grazing and from 17 to 19 g d^{-1} when lucerne hay or pellets were fed at restricted levels. The CH_4 energy loss ranged from 3.0 to 6.4 %GEI (or 4.6 to 5.9 %DEI) at grazing and from 3.8 to 7.7 %GEI (or 8.2 to 9.3 %DEI) when fed indoors at restricted levels.

Table 3.7 Sheep Group 3: Mean daily CH_4 emission (g d^{-1}, % GEI and % DEI) for emission subgroups (low and high) and trials (T_{31}, T_{32}, T_{33}, T_{34}). (n=78 observations; on average = number of replications within sheep within trial x 7 sheep x 4 trials).

Methane Emission	Sheep Subgroups[1] (S)	Trials (T)				
		T_{31}	T_{32}	T_{33}	T_{34}	s.e.[3]
g d^{-1}:						
	Low	23.0 [a]	32.8 [a]	32.0 [a]	27.3 [a]	3.3
	High	37.3 [a]	37.3 [a]	31.3 [a]	36.4 [a]	2.9
	Probability[2]	*0.005*	*0.31*	*0.87*	*0.05*	
% GEI						
	Low	3.0 [a]	5.0 [bc]	3.8 [ab]	3.1 [a]	0.5
	High	5.4 [ab]	6.4 [bc]	4.2 [a]	4.6 [a]	0.4
	Probability[2]	*0.002*	*0.04*	*0.57*	*0.04*	
% DEI						
	Low	3.8 [a]	6.6 [bc]	5.0 [ab]	3.9 [a]	0.6
	High	6.7 [a]	8.5 [b]	5.4 [a]	5.6 [a]	0.5
	Probability[2]	*0.003*	*0.03*	*0.56*	*0.04*	

[1] Means, within a row, with the same letter in their superscript are not significantly different (p>0.05).

[2] Probabilty value for Ho: Low subgroup=High subgroup, within a particular trial.

[3] Standard error of mean.

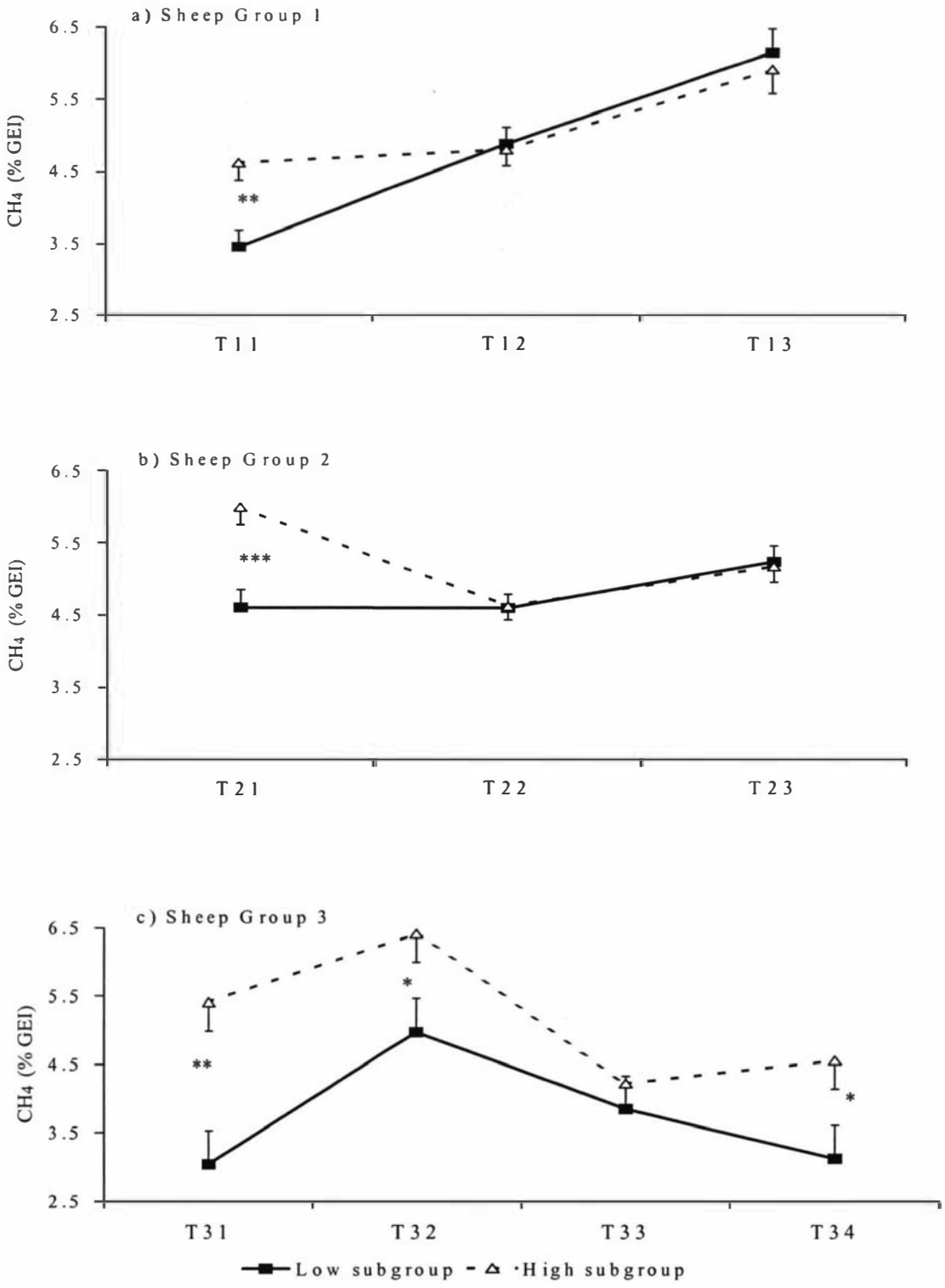

Figure 3.2 CH_4 emission (% GEI) during the trials by low (■—■) and high (Δ---Δ) emission subgroups of sheep belonging to three experimental groups (1, 2 and 3). (levels of significance for differences between the emission subgroups in each trial: •••, $p<0.001$; ••, $p<0.01$; •, $p<0.05$).

Table 3.8 shows the rank order correlation coefficients between trials for CH_4 emission (g d^{-1}, %GEI, %DEI). In general, for groups 1 and 2, the correlation values are very small and not significant, especially for CH_4 emission variables expressed per unit of intake. In contrast, the corresponding correlations for sheep in group 3 were high, and in terms of CH_4 emission per unit of intake, only trials T_{33} and T_{34} did not correlate at all.

Table 3.8 Rank order correlation coefficients[1] between trials for methane emission (g d^{-1}, % GEI and % DEI) for the three groups of sheep.

	g d^{-1}			**% GEI**			**% DEI**		
	Sheep Group 1 (n=8)								
	T_{11}	T_{12}		T_{11}	T_{12}		T_{11}	T_{12}	
T_{12}	0.18 *0.67*			0.14 *0.74*			0.07 *0.87*		
T_{13}	0.11 *0.80*	0.48 *0.23*		0.00 *1.00*	0.33 *0.42*		0.00 *1.00*	0.21 *0.61*	
	Sheep Group 2 (n=10)								
	T_{21}	T_{22}		T_{21}	T_{22}		T_{21}	T_{22}	
T_{22}	-0.20 *0.58*			-0.15 *0.68*			0.08 *0.83*		
T_{23}	0.36 *0.31*	0.18 *0.63*		0.02 *0.96*	0.07 *0.85*		-0.04 *0.91*	-0.01 *0.99*	
	Sheep Group 3 (n=7)								
	T_{31}	T_{32}	T_{33}	T_{31}	T_{32}	T_{33}	T_{31}	T_{32}	T_{33}
T_{32}	0.89 *0.007*			0.86 *0.01*			0.86 *0.01*		
T_{33}	-0.14 *0.76*	0.04 *0.94*		0.54 *0.22*	0.72 *0.07*		0.54 *0.22*	0.72 *0.07*	
T_{34}	0.82 *0.02*	0.64 *0.12*	-0.61 *0.15*	0.71 *0.07*	0.68 *0.09*	0.00 *1.00*	0.71 *0.07*	0.68 *0.09*	0.00 *1.00*

[1] *in italics*, probability values for Ho: $\rho=0$.

3.4.4 The acetate/propionate (A/P) ratio in rumen fluid and CH_4 emission in sheep group 3

The difference in A/P ratio in rumen fluid between the two CH_4 emission subgroups (S) was not significant (p=0.31), but the difference between trials (T) was significant (p=0.001). No SxT interaction effects (p=0.18) upon the A/P ratio were observed. The mean A/P ratios in T_{31}, T_{32}, T_{33} and T_{34} were 3.3, 2.8, 3.0 and 3.6, respectively and all the trials significantly differed from each other (p<0.05) in A/P ratio. Table 3.9 shows the mean A/P ratios for the sheep in the low and high CH_4 emission subgroups in each trial. At all the trials, except T_{34}, the A/P ratios for the low CH_4 emission sheep were lower (approaching statistical significance) than those for the high CH_4 emission sheep. The between-trials change in the A/P ratio for the low and high CH_4 emitters were almost similar.

The coefficients of correlation between the A/P ratios and the CH_4 emission (g d^{-1}, on the last day of measurement) at T_{31}, T_{32}, T_{33} and T_{34} were 0.10 (p=0.83), 0.75 (p=0.05), 0.49 (p=0.26) and 0.45 (p=0.36), respectively.

Table 3.9 Sheep group 3: Mean acetate/propionate ratio (A/P) for CH_4 emission subgroups and trials (n=28 observations, 7 per trial).

CH_4 Emission Subgroups[1]	T_{31}	T_{32}	T_{33}	T_{34}	s.e.[3]
Low	3.2 [a]	2.7 [b]	2.9 [ab]	3.6 [c]	0.1
High	3.5 [a]	2.9 [b]	3.2 [b]	3.6 [a]	0.1
Probability[2]	*0.07*	*0.16*	*0.12*	*0.80*	

[1] Means, within a row, with the same letter in their superscript are not significantly different (p>0.05).
[2] Probability value for Ho: Low subgroup=High subgroup, within a particular trial.
[3] Standard error of mean.

3.5 DISCUSSION

Overall, the quality of feed and the level of feeding in the grazing trials were markedly higher from those in controlled-feeding indoor trials (Tables 3.2, 3.3). Thus, the lower CH_4 emissions per unit of intake (%GEI or %DEI) observed in the grazing trials compared to those from indoor trials might be attributed to the better feeding conditions in the former (Blaxter and Clapperton, 1965). The large between-sheep

variation in CH_4 emission observed in most of the trials (except T_{21} and T_{33}) in this study (Table 3.4) confirm previous findings with both grazing (Lassey et al., 1997; Ulyatt et al., 1999) and pen-fed sheep (Ulyatt et al., 1999). However, the variations reported in this study (Table 3.4) were measured in sheep which had been selected for either low or high CH_4 emission rates.

Sheep CH_4 emission subgroups (S) in both groups 2 and 3 differed in LW in all their respective trials (Table 3.3), with the low CH_4 emitters being heavier than the high emitters. However, per unit of metabolic liveweight ($kg^{0.75}$), the low and high emission subgroups did not differ in their daily feed intakes (MJ GEI $kg^{-0.75}$), except in T_{21} (Table 3.3). LW (or $kg^{0.75}$) was not associated with CH_4 emission expressed per unit of intake (%GEI or %DEI). The latter can be attributed to the fact that daily feed intake and LW (or $kg^{0.75}$) were closely correlated each other (r ranged from 0.70 to 0.90, $p<0.01$).

The analysis of variance of CH_4 emission data for groups 1 (Table 3.5) and 2 (Table 3.6) revealed that the differences in CH_4 emission rates between the low and high emitters were not persistent in the long-term (significant SxT interaction) (Figure 3.2a,b) and this is confirmed by the poor correlation coefficients of rankings between trials (Table 3.8). The lack of persistence might be attributed to the changing feeding conditions between trials. However, even for similar feeding conditions operated in T_{22} and T_{23} (group 2), the correlations between trials were very small. In contrast, with group 3, with all the trials carried out at grazing, no SxT interaction was observed upon CH_4 emission (Table 3.7, Figure 3.2c) and strong between-trials correlation coefficients for CH_4 emission rates were observed (Table 3.8), although this repeatability weakened in the last measurement (T_{34}).

The high repeatability in CH_4 emission between trials observed with group 3, fed *ad libitum* at grazing (Table 3.8), in contrast to those observed with groups 1 and 2 (restricted feeding), suggest that feeding conditions, which maximize voluntary feed intake (e.g. generous allowance of good quality pasture) favors the expression of the between-sheep differences in CH_4 emission (per unit of intake). However it is not clear why these differences tend to disappear in the longer term.

Although low CH_4 emitters in group 3 were consistently heavier than high emitters (Table 3.3), they did not differ in their feed intake per kg of metabolic liveweight ($kg^{0.75}$) (Table 3.3). Nevertheless, consistent differences in CH_4 emission between low and high emitters were observed (Table 3.7). The latter confirms the observation of Blaxter and Clapperton (1965) who found that between-sheep differences in CH_4 emission were still apparent even when sheep differing in LW were fed at similar levels per unit of $LW^{0.73}$.

At all the trials, except T_{34}, low CH_4 emitters sheep in group 3 had lower (approaching statistical significance) A/P ratios than their high counterparts (Table 3.9). However, because a positive correlation between CH_4 emission and the acetate/propionate (A/P) ratio in rumen fluid is expected from the stoichiometry of rumen fermentation (Demeyer and Van Nevel, 1975), the results of the present study (group 3) provide weak confirmation of the latter relationship, with the correlations being positive, but not significant, except in T_{32} ($r=0.75$, $p=0.05$).

In conclusion, this study showed that between-sheep differences in CH_4 emission persisted only under conditions which maximized feed intake (group 3). Thus, the involvement of animal factors such as rumen fractional outflow rate of feed particles and rumen volume in the between-sheep variation in CH_4 emission might be hypothesized. A detailed study preferably under *ad libitum* feeding conditions is required to test the latter hypothesis.

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Chapter 4

Some rumen digestion characteristics related to methane emission in sheep

4.1 ABSTRACT

Ten rumen-cannulated Romney wether sheep were fed two-hourly (1.2 times the maintenance requirement) on chaffed lucerne hay. A 6-day energy and nitrogen balance period was followed by a 8-day digestion dynamics period. Methane (CH_4) emission was measured by the sulphur hexafluoride (SF_6) tracer technique during days 2, 5 and 6 of the balance period and then on days 4 and 7 of the digestion dynamics period. Rumen contents were emptied at days 5 and 8 of the digestion dynamics period. Rumen contents were weighed, samples taken for the analysis of chemical composition, Cr, lignin, particle size, protozoa count, ammonia and VFA concentrations and buffering capacity, and returned to the rumen. Rumen particulate and fluid fractional outflow rates (FOR) were estimated using lignin (ADL) and Cr-EDTA, respectively.

CH_4 emission (g d^{-1}) was positively correlated with organic matter intake (OMI, g d^{-1}) (r=0.67, p=0.04), rumen OM pool size (g) (r=0.84, p=0.002) and the rumen fill (g, wet contents) (r=0.76, p=0.01). Multiple regression analysis showed that CH_4 emission (g d^{-1}) was a function of rumen OM pool size and the molar % of butyrate (R^2=0.88), however OM pool size alone accounted for a large proportion (R^2=0.71) of the variation in CH_4 emission (g d^{-1}).

CH_4 emission (% energy intake, %GEI) was negatively correlated with the particulate FOR (% h^{-1}) (r=-0.75, p=0.01) and buffering capacity of rumen fluid (mmol HCl) (r=-0.72, p=0.02), but positively correlated with the digestibility of cellulose (r=0.66, p=0.04). Multiple regression analysis showed that CH_4 emission (%GEI) was a function of particulate FOR, OMI per kg metabolic liveweight (g $kg^{-0.75}$) and the molar % of butyrate (R^2=0.88). Particulate FOR explained a large proportion (R^2=0.57) of the variation in CH_4 emission (%GEI). Particulate FOR was negatively correlated with rumen fill (g) (r=-0.69, p=0.03) and digestibility of cellulose (r=-0.65, p=0.04).

It was concluded that differences in rumen particulate FOR had a major effect on the differences between sheep in CH_4 emission (%GEI). Sheep with lower particulate FOR (i.e. longer rumen retention times) had larger rumen fills and higher feed digestibilities and CH_4 emissions. If rumen particulate FOR is to be used as a tool for CH_4 mitigation, the repeatability of its relationship to CH_4 emission must be assessed preferably under grazing conditions. For this, non-invasive techniques for field screening of this factor need to be developed.

4.2 INTRODUCTION

The interaction between ruminant animals and rumen microorganisms is clearly symbiotic. The animal provides the microorganisms with a habitat for growth, whilst the microorganisms, in turn, provide the animal with fermentation acids and microbial protein (Hungate, 1966). In the rumen fermentation reactions, reduction of carbon dioxide (CO_2) with hydrogen (H_2) and formation of methane (CH_4) by methanogens has a profound effect on the formation of different end products, the amount of ATP generated and finally the efficiency of rumen microbial yield (Van Nevel and Demeyer, 1996). During fermentation, H_2 is formed in large amounts, but it does not accumulate because it is immediately used by methanogens (Wolin and Miller, 1988). Thus, CH_4 formation may be seen as a major sink into which the H_2 from all rumen organisms drains (Demeyer and Van Nevel, 1975).

Although the end-products of rumen fermentation, such as CH_4, are the result of microbial activity and are influenced by the diet, it is recognized that animal factors such as mastication, salivation and digesta kinetics affect the rate and type of fermentation (Faichney, 1993; Mathison et al., 1995; Wilson and Kennedy, 1996; Varga and Kolver, 1997). Thirty years ago, Blaxter and Clapperton (1965) showed that individual sheep fed the same diet differed significantly in their CH_4 emission rates (% gross energy intake, GEI) and more recently, Lassey et al. (1997) have reported that about 86% of the variation in daily CH_4 emission of grazing sheep is between-animal. This variation might be, at least partially, genetic in origin, but this possibility, which might provide a tool for CH_4 emission control, has not been studied.

Studies both with cattle (Hartnell and Satter, 1979; Ørskov et al., 1988) and with sheep (Hodgson and Thomas, 1975; Faichney, 1993) have shown consistent between-animal differences in the rate of outflow of rumen digesta. In these studies (Hartnell and Satter, 1979; Ørskov et al., 1988; Faichney, 1993), animals with high outflow rates had smaller rumen volumes. Thus, the hypothesis tested in this study was that animal factors such as rumen fractional outflow rate and volume have an influence on the between-sheep variation in CH_4 emission.

4.3 MATERIALS AND METHODS

4.3.1 Experimental design

An experiment involving 10 rumen-cannulated sheep was conducted in May and June 1998 at AgResearch Grasslands, Palmerston North, New Zealand, under controlled indoor conditions, using the protocol given in Figure 4.1. Energy and nitrogen balances were measured for a 6-d period, after which, a 2-d transition period (non-measurement) was allowed before the 8-d digestion dynamics period. In this latter period, Cr-EDTA was continuously infused into the rumen for 8 days and rumen contents were bailed on days 5 and 8. CH_4 emission was measured on days 2, 5 and 6 of the balance period and on days 4 and 7 of the digestion dynamics period.

4.3.2 Animals

The sheep were of the Romney breed, cryptorchids, aged 22 months and weighing 46.9±4.8 (s.d.) kg liveweight (LW). All sheep were fistulated in the rumen and fitted with permanent rubber cannulae (65 mm i.d.). Leakage of rumen contents was minimised by fitting plastisol washers around the cannulae. The sheep were kept in metabolic crates and housed in a naturally well-ventilated building.

The sheep were weighed at the start and end of each measurement period (balance and digestion dynamics) and also immediately before the rumen contents were emptied.

4.3.3 Feed and feeding

The sheep were fed on lucerne (*Medicago sativa*) hay. This contained (per kg DM), 30.2 g N, 423 g neutral detergent fibre (NDF), 359 g acid detergent fibre (ADF), 73 g lignin, 94 g ash and 18.35 MJ of gross energy. The total requirement of hay for the whole experiment was calculated and after chaffing (~50 mm lengths) it was throughly mixed and the individuals' daily requirements were weighed and stored in plastic bags until required. Feeding was at a restricted level (1.2 times the maintenance energy requirements). Automatic overhead feeders delivered the day's ration in 12 feeds, at 2 h intervals. Drinking water was made available *ad libitum*.

PERIODS	Acclimatization	Balance						Transition		Digestion Dynamics							
FEEDING	*ad libitum*	1.2 x maintenance requirements															
DAYS	1 to 21	1	2	3	4	5	6	1	2	1	2	3	4	5	6	7	8
ACTIVITIES		energy balance, nitrogen balance, urine collection for purine derivatives, CH_4 collection: days 2, 5, 6						cleaning, fitting infusion lines		feed intake, Cr-EDTA infusion: days 1 to 8, Rumen contents bailing: days 5 and 8, CH_4 collection: days 4, 7							

Figure 4.1 Experimental design showing the periods, days and the main activities involve.

4.3.4 Fluid marker infusion procedure

The fluid phase marker Cr-EDTA was prepared by the method of Binnerts et al. (1968) and adjusted to pH 6.7. The infusate, containing 380 μg Cr/ml was continuously infused (through the rumen cannulae) for 8 days at a rate of 0.53 ml/min using a peristaltic pump (PLG-multipurpose pump; Dasaga, Heidelberg, Germany). The exact infusion rate was determined for each sheep.

4.3.5 Sample collection procedures

4.3.5.1 Balance period

Samples of feed on offer were taken from the feed batch (whole experiment requirement), before weighing the individual sheep's daily rations (see section 4.3.3). After pooling and mixing the samples obtained, two subsamples were taken for dry matter (DM) determination (100 °C, 48h). Other subsamples (2) were stored at –20 °C for chemical analysis. The amounts of daily feed refusals and faeces outputs were recorded and subsamples taken for DM determinations (100 °C, 48h). Other daily subsamples of feed refusals and faeces were stored frozen (-20 °C). After the collection, all frozen subsamples were pooled within animals, mixed throughly, re-sampled, then freeze-dried, ground through a 1 mm mesh sieve (Wiley Mill, USA) and used for analysis.

Urine was weighed daily from buckets containing sufficient H_2SO_4 to decrease the pH to between 2.5 and 3.0. Samples of the daily urine production (10%) were diluted (1:3, v/v) in water, sub-sampled (10%) and stored (-20 °C) for later analysis of purine derivatives (PD) on samples pooled within sheep. Other samples of the daily urine production (10%) were taken, stored frozen (-20 °C) and later pooled within sheep, freeze-dried and analysed for energy and N contents.

4.3.5.2 Bailing of rumen contents

Sheep ruminoreticulums (hereafter named rumen) were emptied (bailed) on days 5 (morning) and 8 (afternoon) of the digestion dynamics period (Figure 4.1). Rumen bailings took place within between 30 and 60 min after the feed delivery (09:00 h on day 5 or 15:00 h on day 8). Rumen contents were weighed, throughly mixed, and

subsampled before being returned to the rumen. The procedure took about 7 min per animal. Subsamples of digesta were taken for triplicate DM determination (60 °C, 72 h). Other subsamples of digesta were taken and managed in the following way: (1) a subsample (~200 g) was used for immediate pH determination, then stored (-20 °C), freeze dried, ground to pass through a 1 mm sieve and used for chemical analysis, (2) a subsample (~100 g) was stored (-20 °C) and later used for particle size determinations, (3) a subsample (~100 g) was stored (-20 °C) for later analysis of Cr concentration, (4) a subsample (~100 g) was strained through a nylon bag (60 μ mesh) and samples taken for analysis of ammonia (NH_3) and volatile fatty acid (VFA) concentrations, protozoa counting and measurement of buffering capacity.

The rumen fluid samples for NH_3 and VFA analysis were acidified, deproteinised and centrifuged immediately after sampling, using procedures described by Domingue et al. (1991). Samples for protozoa counting were prepared according to Odenyo et al. (1997): 4 ml of strained rumen fluid was added to 16 ml of formal-saline solution (1:5 dilution) and kept at 4 °C until counting.

4.3.5.3 CH_4 measurements

The SF_6 tracer technique (Johnson et al., 1994) was used for CH_4 measurements. All the measurements (Figure 4.1) were carried out while sheep were kept in digestibility crates, using the protocol described by Lassey et al. (1997). Sheep in digestibility crates were placed 2-3 m from each other within the building. The gas collection PVC yokes were suspended towards the rear of the metabolic crate and the sample line from the halter to the yoke was closely attached to the animal's back line to prevent chewing.

4.3.6 Laboratory methods

Samples of feed offered, feed refusals, faeces, and urine were analysed for energy content using an adiabatic bomb calorimeter (Gallenkamp Autobomb - Automatic; London, UK) and for total N by the Kjeldahl method. Crude protein (CP) was calculated as N x 6.25. Organic matter (OM) content of feed on offer, refusals, faeces and rumen contents was determined by ashing in a furnace at 550 °C for 16 h. Neutral detergent fibre (NDF), acid detergent fibre (ADF) and acid detergent lignin (ADL) contents of feed, feed refusals, faeces and rumen contents were determined according

to the methods of Goering and Van Soest (1970). Hemicellulose was calculated as NDF-ADF, whereas cellulose was calculated as ADF-ADL. Chromium concentration in the rumen digesta was analysed using an Inductively Coupled Plasma Emission Spectrometer (ARL 34000).

Urinary purine derivatives (PD), allantoin, xanthine (plus hypoxanthine) and uric acid were respectively determined using the colorimetric, enzymatic and uricase methods of Chen and Gomes (1992).

The pH of rumen contents was determined using a 3020 pH Meter (Jenway Ltd., England). VFA concentrations in rumen fluid were determined by capillary gas chromatography (Carlo Erba GC-5380, Italy). NH_3 concentration in rumen fluid was determined by auto analyser (COBAS, FARA, Basel, Switzerland), using commercially available diagnostic kits (Sigma, St. Louis, USA).

Buffering capacity of rumen fluid was determined according to the method described by Ding et al. (1997). HCl (1M) was added, in quantities of 20 μl at a time, to a 20 ml sample of rumen fluid until pH 4.5 was reached. Buffering capacity was defined as the amount of acid (mmol/l) required to change the pH of 1 ml of rumen fluid by 1 unit (Ding et al., 1997).

The particle size distributions in rumen digesta were determined using a wet sieving apparatus (TBA, Research Engineering Department, England) and following the procedure described by Domingue et al. (1991). The sieve sizes (length of side of square hole) used were: 4.0, 2.0, 1.0, 0.5 and 0.25 mm. Materials retained on the sieves were washed onto weighed filter paper (Whatman No. 21) in a Buchner funnel and dried at 60 °C to constant weigh to determine the dry weight of each particle size fraction. The dry weight of material not retained on the sieves (<0.25 mm particles), was determined by difference from the initial sample dry weight and the sum of recovered particulate DM fractions. Results for each fraction (particulate and soluble) were expressed as the % of the total initial DM in each sample.

For protozoa counting, a 1-ml aliquot of the formalinized rumen fluid sample was pipetted with a wide-orifice pipette into a 20 ml beaker containing 9 ml tap water (1:10 dilution). The diluted sample was pipetted into a counting chamber by a wide-orifice pipette. Protozoa were counted at a magnification of 128x. Each sample was

counted in triplicate, and each counting involved 15 fields. The total number of protozoa was counted, and the numbers of holotrichs and entodiniomorphs were also recorded. Protozoa counts were expressed per ml of rumen fluid.

4.3.7 Calculations

4.3.7.1 Fractional outflow rate (FOR) and mean retention time (MRT) in the rumen

Rumen fractional outflow rate (FOR, h^{-1}), the proportion of a digesta constituent that leaves the rumen per unit time (Faichney, 1980), was calculated using the continuous infusion and total sampling procedure of Faichney (1975). Liquid FOR (*Eq.* 4.1) was calculated with reference to the external marker Cr-EDTA, whereas the internal marker ADL was used to calculate particulate matter FOR (*Eq.* 4.2). Liquid FOR was calculated from the ratio of Cr-EDTA infusion rate (g Cr^{+++} h^{-1}) to the rumen pool size (g) of Cr^{+++}, determined at rumen bailing. The particulate matter FOR from the rumen was calculated from the ratio of faecal ADL flow (g h^{-1}) to the quantity (g, pool size determined at bailing) of ADL in the rumen (Faichney, 1980). It was assumed that the faecal output of ADL represented its abomasal flow.

$$\text{Liquid FOR } (h^{-1}) = \frac{Cr^{+++} \text{ infusion rate (g } h^{-1})}{Cr^{+++} \text{ rumen pool size (g)}} \qquad \textit{Equation } 4.1$$

$$\text{Particulate FOR } (h^{-1}) = \frac{\text{Faeces ADL flow (g } h^{-1})}{\text{ADL rumen pool size (g)}} \qquad \textit{Equation } 4.2$$

The mean retention time (MRT, h) of the liquid and the particulate phases in the rumen were calculated as the reciprocal of their respective FOR (Faichney, 1980). Thus:

$$\text{Liquid MRT (h)} = \frac{1}{\text{Liquid FOR } (h^{-1})} \qquad \textit{Equation } 4.3$$

$$\text{Particulate MRT (h)} = \frac{1}{\text{Particulate FOR } (h^{-1})} \qquad \textit{Equation } 4.4$$

For convenience, values for FOR (*Eqs.* 4.1 and 4.2) were multiplied by 100. Therefore, they read as % h^{-1}.

4.3.7.2 Apparent mean retention time (AMRT) in the rumen

In order to express the DM fractions of the rumen digesta as a proportion of their intakes, the apparent mean rumen retention time (AMRT) (Minson, 1966) of these constituents were calculated:

$$\text{AMRT (h)} = \frac{\text{Rumen pool size (g)}}{\text{Intake (g h}^{-1}\text{)}} \qquad \textit{Equation } 4.5$$

4.3.7.3 Rumen fill

Rumen fill (l or kg, wet contents) is highly correlated to the liveweight (LW) of the animal (Purser and Moir, 1966). In this study, rumen fill was measured as g per head. In addition, rumen fill was expressed as a proportion of the LW of the animal (%LW), but because sheep were fed on LW basis, the latter expression become an alias of the particulate FOR (% h^{-1}). Preliminary analysis showed that rumen fill (%LW) was poorly related to CH_4 emission (g d^{-1} or %GEI), so it was omitted from further analysis.

4.3.7.4 Digesta particle size distribution and modulus of fineness

The particle size of rumen digesta were expressed both as 'modulus of fineness' and as 'cumulative DM'. Modulus of fineness was calculated according to the procedure described by Poppi et al. (1980). Briefly, the DM on each sieve was expressed as a percentage of the total DM retained (≥ 0.25 mm) on the five sieves. Then, these percentages (% total DM retained on each sieve) were multiplied by the number of their respective sieves (sieves were numbered 1 to 5, from the smallest, 0.25 mm to the largest pore size, 4.00 mm), and the sum of these values divided by 100.

Alternatively, the cumulative proportions (% total DM) and pool size of particles > 1mm and < 1mm in the rumen were calculated. According to Poppi et al. (1980) the critical particle size for clearance from the rumen is about 1.2 mm.

4.3.7.5 Microbial nitrogen supply

Based on purine derivatives (PD) excretion, the microbial N supply (g N d^{-1} and g N kg^{-1} digestible organic matter apparently fermented in the rumen, (DOMR)) was

calculated according to the procedures described by Chen and Gomes (1992). Briefly, the total PD excretion (sum of allantoin, uric acid, xanthine and hypoxanthine; mmol d^{-1}) was calculated. Then, based on the daily excretion of PD and accounting for the endogenous contribution of PD, the amount of microbial purines absorbed (mmol d^{-1}) was estimated assuming that 84% of absorbed purines were recovered as PD in urine. Microbial N supply (g N d^{-1}) was calculated assuming: (1) the N content of absorbed purines was 70 mg $mmol^{-1}$, (2) the digestibility of microbial purines was 83%, and (3) the ratio of purine-N:total N in mixed rumen microbes was 11.6:100.

4.3.8 Statistical analysis

Data for variables derived from the two bailings were pooled for each sheep and the mean values calculated. Similarly, CH_4 emission values were pooled for each sheep and the mean values calculated. Mean values (and standard deviations) for all the variables measured were calculated.

The relationships between the CH_4 emission (expressed either as g d^{-1} or % gross energy intake, %GEI) and each variable measured, were assessed by correlation analysis (SAS, 1985). In addition, multiple regression analysis of CH_4 emission, expressed either as g d^{-1} or %GEI, upon the other variables was carried out using the forward model-selection method of the stepwise procedure of SAS (1985). In order to guard against selecting too many variables in the multiple regression model, the level of significance for the SLENTRY criteria for the stepwise procedure (SAS, 1985) was set at $p<0.10$.

In the multiple regression analysis of CH_4 emission as either g d^{-1} or %GEI, the variables included in the model-selection procedure were almost the same. Exceptions were that in the multiple regression analysis of CH_4 emission (%GEI), the variables OM intake (g) per kg metabolic liveweight ($kg^{0.75}$) (g $kg^{-0.75}$), microbial N yield (g kg^{-1} DOMR) and the AMRT of OM (h) were included instead of their corresponding absolute values (OM intake, g d^{-1}; microbial N yield, g d^{-1} and OM pool size, g, respectively). The AMRT (*Eq.* 4.5) is an expression of the rumen pool size of a particular constituent of digesta, adjusted for its intake.

When not otherwise stated, the number of observations was 10 (mean values for ten sheep).

4.4 RESULTS

4.4.1 Daily feed intake and apparent digestibility.

Daily feed intake (per animal and per $kg^{0.75}$) and the corresponding apparent digestibilities of dry matter (DM), organic matter (OM) and energy are shown in Table 4.1. The between-sheep coefficients of variation (CV) for daily feed intake, per head and per $kg^{0.75}$, were 10 and 7%, respectively. The mean apparent digestibilities of feed DM, OM and energy were 59.1, 60.4 and 56.7 %, respectively, and the variation in feed digestibility between-sheep was relatively small.

Table 4.1 Mean (±s.d.) daily feed intake (per head and per kg metabolic liveweight, $kg^{0.75}$) and apparent digestibilities.

	Daily Intake	Apparent Digestibility (%)
Dry matter (DMI)		59.1±1.8
g d^{-1}	1083±114	
g $kg^{-0.75}$	60.5±4.5	
Organic matter (OMI)		60.4±1.9
g d^{-1}	987±92	
g $kg^{-0.75}$	55.2±4.0	
Energy (GEI)		56.7±1.9
MJ d^{-1}	19.9±2.1	
MJ $kg^{-0.75}$	1.1±0.1	

4.4.2 Energy and nitrogen balances, and microbial N yield

Of the gross energy intake (Table 4.2), 45.9% was metabolised (8.4 MJ/kg DM); whereas the excretion of energy in faeces, urine and CH_4 were 43.3, 5.6 and 5.2 % of the GEI, respectively. With respect to the N balance, urinary excretion of N represented 67.4% and faecal N 28.5% of the N intake. N retention ranged from –3.8 to 13.6% of the amount eaten.

The mean microbial N yield was 11.5 g d^{-1} or 29.6 g kg^{-1} DOMR (Table 4.2). The between-sheep CV in microbial N yield (g kg^{-1} DOMR) was 17%.

Table 4.2 Mean (±s.d.) daily balances and partitioning (% of intake) of energy (MJ) and nitrogen (g) and microbial nitrogen yield (g d^{-1} and g kg^{-1} DOMR).

	Energy and Nitrogen Partition	
Energy Balance	MJ d^{-1}	% of intake
Intake	19.9±2.1	100
Excretion		
Faeces	8.6±1.1	43.3±1.9
Urine	1.1±0.1	5.6±0.3
CH_4	1.0±0.1	5.2±0.5
Metabolizable	9.1±1.0	45.9±1.9
Nitrogen Balance	g d^{-1}	% of intake
Intake	32.9±2.9	100
Excretion		
Faeces	9.4±1.1	28.5±1.4
Urine	22.2±2.7	67.4±3.6
Retained	1.3±1.3	4.1±4.2
Microbial Nitrogen Yield		
g d^{-1}	11.5±2.4	
g kg^{-1} DOMR[/]	29.6±4.9	

[/] DOMR, digestible organic matter apparently fermented in the rumen, estimated as 0.65 DOMI, digestible OM intake (Chen and Gomes, 1992).

4.4.3 Rumen fermentation parameters and protozoa count.

Table 4.3 shows the rumen pH, NH_3 concentration (mg l^{-1}), total VFA concentration (mmol l^{-1}), the molar proportions of acetate, propionate and butyrate, the acetate/propionate ratio, the buffering capacity of rumen fluid (mmols HCl) and the protozoa count. Except for protozoa count, the between-sheep variation for these parameters was small. Almost 95% of the protozoal population were entodiniomorphs, these being mostly small *Entodinium*.

Table 4.3 Mean (±s.d.) values for rumen fermentation parameters (pH, NH_3, VFA), buffering capacity of rumen fluid and protozoa count.

Parameter	Mean±s.d.
pH	6.78±0.06
NH_3 (mg l^{-1})	284±18
VFA (mmol l^{-1})	119±13
Acetate (mol %)	68.4±0.7
Propionate (mol %)	19.0±0.9
Butyrate (mol %)	8.2±0.3
Acetate/Propionate	3.6±0.2
Buffering capacity (mmols HCl)	39.3±2.1
Protozoa count (10^5 ml^{-1})	
Holotrichs	0.248±0.082
Entodinomorphs	4.612±0.951
Total	4.859±0.981

4.4.4 Rumen fill, pool sizes and particle size distribution.

Table 4.4 shows the rumen fill (g and %LW), the rumen pool sizes (g) of various constituents and the particle size distribution. The mean rumen fill (g, liquid + DM) was 4790 g and it represented 10.4 % of the LW of the animal. The liquid component of the rumen accounted for 88.5 % of the total contents. 76.8% of the rumen DM content was below 1.0 mm and the modulus of fineness of the particles (>0.25 mm) was 2.4.

Table 4.4 Mean (± s.d.) rumen fill, pool sizes and particle size distribution.

	Mean±s.d.
Rumen fill (wet digesta):	
g	4790±610
% LW	10.43±1.5
Rumen pool size (g):	
Liquid	4243±519
DM	546±99
OM	482±90
NDF	303±65
ADF	238±50
Hemicellulose	65±16
Cellulose	163±35
Lignin	75±15
Particle size distribution in rumen:	
Particles > 1.00 mm	
pool size (g DM)	129±43
% (of total DM pool)	23.2±5.2
Particles <1.00 mm	
pool size (g DM)	417±69
% (of total DM pool)	76.8±5.2
Modulus of fineness	2.4±0.2

4.4.5 Rumen digestion kinetics

The values for rumen fractional outflow rates (FOR) and mean retention times (MRT) are shown in Table 4.5. The mean FOR for the liquid and the particulate phases of the digesta were 13.6 and 4.1 % h^{-1}, respectively, whereas the corresponding MRT values were 7.7 and 25.4 h, respectively. The coefficients of variation (between-sheep) for these parameters were 20%.

The apparent mean retention times (AMRT) of digesta constituents are also shown in Table 4.5. Although the AMRTs of digesta constituents were calculated in order to adjust the rumen pool sizes for their intakes (see section 4.3.7.2), their values (Table

4.5) indicate that hemicellulose and lignin were the most recalcitrant components for rumen clearance.

Table 4.5 Mean (± s.d.) values for liquid and particulate fractional outflow rates (FOR), liquid and particulate mean retention times (MRT) and apparent mean retention times (AMRT) of some digesta constituents.

	FOR ($\% h^{-1}$)	**MRT** (h)	**AMRT** (h)
Liquid	13.6±2.8	7.7±1.5	
Particulate	4.1±0.8	25.4±4.8	
DM			12.4±1.8
OM			12.0±1.8
NDF			16.6±3.5
ADF			15.3±3.1
Hemicellulose			25.3±8.2
Cellulose			13.0±2.6
Lignin			24.5±6.2

4.4.6 CH_4 emission rates

Table 4.6 shows the mean daily CH_4 emission rates and the variation between-sheep expressed as a percentage of the total variation. The sheep produced 18.7 g CH_4 daily. When expressed per unit of intake, the CH_4 emission was 17.3 g kg^{-1} DMI, 19.3 g kg^{-1} OMI and 5.2 % GEI. The proportion of the total variation in CH_4 emission (per unit of intake) attributed to the between-sheep source was 62%.

Table 4.6 Mean (± s.d.) daily CH_4 emission and between-sheep variation (as a % of total variation) in CH_4 emission. (n=50 observations; 5 per sheep).

Daily CH_4 emission	**Mean±s.d.**	**Variation between sheep (%)**
g	18.7±2.6	70
g kg^{-1} DMI	17.3±1.8	61
g kg^{-1} OMI	19.3±2.0	62
% GEI	5.2±0.5	62

4.4.7 Relationships between the CH_4 emission and the other rumen digestion variables

Table 4.7 shows the coefficients of correlation (r) between CH_4 emission (g d^{-1} and % GEI) and the other measured variables. CH_4 emission (g d^{-1}) was positively related to feed intake (OMI, g; r=0.67, p=0.04), rumen pool size (OM pool, g; r=0.84, p=0.002) and rumen fill (g, r=0.76, p=0.01). The correlations between CH_4 emission (g d^{-1}) and urinary N partition (% of N intake; r=0.60, p=0.07), N retention (% of N intake; r=-0.54, p=0.11), microbial N yield (g d^{-1}; r=0.54, p=0.11), and molar proportion of butyrate (mol %; r=0.54, p=0.10) approached significance (Table 4.7).

CH_4 emission expressed per unit of intake (%GEI) was negatively related to the particulate FOR (% h^{-1}; r=-0.75, p=0.01) and buffering capacity (mmol HCl; r=-0.72, p=0.02), but positively related to the AMRT of organic matter (AMRT OM, h; r=0.70, p=0.03) and the apparent digestibility of cellulose (%; r=0.66, p=0.04). No significant relationships were found between CH_4 emission (%GEI) and the apparent digestibility of other dietary constituents.

Table 4.7 Coefficients of correlation[1] between the CH_4 emission (g d^{-1} and %GEI) and the other ruminal measurements[2].

Variables	CH_4 (g d^{-1})		CH_4 (% GEI)	
	r	*probability*	r	*probability*
Intake				
Organic matter (g d^{-1})	0.67	*0.04*		
Organic matter (g $kg^{-0.75}$)			-0.52	*0.12*
Rumen organic matter pool (g)	0.84	*0.002*		
AMRT of OM (h)			0.70	*0.03*
Rumen fill (g)	0.76	*0.01*	0.38	*0.27*
Rumen FOR (% h^{-1})				
Liquid	-0.03	*0.94*	-0.22	*0.55*
Particulate	-0.40	*0.25*	-0.75	*0.01*
Apparent digestibility (%)[3]				
Organic matter	-0.05 ✓	*0.90*	0.42	*0.23*
Cellulose	-0.02	*0.95*	0.66 ✓	*0.04*
Energy partition (% of intake)				
Faeces	0.05	*0.88*	-0.35	*0.33*
Urine	0.18	*0.62*	0.34	*0.34*
Metabolisable	-0.20	*0.58*	0.05	*0.88*
N partition (% of intake)				
Faeces	0.06	*0.88*	-0.51	*0.13*
Urine	0.60	*0.07*	0.19	*0.61*
Retained	-0.54	*0.11*	0.01	*0.98*
Microbial N yield				
g d^{-1}	0.54	*0.11*		
g kg^{-1} DOMR			-0.28	*0.44*
pH	-0.03	*0.93*	0.41	*0.24*
Buffering capacity (mmol HCl)	-0.05	*0.89*	-0.72	*0.02*
NH_3				
mg l^{-1}	-0.33	*0.36*	0.05	*0.89*
mg g^{-1} N intake	-0.10	*0.78*	0.44	*0.21*
VFA				
Acetate (mol %)	-0.25	*0.49*	-0.27	*0.44*
Propionate (mol %)	0.06	*0.86*	-0.17	*0.64*
Butyrate (mol %)	0.54	*0.10*	0.48	*0.16*
Acetate/Propionate	-0.11	*0.76*	0.07	*0.84*
Particle size of digesta				
> 1.0 mm (% of DM pool)	0.39	*0.27*	0.25	*0.48*
Modulus of fineness	0.41	*0.23*	0.21	*0.57*
Protozoa count (10^5 ml^{-1})				
Holotrichs	0.10	*0.77*	0.33	*0.35*
Total	-0.06	*0.88*	0.19	*0.59*

[1] For variables: intake, rumen pool size, AMRT and apparent digestibility, only the digesta constituent with the highest r value was tabulated. Autocorrelated variables not tabulated (e.g. FOR vs MRT).

[2] Only the tabulated variables were included in the multiple regression of CH_4 emission (g d^{-1} or %GEI).

[3] Only the digestibility variable with ✓ was included in the regression analysis of CH_4 emission (g d^{-1} or %GEI).

When the variables listed in Table 4.7 were used in the stepwise procedure for multiple regression analysis, the results were as follows:

(A) CH_4 expressed as g d^{-1}

From all the variables included (Table 4.7), only two variables were selected into the regression model to best explain the variation in CH_4 emission (g d^{-1}). The first variable selected was the organic matter pool size (OM pool, g), which explained 71% of the total variation. The second variable selected to enter the model was the molar proportion of butyrate (mol %), which increased the variation explained by the model to 88%. Thus, the multiple regression model to best explain CH_4 emission (g d^{-1}) was as shown by *Eq.* 4.6:

CH_4 (g d^{-1}) = −14.4 + 0.02 (s.e. 0.003) OM pool (g)
+ 2.91 (s.e. 0.898) Butyrate (mol %) ; R^2=0.88; p=0.0005 *Equation* 4.6

(B) CH_4 expressed as %GEI

From all the variables included (Table 4.7), three were selected into the regression model to best explain the variation in CH_4 emission (%GEI). The first variable to be selected was the particulate phase FOR (% h^{-1}) which explained 57% of the total variation. The second variable selected was the OM intake (OMI, g $kg^{-0.75}$), which together with particulate FOR explained 73% of the variation in CH_4 emission. The third and last variable selected to enter the model was the molar proportion of butyrate (mol %), which together with the other two variables accounted for 88% of the variation in CH_4 emission (%GEI). The multiple regression model to best explain the variation in CH_4 emission (%GEI) was as shown by *Eq.* 4.7:

CH_4 (%GEI) = 4.21 − 0.36 (s.e. 0.08) Particulate FOR (% h^{-1})
− 0.03 (s.e. 0.01) OMI (g $kg^{-0.75}$)
+ 0.52 (s.e. 0.20) Butyrate (mol %) ; R^2=0.88; p=0.004 *Equation* 4.7

4.5 DISCUSSION

The between-sheep coefficient of variation (CV) in the absolute daily feed intake (DM or OM, g d^{-1} or GE, MJ d^{-1}) was 10% (Table 4.1), similar to the variation in LW. In this study, sheep were fed at 1.2 times their maintenance requirements. Nevertheless, the between-sheep variation in daily feed intake per kg metabolic

liveweight ($kg^{0.75}$) was still 7% (CV), a consequence of the differences between-sheep in the ratio of feed DM eaten to that offered, which ranged from 0.88 to 0.98. Nevertheless, a high correlation (r=0.71, p=0.02) was found between the daily feed intake (g DM, g OM or MJ energy) and $kg^{0.75}$.

In general, the rumen digestion parameters were in the range reported in the literature for lucerne hay fed sheep (e.g. Egan et al., 1975, Ulyatt et al., 1984; Domingue et al., 1991; Nandra et al., 1993; de Vega et al., 1998). The NH_3 concentration in the rumen fluid was well above the value of 190 mg NH_3-N, the suggested threshold required for optimal fibre digestion (Mehrez et al., 1977). Nevertheless, two sheep approached a negative N balance (<1% of N intake) as a consequence of low feed intake.

In this study, the between-sheep variation in CH_4 emission accounted for 70% of the total variation in daily CH_4 emission (g d^{-1}) (Table 4.6), a value which is slightly lower than that (86%) reported by Lassey et al. (1997) for grazing sheep, but within the range cited by Ulyatt et al. (1999). The restricted and controlled feeding conditions imposed in the present study might have contributed to the lower between-sheep variation in CH_4 emission.

The CH_4 emission (g d^{-1}) was positively and significantly related to the pool size of digesta constituents (e.g. OM pool, g; r=0.84, p=0.002), to rumen fill (weight wet digesta, g; r=0.76, p=0.01) and to feed intake (e.g. OMI, g; r=0.67, p=0.04) (Table 4.7). In agreement with the observations of Purser and Moir (1966), rumen pool sizes of feed constituents were positively related not only to their intakes (r= 0.60 to 0.65, p<0.04), but also to the rumen fill (r= 0.91 to 0.94, p<0.0002). These relationships suggest that larger feed intakes were associated with an increased physiological capacity of the rumen, which allowed longer retention times and higher digestion rates (Grovum, 1984), thus resulting in larger CH_4 emissions. It is well established (e.g. Blaxter and Clapperton, 1965) that the absolute amount of CH_4 production (g d^{-1}) increases with absolute feed intake (g d^{-1}). Nevertheless, in the multiple regression model of CH_4 emission (g d^{-1}) (*Eq.* 4.6), OM pool size (g) was identified as being more important than feed intake (OMI, g) and rumen fill (g). Rumen OM pool size alone accounted for 71% of the total variation in CH_4 emission (g d^{-1}).

CH_4 (g d^{-1}) was positively but not significantly correlated with urinary N excretion (% of N intake; r=0.60, p=0.07) (Table 4.7). It has been shown that rumen protozoa population contributes positively to CH_4 emission (Jouany and Lassalas, 2000) and urinary N loss (Jouany, 1995). Nevertheless, no relationship between CH_4 emission and protozoa count was found in the present study (Table 4.7), and urinary N loss was not selected in the multiple regression model of CH_4 emission (g d^{-1}) (*Eq.* 4.6).

CH_4 emission (g d^{-1}) was not related to either the concentrations or molar proportions of acetate or propionate, but it was positively and weakly correlated (r=0.54, p=0.10) with the molar proportion of butyrate (Table 4.7). A positive relationship between CH_4 emission and butyrate (mol %) was also reported by Whitelaw et al. (1984). Acetate and butyrate formation in the rumen provide the major sources of H_2 for methanogenesis (Wolin, 1960) and acetate is considered to be the major precursor of butyrate during rumen fermentation (Russell and Wallace, 1988). In the multiple regression analysis of CH_4 emission (g d^{-1}), the molar proportion of butyrate (mol %) was the second variable selected in the model (*Eq.* 4.6) and accounted for 7.4% of the variation in CH_4 emission (g d^{-1}). Ciliates have been associated with an increased concentration of butyrate in the rumen fluid (Whitelaw et al., 1984; Hegarty et al., 1994; Jouany and Lassalas, 2000), but no evidence for this was found in the present study. However, it is possible that the straining of rumen digesta through a nylon bag (60 μ mesh) (see section 4.3.5.2) might have excluded some protozoa from the samples of rumen fluid. The molar proportion of butyrate (mol %) was also selected (ranked third and last) in the multiple regression model of CH_4 emission (%GEI) (*Eq.* 4.7), explaining an extra 14.2% of the total variation in CH_4 emission (%GEI).

CH_4 emission (%GEI) (Table 4.7) was positively related to the AMRT of OM (the rumen pool size of OM, adjusted for its intake) (r=0.70, p=0.03) and the apparent digestibility of cellulose (r=0.66, p=0.04), but negatively related to the particulate FOR (r=-0.75, p=0.01). These relationships suggest that longer retention times of feed in the rumen were associated with greater digestibility of cell walls and therefore greater CH_4 emissions. However, from the latter variables, the particulate FOR (% h^{-1}) was identified by the multiple regression analysis as having the strongest relationship to CH_4 emission (%GEI) (*Eq.* 4.7), explaining 57% of the total variation. The influence of particulate FOR on CH_4 emission is in agreement with previous

observations (e.g. Demeyer and Van Nevel, 1975; Okine et al., 1989), i.e. the higher the particulate FOR, the lower the CH_4 emission due to the shorter time that feed particles are exposed to microbial fermentation.

CH_4 emission (%GEI) was negatively correlated with the buffering capacity of rumen fluid (r=-0.72, p=0.02) (Table 7.4). In this study, lucerne hay was used as a sole feed and no between-sheep variation in pH was observed (Table 4.3). Therefore, under these conditions is unlikely that buffering capacity directly influenced the rate of CH_4 emission, but probably reflected the relationship between the rumen particulate FOR and CH_4 emission. Saliva is an important source of buffer in the rumen system (Ding et al., 1997) and the rate of saliva production influences the rumen dilution rate (Harrison et al., 1975; Sibanda et al., 1997). In the present study, buffering capacity was not correlated to liquid FOR (r=0.26, p=0.46), but was positively and weakly correlated (r=0.57, p=0.08) to particulate FOR. Buffering capacity was not identified in the multiple regression model of CH_4 emission (%GEI) as accounting for more variation than particulate FOR alone.

From the apparent digestibilities of dietary constituents, only the apparent digestibility of cellulose was correlated (r=0.66, p=0.04) with CH_4 emission (%GEI) (Table 4.7). This is in line with the estimation made by Moe and Tyrrell (1980) that potential CH_4 from 1 kg of digested cellulose is 5 and 3 times greater than from similar amounts of soluble residues and hemicellulose, respectively. The positive relationship between the apparent digestibility of cellulose and CH_4 emission (%GEI) is in agreement with the observations of Blaxter and Clapperton (1965) for a restricted feeding level. However at higher feeding levels, this relationship would be negative due to shorter rumen retention time (Blaxter and Clapperton, 1965). The apparent digestibility of cellulose was not identified in the multiple regression model of CH_4 emission (%GEI), because its effect was probably overshadowed by the effect of particulate FOR.

The correlation between the CH_4 emission (%GEI) and the OM intake per kg of metabolic liveweight (g $kg^{-0.75}$) approached significance (r=-0.52, p=0.12) (Table 4.7). OM intake (OMI, g $kg^{-0.75}$) was the second variable selected to enter the multiple regression model of CH_4 emission (%GEI) (*Eq.* 4.7), accounting for an extra 16% of the total variation in CH_4 emission (%GEI). CH_4 energy loss (%GEI) decreased as the

level of feed intake (OMI, g $kg^{-0.75}$) increased, a relationship also observed in other studies (Blaxter and Clapperton, 1965; Pelchen and Peters, 1998).

The quantitative interaction between the fractional digestion and passage rates determines the digestibility in the rumen (Poppi et al., 2000). In the present study, with restricted feeding conditions, particulate FOR (% h^{-1}) was the major factor involved in the between-sheep variation in CH_4 emission (%GEI). Particulate FOR not only correlated negatively with rumen fill (g) (r=-0.69, p=0.03), but also with cellulose digestibility (r=-0.65, p=0.04). Thus, larger rumen fills were associated with longer retention times of feed in the rumen and consequently greater fibre digestibilities and CH_4 emissions. Whether these interrelationships, observed at restricted feeding conditions, are the same under *ad libitum* feeding conditions or not, is unknown. Chapter 3 of this thesis showed that the between-sheep differences in CH_4 emission were repeatable only under generous grazing conditions, but not under indoor restricted feeding, which suggests that the impact of particulate FOR upon CH_4 emission was enhanced under generous grazing conditions.

In conclusion, although there is no doubt that CH_4 is produced by microbes, this study has demonstrated that rumen particulate fractional outflow rate (particulate FOR), an animal factor, had a major effect on the between-sheep variation in CH_4 emission (%GEI). If this relationship was persistent in the long term and the rumen fractional outflow rate was heritable, animal breeding might be effective as a tool to reduce CH_4 emission. However, since the effects of this animal factor on the variation in CH_4 emission are likely to be enhanced under *ad libitum* grazing conditions, non-invasive techniques for field screening of this factor need to be developed.

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Chapter 5

Validity of liveweight change under short-term grazing as an index of rumen volume, and a study of its effect upon methane emission in sheep

5.1 ABSTRACT

Two experiments (1 and 2) were conducted in order to find a simple LW-based index for screening of grazing sheep for rumen particulate fractional outflow rate (FOR) or rumen volume. Experiment 1 was carried out with 8 rumen-fistulated wethers grazing perennial ryegrass/white clover pasture under generous herbage allowance. A fasting and subsequent short-term grazing procedure (F/G procedure) was used. At the end of the evening grazing (T_0), sheep were fasted (feed and water deprived) indoors for 16 h, at the end of which (T_1) they were let out to graze for 3.5 h. At the end of grazing (T_2), sheep were again brought indoors. Liveweight (LW) and rumen fill (g, wet and DM contents by emptying) were measured at T_0, T_1 and T_2. It was assumed that rumen fill at T_0 represented its maximum fill (MaxFill, a measure of its volume), and that the changes in rumen DM fill (g) during fasting (T_0-T_1) and grazing (T_2-T_1) represented the rumen clearance rate (a measure related to particulate FOR) and the herbage intake rate, respectively. LW change during grazing (ΔLwGraz) was significantly and negatively correlated with MaxFill (r=-0.69, p=0.06 and r=-0.89, p=0.003, for wet and DM fill, respectively), whereas no other significant relationships between the LW and rumen fill measurements were observed.

In Experiment 2, based on the results of Experiment 1, a field screening of ΔLwGraz, using the F/G procedure (F/G-1), was carried out with a flock of 186 Romney ewe lambs, and ten sheep with 'small' and ten sheep with 'large' ΔLwGraz were selected. Within a period of 2 months, 3 additional measurements of ΔLwGraz (F/G-2, F/G-3 and F/G-4) were carried out with the selected 20 sheep. Immediately after F/G-3, an indoors study (lucerne chaff fed once a day) took place in order to compare the 'small' and 'large' ΔLwGraz sheep (as ranked at F/G-3) for their rates of CH_4 emission, voluntary feed intake, rate of feed intake, apparent feed digestibility and protozoa count. Experiment 2 showed that ΔLwGraz was not repeatable from one measurement to the next, and sheep ranked 'small' or 'large' for their ΔLwGraz did not differ for their rates of CH_4 emission or the other variables measured indoors. It was concluded that the simple measurement of ΔLwGraz can not be used as a repeatable index of rumen volume. However, if the major weight losses (e.g. faeces, urine) and inputs other than feed (e.g. water intake) could be accounted for, ΔLwGraz might prove to be a useful tool for screening of sheep for differences in rumen volume.

5.2 INTRODUCTION

It was found (Chapter 4 of this thesis) that under restricted indoor feeding conditions, rumen pool size of organic matter (g, OM pool) was the major factor responsible for the between-sheep variation in CH_4 emission (g d^{-1}), accounting for 71% of the total variation. For CH_4 emission per unit of gross energy intake (%GEI), the rumen particulate fractional outflow rate (particulate FOR, % h^{-1}) accounted for 57% of the total variation. Rumen fill (g, wet digesta) was positively related to rumen OM pool size (r=0.94, p=0.0001), but negatively related to particulate FOR (r=-0.69, p=0.03). Other studies, also under controlled conditions, both with sheep (Hodgson and Thomas, 1975; Faichney, 1993) and with cattle (Hartnell and Satter, 1979; Ørskov et al., 1988) have also found consistent between-animal differences in rumen outflow rate, and that animals with high outflow rates had smaller rumen fills (Hartnell and Satter, 1979; Ørskov et al., 1988; Faichney, 1993). Experimental animals selected for contrasting rumen particulate FOR or rumen volumes would be very useful to assess the effectiveness of using between-animal variation in these characteristics in the control of CH_4 emission. However, the estimation of these animal-related traits is difficult under grazing conditions. Thus, a simple screening index which is closely related to these animal traits and which can be carried out in the field is required.

It is well established (e.g. Champion et al., 1994) that most of the grazing activity of sheep takes place during daylight hours, with peaks at sunrise and sunset. Thomson et al. (1985) reported that the diurnal pattern of change in the rumen fill was similar to that of grazing activity, with maximum rumen fills (g wet or dry contents) observed at the end of the afternoon grazing period. Thus, measurement of rumen fill at this time should provide a direct measure of its maximum fill (rumen volume). Alternatively, based on the observation that intake rate is positively correlated with rumen volume (Purser and Moir, 1966a), the intake rate measured as the liveweight (LW) change during a short-term grazing period (Penning and Hooper, 1985; McGilloway et al., 1999) should be correlated to rumen volume. On the other hand, if at the end of the afternoon grazing (maximum rumen fill), the sheep is deprived of feed and water overnight, the change in rumen dry matter pool size during this period of fasting would be a measure of its clearance rate. Clearance rate is a measure positively correlated with the particulate FOR (e.g. Hodgson and Thomas, 1975).

LW is a simple animal measurement. In an attempt to find a practical tool for screening grazing sheep for their rumen particulate FOR or rumen volumes, this study tested the adequacy of the LW change (kg) during overnight fasting or the LW change (kg) at short-term grazing (re-feeding), following overnight fasting, as indices of rumen particulate FOR and rumen volume, respectively. The chosen LW-based index was assessed for both its repeatability and association with CH_4 emission.

5.3 MATERIALS AND METHODS

5.3.1 Experimental design

Two experiments were conducted at AgResearch Grasslands, Palmerston North, New Zealand. Experiment 1 was carried out from 15th December 1998 to 5th January 1999, whereas Experiment 2 was carried out from 20st June 1999 to 11th August 1999. Experiment 1 (Figure 5.1) was planned to test the adequacy of the LW change (kg) during overnight fasting (hereafter named **ΔLwFast**) or the LW change at short-term grazing, following overnight fasting (hereafter named **ΔLwGraz**), as indices of rumen particulate FOR and rumen volume, respectively.

Experiment 2 (Figure 5.2) was based on the outcomes of Experiment 1 and involved: (1) the screening of a large flock of sheep for ΔLwGraz and subsequent selection of 10 'small' and 10 'large' ΔLwGraz sheep; (2) three other subsequent assessments of the ΔLwGraz in the selected sheep (Figure 5.2 a); and (3) a comparison of the selected 10 'small' and 10 'large' ΔLwGraz sheep for their rates of CH_4 emission under controlled indoor conditions (hereafter named 'indoors trial').

The indoors trial (Figure 5.2 b) was conducted while sheep were kept in digestibility crates. After a 14-d acclimatisation period, the voluntary intake (VFI) of lucerne chaff was measured for 5 days (days 15 to 19). A 7-d digestibility period (days 22 to 28) followed the VFI measurement period. Intake rate and CH_4 emission were measured during the digestibility period, at the end of which (days 29 and 30) rumen contents were sampled for protozoa counts.

(a)

Periods	**Fasting (16 h)**																**Grazing (3.5 h)**			
Date	29 December '98					30 December '98														
Time (hrs.)	18:00	19:00	20:00	21:00	22:00	23:00	00:00	01:00	02:00	03:00	04:00	05:00	06:00	07:00	08:00	09:00	10:00	11:00	12:00	13:00
	T_0															**T_1**				**T_2**
Measurements[1]	LW_0															LW_1				LW_2

[1] Liveweight (LW) was measured at the start of fasting (T_0), end of fasting (T_1) and end of grazing (T_2).

(b)

Periods	**Fasting (16 h)**																**Grazing (3.5 h)**			
Date	4 January '99					5 January '99														
Time (hrs.)	19:00	20:00	21:00	22:00	23:00	00:00	01:00	02:00	03:00	04:00	05:00	06:00	07:00	08:00	09:00	10:00	11:00	12:00	13:00	14:00
	T_0															**T_1**				**T_2**
Measurements[1]	LW_0															LW_1				LW_2
	B_0															B_1				B_2

[1] Liveweight (LW) measurement and rumen contents bailing (B) were carried out at the start of fasting (T_0), end of fasting (T_1) and end of grazing (T_2).

Figure 5.1 Experiment 1: **(a)** Preliminary observation of liveweight (LW) changes during fasting (ΔLwFast) and during subsequent short-term grazing (ΔLwGraz), and **(b)** Test of adequacy of ΔLwFast and ΔLwGraz as an index of rumen particulate FOR and rumen volume, respectively.

(a)

Trials	F/G -1[1]	F/G -2[1]	F/G -3[1]	Indoor trial	F/G -4[1]
Location	AgResearch Ballantrae	AgResearch Grasslands			
Number of sheep	168	20 (selected at F/G-1)			
Date	20 & 21 June '99	26 & 27 June '99	29 & 30 June '99	2 to 31 July '99	10 & 11 August '99
Fasting and grazing (h)	17 and 4	16 and 3.5	16 and 3.5		16 and 3.5
Start fasting (hrs.)	15:00	17:00	17:00		18:00

[1] Fasting/Grazing (F/G) procedure: Sheep were fasted overnight and then allowed to graze for a short-term. The LW change during the short-term grazing (**ΔLwGraz**) was measured.

(b)

Periods	Acclimatisation					Voluntary feed intake					Transition		Feed digestibility							Protozoa sampling	
Feeding	ad libitum (15% refusal), once a day										restricted (1 x maintenance), once a day										
Days	1	2	...	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	29	30
Activities	Acclimatisation					Feed intake, Training harnesses for gas collection.					nil		Feed intake, Feed digestibility, Intake rate: days 25 to 28, CH_4 emission: days 22 to 25.							Rumen contents sampling by stomach tube	

Figure 5.2 Experiment 2: **(a)** Experimental designs for the initial (F/G-1, after which 20 sheep were selected) and subsequent (F/G-2, F/G-3, F/G-4) grazing trials where the ΔLwGraz were measured, and **(b)** The indoors trial conducted with the selected sheep.

5.3.2 Methodology

5.3.2.1 Experiment 1

Experiment 1 involved ten rumen-fistulated (i.d. 65 mm) Romney wethers (28 months old, 69.5±7.5 kg LW), which were grazed on perennial ryegrass/white clover pasture.

Starting on 15 December 1998, the early morning grazing behaviour of the sheep was observed, following a period of overnight fasting (indoors in digestibility crates, with feed and water deprived). Sheep grazed continuously for about 3.5 hours and then started to ruminate. At 4 hours after the grazing was started, all sheep were ruminating and drank water. Similar behaviour was observed when the procedure was repeated two days later.

Based on the above observation, on 29 December, 1998, the same 10 sheep were again fasted for 16 h (from 18:00 h to 10.00 h of the next day) (see Figure 5.1 a). They were then allowed to graze for 3.5 h. LW was measured at the start and the end of fasting, and at the end of grazing. A close relationship ($r=0.89$, $p=0.0005$) was observed between ΔLwFast (a decrease) and ΔLwGraz (an increase).

The test of adequacy of ΔLwFast and ΔLwGraz as indices of rumen particulate FOR and rumen volume, respectively, was carried out from 4 to 5 January 1999 (Figure 5.1 b). Six weeks before the test, a paddock of perennial ryegrass/white clover pasture was grazed by cows and then topped (above 7 cm) to remove stem material. At the time of the test the herbage mass was approximately 2000 kg DM ha^{-1}. The sheep started grazing in the prepared paddock 3 days before the test, and grazed a fresh area of pasture each day. Daily herbage allowance was set to give about 10-12% of their liveweight (LW) in total herbage dry matter (DM) offered, in order to maximise intake (Hodgson, 1990). Drinking water was freely available in the grazing area.

The test involved a period of 16 h fasting (indoors, with feed and water deprived), followed by 3.5 h grazing period (hereafter named **Fasting/Grazing** procedure, **F/G)** (see Figure 5.1 b). Sheep were brought indoors at 19:00 h (T_0), housed in digestibility crates and fasted until 11:00 h on the following day (T_1), at which time they were let out to graze until 14:30 h (T_2) (3.5 h net grazing period). LW was measured at T_0, T_1

and T_2 (±0.1 kg). Immediately after each LW measurement, rumen contents were emptied (bailed). Rumen emptying, weighing, sampling and returning the contents back to the rumen, were arranged in such a way that the whole task was completed in the 10 sheep within 30 minutes. Rumen contents were sampled for DM determination, by drying the samples at 60 °C for 72 h.

The following assumptions were made: (1) rumen fill measurements at T_0 represented maximum rumen fill values (**MaxFill**); and (2) rumen fill and LW measurements at T_1 represented the values for both the end of fasting and the start of grazing.

The index for particulate FOR was assessed from the relationship of the ΔLwFast to the change (g, lost) in the dry (DM) rumen fill contents during the overnight fasting (i.e. the clearance rate at fasting, hereafter named **RCRFast**). The index for rumen volume was assessed from the relationship of the ΔLwGraz to both (1) the MaxFill (g, wet or dry (DM) rumen contents) and (2) the change (g, gained) in the dry (DM) rumen fill contents during the short-term grazing (i.e. the intake rate at grazing, hereafter named **IRGraz**).

5.3.2.2 Experiment 2

5.3.2.2.1 Grazing trials and repeatability of ΔLwGraz measurements

On 21 June 1999, a flock of 186 Romney ewe lambs (10 months old, 32.5±3.4 kg LW), from a hill country farm (AgResearch Ballantrae, Woodville, Manawatu, NZ), were fasted for 17 h (from 15:00 h to 08:00 h the next day), after which they were allowed to graze for 4 h (**F/G-1**, see Figure 5.2 a). LW was measured at the start and the end of fasting, and at the end of grazing. Ten sheep with the smallest (0.59±0.09 kg) and 10 sheep with the largest (2.52±0.09 kg) ΔLwGraz were selected from sheep with LW in the mid range of the whole flock (31 to 34 kg). At this stage (F/G-1), there was a significant difference (p=0.0001) between the two groups of sheep in their ΔLwGraz values.

The selected sheep were brought to AgResearch Grasslands, Palmerston North, and their ΔLwGraz were measured in three other trials (**F/G-2**, **F/G-3**, **F/G-4**; Figure 5.2 a) carried out under more controlled grazing conditions on a prepared paddock, with a herbage mass of 2000 kg DM ha^{-1}. Three days before the commencement of each

trial, the sheep grazed a fresh area of pasture each day. Daily herbage allowance was set to maximise intake (Hodgson, 1990). Drinking water was freely available in the grazing area. The periods of fasting and grazing and the time at which fasting started were similar for trials F/G-2, F/G-3 and F/G-4 (Figure 5.2 a). F/G-4 took place 10 days after the indoor trial was completed (Figure 5.2 a).

5.3.2.2.2 The indoors trial

Feed and feeding:

The sheep were fed on chaffed (~50 mm length) lucerne (*Medicago sativa*) hay. This contained (per kg DM), 38.2 g N, 367 g neutral detergent fibre (NDF), 290 g acid detergent fibre (ADF), 97 g ash and 18.5 MJ of gross energy.

The hay was fed once a day at 08.00 h. During the VFI period (Figure 5.2 b), the feeding level was *ad libitum* (allowing 15% refusals). During the measurement of digestibility and the protozoa sampling periods, feeding level was restricted to the estimated maintenance energy requirements (Figure 5.2 b). Drinking water was made available *ad libitum* during the whole trial.

Measurements and sample collection procedures:

Voluntary feed intake (VFI).- The total amount of feed required for the whole VFI period was estimated, prepared (chaffed) and after thorough mixing, duplicate samples were taken for dry matter (DM) determination (100 °C, 48 h). Other subsamples (2) were stored at –20 °C for chemical analysis. The amounts of feed refused were recorded daily and samples taken for daily DM determination (100 °C, 48 h). Other daily samples of feed refusals were stored frozen (-20 °C). After the collection, all frozen samples were pooled within animals, mixed thoroughly and re-sampled, then freeze dried, ground through a 1-mm mesh sieve (Wiley Mill, USA) and used for analysis.

Apparent feed digestibility and rate of intake.- The procedures for sampling and the management of samples of feed offered and refused were the same as for the VFI period (see previous section). Faeces output was recorded daily and samples taken for daily DM determination (100 °C, 48 h). Other daily samples of the faeces were stored frozen (-20 °C) and later pooled within animals, mixed throughly, re-sampled, freeze-dried and ground (1 mm mesh sieve) for chemical analysis.

Intake rate (IR) was measured during the last four days of the feed digestibility period (Figure 5.2 b). The feed remaining (g, wet) in the feeding tray was weighed at 4.5 h after feeding. The feed intake (g, wet) during this time (4.5 h) was calculated by difference from the feed offered. Intake rate (%) was calculated as the proportion of the daily ration eaten in 4.5 h, on wet weight basis.

CH_4 emission measurements.- CH_4 emission was measured by the sulphur hexafluoride (SF_6) tracer technique (Johnson et al., 1994). CH_4 measurements were carried out during the first 4 days of the digestibility period (Figure 5.1), with sheep in digestibility crates. The protocol followed for the CH_4 measurements was described by Lassey et al. (1997). Sheep in digestibility crates were placed 2-3 m from each other within a well-ventilated building. The gas collection PVC containers were suspended in the rear end of the crates and the sample conduction line was closely attached to the animal's back line to prevent chewing.

Sampling of rumen contents for protozoa count.- At the end of the feed digestibility period, rumen contents were sampled on two consecutive days using a stomach tube. Samplings took place between 2.5 to 3.0 h post feeding. About 15-20 ml of rumen contents were collected, which took about 2 min per animal.

Samples for protozoa counting were prepared according to Odenyo et al. (1997), but using whole rumen contents (unstrained). Four ml of sample was added to 16 ml of formal-saline solution (1:5 dilution) and stored at 4 °C until the protozoa were counted.

Laboratory methods:

Samples of feed offered, feed refused and faeces were analysed for energy content using an adiabatic bomb calorimeter (Gallenkamp Autobomb; Loughborough, Leics, UK). Organic matter (OM) content of feed on offer, refusals and faeces was determined by ashing in a furnace at 550 °C for 16 h.

Protozoa counting was carried out after a further dilution (1:10) of the preserved samples. A 1-ml aliquot of the formalinized rumen contents sample was pipetted with a wide-orifice tube into a 20 ml beaker containing 9 ml tap water. The diluted sample was pipetted into a counting chamber by a wide-orifice pipette. Protozoa were counted at a magnification of 128x. Each sample was counted in triplicate, and each counting involved 15 fields. The total number of protozoa was counted, and the numbers of holotrichs and entodiniomorphs were also recorded. Protozoa counts were expressed per ml of rumen fluid.

5.3.3 Statistical analysis

5.3.3.1 Experiment 1

The relationships between the LW changes (kg) (ΔLwFast or ΔLwGraz) and the changes in rumen fill (g, wet and dry) at fasting or short-term grazing were analysed by correlation analysis. Index for rumen particulate FOR was assessed from the relationship of the ΔLwFast to RCRFast (change in dry rumen fill during fasting, g DM). Index for rumen volume was assessed from the relationship of ΔLwGraz to both (1) the MaxFill (g, wet or dry rumen contents at the start of fasting) and (2) IRGraz (change in dry rumen fill during grazing, g DM) (see section 5.3.2.1).

5.3.3.2 Experiment 2

Grazing trials:

Since ΔLwGraz (kg) on the selected 20 sheep were measured in four trials (F/G-1, F/G-2, F/G-3 and F/G-4; see Figure 5.2 a), correlation analysis between the trials were carried out in order to assess the repeatability of the ΔLwGraz measurements.

Indoors trial:

Analysis of variance of data for voluntary feed intake (VFI), intake rate (IR), apparent feed digestibility, CH_4 emission rate and protozoa count was carried out using the GLM procedure of SAS (SAS, 1985). Effects of the sheep groups (G, 'small' and 'large' ΔLwGraz) were tested using the sheep (S) within group [S(G)] component as the error term.

Since F/G-1 was carried out on hill country (AgResearch, Ballantrae), and because for logistic reasons, fasting started early in the afternoon (15.00 h) and tagging and weighing took nearly 2 h, rumen fill was probably not at its maximum value for all sheep at the start of fasting. Thus, considering that F/G-3 was carried out at more controlled grazing conditions and immediately before the indoors trial took place (Figure 5.2 a), sheep groups (G) used in the analysis of variance of the indoors trial data were as re-ranked (10 'small' and 10 'large' ΔLwGraz) on the basis of data recorded in F/G-3. At this stage (F/G-3), sheep groups ('small and 'large') differed significantly (2.0±0.14 vs 3.4±0.14 kg; p=0.0001) in their ΔLwGraz.

5.4 RESULTS

5.4.1 Experiment 1

Table 5.1 shows the mean (± standard deviation) values for liveweight (LW, kg) and rumen fill (g, wet and dry matter (DM) contents) at the start of fasting (T_0), the end of fasting (T_1), the end of grazing (T_2) and their changes during fasting (T_0-T_1) and grazing (T_2-T_1). The main correlations between the rumen fill (g, wet or DM contents) measurements at the start of fasting (T_0) and their changes during fasting (T_0-T_1) and grazing (T_2-T_1) are presented in Table 5.2. Because two sheep lost their rumen cannulae during the overnight fasting (T_0-T_1), data are presented for only n=8 sheep. The between-sheep variation (coefficient of variation) for LW, wet and DM rumen fill at the start of fasting (T_0) were 10, 14 and 20%, respectively. At this stage (start of fasting), the wet and DM rumen fill (MaxFill) represented respectively 13.0±2.4 and 1.3±0.3 % of the corresponding LW of the animal.

Table 5.1 Experiment 1: Mean (± s.d.) liveweight (kg head^{-1}) and rumen fill (g, wet and DM contents) at the start of fasting (T_0, maximum fill), end of fasting (T_1) and end of grazing (T_2) and their changes[1] during fasting (T_0-T_1, a decrease) and grazing (T_2-T_1, an increase). (n=8 sheep).

	Mean±s.d
Liveweight (kg head^{-1}):	
T_0	67.8±6.7
T_1	62.3±6.7
T_2	65.2±6.5
T_0-T_1 (ΔLwFast)	5.55±0.56
T_2-T_1 (ΔLwGraz)	2.91±0.63
Rumen fill, wet (g):	
T_0 (wet MaxFill)	8720±1231
T_1	6550±1004
T_2	7660±1233
T_0-T_1	2170±666
T_2-T_1	1110±511
Rumen fill, DM (g):	
T_0 (DM MaxFill)	843±178
T_1	294±69
T_2	607±106
T_0-T_1 (RCRFast)	549±141
T_2-T_1 (IRGraz)	312±56

[1] **ΔLwFast**, change in LW during overnight fasting; **ΔLwGraz**, change in LW during short-term grazing, following fasting; **MaxFill**, wet or DM maximum rumen fill (g) at the start of fasting (T_0); **RCRFast**, loss in rumen DM fill during fasting, a measure of the rumen DM clearance rate (g DM period^{-1}); **IRGraz**, gain in rumen DM fill during short-term grazing, a measure of the rate of intake (g DM period^{-1})

Table 5.2 shows that the rumen fill (both wet and dry) at the start of fasting (T_0) (MaxFill), was highly and positively correlated to both the loss (at fasting) and gain (at grazing) of the DM fill rather than to the corresponding changes in wet fill. The relationship between the loss of DM fill (g) during fasting (RCRFast) and the gain in DM fill (g) during grazing (IRGraz) was positive and aproached statistical significance (r=0.65, p=0.08), whereas that for the wet rumen fill was poor (r=0.44, p=0.28).

Table 5.2 Experiment 1: Coefficients of correlation[1] between the rumen fill (g, wet and DM) at the start of fasting (T_0, maximum fill) and changes in rumen fill (g, wet or DM) during the overnight fasting (T_0-T_1, loss in fill) or short-term grazing (T_2-T_1, gain in fill). (n=8 sheep).

	Fill loss (g) at fasting (T_0-T_1)		**Fill gain (g) at grazing (T_2-T_1)**	
	Wet	DM[3]	Wet	DM[4]
Fill (g) at start fasting (T_0) (MaxFill)[2]:				
Wet	0.58 *0.13*	0.71 *0.05*	0.44 *0.28*	0.67 *0.06*
DM	0.31 *0.45*	0.93 *0.0007*	0.60 *0.12*	0.68 *0.06*
Fill gain (g) at grazing (T_2-T_1):				
Wet	0.44 *0.28*			
DM[4]		0.65 *0.08*		

[1] *In italics*, probability values for H_0: ρ=0.

[2] Maximum Rumen fill stage (**MaxFill**).

[3] Represents the rumen DM clearance rate (digestion and passage) during fasting (**RCRFast**, g DM period^{-1}).

[4] Represents the intake rate of DM during the short-term grazing period (**IRGraz**, g DM period^{-1}).

Table 5.3 shows the coefficients of correlation of the changes in liveweight (LW) during fasting (ΔLwFast) or subsequent short-term grazing (ΔLwGraz) with the rumen fill (g, wet or DM contents) at the start of fasting (MaxFill) and the changes in rumen DM fill during fasting (RCRFast) or subsequent grazing (IRGraz), which were used to test their adequacy as indices of rumen particulate FOR and rumen volume. ΔLwFast was not correlated with the loss in rumen DM fill during fasting (RCRFast). ΔLwGraz was significantly and negatively correlated with both the wet and DM rumen fills (g) at the start of fasting (MaxFill) (r=-0.69, p=0.06 and r=-0.89, p=0.003, respectively). A low and non-significant correlation (r=-0.45, p=0.27) was observed between the ΔLwGraz and the gain in rumen DM fill during grazing (IRGraz). Thus, ΔLwGraz appears to be an index for rumen volume (MaxFill measured at start of fasting, T_0), but the lack of any relationship between ΔLwFast and RCRFast (Table 5.3) suggest that ΔLwFast was not an index of particulate FOR.

Table 5.3 Experiment 1: Coefficients of correlation[1] of the changes in liveweight (LW) during fasting (ΔLwFast) or subsequent short-term grazing (ΔLwGraz) with the rumen fill variables[2] (maximum fill and changes in DM fills at fasting or grazing). (n=8 sheep) (blank cells, not determined because of lack of relevance to this study).

	LW change (kg)	
	Fasting (ΔLwFast)	**Grazing (ΔLwGraz)**
A. Index for rumen particulate FOR[3]:		
1. Loss in rumen fill (g) at fasting (**RCRFast**):		
DM	-0.03 *0.94*	
B. Index for rumen volume:		
1. Rumen fill (g) at start fasting (**MaxFill**):		
Wet		-0.69 *0.06*
DM		-0.89 *0.003*
2. Gain in rumen fill (g) at grazing (**IRGraz**):		
DM		-0.45 *0.27*

[1] *In italics*, probability values for H_0: $\rho=0$.

[2] **MaxFill**, maximum rumen fill (g, wet and DM) at start of fasting; **IRGraz** (g DM period^{-1}), gain in rumen DM fill during short-term grazing, following fasting; **RCRFast** (g DM period^{-1}), loss in rumen DM fill during overnight fasting.

[3] It was assumed that during fasting the rumen DM clearance rate (RCRFast) was positively correlated with the particulate FOR.

5.4.2 Experiment 2

5.4.2.1 Repeatability of the ΔLwGraz measurements

The outcomes of Experiment 1 suggested that ΔLwGraz might be a suitable index for rumen volume. Table 5.4 shows the between trials (F/G-1, F/G-2, F/G-3 and F/G-4, see Figure 5.2a for details) coefficients of correlation for the ΔLwGraz measurements. As previously indicated (Section 5.3.2.2.1), F/G-4 took place 10 days after the indoor trial was finished. From the four occasions on which ΔLwGraz were measured, only the initial (F/G-1) and the third (F/G-3) measurements were positively related (r=0.61, p=0.004), whereas no relationships among the other measurements were found (Table 5.4). Thus, ΔLwGraz was not repeatable from one trial to the next.

Table 5.4 Experiment 2 (grazing trials): Between-trials (F/G-1, F/G-2, F/G-3 and F/G-4; for details see Figure 5.2a) coefficients of correlation[1] for ΔLwGraz[2] measurements. (n=20 sheep).

	F/G-1	F/G-2	F/G-3
F/G-2	-0.08 *0.74*		
F/G-3	0.61 *0.004*	0.05 *0.84*	
F/G-4	-0.02 *0.95*	0.05 *0.83*	-0.26 *0.30*

[1] *In italics*, probability values for H_0: $\rho=0$.

[2] ΔLwGraz, the change in liveweigh (LW) during a short-term grazing, following overnight fasting.

5.4.2.2 Indoors trial

Table 5.5 shows the mean voluntary feed intake (VFI) of dry matter (DMI), organic matter (OMI) and gross energy (GEI), both per head (d^{-1}) and per kg metabolic liveweight ($kg^{0.75}$) for the 'small' and 'large' ΔLwGraz groups of sheep (re-ranked into groups after F/G-3). Table 5.6, in turn, presents the mean apparent digestibilities of feed DM, OM and energy, the feed intake rate (IR) and the protozoa counts. The CH_4 emission values, both absolute (g d^{-1}) and per unit of gross or digestible feed intake, for the two groups of sheep are presented in Table 5.7. It must be noted that Tables 5.6 and 5.7 present values for variables measured at restricted feeding level (maintenance requirements). Overall, no differences between the 'small' and 'large' ΔLwGraz groups were found for the variables measured (Tables 5.5, 5.6 and 5.7).

Table 5.5 Experiment 2 (indoors trial): Voluntary feed intake (VFI, per day and per kg metabolic liveweight, $kg^{0.75}$) of dry matter (DMI), organic matter (OMI) and gross energy (GEI) by sheep re-ranked 'small' or 'large' for their ΔLwGraz[1] after F/G-3. (n=number of observations; 5 per sheep).

VFI	n	'Small' ΔLwGraz	'Large' ΔLwGraz	s.e[2]	*p*[3]
DMI					
g d^{-1}	100	1111	1073	76	*0.72*
g $kg^{-0.75}$	100	79.7	76.6	4.9	*0.65*
OMI					
g d^{-1}	100	1012	977	69	*0.73*
g $kg^{-0.75}$	100	72.6	69.8	4.4	*0.65*
GEI					
MJ d^{-1}	100	20.7	20.0	1.4	*0.73*
g $kg^{-0.75}$	100	1.5	1.4	0.09	*0.65*

[1] ΔLwGraz: LW change (kg) at short-term grazing, following overnight fasting, as measured at F/G-3 (see Figure 5.2a for details).

[2] s.e., standard error of means; [3] *p*, probability value for difference of means.

Table 5.6 Experiment 2 (indoors trial): Apparent digestibilities (% of intakes) of dry matter (DM), organic matter (OM) and energy, intake rate (IR, %), and protozoa count (10^5 ml^{-1}) for sheep re-ranked 'small' or 'large' for their ΔLwGraz[1] after F/G-3. All measured at restricted (maintenance) feeding level. (n=number of observations: 1, 4 and 2 per sheep for digestibility, IR and protozoa count, respectively).

	n	'Small' ΔLwGraz	'Large' ΔLwGraz	s.e[2]	*p*[3]
Apparent Digestibility (%):					
DM	20	64.3	63.9	0.3	*0.48*
OM	20	65.4	65.0	0.3	*0.47*
Energy	20	61.8	61.4	0.4	*0.52*
IR (%)[4]:					
4.5 h	80	93.9	93.9	1.75	*0.99*
Protozoa Count (x 10^5 ml^{-1}):					
Holotrichs	40	0.058	0.030	0.031	*0.55*
Entodiniomorphs	40	2.117	2.274	0.312	*0.73*
Total	40	2.158	2.305	0.314	*0.75*

[1] ΔLwGraz: LW change (kg) at short-term grazing, following overnight fasting, as measured at F/G-3 (see Figure 5.2a for details).

[2] s.e., standard error of means; [3] *p*, probability value for difference of means.

[4] Feed intake rate, proportion of the daily intake (wet basis) eaten during the first 4.5 h post-feeding.

Table 5.7 Experiment 2: CH_4 emission (g d^{-1}, and per unit of gross or digestible feed intake) by sheep re-ranked 'small' or 'large' for their ΔLwGraz[1] after F/G-3. All measured at restricted (maintenance) feeding. (n=number of observations; 4 per sheep).

CH_4 Emission	n	'Small' ΔLwGraz	'Large' ΔLwGraz	s.e.[2]	*p*[3]
g d^{-1}	80	11.5	11.6	0.9	*0.90*
Per Gross Feed Intake[4]:					
g kg^{-1} DMI	80	14.8	15.0	1.2	*0.92*
g kg^{-1} OMI	80	16.3	16.4	1.3	*0.92*
% GEI	80	4.4	4.5	0.3	*0.92*
Per Digestible Feed Intake[5]:					
g kg^{-1} DDMI	80	23.0	23.4	1.8	*0.88*
g kg^{-1} DOMI	80	24.9	25.3	2.0	*0.88*
% DEI	80	7.2	7.3	0.6	*0.87*

[1] ΔLwGraz: LW change (kg) at short-term grazing, following overnight fasting, as measured at F/G-3 (see Figure 5.2a for details).

[2] s.e., standard error of means; [3] *p*, probability value for difference of means.

[4] DMI=dry matter intake, OMI=organic matter intake, GEI=gross energy intake.

[5] DDMI=digestible DM intake, DOMI=digestible OM intake, DEI=digestible energy intake.

Table 5.8 shows the relationships between the ΔLwGraz (as measured at F/G-3), the VFI of organic matter (OMI, g d^{-1}), CH_4 emission (g d^{-1}), feed intake rate (IR, % wet ration eaten during 4.5 h), apparent organic matter digestibility (OMD, %) and total protozoa count (holotrichs plus entodiniomorphs, 10^5 ml^{-1}). The lack of relationships between ΔLwGraz and the other variables measured confirm the results of the analysis of variance (previous paragraphs). A significant positive correlation was observed between the VFI and the IR. These two variables, VFI and IR, were negatively correlated with the protozoa count (r=-0.48, p=0.03 and r=-0.42, p=0.07), respectively. No other relationship was significant (Table 5.8).

Table 5.8 Experiment 2. Coefficients of correlation[1] between the ΔLwGraz[2] (as measured at F/G-3, grazing), and the indoors measured variables: voluntary feed intake (VFI, g OM d^{-1}), CH_4 emission (g d^{-1}), intake rate (IR, %), apparent OM digestibility (OMD, %) and protozoa count (10^5 ml^{-1}). (n=20 sheep).

	VFI (g OM d^{-1})	**CH_4**[3] (g d^{-1})	**IR**[3] (%)	**OMD**[3] (%)	**Protozoa**[3] (10^5 ml^{-1})
ΔLwGraz (kg)	-0.13 *0.59*	0.13 *0.58*	0.13 *0.59*	0.01 *0.96*	-0.06 *0.81*
VFI	1.0 *0.0*	-0.23 *0.33*	0.51 *0.02*	0.11 *0.65*	-0.48 *0.03*
CH_4		1.0 *0.0*	0.05 *0.83*	0.18 *0.44*	-0.04 *0.86*
IR			1.0 *0.0*	0.33 *0.15*	-0.42 *0.07*
OMD				1.0 *0.0*	0.12 *0.63*

[1] *In italics*, probability values for H_0: $\rho=0$.

[2] ΔLwGraz: LW change (kg) at short-term grazing, following overnight fasting, as measured at F/G-3 (see Figure 5.2a for details).

[3] Variables measured at restricted feeding level (maintenance requirements).

5.5 DISCUSSION

Experiment 1 showed that rumen fill (g, wet or dry contents) at the start of fasting (MaxFill), a direct measure of the rumen volume (Purser and Moir, 1966a), was directly related to the changes (g) in rumen DM fill during fasting (RCRFast) or grazing (IRGraz), but was not related to changes in wet fill (Table 5.2). The RCRFast and the IRGraz were positively correlated with each other (r=0.65, p=0.08). These relationships suggest that when sheep were refed at generous grazing conditions, after overnight fasting, their rates of intake (IRGraz, g DM/period) was driven to compensate the DM lost (by digestion and passage, RCRFast) during the previous fasting period.

Based on the above relationships and assuming that both the wet and DM rumen fills at T_0 (MaxFill) were direct measures of rumen volume (Purser and Moir, 1966a), it can be argued that IRGraz was positively associated with rumen volume, a relationship also found under indoors *ad libitum* feeding conditions (Purser and Moir, 1966a). In addition, the positive relationship between MaxFill (wet or DM) and

RCRFast suggest that the larger the rumen volume the higher was the rumen DM clearance rate (and probably higher particulate FOR).

Experiment 1 was designed to find a simple LW-based index for screening grazing sheep for differences in rumen particulate FOR or rumen volume. The ΔLwFast was not related to RCRFast (Table 5.3), which invalidated its use as an index of rumen particulate FOR. On the other hand, although ΔLwGraz and IRGraz (Table 5.3) were not correlated with each other, a negative and significant correlation between ΔLwGraz and MaxFill (wet or DM) was observed, thus suggesting that ΔLwGraz could be used as an index of rumen volume. However, from the positive relationship observed between IRGraz and MaxFill (Table 5.2), a positive relationship between ΔLwGraz and MaxFill was also expected, but in fact this relationship was negative (r=-0.83, p=0.003 for DM fill) (Table 5.3). It is not clear whether this apparent discrepancy had a physiological basis, or it was an artifact of the ΔLwGraz measurement. During grazing, the between-sheep variation for the change in wet rumen fill (g) was much larger (CV=46%) than that for rumen DM fill (IRGraz) (CV=18%) (Table 5.1), which may be related to the rapid rate of fluid movement across the rumen, especially under non steady-state conditions (Tulloh et al., 1965).

Grazing trials of Experiment 2 (Table 5.4) showed that the sheep selected (at F/G-1, see Figure 5.2a) for 'small' or 'large' ΔLwGraz did not maintain their rankings between consecutive measurements (F/G-1, F/G-2, F/G-3 and F/G-4), which invalidates the use of ΔLwGraz as a index of rumen volume. Rumen volume and digesta kinetics of grazing sheep can change within a day and with seasons (Corbett and Pickering, 1983; Thomson et al., 1985). With this knowledge, ΔLwGraz measurements in this study were carried out within a short period and under controlled grazing conditions. However, variations in water intake, defecation and urination might be responsible for the lack of repeatability in the ΔLwGraz measurements. If it was possible to account for weight losses and gains through these other routes, and for LW to be measured more accurately (e.g. Penning and Hooper, 1985; McGilloway et al., 1999), then it may be possible to obtain more accurate relationships between ΔLwGraz and the rumen parameters.

Results of the indoors trial of Experiment 2 (Tables 5.5, 5.6 and 5.7) showed that sheep ranked 'small' or 'large' ΔLwGraz (rankings based in F/G-3 trial, see Section 5.3.3.2.2) did not differ from each other either for CH_4 emission, or for the other variables measured (VFI, apparent digestibility, intake rate or protozoa count). This is confirmed by the poor correlations observed between ΔLwGraz (rankings based in F/G-3 trial) and the other measured variables (Table 5.8).

Independently from the ΔLwGraz rankings, the relationships between the variables measured in the indoors trial (Table 5.8) showed that the IR of lucerne hay was positively related to its VFI ($r=0.51$, $p=0.02$), which confirms the findings of Purser and Moir (1966a). Purser and Moir (1966b) found that the IR was positively related to rumen volume, so that sheep with small rumen volumes and slow rates of intake exhibited greater protozoal concentrations than those with large rumen volumes. Rumen volume was not measured in the present study, but based on the results of Purser and Moir (1966a,b), it may be argued that in the present study rumen volume was involved in the relationships between protozoa concentration, IR and VFI (Table 5.8). Different ecological rumen environments have been suggested to exist in animals which differ in rumen volume (Purser and Moir, 1966a; Hodgson and Thomas, 1975). Nevertheless, IR (which is associated with rumen volume) was not related to CH_4 emission (Table 5.8). It had been observed previously (Chapter 3, this thesis) that the between-sheep differences in CH_4 emission was depressed at restricted feeding levels. Similar decreases in differences between-animals may have occurred in this study.

In conclusion, based on LW and rumen fill measurements carried out within a single 24-h period, this preliminary study found that the rate of LW change during short-term grazing, following an overnight fasting (ΔLwGraz), was related to the rumen volume (g, wet and DM contents at maximum fill). However, because ΔLwGraz was not repeatable in time, its use as an index for field screening purposes is not recommended. However it is possible that if the major weight losses (e.g. faeces, urine) and inputs other than feed (e.g. water intake) could be accounted for, the ΔLwGraz might prove to be a useful tool for screening of sheep for rumen volume.

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Chapter 6

Methane emission by alpaca and sheep fed on lucerne hay or grazed on pastures of ryegrass/white clover or birdsfoot trefoil

6.1 ABSTRACT

Alpaca (*Lama pacos*) have lower fractional outflow rate of feed particles (particulate FOR) from their forestomachs than sheep (San Martin, 1987). Based on this knowledge, this study aimed to compare these species in relation to methane (CH_4) emission and other digestion parameters under three successive experiments (1, 2 and 3): Experiment 1, lucerne hay fed indoors; Experiment 2, grazed on ryegrass/white clover pasture (RG/WC), and Experiment 3, grazed on birdsfoot trefoil (*Lotus corniculatus*) pasture (Lotus). Six male alpaca and six castrated Romney sheep were simultaneously and successively fed on the forages either *ad libitum* or at generous herbage allowances (grazing). CH_4 emission (using the sulphur hexafluoride tracer technique), voluntary feed intake (VFI), diet quality, and protozoa counts and volatile fatty acids (VFA, molar%) in samples of forestomach contents (obtained by stomach tube) were determined. In addition feed digestibility, water intake, energy and nitrogen (N) balances and microbial N yield (using purine derivatives excretion) were measured in Experiment 1.

Diets selected by alpaca were of lower quality than those selected by sheep, and the voluntary intakes of dry matter per kg of metabolic liveweight (DMI, g $kg^{-0.75}$) were consistently lower ($p<0.001$) for the alpaca than for the sheep (38.8 vs 74.0, 33.5 vs 70.0 and 40.3 vs 128.0 on lucerne hay, RG/WC and Lotus, respectively). Alpaca and sheep did not differ ($p>0.05$) in their CH_4 emissions (% gross energy intake, %GEI) when fed on lucerne hay (5.1 vs 4.7), but they did differ when fed on RG/WC (9.4 vs 7.5, $p<0.04$) and Lotus (6.4 vs 2.7, $p<0.001$). In addition to the depressed CH_4 emission (%GEI) by sheep grazing Lotus, protozoa populations in these sheep increased four-fold in comparison to the other forages. On lucerne hay and Lotus, but not on RG/WC, the animal species differed in the acetate/propionate ratio in forestomach fluid, this being greater for alpaca than sheep. When fed on lucerne hay, alpaca and sheep did not differ in water intake, N balance or microbial N yield. However alpaca had higher ($p<0.05$) NDF digestibilities (47.8 vs 46.1%), but lower ($p<0.01$) urinary energy losses (5.2 vs 5.8 %GEI) than sheep. Particulate FOR was not measured directly, but differences between animal species in: (1) chemical composition of their diets (more fibrous in alpaca, requiring more time to digest), (2) VFI (lower in alpaca), and (3) digestibility of cell walls (higher in alpaca), are consistent with alpaca having a lower particulate FOR than the sheep. Thus, differences between these species in particulate FOR might have been the underlying physiological mechanism responsible for the differences in CH_4 emission (%GEI). However, it was not possible to separate the possible effects of VFI, diet quality and animal species upon CH_4 emission (%GEI). More controlled studies are required to identify the factor (s) responsible for the depressed CH_4 emission (%GEI) observed in sheep when they were grazed on Lotus.

6.2 INTRODUCTION

Chapter 4 of this thesis showed that rumen particulate fractional outflow rate (particulate FOR) was a major contributor to the differences between sheep in methane (CH_4) emission. Sheep with lower particulate FORs (i.e. longer retention times) had larger rumen fills and higher feed digestibilities and CH_4 emissions. The direct measurement of the particulate FOR and rumen fill is much more difficult under grazing conditions than controlled conditions and Chapter 5 of this thesis showed that liveweight-based indices were not effective in accounting for these animal factors. The study of CH_4 emission rates by species or breeds differing in these animal factors might yield further insights of their involvement in CH_4 emission.

South American camelids (SAC: llama, *Lama glama*; alpaca, *L. pacos*; guanaco, *L. guanicoe*; and vicuña, *L. vicugna*) differ from sheep in the structure and function of their digestive system and therefore in their nutritional strategies (Vallenas et al., 1971; Heller et al., 1986; San Martin and Bryant, 1989). Most of the comparative studies, under both penned and grazing conditions have shown that SAC digest plant cell walls more efficiently than sheep (San Martin and Bryant, 1989; Warmington et al., 1989; Dulphy et al., 1994; Lemosquet et al, 1996; Dulphy et al., 1997). This higher digestibility was attributed to a lower particulate FOR (San Martin and Bryant, 1989; Dulphy et al. 1994; Dulphy et al., 1997; Lemosquet et al., 1996; Raggi and Ferrando, 1998).

Based on the knowledge that alpaca and sheep differ in their forestomach particulate FOR, this study compared these species for their CH_4 emissions under successive feeding on three different forages: (a) a lucerne hay fed indoors, (b) grazing on perennial ryegrass/white clover pasture (RG/WC), and (c) grazing on birdsfoot trefoil pasture (Lotus).

6.3 MATERIALS AND METHODS

6.3.1 Experimental design

The study was carried out from October to December 1999 and involved three successive experiments (1, 2 and 3; Figure 6.1), during which 6 alpaca and 6 sheep were fed *ad libitum* indoors (Experiment 1), or grazed under generous pasture

allowances (Experiments 2 and 3). All 12 animals were fed on the same forage at the same time, and the three periods were run consecutively.

Experiment 1 (indoors) was carried out at AgResearch Grasslands, Palmerston North, NZ, when chaffed lucerne (*Medicago sativa*) hay was fed. This experiment (1) was 29 days in duration, and the measurements were voluntary feed intake (VFI), CH_4 emission, energy and nitrogen (N) balance, microbial N yield, water intake, protozoa count and molar % of volatile fatty acids (VFA, mol %). Details are shown in Figure 6.1.

During Experiments 2 and 3 the animals were grazed on ryegrass/white clover (*Lolium perenne/Trifolium repens*) pasture (hereafter named 'RG/WC') and birdsfoot trefoil (*Lotus corniculatus* cv. Grasslands Goldie) pasture (hereafter named 'Lotus'), respectively at Massey University, Palmerston North, NZ. Each of these Experiments (2 and 3) was 21 days. Measurements during Experiments 2 and 3 included: diet composition, faeces output, CH_4 emission, protozoa count and molar % of VFAs, and the details are shown in Figure 6.1.

6.3.2 Animals

Male alpaca (6) of the Huacaya breed (61.4±10.5 (SD) kg) and castrated Romney sheep (6) (43.0±1.8 kg) were used. At the start of the study, the alpaca were 18 months old, and the sheep 15 months old. All animals had been grazing ryegrass/white clover pasture before the commencement of the study.

During Experiment 1, where lucerne chaff was fed indoors, the animals were housed individually in digestibility crates placed 3 m from each other within a well ventilated building. One side of the building was used for alpaca, whereas sheep occupied the other side. The camelids were housed in crates of similar design to those described by Milne et al. (1978) for red deer, with internal dimensions of 1.72 m (length), 1.52 m (height) and 1.11 m (width). One side of the crate was movable, so the width was decreased to 0.75 m to prevent the alpaca from turning around. The sheep crates were of standard design. Faeces and urine of both species were collected on an underlying steel chute, and the urine was immediately drained into a bucket containing 100 ml of 50% sulphuric acid.

Experiment 1: Fed indoors on lucerne hay

System (dates)	Indoors in digestibility crates (2 to 30 October, 1999)																			
Feed and feeding	chaffed lucerne hay, ad libitum, once a day feeding																			
Days	1	2	3	...	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	29
Activities	Acclimatisation: - training for CH_4 collection harness						VFI measurement						Energy and N balance and: - CH_4 collection: days 22 to 25 - purine derivatives excretion - water intake						Forestomach contents sampling: - Protozoa count - Molar % VFAs	

Experiment 2: Grazing on ryegrass/white clover pasture (RG/WC)

System (dates)	Grazing (2 to 22 November, 1999)										
Grazing	controlled grazing (daily rotation, generous herbage allowance): alpaca and sheep grazed separately										
Days	1	2		14	15	16	17	18	19	20	21
Activities	Acclimatisation: - training for faecal collection and CH_4 collection harnesses					Sample collection: - CH_4 collection: days 16 to 19 - total collection of faeces - herbage sampling				Forestomach contents sampling: - Protozoa count - Molar % VFAs	

Experiment 3: Grazing on birdsfoot trefoil pasture (Lotus)

System (dates)	Grazing (23 November to 13 December, 1999)										
Grazing	controlled grazing (daily rotation, generous herbage allowance): alpaca and sheep grazed separately										
Days	1	2		14	15	16	17	18	19	20	21
Activities	Acclimatisation: - training for faecal collection and CH_4 collection harnesses					Sample collection: - CH_4 collection: days 16 to 19 - total collection of faeces - herbage sampling				Forestomach contents sampling: - Protozoa count - Molar % VFAs	

Figure 6.1 Experimental design for the three subsequent experiments (1, 2 and 3) in which six alpaca and six sheep were compared.

6.3.3 Forages and feeding management

6.3.3.1 Experiment 1: Fed indoors on lucerne hay

Experimental animals were fed lucerne hay *ad libitum* (allowing 5-10% refusal). The hay was chaffed (~50 mm lengths) before feeding. Feeding level during days 22 to 29 (the digestion trial and period of sampling of rumen contents) (Figure 6.1) was fixed at 1.05 times the individual's average intake observed during the VFI measurement. The daily ration was fed at 08:00 h and drinking water was given *ad libitum*.

6.3.3.2 Experiment 2: Grazing on ryegrass/white clover pasture (RG/WC)

Two 0.4 ha paddocks (1 and 2) of RG/WC pasture were selected for uniformity of herbage composition. Each paddock was subdivided into two plots (using a portable fence) in the direction of the prevailing wind and the animal species (alpaca or sheep) randomly allocated to the plots within one paddock. Thus, alpaca and sheep were grazed on paired plots as separate flocks. Within each plot, a fresh strip of pasture was grazed each day. Daily herbage allowance was controlled by electric fences (back and front) to offer about 12% of their body weight in total herbage dry matter (DM) each day, in order to maximise intake (Hodgson, 1990).

Paddock 1 was grazed first, when it was in the stage of flowering. The whole of paddock 1 and one third of paddock 2 were grazed during the acclimatisation of experimental animals (d 1 to 15), while measurements and sample collections were carried out while the animals were grazed in paddock 2, when the pasture was also in flowering. During the animal measurements, herbage mass was 3490±346 kg DM ha^{-1}, composed of perennial ryegrass (75%), white clover (15%) and other species (10%; *Holcus lanatus*, *Agrostis capillaris*, etc.).

6.3.3.3 Experiment 3: Grazing on birdsfoot trefoil pasture (Lotus)

Two paddocks (0.4 ha each) of Lotus pasture were manually prepared with the aim of providing a pure stand of this pasture and at the same time avoiding the use of herbicides, residues of which might affect methanogenesis.

The selected paddocks contained overgrowth pasture, but in vegetative stage. There were weeds in either senescent (mostly grasses: ryegrass and annual poa) or

vegetative (mostly of the Compositeae family) stages. The weeds were pulled out manually by their roots, but grasses were manually cut 5 cm above ground level. Weeding took place about 2 days before the animals were due to graze the strip.

After weeding, the total herbage mass was 5,680±437 kg DM ha^{-1}, of which 53, 42 and 5% were stems and green leaf of Lotus and senescent weeds (mostly stems of grasses), respectively. Because of the high proportion of stem material, a very generous daily pasture allowance was offered, equivalent to 12% of LW in the form of leaf DM. The subdivision of paddocks and grazing management were similar to that for the RG/WC pasture (see Section 6.3.3.2).

6.3.4 Measurements and sample collection procedures

6.3.4.1 Experiment 1: Fed indoors on lucerne hay

The total amount of feed required for all the measurement phases of Experiment 1 (d 16 to 29) was estimated, prepared (chaffed) and after thorough mixing, duplicate samples were taken for dry matter (DM) determination (100 °C, 48 h). Other subsamples (2) were stored at –20 °C for chemical analysis. The amounts of feed refused were recorded daily and samples taken for daily DM determination (100 °C, 48 h). Other samples of daily feed refusals were stored frozen (-20 °C). After the collection, all frozen samples were pooled within animals, mixed thoroughly and re-sampled, then freeze dried, ground through a 1 mm mesh sieve (Wiley Mill, USA) and used for analysis.

Daily intakes of drinking water and outputs of faeces and urine were measured during the energy and N balance measurement phase (d 22 to 27). Samples of faeces were taken for daily DM determination (100 °C, 48 h). Other daily samples of the faeces were stored frozen (-20 °C) and later pooled within animals, mixed throughly, re-sampled, freeze-dried and ground (1 mm mesh sieve) for chemical analysis. Samples (10%) of the daily urine production (after acidification) were diluted (1:3, v/v) in water, sub-sampled (10%) and stored (-20 °C) for later analysis of purine derivatives (PD) on samples pooled within each animal. Other samples (10%) of the daily urine production were taken, stored frozen and later pooled within animal, freeze-dried and analysed for energy and N contents. No allowance for evaporative loss of drinking water was made.

Daily CH_4 emission was measured for four days (d 22 to 25, see Figure 6.1a) by the sulphur hexafluoride (SF_6) tracer technique (Johnson et al., 1994a), while animals were in digestibility crates, using the protocol described by Lassey et al. (1997). The gas collection PVC containers were suspended at the rear end of the crates and the sample conduction line was closely attached to the animal's back line to prevent chewing.

Rumen contents were sampled on two consecutive days (d 28 and 29) using a stomach tube. Samplings took place between 2.5 to 3.0 h post feeding. About 15-20 ml of rumen contents were collected and this task took about 2 min per animal. Samples for protozoa counting were prepared according to Odenyo et al. (1997), but using whole rumen contents (unstrained). Four ml of sample was added to 16 ml of formal-saline solution (1:5 dilution) and stored at 4 °C until counting. Samples of rumen contents for VFA analysis were acidified, deproteinised and centrifuged immediately after sampling, using procedures described by Domingue et al. (1991).

6.3.4.2 Experiments 2 (grazing on RG/WC) and 3 (grazing on Lotus)

Similar methods for collection of samples and their management were used in Experiments 2 and 3.

Samples of pasture on offer were obtained daily before animals entered the allocated pasture strips. Four (2 for each animal species) 0.10 m^2 quadrates (0.40x0.25m) were cut at ground level, weighed, pooled, and subsampled for DM determination. Other daily samples of the daily pooled material were stored (-20 °C) for later within-period pooling, freeze drying, grinding (1 mm mesh) and chemical analysis.

For each animal species, samples of the herbage which was grazed (diet) were collected from within three 0.5 m^2 protected (using 1.0x0.5 m wire cages) areas. The samples were taken to simulate the diet selected by the animals grazing that strip. Daily samples were stored (-20 °C) and later pooled within animal species, freeze dried, ground and used for chemical analysis.

In both Experiments 2 and 3, daily CH_4 emission was measured for four consecutive days (d 16 to 19, see Figure 6.1b,c), using the SF_6 tracer technique. A minimum of 3 successful CH_4 emission sampling days was required from each animal.

Total faeces outputs by the grazing animals were collected twice-daily using a harness and canvas bag. Collection of faeces was delayed by 1 d relative to the collection of samples for CH_4 measurement. Faeces from each animal were weighed, pooled within each day and sampled for DM determination (100 °C, 48 h). Other subsamples (10%) of the daily faeces output were stored (-20 °C) and later pooled within animal species, sub-sampled, freeze dried, ground and used for chemical analysis.

Daily dry matter intakes (DMI) by each individual alpaca and sheep were estimated from the *in vitro* pasture dry matter (DM) digestibilities (DMD) in conjunction with the total faecal DM output by the individual animals.

Each animal was weighed (LW, kg) at the start and the end of the sample collection phases of each of the experiments (1, 2 and 3).

6.3.5 Laboratory methods

Samples of lucerne chaff (both offered and refused), pasture (both that on offer and the diet selected), faeces, and urine were analysed for energy content using an adiabatic bomb calorimeter (Gallenkamp Autobomb; Loughborough, Leics, UK) and for total N by the Kjeldahl method. Organic matter (OM) content of lucerne hay and pasture samples, and faeces was determined by ashing in a furnace at 550 °C for 16 h, whereas their neutral detergent fibre (NDF) and acid detergent fibre (ADF) contents were determined according to the methods of Goering and van Soest (1970).

Samples of diet selected on RG/WC and Lotus pastures underwent *in vitro* digestibility determinations by the enzymatic method of Roughan and Holland (1977). The *in vitro* values were then adjusted using either ryegrass/white clover or birdsfoot trefoil standards with known *in vivo* digestibility values.

Samples of the pasture (RG/WC and Lotus) on offer and diet selected were analysed for extractable and bound condensed tannins (CT) using the butanol-HCl procedure of Terrill et al. (1992a).

Urinary purine derivatives (PD), allantoin, xanthine (plus hypoxanthine) and uric acid were respectively determined using the colorimetric, enzymatic and uricase methods of Chen and Gomes (1992). PD excretion was used to estimate the microbial N yield,

according to the procedures described by Chen and Gomes (1992) (see Chapter 4 of this thesis for details).

VFA concentrations in rumen fluid were determined by capillary gas chromatography (Carlo Erba GC-5380, Italy). The molar proportions (mol %) of acetate, propionate and butyrate, and the acetate/propionate (A/P) were calculated.

For protozoa counting, a 1-ml aliquot of the formalinized rumen contents sample was pipetted with a wide-orifice pipette into a 20 ml beaker containing 9 ml tap water (1:10 dilution). The diluted sample was pipetted into a counting chamber by a wide-orifice pipette. Protozoa were counted at a magnification of 128x. Each sample was counted in triplicate, and each counting involved 15 fields. The total number of protozoa was counted, and the numbers of holotrichs and entodiniomorphs were also recorded. Protozoa counts were expressed per ml of rumen contents.

6.3.6 Data calculation and statistical analysis

Data for voluntary dry matter intake (DMI, g) were expressed per animal and day (d). In addition, the voluntary intakes of dry matter (DMI, g), organic matter (OMI, g), gross energy (GEI, MJ), neutral detergent fibre (NDFI, g) and nitrogen (NI, g) were expressed per kg metabolic liveweight ($kg^{0.75}$). Data for CH_4 emission (g) were expressed per animal and day (d) as well as per unit of GEI (% GEI) and digestible NDFI (DNDFI; g kg^{-1}).

In this study the same animals were successively fed on three different forages, and although the animals were male and adult, the time and forage effects were confounded, making it statistically impossible to test the effects of forages and forages x animal species. Thus, since the major objective of this study was to compare the two animal species (alpaca and sheep) for their rates of CH_4 emission, data (for CH_4, VFI, protozoa and VFA concentration) were analysed within each experiment (or forage) using Proc GLM of SAS (SAS, 1985). Data for apparent feed digestibility, energy and N balance, apparent water intake and microbial N yield, all measured only in Experiment 1 (indoors on lucerne hay), were also subjected to similar analysis of variance. The data are presented as the least squared means, tested with the PDIFF option in SAS (1985).

Hereafter, the three forages: lucerne hay, RG/WC and Lotus, will be used in reference to Experiments 1, 2 and 3, respectively.

6.4 RESULTS

6.4.1 Experiment 1: Fed indoors on lucerne hay

Diet quality and voluntary feed intake (VFI).- The quality of the diet eaten by the alpaca was slightly lower (higher fiber, but low N contents) than that of sheep, but similar for *in vivo* OMD (%) (Table 6.1).

Alpaca were heavier ($p<0.01$) than sheep (63.3 vs 43.3 kg head^{-1}) (Table 6.2). Sheep ate significantly ($p<0.01$) more feed, both per animal and day (d) and per kg metabolic liveweight (kg$^{0.75}$), than alpaca (Table 6.2). Per kg$^{0.75}$ the mean feed intakes of sheep were about twice those of the alpaca.

CH_4 emission, and concentrations of volatile fatty acid (VFA) and protozoa.- The absolute amount (g d^{-1}) of CH_4 produced by alpaca was slightly lower, but not significantly ($p>0.05$), than that by sheep (14.9 vs 18.8). There was no difference ($p>0.05$) between the animal species in their CH_4 emissions expressed per unit of intake (Table 6.2).

There was a difference ($p<0.05$) between the animal species in the ratio of acetate/propionate, but not in butyrate concentration (mol %) (Table 6.2). The mean A/P ratios for alpaca and sheep were 3.0 and 2.3, respectively.

No holotrich protozoa were found in the forestomach contents of alpaca (Table 6.2), whereas holotrichs accounted for 1.0 % of the total protozoa concentrations in the rumen contents of sheep. Sheep had significantly higher ($p<0.01$) counts (10^5 ml^{-1}) of both entodinomorphids and total protozoa than alpaca.

Table 6.1 Chemical composition (g kg^{-1} DM) and apparent *in vitro* organic matter digestibility (OMD, %) of the forage on offer and of the diet selected by alpaca and sheep during lucerne hay feeding or grazing on ryegrass/white clover pasture (RG/WC) or birdsfoot trefoil pasture (Lotus). (n=1, but n=6 for diets in Experiment 1).

	Forage on offer	Diet selected	
		Alpaca	Sheep
	Experiment 1: Fed indoors on lucerne hay		
Organic matter (OM)	909	912	909
Total nitrogen (N)	36.5	36.7	38.3
Neutral detergent fibre (NDF)	384	394	380
Acid detergent fibre (ADF)	316	332	313
OM digestibility (OMD, %)	65.1[1]	65.1[2]	65.0[2]
	Experiment 2: Grazing on RG/WC		
Organic matter (OM)	909	905	898
Total nitrogen (N)	24	26	38
Neutral detergent fibre (NDF)	491	486	360
Acid detergent fibre (ADF)	300	303	242
Condensed tannins (CT):			
Extractable	0.5	0.6	0.5
Protein-bound	0.4	0.2	0.3
Fibre-bound	0.0	0.1	0.0
Total CT	0.9	0.9	0.8
OM digestibility (OMD, %)	72.0[1]	67.7	76.6
	Experiment 3: Grazing on Lotus		
Organic matter (OM)	926	921	919
Total nitrogen (N)	28	32	43
Neutral detergent fibre (NDF)	422	380	249
Acid detergent fibre (ADF)	344	282	199
Condensed tannins (CT):			
Extractable	13.0	12.0	25.6
Protein-bound	10.6	9.8	17.0
Fibre-bound	1.8	1.7	0.9
Total CT	25.4	23.5	43.6
OM digestibility (OMD, %)	63.0[1]	68.6	80.0

[1] *In vitro* digestibility by NIR. [2] *In vivo* values.

Table 6.2 Experiment 1: Fed indoors on lucerne hay. Liveweight (LW), voluntary feed intake (VFI[1]), CH_4 emission[2], volatile fatty acid (VFA) concentrations and protozoa counts for alpaca and sheep. (error degrees of freedom=10).

	ALPACA	SHEEP	Standard Error	*Probability*
LW (kg head^{-1})	63.3	43.3	3.6	0.005
VFI:				
DMI (g d^{-1})	844	1251	86	0.007
DMI (g kg$^{-0.75}$)	38.8	74.0	4.0	0.0002
GEI (MJ kg$^{-0.75}$)	0.74	1.36	0.07	0.0002
NI (g kg$^{-0.75}$)	1.4	2.7	0.1	0.0001
CH_4 emission:				
g d^{-1}	14.9	18.8	1.7	0.13
%GEI	5.1	4.7	0.3	0.40
g kg^{-1} DNDFI	92.0	92.5	6.5	0.96
VFA:				
Acetate/Propionate (A/P)	3.0	2.3	0.1	0.005
Butyrate (mol %)	6.9	6.5	0.7	0.68
Protozoa counts (10^5 ml^{-1}):				
Holotrichs	0	0.04	0.02	0.19
Entodinomorphs	2.08	3.80	0.4	0.009
Total	2.08	3.84	0.4	0.008

[1] VFI, expressed per animal and day (d) and per kg metabolic liveweight (kg$^{0.75}$).

[2] CH_4 emission expressed per animal and day (d) and per unit of intake (%GEI or kg DNDFI).

Abbreviations: DMI, GEI, DNDFI, NI are intakes of dry matter, gross energy, digestible neutral detergent fibre and nitrogen, respectively.

Apparent feed digestibilities and drinking water intake.- There were no differences between the animal species in their apparent digestibilities of dry matter (DM), organic matter (OM), gross energy (GE) or nitrogen (N) (Table 6.3). However, alpaca were more efficient ($p<0.05$) than sheep in digesting both neutral detergent fibre (NDF) and acid detergent fibre (ADF).

Daily drinking water intakes by alpaca and sheep were similar (4980 and 4741 ml d^{-1}, respectively) and there were no differences ($p>0.05$) between animal species for water intakes relative to metabolic liveweight (kg$^{0.75}$) or DMI (Table 6.3).

Table 6.3 Experiment 1: Fed indoors on lucerne hay. Apparent *in vivo* digestibilities (%) and water intakes by alpaca and sheep. (error degrees of freedom=10).

	ALPACA	SHEEP	Standard Error	*Probability*
Apparent digestibilities (%)[1]:				
DM	63.6	63.9	0.4	0.54
OM	65.1	65.0	0.4	0.85
GE	62.7	61.9	0.4	0.16
N	74.7	74.8	0.4	0.89
NDF	47.8	46.1	0.6	0.05
ADF	52.6	50.3	0.6	0.02
Drinking water intake:				
ml d^{-1}	4980	4741	1234	0.89
ml $kg^{-0.75}$	218	281	44	0.31
ml g^{-1} DMI	4.04	5.5	1.0	0.31

[1] Abbreviations: DM, dry matter; OM, organic matter; GE, gross energy; N, nitrogen; NDF, neutral detergent fibre; ADF, acid detergent fibre.

Energy and nitrogen (N) balances and microbial N yield.- The daily gross energy intake (GEI) by sheep (22.0 MJ) was significantly higher (p=0.01) than that by alpaca (16.0 MJ) (Table 6.4). Although the GEI loss as CH_4 did not differ between animal species (4.7 vs 5.1 %GEI, for sheep and alpaca, respectively) (Table 6.2), the urinary energy loss was significantly (p=0.007) greater in sheep (5.8 %) than in alpaca (5.2 %) (Table 6.4). Alpaca metabolised a slightly greater proportion (53 %) of their GEI than sheep (51.4%), but this difference was not significant (p=0.12).

The intake of nitrogen (N, g d^{-1}) by sheep was also significantly (p=0.005) higher (45.1 g) than that by alpaca (31.2 g) (Table 6.4), but there no differences (p>0.90) in the N partitioning between the animal species.

The daily microbial N yield (g) was significantly (p=0.006) higher in sheep than in alpaca (14.3 vs 9.5 g) (Table 6.5). Nevertheless, when microbial N yield was expressed per kg of digestible OM apparently fermented in the rumen (DOMR), there were no differences between the animal species.

Table 6.4 Experiment 1: Fed indoors on lucerne hay. Energy and nitrogen (N) balances, and microbial N yield by alpaca and sheep. (error degrees of freedom=10).

	ALPACA	SHEEP	Standard Error	*Probability*
Energy balance:				
Intake MJ d^{-1}	16.0	22.0	1.4	0.01
Partition (% of intake)				
Faeces	37.3	38.1	0.4	0.16
Urine	5.2	5.8	0.1	0.007
Metabolisable	53.0	51.4	0.6	0.12
Nitrogen balance:				
Intake g d^{-1}	31.2	45.1	2.8	0.005
Partition (% of intake)				
Faeces	25.3	25.2	0.4	0.89
Urine	57.4	57.8	2.5	0.93
Retained	17.2	17.0	2.7	0.95
Microbial N yield:				
g d^{-1}	9.5	14.3	1.0	0.006
g kg^{-1} DOMR[1]	29.1	31.8	2.1	0.37

[1] DOMR, digestible organic matter apparently fermented in the rumen, estimated as 0.65DOMI, digestible OM intake (Chen and Gomes, 1992).

6.4.2 Experiment 2: Grazing on ryegrass/white clover pasture (RG/WC)

Diet quality and voluntary feed intake (VFI).- The quality of the RG/WC diet selected by alpaca was much lower than that selected by sheep (Table 6.1). For example, the N and NDF contents were lower and higher, respectively in the diet of alpaca than in the diet of sheep. Accordingly, the OMD (%) of the sheep diet was higher than that of alpaca (76.6 vs 67.7 %) (Table 6.1). As expected the condensed tannins (CT) concentrations in the forage on offer and in the diets selected were low.

Alpaca were heavier ($p<0.01$) than sheep (65.1 vs 46.4 kg $head^{-1}$) (Table 6.5). The VFI of sheep, both per animal and day (d) and per kg metabolic liveweight ($kg^{0.75}$), were significantly ($p<0.001$) higher than of alpaca (Table 6.5). For example, per $kg^{0.75}$ the GEI (MJ) and NI (g) of sheep were respectively 2.2 and 3 times higher than those of alpaca.

CH_4 emission, and concentrations of volatile fatty acid (VFA) and protozoa.- The CH_4 emission of alpaca, per day (g d^{-1}), was lower (p=0.02) than that of sheep (22.6 vs 31.1) (Table 6.5). There was a difference (p<0.05) between the animal species in their CH_4 emissions expressed as a proportion of the GEI, but not (p>0.05) when expressed per kg of DNDFI (Table 6.5). There was no difference (p>0.05) between the species either in A/P or in butyrate concentration (mol %) (Table 6.5).

No holotrich protozoa were found in the forestomach contents of alpaca (Table 6.5), whereas in sheep holotrichs accounted for less than 1.0 % of the total protozoa counts. Nevertheless, no differences (p>0.05) between the animal species were found in the counts of protozoa in their forestomachs (Table 6.5).

Table 6.5 Experiment 2: Grazing on RG/WC pasture. Liveweight (LW), voluntary feed intake (VFI[1]), CH_4 emission[2], volatile fatty acid (VFA) concentrations and protozoa counts for alpaca and sheep. (error degrees of freedom=10).

	ALPACA	SHEEP	Standard Error	*Probability*
LW (kg $head^{-1}$)	65.1	46.4	3.2	0.002
VFI:				
DMI (g d^{-1})	761	1241	65	0.0004
DMI (g $kg^{-0.75}$)	33.5	69.8	3.3	0.0001
GEI (MJ $kg^{-0.75}$)	0.61	1.32	0.06	0.0001
NI (g $kg^{-0.75}$)	0.9	2.7	0.1	0.0001
CH_4 emission:				
g d^{-1}	22.6	31.1	2.2	0.02
%GEI	9.4	7.5	0.5	0.04
g kg^{-1} DNDFI	95.2	103.1	10.1	0.61
VFA:				
Acetate/Propionate (A/P)	2.7	2.9	0.1	0.33
Butyrate (mol %)	12.0	11.0	0.7	0.32
Protozoa counts (10^5 ml^{-1}):				
Holotrichs	0	0.04	0.02	0.19
Entodinomorphs	4.20	4.05	0.8	0.90
Total	4.20	4.09	0.8	0.93

[1] VFI, expressed per animal and day (d) and per kg metabolic liveweight ($kg^{0.75}$).

[2] CH_4 emission expressed per animal and day (d) and per unit of intake (%GEI or kg DNDFI).

Abbreviations: DMI, GEI, DNDFI, NI are intakes of dry matter, gross energy, digestible neutral detergent fibre and nitrogen, respectively.

6.4.3 Experiment 3: Grazing on birdsfoot trefoil pasture (Lotus)

Diet quality and voluntary feed intake (VFI).- As in the case of RG/WC pasture, the quality of the Lotus diet eaten by alpaca was much lower than that eaten by sheep (lower N, but higher NDF concentrations) (Table 6.1). The estimated OMD (%) of the alpaca diet was much lower than that of sheep (68.6 vs 80.0%) (Table 6.1). The concentration of CT in the diet selected by sheep was about twice that in the diet of alpaca or in the forage on offer (Table 6.1).

As expected alpaca were heavier ($p<0.01$) than sheep (63.1 vs 47.2 kg head^{-1}) (Table 6.6). The VFI of sheep, both per animal and day (d) and per kg metabolic liveweight (kg$^{0.75}$), were much higher ($p<0.0001$) than of alpaca (Table 6.6). For example, the GEI (MJ kg$^{-0.75}$) and NI (g kg$^{-0.75}$) of sheep were 3.3 and 4.2 times higher, respectively than those of alpaca (Table 6.6).

CH_4 emission, and concentrations of volatile fatty acid (VFA) and protozoa.- The CH_4 emission of alpaca, per day (g d^{-1}), was similar ($p=0.30$) to that of sheep (19.1 vs 22.0) (Table 6.6). However, per unit of intake, the CH_4 emissions of alpaca were much higher ($p<0.0001$) than of sheep (e.g. 6.4 vs 2.7 %GEI) (Table 6.6).

There was a difference ($p<0.001$) between the animal species in A/P (3.4 vs 2.6; for alpaca and sheep, respectively), and the butyrate concentration (mol %) in the rumen contents of sheep were slightly higher ($p=0.07$) than in alpaca (Table 6.6).

As observed in the other two forages (lucerne hay and RG/WC), no holotrich protozoa were found in the forestomach contents of alpaca (Table 6.6) and holotrichs accounted for less than 1.0 % of the total protozoa counts in sheep. Animal species significantly differed in their counts of holotrichs ($p=0.06$), entodinomorphs ($p=0.001$) and total numbers of protozoa ($p=0.001$) (Table 6.6). The total concentration of protozoa (10^5 ml^{-1}) in sheep was 3.5 times higher than in alpaca (Table 6.6).

Table 6.6 Experiment 3: Grazing on Lotus pasture. Liveweight (LW), voluntary feed intake (VFI[1]), CH_4 emission[2], volatile fatty acids (VFA) concentrations and protozoa counts for alpaca and sheep. (error degrees of freedom=10).

	ALPACA	SHEEP	Standard Error	*Probability*
LW (kg head^{-1})	63.2	47.2	3.2	0.005
VFI:				
DMI (g d^{-1})	902	2303	119	0.0001
DMI (g kg$^{-0.75}$)	40.3	127.9	5.5	0.0001
GEI (MJ kg$^{-0.75}$)	0.77	2.53	0.11	0.0001
NI (g kg$^{-0.75}$)	1.3	5.5	0.2	0.0001
CH_4 emission:				
g d^{-1}	19.1	22.0	2.0	0.30
%GEI	6.4	2.7	0.2	0.0001
g kg^{-1} DNDFI	152.0	70.0	6.0	0.0001
VFA:				
Acetate/Propionate (A/P)	3.4	2.6	0.1	0.001
Butyrate (mol %)	11.6	13.7	0.7	0.07
Protozoa counts (10^5 ml^{-1}):				
Holotrichs	0	0.12	0.04	0.06
Entodinomorphs	4.7	16.4	1.9	0.001
Total	4.7	16.5	1.9	0.001

[1] VFI, expressed per animal and day (d) and per kg metabolic liveweight (kg$^{0.75}$).

[2] CH_4 emission expressed per animal and day (d) and per unit of intake (%GEI or kg DNDFI).

Abbreviations: DMI, GEI, DNDFI, NI are intakes of dry matter, gross energy, digestible neutral detergent fibre and nitrogen, respectively.

6.5 DISCUSSION

6.5.1 Diet selection

Within each of the three forages, the diet selected by the alpaca was of lower quality than that selected by sheep (Table 6.1). The higher OM digestibility (OMD %) for sheep diets, especially under grazing conditions, might be attributed to the selection of particular plant parts (and plant species) which were higher in N but lower in fibre than the forage on offer. Even when fed on lucerne hay, sheep preferred the leafier material, whereas alpaca preferred the stalkier portions. The latter confirms the

feeding preferences observed by Warmington et al. (1989) when llama x guanaco crosses and sheep were fed on ryegrass straw.

At grazing, the differences between species in diet selection were even greater. On RG/WC, sheep selected mostly white clover and grass green leaf, whilst on Lotus, which was almost a pure stand, the sheep diet was composed entirely of Lotus leaves. In marked contrast, on RG/WC, the alpaca avoided white clover but they grazed patches of pure grass completely to ground level. On Lotus, alpaca selected primarily the senescent grass material (weeds), but because of the low availability of this material, Lotus stem and leaf materials were also eaten. The differences between species in selective grazing was very evident in this study and agree with the results from other studies with alpaca and sheep (Sharp et al., 1995) or guanacos and sheep (Bakker et al., 1997; Fraser, 1998; Fraser and Gordon, 1997).

6.5.2 Voluntary feed intake (VFI)

On all the three forages, VFI, both per animal and day (d) and per kg metabolic liveweight ($kg^{0.75}$), was consistently lower ($p<0.001$) in alpaca than in sheep (Tables 6.2, 6.5 and 6.6). This is consistent with a lower forestomach particulate FOR in alpaca (San Martin, 1987). The following discussion centred on intake of DM (DMI, $g\ kg^{-0.75}$).

On lucerne hay (Table 6.2), alpaca DMI was 48% less than that of sheep (38.8 vs 74 g $kg^{-0.75}$). Although this difference in VFI is in the upper end of the range of intake differences quoted by San Martin and Bryant (1989), it contrasts with the lack of any difference in VFI observed when SAC (other than alpaca) and sheep were fed on forages (Warmington et al., 1989; Lemosquet et al., 1996; Fraser and Baker, 1998). The DMI by alpaca on lucerne hay was similar to that (39.0 g $kg^{-0.75}$) of llama x guanaco crosses offered ryegrass straw (Warmington et al., 1989), but lower than those (range 43 to 60 g $kg^{-0.75}$) observed in alpaca (López et al., 1998) and other SAC (llamas and guanacos) (Dulphy et al., 1997; Lemosquet et al., 1996; Fraser and Baker, 1998) fed on forages. On the other hand, the DMI by sheep fed on lucerne hay (74 g $kg^{-0.75}$) (Table 6.2) was higher than the intakes (range 36 to 65 g $kg^{-0.75}$) recorded when grass hays were fed (Warmington et al., 1989; Lemosquet et al., 1996), but within the range reported by San Martin and Bryant (1989).

The DMI (g $kg^{-0.75}$) of alpaca on RG/WC and Lotus were 52 and 69 % less than those of sheep (Tables 6.5 and 6.6). These differences were higher than that (37%), found between alpaca (57.6 g $kg^{-0.75}$) and sheep (91.4 g $kg^{-0.75}$) grazing on a ryegrass-predominant pasture (San Martin and Bryant, 1989).

The DMI of alpaca was relatively constant for all three forages fed (38.8, 33.5 and 40.3 g $kg^{-0.75}$, on lucerne hay, RG/WC and Lotus, respectively) (Tables 6.2, 6.5 and 6.6). In contrast, the DMI of sheep was extraordinarily high when they were grazed on Lotus (128.0 g $kg^{-0.75}$). As stated in Section 6.3.4.2, DMI under grazing was estimated from the total collection of faeces (g DM d^{-1}) and the *in vitro* DM digestibility of the forage eaten. It is unlikely that unrepresentative samples of the sheep Lotus diets were taken. Firstly, the pasture offered was prepared to a condition of almost pure Lotus, with only 5% of weeds. Secondly, because leaf availability was high (herbage allowance was calculated on the basis of leaf DM, rather than whole herbage DM), occasional non-systematic observations showed that the sheep grazed almost exclusively on leaves. Consequently, the leaves were sampled to represent the sheep diet. Waghorn et al. (1997) stated that on CT-containing temperate forages, sheep and cattle select leaves in preference to stems.

In this study, the *in vitro* digestibilities of Lotus samples were adjusted using *Lotus corniculatus* standards of known *in vivo* digestibilities. The DM digestibility (DMD %) estimated for the sheep diet on Lotus was 78.7% (or 80.0% OMD), which was slightly higher than that (76.6 %) measured *in vivo* for the same forage (*Lotus corniculatus* cv. Grasslands Goldie), fed near to *ad libitum* (Wang et al., 1994). However, *in vitro* OMD of 81% was reported for grazing sheep on the same cultivar of Lotus (Douglas et al., 1995). It is expected in sheep, that selective grazing under generous herbage allowance would yield highly digestible diets.

Forages containing more than 55 g CT kg^{-1} DM might depress VFI (Waghorn et al., 1997; Barry and McNabb, 2000). Douglas et al. (1995) reported a concentration of 57.3 g CT kg^{-1} DM in diets of sheep grazing Lotus, but the VFI of Lotus was even greater ($p<0.05$) than that of lucerne or Lotus x lucerne pastures. Similarly, Terrill et al. (1992b) reported that grazing sheep had higher ($p<0.001$) DMI on sulla (*Hedysarum coronarium*; 36 g CT kg^{-1} DM on diet) than on RG/WC pasture (132 vs 90 g $kg^{-0.75}$). The CT concentration in sheep diets determined in the present study was

relatively low, 43.6 g kg^{-1} DM (Table 6.1). So, the depressing effect of CT in Lotus on VFI probably did not occur in this study.

It is well established (Blaxter and Wainman, 1964; Colucci et al., 1990; Moss et al., 1995) that at high feeding levels the digestibility of forages is depressed by 2 to 3 units (%). In the present study, where there was a high herbage allowance, a discount of the *in vitro* DMD of Lotus for this depression would decrease the DMI of sheep by 12.4% (from 128 to 112 g $kg^{-0.75}$), which is still a high DMI value.

In summary, the feed intakes (per $kg^{0.75}$) recorded in the present study for alpaca were at the lower end of VFI reported in the literature; whereas those of sheep were relatively high. Thus, the resulting differences between the animal species in VFI were large. The lowest intake recorded in the literature for alpaca was 28.8 g OM $kg^{-0.75}$ when they were fed on ryegrass hay (Reiner et al., 1987), but the animals lost weight. In the present study alpaca maintained their LW with an average OMI of 34 g $kg^{-0.75}$, whereas sheep gained LW with an average OMI of 82.5 g $kg^{-0.75}$.

6.5.3 CH_4 emission, volatile fatty acid (VFA) and protozoa concentrations

Interpretation of the effects of animal species upon CH_4 emission (Tables 6.2, 6.5 and 6.6) was complicated because these effects were confounded with those of the chemical composition of the diets eaten (Table 6.1) and VFI (Tables 6.2, 6.5 and 6.6).

Mean CH_4 emissions by sheep (g d^{-1}) were within the range reported in the literature (Blaxter and Clapperton, 1965; Pelchen and Peters, 1998; Ulyatt et al., 1999). However, as %GEI, the CH_4 emitted by sheep in this study was relatively lower than those reported in the literature (e.g. Pelchen and Peters, 1998), which might be attributed to the effects of the *ad libitum* feeding (Blaxter and Clapperton, 1965) and the technique (SF_6 tracer) used for CH_4 measurement (slightly lower CH_4 values) (Chapter 2, this thesis; Johnson et al., 1994b; McCaughey et al., 1999). Similarly, the CH_4 emission (%GEI) recorded in alpaca on lucerne hay (5.1%) (Table 6.2) was much lower than those (range 6.0 to 8.3 %) reported for llamas fed on mixed diets (Schneider et al., 1974; Carmean et al., 1992). No other report of CH_4 emission by SAC was found in the literature, neither were other reports of CH_4 emission by animals grazing on CT-containing forages found.

On all the three forages the absolute amounts (g d^{-1}) of CH_4 emitted by sheep were slightly higher (significant only on RG/WC) than those by alpaca (Tables 6.2, 6.5 and 6.6). This can be attributed to the higher feed intakes (g d^{-1}) observed in sheep (Tables 6.2, 6.5 and 6.6). On the other hand, except on lucerne hay (Table 6.2), the proportions of the GEI loss in CH_4 (%GEI) were significantly lower ($p<0.05$) in sheep than in alpaca (Figure 6.2a). The latter is in agreement with the earlier findings by Blaxter and Clapperton (1965) that CH_4 emission (%GEI) decreases with increasing feed intake (relative to maintenance requirements) and with increasing diet digestibilities. In fact, in the present study, except on lucerne hay, the diets selected by sheep contained less cell walls (NDF), but more N than those of alpaca (Table 6.1).

Within animal species, GEI (MJ $kg^{-0.75}$) of lucerne hay and RG/WC were relatively similar (0.74 and 0.61 for alpaca, and 1.36 and 1.32 for sheep) (Figure 6.2a). Despite that, the CH_4 emissions (%GEI) of both animal species on RG/WC were higher than those observed on lucerne hay (9.4 vs 5.1 in alpaca and 7.5 vs 4.7 in sheep) (Figure 6.2a). In both animal species, the intake of digestible NDF (DNDFI, g d^{-1}) on RG/WC were higher ($p<0.001$) than those on lucerne hay (240.1 vs 161.5 in alpaca and 311.8 vs 208.7 in sheep; not tabulated). Since DNDFI is rich in the most methanogenic carbohydrates (cellulose and hemicellulose) (Moe and Tyrrell, 1980), the higher CH_4 emissions (%GEI) observed on RG/WC may be attributed to the increased DNDFI observed both in sheep and alpaca.

CH_4 emission per unit DNDFI (g kg^{-1} DNDFI) did not differ ($p>0.05$) between animal species either on lucerne hay (Table 6.2) or RG/WC (Table 6.5), which is in agreement with the concept that CH_4 emission is mainly a function of cell wall digestion (Moe and Tyrrell, 1980). In fact, when data for lucerne hay and RG/WC were pooled within animal species, the only intake variable correlated to CH_4 emission (g d^{-1}) was DNDFI (g d^{-1}). The coefficients of correlation between these variables were 0.87 ($p=0005$) and 0.63 ($p=0.03$) for alpaca and sheep, respectively.

When fed on lucerne hay or grazed on Lotus, sheep had lower acetate/propionate ratios (A/P) in their forestomach fluid than alpaca (Tables 6.2 and 6.6). However, the positive relationship between A/P and CH_4 emission (%GEI) (Demeyer and van Nevel, 1975) was evident only on Lotus (Figure 6.2b).

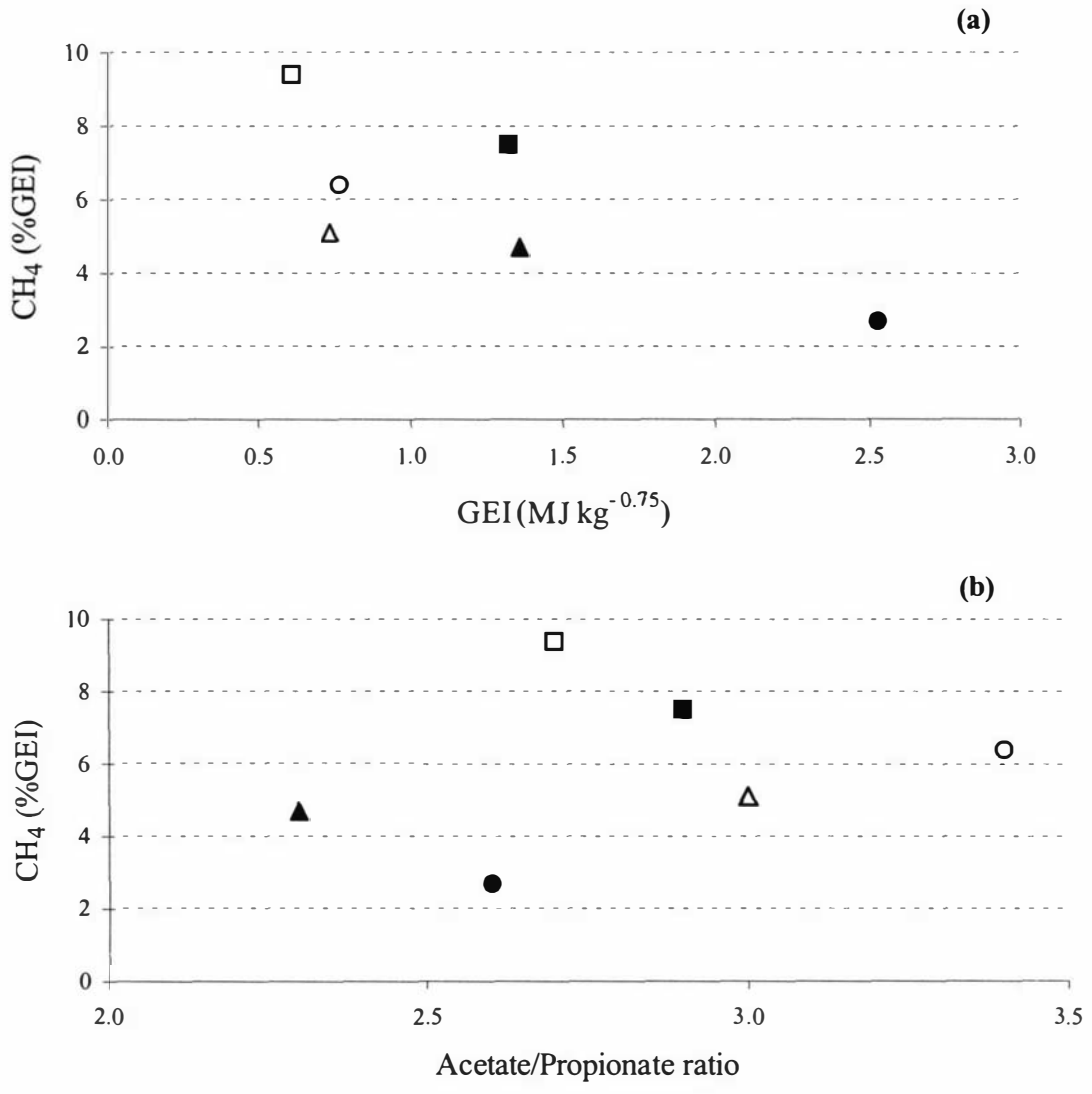

Figure 6.2 Relationships between the mean CH_4 emissions (%GEI) and **(a)** gross energy intake (GEI, MJ $kg^{-0.75}$), and **(b)** acetate/propionate ratio in forestomach fluid, for alpaca (Δ,□, O) and sheep (▲, ■, ●) when fed on lucerne hay (Δ,▲) or grazed on RG/WC (□,■) or Lotus (O, ●) pastures.

In alpaca only, the estimated NDF digestibility (%) of the Lotus diet was much lower than that measured on lucerne hay (38.4 vs 47.8%). It is not clear whether this lower NDF digestibility (%) was a consequence of their selection of dead material (high in non-digestible material) (see Section 6.5.1) or a negative consequence of CT on cellulolysis (Foley et al., 1999). It has been documented (Fraser and Gordon, 1997), that SAC strongly avoid dicotyledonous plants, therefore their forestomach ecosystem might be more sensitive than ruminants to plant secondary metabolites.

The CH_4 emission (%GEI) by sheep on Lotus (2.7%) was significantly lower than that by alpaca (6.4%) and much lower than those by sheep on lucerne hay (4.7 %) or RG/WC (7.5 %) (Figure 6.2a). It confirms earlier observations by Waghorn (1996), who found that sheep fed indoors on *Lotus pedunculatus* (80 g CT kg^{-1} DM) produced less CH_4 than when fed on perennial ryegrass or lucerne pastures (3.9, 6.2 and 5.9 %GEI, respectively). Similar responses in CH_4 emission were also observed when dairy cows were fed silages of perennial ryegrass or *Lotus pedunculatus* (S. Woodward and G.C. Waghorn, personal communication). In addition, *in vitro* studies with other CT-containing plant species such as *Mangifera indica* (Finger et al., 1998) and sainfoin (*Onobrychis viciifolia*) (McMahon et al., 1999), have also found depressing effects on CH_4 production.

The depressed CH_4 emission (%GEI) by sheep on Lotus (Figure 6.2a) cannot entirely be attributed only to the effects of their high intakes (Figure 6.2a) of high quality diets (Table 6.1) (Blaxter and Clapperton, 1965), but probably also the action of some compound (s) in Lotus contributed to the low values. It is recognized (Foley et al., 1999) that if tannins are present in a plant, then non-tannin phenolics are also present. Thus, whether CT or other compounds in Lotus contributed to the lower CH_4 emission observed in sheep remains to be determined together with its mechanism of action.

The protozoal population in sheep grazing Lotus was 3.5-4.0 times higher than those in alpaca or those in sheep on the other forages (Figure 6.3a). This is in agreement with the three times higher protozoal population observed in sheep grazing on sulla than in those grazing on sulla but supplemented with polyethylene glycol (Terrill et al., 1992b). It is not clear the reasons for the increased ciliate numbers on Lotus, but probably it was due to the better quality of the diet, compared to those on the other forages (Jouany, 1989). *In vitro* studies have shown a consistent negative relationship between ciliate numbers and protein solubility (Michalowski, 1989) and it is known that CT reduces proteolysis in the rumen (Barry and McNabb, 2000).

The absence of holotrichs in the forestomach contents of alpaca (Tables 6.2, 6.5 and 6.6) may be due to the poor availability of soluble carbohydrates in their diets (Jouany, 1995).

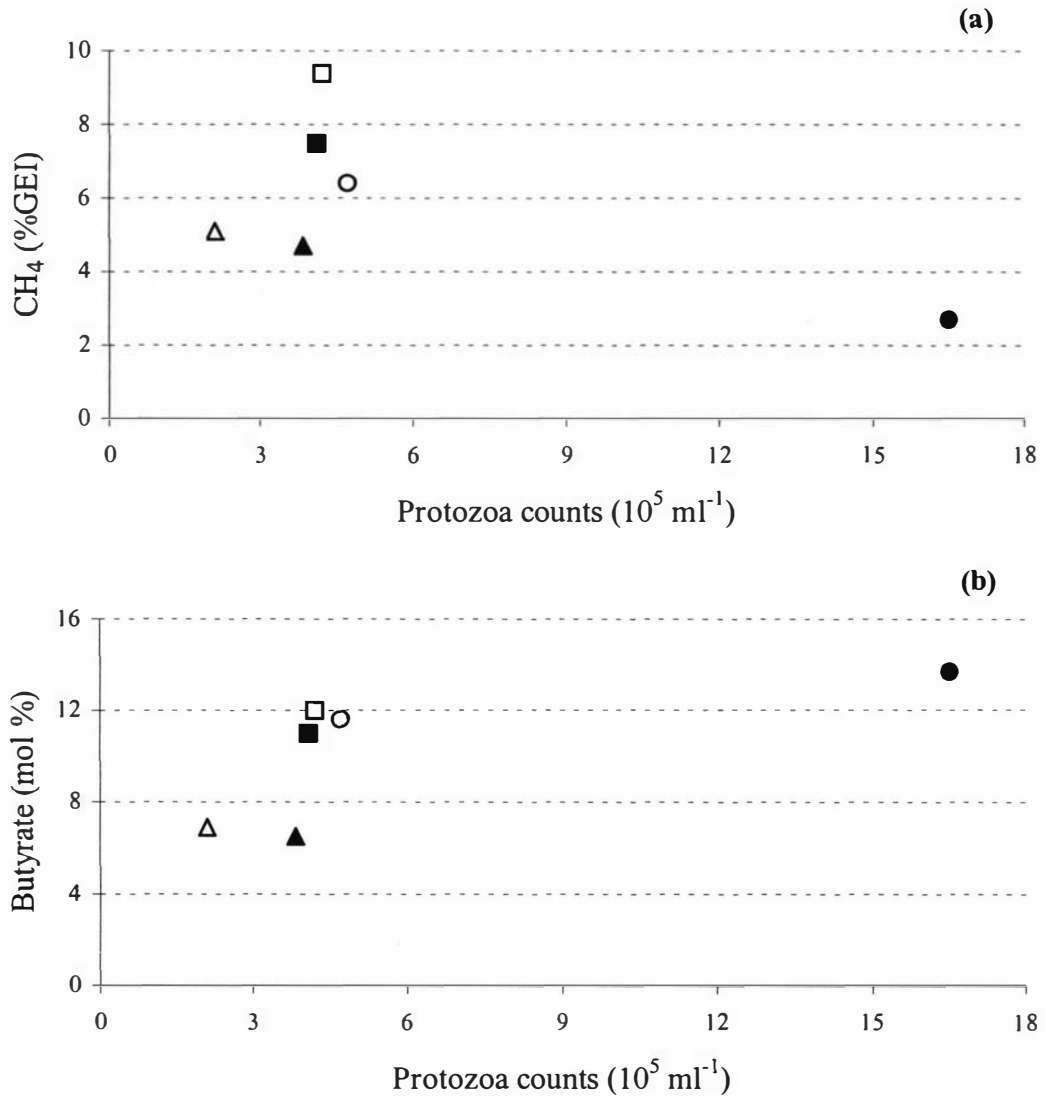

Figure 6.3 Relationships between the mean protozoa counts (10^5 ml^{-1}) and **(a)** CH_4 emissions (%GEI), and **(b)** butyrate molar proportions (mol %) for alpaca (Δ,□, O) and sheep (▲, ■, ●) when they were fed on lucerne hay (Δ,▲) or grazed on RG/WC (□,■) or Lotus (O, ●) pastures.

It is well documented (e.g. Jouany and Lassalas, 2000) that, by virtue of the inter-species H_2 transfer, more CH_4 (%GEI) is lost when protozoa are present in the rumen, and the larger the population of protozoa the greater is the effect. Except for sheep grazed on Lotus, the results of the present study (Figure 6.3a) confirm the latter relationship. Reasons for the increased protozoal population, but depressed CH_4 emission (%GEI) observed in sheep grazed on Lotus (Figure 6.2a) are unknown. Probably, some compound in Lotus prevented for example the occurrence of the physical association between ciliates and methanogens (Ushida et al., 1997), necessary for the transfer of H_2.

In agreement with other authors (Whitelaw et al., 1984; Hegarty et al., 1994), a positive association between ciliate numbers and the molar % of butyrate was observed in the present study (Figure 6.3b). In addition, in both alpaca and sheep, the molar % of butyrate were relatively higher under grazing conditions (RG/WC and Lotus) than on lucerne hay (Figure 6.3b). Holden et al. (1994) also observed increased molar % of butyrate in grazing cows compared to those fed on hay.

6.5.4 Feed digestibility, water intake, energy and N balances, and microbial yield, measured only in Experiment 1 (indoors on lucerne hay)

Compared to sheep, alpaca digested a significantly greater proportion (%) of the feed NDF and ADF (Table 6.3), which confirms the belief that SAC are more efficient in their ability to digest cell walls than sheep (San Martin and Bryant, 1989; Lemosquet et al., 1996; Dulphy et al., 1997). The mechanism for this greater efficiency was attributed to the low fractional outflow rate of feed particles from their forestomach (Lemosquet et al., 1996; Dulphy et al., 1997), which would also explain in part the lower VFI observed in this and other studies (e.g. San Martin and Bryant, 1989; Lemosquet et al., 1996).

Drinking water intake (ml g^{-1} DMI) by alpaca was slightly lower (but not significantly) than that by sheep (Table 6.3). Warmington et al. (1989) reported that llama x guanaco crosses had lower intakes of water than sheep.

There was no difference between the alpaca and sheep in the energy loss in CH_4 (%GEI) (Table 6.2), but alpaca had lower losses (%GEI) of urinary energy than sheep, resulting in slightly greater (but not significant, p=0.12) availability of metabolisable energy (ME, %GEI) (53.0 vs 51.4%) (Table 6.4). Carmean et al. (1992) determined that llamas required 0.353 MJ ME $kg^{-0.75}$ for maintenance, which is similar to the 0.392 MJ ME $kg^{-0.75}$ eaten by the alpaca in the present study, while they maintained their LW.

No differences were found between alpaca and sheep in nitrogen (N) balance (Table 6.4), which disagrees with previous findings that SAC are more efficient in conserving N (Dulphy et al., 1997; Lemosquet et al., 1996; Warmington et al., 1989). The latter is probably correct on low N diets, but not on diets high in N, such as lucerne hay. As a consequence of greater feed intake, the sheep had higher microbial

N flows ($g\ d^{-1}$) than the alpaca, however this difference disappeared when microbial N flow was expressed per unit of digestible OM apparently fermented in the forestomach (DOMR) (Table 6.4).

In conclusion, observations such as the differences between animal species in: (1) chemical composition of diets selected (more fibrous in alpaca, requiring more time to digest), (2) VFI (lower in alpaca, reflecting more time spent in the forestomach), and (3) digestibility of cell walls (higher in alpaca, a consequence of longer retention times in forestomach), are consistent with alpaca having a lower particulate FOR than the sheep. This, and the fact that alpaca and sheep differed in CH_4 emissions (%GEI), suggest that differences between these species in particulate FOR from their forestomach might have been the underlying physiological mechanism responsible for the differences in CH_4 emission (%GEI) (Chapter 4, this thesis; Demeyer and Van Nevel, 1975; Okine et al., 1989). However, since VFI and diet quality also differed between animal species, it was impossible to determine the effect of animal species on CH_4 emission independently of the effects of differences in diet quality and intake. The low CH_4 emission (%GEI) observed on sheep grazing Lotus deserves further study in order to determine the reasons and mechanisms for this. Finally, the results of this study support the belief that South American camelids have adapted to the highly fluctuating supply of poor quality forages in the Andes by reducing their intake and decreasing the particulate FOR from their forestomach. Thus, compared to sheep, their higher ability to digest structural carbohydrates is associated with relatively higher CH_4 emission (%GEI).

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Chapter 7

General Discussion

7.1 INTRODUCTION

Rumen methanogenesis represents a loss of between 2 to 15% of the energy intake by the animal (Johnson et al., 1991; Pelchen and Peters, 1998), and methane (CH_4) has been implicated in the global warming phenomenon (IPCC, 1996). Therefore, any reduction of ruminant CH_4 emission would have both environmental and nutritional benefits. However, with the exception of increased feeding intensity (i.e. reduction in CH_4 per unit of product), most of the technologies available to reduce ruminant CH_4 emission (see Chapter 1, Section 1.4) are viewed with pessimism (McAllister et al., 1996; van Nevel and Demeyer, 1996; Meeks and Bates, 1999). Therefore, the development of cost-effective strategies to mitigate CH_4 production in ruminants, without causing a negative impact on animal production, especially for those based on forages, continues to be a major challenge.

Differences between animals is a recognized source of variability in experiments, and statistical designs have been developed to minimise this source of variability in order to increase the chances of detecting significant treatments effects (Grovum, 1984). Based on the large between-sheep (within a breed) variation in CH_4 emission observed both under controlled (e.g. Blaxter and Clapperton, 1965; Demeyer and Van Nevel, 1975) and grazing (Lassey et al., 1997) conditions, this thesis studied the nature and causes of between-sheep variation in CH_4 emission, with the objective of using it as a tool to reduce CH_4 emission. In addition, alpaca were compared to sheep in one experiment because these species were known to differ in their forestomach outflow rates (San Martin, 1987). Most of the work in this study relied on the sulphur hexafluoride (SF_6) tracer technique for CH_4 measurement (Johnson et al., 1994a), and this was compared with the calorimetric chamber method.

The results of each of the experiments in this study have been presented in their respective chapters, each of which included specific discussions of the topics investigated. This General Discussion integrates the main outcomes of the study and discusses them from a broader perspective.

7.2 THE SULPHUR HEXAFLUORIDE (SF_6) TRACER TECHNIQUE FOR CH_4 MEASUREMENT IN SHEEP

The development of the SF_6 tracer technique (Johnson et al., 1994a) was claimed to fulfill the requirements of a technique for CH_4 measurement from individual animals under production conditions (Johnson et al., 1994a; Ulyatt et al., 1999). The technique has been validated against the calorimetry chamber technique using penned cattle (Johnson et al., 1994b), and since then it has been widely used for CH_4 measurement in grazing sheep, dairy cattle, beef cattle and water buffalo (see Section 1.5.2.2.1, Chapter 1).

Johnson et al. (1994b) examined the validity of the SF_6 tracer technique for CH_4 measurement by comparing 55 measurements made with the tracer (with cattle in pens) to those obtained from 25 measurements using open circuit respiration chambers. They found that whereas the tracer mean estimates were 93% of those in the chambers, these values were not significantly different. In the present thesis (Chapter 2), a similar evaluation was carried out with penned sheep. From the three trials involved, only one trial (T_{23}) produced conclusive results. T_{23} was carried out with 10 sheep, and involved 40 and 24 CH_4 measurements with the tracer and chamber techniques, respectively. The CH_4 emission determined by the tracer method was 96% (when expressed per animal and day, g d^{-1}) and 89% (when expressed per unit intake, g kg^{-1} DMI) of the corresponding chamber values. The CH_4 emission values determined by both techniques were highly correlated (r=0.93, p=0.001 for g d^{-1} and r=0.79, p=0.02 for g kg^{-1} DMI) (Chapter 2, this thesis). The results of this thesis (Chapter 2) and those of Johnson et al. (1994b) suggest that under controlled conditions, the SF_6 technique accounts for about 90-93% of the total digestive tract CH_4 production.

From the CH_4 production and dynamics studies of Murray et al. (1976), on which the tracer technique is based, it is expected that this technique should account for ~98% of the total CH_4 production (Section 1.5.2.2.1, Chapter 1). According to Murray et al. (1976), 87 and 13% of the total CH_4 was produced in the rumen and hindgut, respectively, and that whereas 95% of the rumen CH_4 was excreted by eructation, only 11% of the hindgut CH_4 was excreted through the flatus, the rest being excreted through the lungs (breath). Therefore, in theory, less than 2% of the total CH_4

production (i.e. the flatus CH_4) would not be accounted by the tracer technique. However, the importance of the hindgut as a source of CH_4 appears be greater under conditions of high rumen outflow rates. For example, Kennedy and Milligan (1978) showed that as a consequence of cold conditions and high feeding level, the hindgut accounted for up to 32% of the total CH_4 production. A similar situation may occur in ruminants fed on lush pasture, where very soft faeces, indicative of increased hindgut fermentation (Ørskov, 1994), are often observed. Consequently, it might be possible that at high rumen outflow rates, the flatus CH_4 excretion is more important than that reported by Murray et al. (1976), thus increasing the proportion of the total tract CH_4 production which is not accounted for by the tracer technique.

Table 7.1 summarizes the CH_4 emission values (% GEI) for sheep found in the present thesis and compares them with some from the literature. In general, the CH_4 emission values determined in this thesis (using both tracer or calorimetry techniques) are in agreement with others in New Zealand (Lassey et al., 1997; Judd et al., 1999), but they are at the low end of the range (2-15%) reported by Pelchen and Peters (1998). The relatively low values of CH_4 emission (%GEI) observed in the present study (and in New Zealand) may be due to various reasons, including breed (Blaxter and Wainman, 1964; Chandramoni et al., 2000). In New Zealand, all the CH_4 measurements (> 1000 observations) (this study; Lassey and Ulyatt, 1999; Judd et al., 1999) have been carried out with Romney sheep, and the mean CH_4 emission found was 4 %GEI (Lassey and Ulyatt, 1999).

Other factors responsible for the low CH_4 emission (%GEI) observed in this study may be the high crude protein (CP) contents of the forages used (lucerne hay or fresh pasture), because CP intake has been shown to have a negative impact on CH_4 production (Holter and Young, 1992; Pelchen and Peters, 1998). In addition, measurements at grazing used high herbage allowances and Table 7.1 shows that CH_4 emission (%GEI) measured at grazing tends to decrease as DMD (%) increases. High feeding levels with good quality feeds reduce the rate of CH_4 production (%GEI) (Blaxter and Clapperton, 1965), through a less methanogenic fermentation (Demeyer and van Nevel, 1975) and lower digestion of cell walls due to higher rumen outflow rates (Demeyer and van Nevel, 1975).

Table 7.1 CH_4 emissions (% gross energy intake, %GEI) measured by the SF_6 tracer or respiration chamber methods and between-animal variation (% of total variation) for grazing or penned sheep. (DMD; dry matter digestibility, %). (Blank cells, not reported).

SHEEP	n[1]	Feed and feeding	DMD (%)	CH_4 (%GEI)	Variation (% total)	Reference
By the SF_6 tracer technique						
a) Grazing						
Hoggets	7	lucerne (restricted)	70	6.6	50	Leuning et al., 1999
Cryptorchids	52	ryegrass/white clover	75	4.6	88	Lassey et al., 1997
Wethers	10	ryegrass/white clover	81	4.2		Lassey & Ulyatt, 1999
Wethers	11	ryegrass/white clover	81	3.6	88	Judd et al., 1999
Cryptorchids	10	ryegrass/white clover	80	5.3	35	this thesis, Chapter 3
Cryptorchids	20	ryegrass/white clover	83	4.2	84	C.Pinares, unpublished
Cryptorchids	7	ryegrass/white clover	78	5.7	78	this thesis, Chapter 3
Cryptorchids	7	ryegrass/white clover	80	4.0	45	this thesis, Chapter 3
Cryptorchids	7	ryegrass/white clover	83	3.8	88	this thesis, Chapter 3
Cryptorchids	6	ryegrass/white clover	72	7.5	67	this thesis, Chapter 6
b) Penned						
Mature	2				95	Ulyatt et al., 1999
Mature	6				71	Ulyatt et al., 1999
Cryptorchids	10	lucerne hay, restricted	59	5.9	62	this thesis, Chapter 3
Ewe lambs	20	lucerne hay, restricted	64	4.5	84	this thesis, Chapter 5
Cryptorchids	6	lucerne hay, *ad libitum*	64	4.7	45	this thesis, Chapter 6
By calorimetry chamber technique						
Cryptorchids	8	lucerne pellets, restricted	60	4.9	92	this thesis, Chapter 3
Cryptorchids	8	pasture, restricted	73	6.0	88	this thesis, Chapter 3
Cryptorchids	10	lucerne hay, restricted	56	4.6	78	this thesis, Chapter 3
Cryptorchids	8	lucerne hay, restricted	56	5.9	68	this thesis, Chapter 2
Wethers	4	dried lucerne, restricted	70	6.4		Moss et al., 2000
Rams	4	oat hay, restricted	58	3.9		Chandramoni et al., 2000
Wethers	3	grass hay, restricted	61	6.7		Blaxter &Wainman, 1964
Wethers	3	grass hay, high level	59	6.5		Blaxter &Wainman, 1964
Wethers	6	grass pellets, restricted	57	6.1		Kennedy&Milligan, 1978
All sheep[2]	1137	various		7.2±1.7		Pelchen & Peters, 1998

[1] n=number of animals.

[2] Mean±s.d. for 1137 observations from all age sheep fed with different rations. Data from literature review.

Ulyatt et al. (1999) have quoted the difficulty that various researchers experienced in matching the SF_6 tracer and calorimetry chamber CH_4 values. Similar difficulty was also found when the polytunnel system and the chamber techniques were compared (Murray et al., 1999). However, it seems more pertinent to evaluate the SF_6 tracer technique, which was developed for CH_4 measurements under production situations, against other field techniques, rather than by comparison with the standardised calorimetry chamber technique. In this respect, two separate studies with grazing sheep (Judd et al., 1999 and Leuning et al., 1999) compared the SF_6 tracer (individuals-based) and micrometeorological (group-based) techniques. Both, these studies found close relationships between the techniques for the mean CH_4 emission values (%GEI). Similar agreement between the mean CH_4 emission values was found when the SF_6 gas was used as a tracer for CH_4 measurement on individual cows or as a tracer in a mass-balance technique (applied indoors or on pasture) (Johnson et al., 1998).

It is now well established (Chapter 2, this thesis; Lassey and Ulyatt, 1999; Ulyatt et al., 1999) that permeation rates (PR) of SF_6 from permeation tubes decline with time of deployment in the rumen. Monitoring of weight losses of permeation tubes recovered from the rumen showed that PR of SF_6 fits a quadratic relationship rather than a linear relationship as originally assumed (K.R. Lassey, personal communication). Whether this pattern of permeation is a result of the effects of the rumen environment or not is not clear. Nevertheless, to obtain good results the tubes must not be deployed for too long in the rumen.

In conclusion, this study confirms the findings of previous studies (Johnson et al., 1994b; Johnson et al., 1998; Lassey et al., 1997; Lassey and Ulyatt, 1999; Ulyatt et al.,1999; Judd et al., 1999; Leuning et al., 1999) that the SF_6 tracer technique provides an effective method for measuring CH_4 emission by individual animals both under indoor and grazing conditions. Nevertheless, the effects of feeding level upon the efficiency of CH_4 measurement by this technique need to be determined in order to make corrections if necessary.

7.3 BETWEEN-SHEEP VARIATION IN CH_4 EMISSION AND PERSISTENCE OF RANKINGS FOR RATE OF CH_4 EMISSION

Lassey et al. (1997) reported that 88% of the variation in CH_4 emission from grazing sheep was between-sheep. The results found in the present study and those from other studies (Table 7.1) confirm the importance of the between-sheep variation in CH_4 emission (% GEI), which generally exceeded 70% (Table 7.1).

If the between-sheep variation in CH_4 emission is to be used as a tool for breeding for low CH_4 emission, a necessary condition is that the ranking of sheep for low or high CH_4 emission must be persistent in the long term, and the mechanism responsible for such variation must be heritable. Chapter 3 of this thesis studied the persistence of this variation with three different groups of sheep. Each group comprised sheep initially selected for low or high CH_4 emissions rates (%GEI). The results of the persistence studies showed that low and high CH_4 emitters did not persist in their rates of CH_4 emission when they were brought from pasture to restricted indoor feeding conditions. In contrast, sheep did persist in their CH_4 emission rankings when maintained at grazing under generous pasture allowance, although the persistence weakened with time.

Lassey et al. (1997) reported that grazing sheep identified as low or high CH_4 emitters, from within a flock of 50, maintained their rankings of CH_4 emission during a period of 3 weeks. Other studies with sheep (Martin et al., 2000; Mbanzamihigo et al., 2000) and cattle (Johnson et al., 1998) have also observed consistent between-animal variation in CH_4 emission under *ad libitum* feeding conditions. Thus, the fact that between-sheep variation in CH_4 emission (%GEI) was persistent only under grazing conditions in this study (Chapter 3) suggests that feeding conditions which maximize feed intake (e.g. generous allowance of good quality pasture under grazing) favour the expression of the mechanism(s) responsible for the between-sheep differences in CH_4 emission. However, it is not clear why the persistence in CH_4 emission weakened with time.

Chapter 4 of this thesis identified rumen particulate fractional outflow rate (particulate FOR; i.e. the reciprocal of retention time) as a major contributor to the between-sheep variation in CH_4 emission (%GEI), and also that particulate FOR was negatively related to rumen fill. Seasonal changes in rumen outflow rate and fill have been

observed in grazing sheep (Corbett and Pickering, 1983) and cattle (Tulloh, 1966), and it is likely that these changes are not only related to seasonal changes in pasture quality but also the botanical composition of the pasture on offer (Cruickshank, 1986; Barton and Ulyatt, 1963; Johns et al., 1963; Ulyatt, 1971). The CH_4 emission persistence study under grazing conditions (Chapter 3) was carried out from October (Spring) to February (Summer) and it was impossible to maintain a uniform pasture quality between trials (Sheep Group 3, Table 3.2, Chapter 3). Thus, the weakened persistence of the between-sheep CH_4 emission under grazing may have been due to the sensitivity of rumen fill and outflow rates to the changes in herbage quality and botanical composition over the period of the study or perhaps there was between-sheep differences in diet selection and this probably was affected by the seasonal changes in pasture quality and composition.

7.4 THE EFFECT OF RUMEN PARTICULATE FRACTIONAL OUTFLOW RATE (Particulate FOR) ON BETWEEN-SHEEP VARIATION IN CH_4 EMISSION AND ROLE IN CH_4 MITIGATION

The effect of rumen fractional outflow rate upon the rate of CH_4 production is well established (e.g. Okine et al., 1989; McAllister et al., 1996; van Nevel and Demeyer, 1996). For example, when rate of passage of feed particles was increased by 63%, CH_4 production was reduced by 29% (Okine et al., 1989). Structural carbohydrates (cellulose, hemicellulose) are fermented at slower rates than non-structural carbohydrates (starch, sugars) and yield more methane per unit of substrate fermented (Czerkawski, 1969; Holter and Young, 1992; Moe and Tyrrell, 1979). Thus, lower rumen particulate FOR increase the rate of CH_4 emission probably by allowing an increased digestion of structural carbohydrates.

Chapter 4 of this thesis showed that, under restricted feeding conditions (1.2 times maintenance) on lucerne hay, rumen particulate FOR accounted for 57% of the between-sheep variation in CH_4 emission (%GEI). Particulate FOR was negatively correlated with rumen fill (g) ($r=-0.69$, $p=0.03$) and digestibility of cellulose ($r=-0.65$, $p=0.04$). The latter relationships suggest that sheep with lower particulate FOR (i.e. longer rumen retention times) had larger rumen fills and higher cellulose digestibilities and CH_4 emissions (%GEI). Whether the negative relationship between particulate FOR and CH_4 emission (%GEI) is maintained under *ad libitum* feeding conditions is unknown. However, studies with sheep (Lambourne, 1956; Faichney,

1993) and cattle (Campling et al., 1961; Ørskov et al., 1988) reported that rankings of animals on the basis of rumen outflow rates were maintained across diets and feeding levels. In addition, Moe and Tyrrell (1979) reported that the impact of cellulose degradation on CH_4 emission rate was greater at high intakes than at intakes below 1.5 times maintenance.

Although the decreased CH_4 production associated with increased particulate FOR from the rumen might be regarded primarily as a result of decreased cell wall digestion, a partial shift to a propionate-type fermentation with increasing paticulate FOR, a relationship commonly observed when different ratios of forage to concentrate are fed (e.g. Ørskov et al., 1968), was also suggested as a contributor (Demeyer and van Nevel, 1975; Kennedy and Milligan, 1978; Moe and Tyrrell, 1979; Okine et al., 1989; McAllister et al., 1996). On the other hand, increased dilution rates of the rumen (the fractional rate of clearance of fluid from the rumen) have been shown be associated with lowered propionate (mol %) concentrations (Harrison et al., 1975; Hodgson and Thomas, 1975; Baker, 1997). In the present study (Chapter 4), however, no relationships between particulate FOR or liquid FOR and propionate (mol %) or acetate/propionate ratio (A/P) were observed.

The results of the comparative study (Chapter 6, this thesis) of CH_4 emission rates between sheep and alpaca suggested that forestomach particulate FOR was the underlying mechanism by which these animal species differed in CH_4 emission (%GEI). Interestingly, when data for lucerne hay and ryegrass/white clover pasture were pooled within animal species, digestible neutral detergent fibre intake (DNDFI, g d^{-1}) was the only intake variable (of those measured) significantly related to CH_4 emission (g d^{-1}). The influence of DNDFI (g d^{-1}) on CH_4 emission (g d^{-1}) was greater and more important in alpaca than in sheep (Figure 7.1), suggesting that the impact of cell wall digestion on the rates of CH_4 emission (Moe and Tyrrell, 1979) was greater in alpaca than in sheep. Probably, other components (e.g. soluble carbohydrates or lipids) in sheep diets also were important contributors to CH_4 emission.

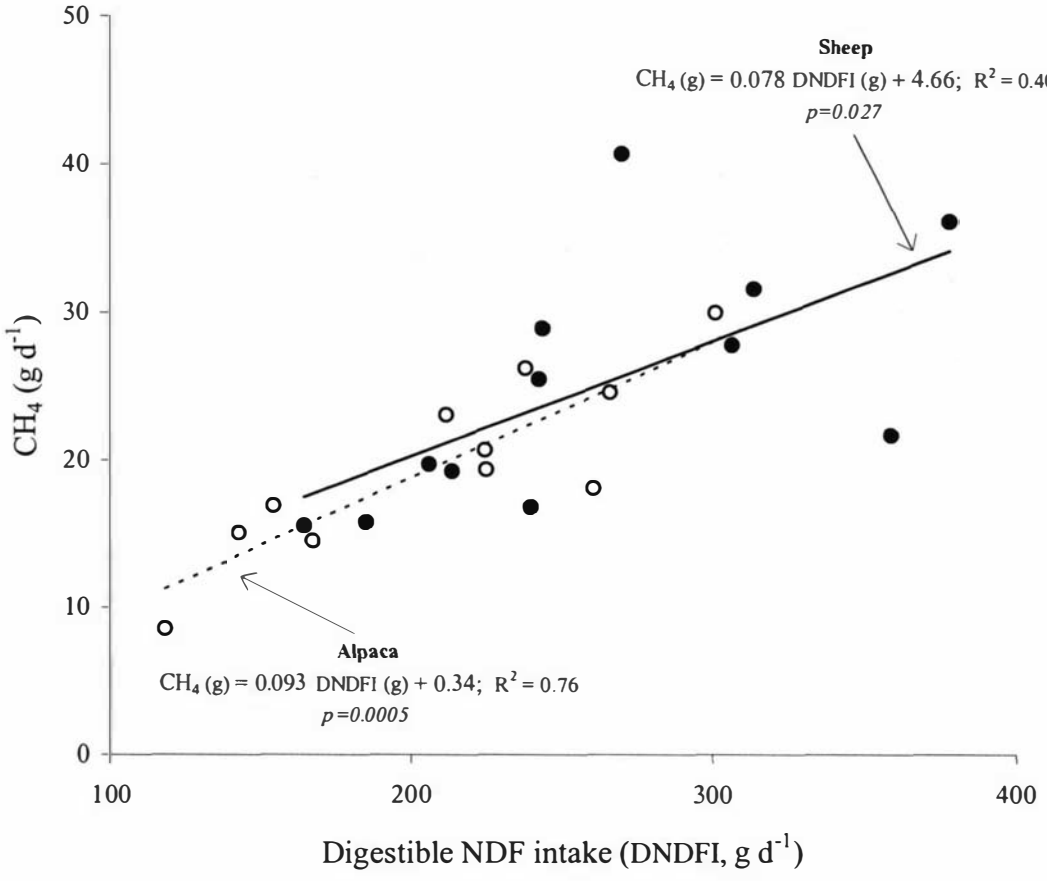

Figure 7.1 Relationship between digestible neutral detergent fibre intake (DNDFI, g d^{-1}) and CH_4 emission (g d^{-1}) for alpaca (O) and sheep (●), when data for lucerne hay and ryegrass/white clover pasture were pooled within animal species.

Between-animal variation in rumen fractional outflow rates have been documented both in sheep and cattle (see Section 4.2, Chapter 4) and Faichney (1993) stated that this variation can account as much as 80-90% of the total variation. There is a growing evidence that rumen fill (volume) is a determinant of rumen fractional outflow rate (Ørskov et al., 1988; Faichney, 1993; Chapter 4, this thesis), i.e. animals with smaller rumen volumes have higher rumen fractional outflow rates, and *viceversa*. Passage of feed particles from the rumen is a function of their size and functional specific gravity (FSG) (Murphy and Kennedy, 1993) and particle comminution, especially during rumination is of major importance for both particle size reduction and FSG (Poncet, 1991). It is not known whether between-animal differences in rumen outflow rate are due to differences in feed comminution. If so, greater efficiency in feed comminution would be expected in animals with high rumen outflow rates (Grovum, 1984). Alternatively, between-animal differences in: (1) water and saliva inflow into the rumen (particles must leave the rumen in liquid

suspension), (2) microbial activity (which facilitates comminution), (3) reticulo-rumen motility (propulsive activity) and (4) degree and frequency of opening of the reticuloomasal orifice (i.e. differences in particle size threshold to passage) may be suggested as the mechanisms responsible for the between-animal differences in particulate FOR.

There is no report in the literature on the persistence or heritability of rumen outflow rate or rumen volume. It was intended in the present study to select sheep for contrasting rumen outflow rates or rumen volumes (correlated variables, Chapter 4, this thesis) and monitor the persistence of these characteristics and their relationship to CH_4 emission, but this was not successful because the field index (change in liveweight during short-term grazing) which was found to be correlated with rumen volume was not repeatable (Chapter 5). Future developments in this field will be useful in testing the effectiveness of using these animal factors as tools for CH_4 mitigation. Assuming that rumen outflow rate and rumen volume are heritable traits, breeding for two contrasting lines of animals, high or low rumen outflow rates, would produce the following characteristics:

(1) High rumen outflow rate (small rumen volume) line:

- relatively high *ad libitum* intakes of forages, especially high quality pasture;
- lower feed digestibilities (because of shorter retention times), but the higher intakes would compensate for that disadvantage;
- higher absolute CH_4 emissions (g d^{-1}), but lower per unit of intake (%GEI);
- higher microbial cell growth and flow to the intestines (Figure 1.2, Chapter 1; Hespell and Bryant, 1979), especially at *ad libitum* feeding;
- higher productivity when fed *ad libitum* on good quality forages. For example, lambs would have higher liveweight gains and they would reach slaughter weight at a shorter time.

(2) Low rumen outflow rate (large rumen volume) line:

- relatively high *ad libitum* intakes of poor quality forages;
- higher feed digestibilities due to prolonged retention times;
- lower CH_4 emissions (g d^{-1}), but higher emissions per unit of intake (%GEI);
- lower microbial cell growth and flow to the intestines. However, at restricted feeding levels, they would have an efficient microbial cell synthesis due to the microbial growth rate hysteresis (Baker and Dijkstra, 1999).
- lower productivity, but they would have outstanding efficiency of feed conversion on poor quality forages.

Up to now, the most cost-effective natural option for ruminant CH_4 mitigation appears to be the dilution of the CH_4 emission associated with the maintenance requirements, by increasing animal productivity, i.e. the increase in animal product output per unit of CH_4 output (Meeks and Bates, 1999; Johnson et al., 2000). In broad terms, this option implies that animal and feed resources must be fully matched (Ørskov, 1989). Thus, high rumen outflow rate animals would be highly efficient under systems of high quality forages. On the other hand, low rumen outflow rate animals would have a major role on poor quality roughages, and probably the latter is already working in most of the ruminant production systems in the developing countries.

7.5 THE ROLE OF *Lotus corniculatus* IN CH_4 MITIGATION

Chapter 6 of this thesis showed that CH_4 emission (%GEI) by sheep grazing *Lotus corniculatus* pasture (Lotus) was very low compared to their corresponding emissions on lucerne hay or ryegrass/white clover pasture. Sheep diets on Lotus were of higher quality with intakes being twice those on the other two forages, and although these factors are known to reduce CH_4 emission (%GEI) (Blaxter and Clapperton, 1965) it is possible that some compounds (e.g. condensed tannins) present in Lotus contributed to the depressed CH_4 emission (%GEI). The depressed CH_4 emission (%GEI) observed on Lotus confirms other observations both *in vivo* with *Lotus pedunculatus* (Waghorn, 1996; S. Woodward and G.C. Waghorn, personal communication) and *in vitro* with other condensed tannin (CT)- containing plant species such as *Mangifera indica* (Finger et al., 1998) and sainfoin (*Onobrychis viciifolia*) (McMahon et al., 1999).

Assuming that CT in Lotus contributed to the depressed CH_4 emission (%GEI) observed in sheep in the present study, the mechanisms of action may include: (1) hydrogen deprived methanogens; plant secondary metabolites, which require biohydrogenation in the rumen, may act as alternative electron sinks (Baker, 1997), (2) anti-Archaeal effect; similar to the bacteriostatic effects of CT on cellulolytic bacteria and formation of tannin-enzyme complexes (Bae et al., 1993). For example, reduction in concentration of methanogens was observed when cattle were relocated from pasture to tagasaste (*Chameacytisus proliferus*), a CT-containing browse species (Thyer et al., 2000), (3) enhanced rumen fractional outflow rates (i.e. depressed digestibilities) as suggested by (McMahon et al., 1999). However, studies involving

CT-acting and CT-inactivated sheep (by polyethylene glycol supplementation) (Terrill et al., 1992; Wang et al., 1994) do not support the latter hypothesis, (4) a poor transfer of H_2 from H_2-producer species (e.g. from ciliates) to methanogens.

In the present study (Chapter 6), the protozoal population on sheep grazing Lotus was four-fold larger compared to the numbers in sheep fed on lucerne hay or ryegrass/white clover pasture, this being in agreement with a three-fold higher protozoal population observed on sheep grazing sulla (*Hedysarum coronarium*, 36 g CT kg^{-1} DM) (Terrill et al., 1992). The increased ciliate population, but depressed CH_4 emission (%GEI) observed in the present study (Chapter 6) contrasts with the belief (e.g. Jouany and Lassalas, 2000) that more CH_4 (%GEI) is produced when protozoa are present in the rumen and the larger the population of protozoa the greater is the effect. Hydrogen is a major source of energy for methanogens and rumen ciliate protozoa are the most potent hydrogen producers. In order to increase the efficiency of hydrogen uptake, methanogens are closely attached to ciliates (Ushida et al., 1997) and this mechanism has been suggested to be responsible for between 25 to 37% of the rumen CH_4 production (Finlay et al., 1994; Newbold et al., 1995). Possibly, some compound in Lotus had an adverse effect on the physical relationship between methanogens and ciliates.

Numerous studies carried out during the last two decades (Barry and McNabb, 2000; Waghorn et al., 1997) have shown the potential benefits of CT-containing temperate legumes for a sustainable pastoral livestock system in these regions. Among the benefits of CT-forages are: prevention of bloat (e.g. McMahon et al., 1999), control of endoparasitism burden (e.g. Niezen et al., 1995), and improved liveweight gain, wool production, milk yield and fertility (Min, 1999; Wang, 1995), resulting from increased efficiency of nitrogen utilization. The results of the present study (Chapter 6) showed another benefit of CT-containing forages, decreased CH_4 emission. These benefits, will likely encourage the introduction of these forage species in farming situations.

In conclusion, this thesis showed that rumen particulate fractional outflow rate (particulate FOR) was a major factor responsible for the between-sheep variation in CH_4 emission. The persistence of this latter relationship and its heritability are not known. However, since sheep ranked initially low or high for CH_4 emission rates (%GEI) persisted in their rankings only under generous grazing conditions, and this

persistence weakened with time, it is suggested that changes in diet selection or particulate FOR may have occurred as a result of seasonal changes in pasture quality during the study. The clear differences in CH_4 emission (%GEI) between alpaca and sheep, two animal species with known differences in their forestomach particulate FOR, found in this study also support the role of particulate FOR on CH_4 emission (%GEI). Future development of techniques able to measure between-sheep differences in particulate FOR or rumen volume under grazing conditions will be of great value in the assessment of the benefits of these animal factors for CH_4 mitigation. The CH_4 depressing effect of *Lotus corniculatus* shown in this study adds another benefit of CT-containing temperate forages in sustainable pastoral production systems.

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