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Dietary manipulation to reduce methane production from ruminants and the impact on milk fatty acid profile

Joyce Mufungwe
(BVM, MSc)

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Declaration

The work in this thesis is original. None of this work has been presented in any previous application for any degree or qualification.

Joyce Mufungwe

Abstract

Methane is one of the greenhouse gases that causes global warming and has been listed for reduction within the UK. Under the UK climate change act (2008), the UK is committed to reducing GHG emissions by 80% against the 1990 baseline by the year 2050 with a minimum of 34% reductions to be achieved by the year 2020. This thesis presents results of assessments of various dietary manipulations including use of starch sources, use of oil sources and grazing with or without supplementation on methane production and productivity and the impact on milk fatty acid profile in dairy cows.

The *in vitro* study was initiated to assess the effects of starch and oil source on *in vitro* fermentation characteristics and methane production. Results showed that the three starch sources, wheat, barley and maize differed in their cumulative and rate of gas production. Wheat produced the highest and maize the lowest cumulative and rates of gas production. Methane production did not differ among the starch sources. Among the oil sources, carvacrol, linseed oil and fish oil when added at the same level of supplementation differed in fermentation characteristics and on methane production. However, when methane production was expressed per time of incubation, variations in methane production were observed when compared to the control. Carvacrol reduced methane production by 50-80% at all time periods while linseed oil only reduced methane production by 20% at 36-48 hrs of *in vitro* incubation and fish oil either had no effect or increased methane production. Results of the *in vitro* study were used to establish the treatments for the first *in vivo* study.

The effects of starch and oil source on methane production, productivity and milk fatty acid profile in dairy cows were examined in a 4X4 Latin square design. Wheat and maize based concentrates were used as starch sources and Megalac and sunflower oil were used as oil sources. Sunflower oil was not effective at reducing methane production in cows, but did alter the milk FA profile by increasing the PUFA content and reduced the palmitic acid content. Maize based concentrates were effective in reducing methane output when results were expressed as g/d and g/kg milk yield and improved the energy balance of the cows as evidenced by the positive condition score change, and also reduced plasma 3-OHB concentration. The starch and oil source acted independently, with no interaction observed on methane production and milk FA profile.

The third experiment was a grazing trial which examined the effect of time of pasture access with or without TMR supplementation on methane production, productivity and milk fatty acid profile of high yielding dairy cows. Grazing, regardless of the time of access, reduced methane production when expressed as g/kg DM intake and g/kg milk yield. Productivity of cows that grazed during the day with access to TMR was similar to continuously housed animals, while in the rest of the grazing groups, milk production was lower. Grazing also increased the long chain FAs in milk fat and reduced concentration of palmitic acid.

In conclusion, a variety of dietary manipulations can have a significant impact in reducing methane emissions. Conclusions drawn from the project are that, maize as opposed to wheat based concentrates reduce methane production and improves condition score of the cows. Purified sunflower oil supplementation reduces intake, does not reduce methane production but improves the fatty acid profile of the milk. When grazing high yielding cows, grazing during the day with TMR supplementation is recommended as this does not compromise milk production. Methane production per unit of DM intake and per unit of milk yield is lowered regardless of the time of grazing.

Statement of publications and conferences attended

During the period of study, the author presented the following papers at conferences;

J Mufungwe, J A Huntington, R G Wilkinson, K J Hart, C G Bartram and L A Sinclair (2013). The effect of oil and starch source of performance, methane production and milk fatty acid profile of Holstein dairy cows. *Proceedings of the British Society of Animal Science, Annual conference 2013.* Vol 4 part 1 pp 027. ISSN 2040-4700

Mufungwe J, Rutter S M, Birch S, Huntington J A, Wilkinson R G, Sinclair L A (2014). Influence of time of access to pasture and provision of a total mixed ration on the intake, milk fatty acid profile and methane production of high yielding dairy cows. *Proceedings of the British Society of Animal Science, Annual conference 2014.* April 2014 Vol 5 part 1 pp 96. ISSN 2040-4700

The following poster paper was presented at the International Grassland Congress in Sydney, Australia:

Joyce Mufungwe, Mark S. Rutter, Robert G Wilkinson, Jim A. Huntington. and Liam A. Sinclair (2013). The effect of time of access to pasture and the provision of TMR on performance and methane production of high yielding dairy cows. *2013 Proceedings of the 22nd International Grassland Congress*, 1384-1385.

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Abbreviations and Acronyms

3OHB	betahydroxybutyrate
Anova	analysis of variance
CH_4	methane gas
CO_2	carbon dioxide
CLA	conjugated linoleic acid
CP	crude protein
CTAB	cetyltrimethylammoniumbromide
CTR	control
DM	dry matter
DPA	decosapentanoic acid
Dig	digestibility
°C	degree celcius
EPA	eicosapentanoic acid
GE	gross energy
FAs	fatty acids
FAME	fatty acid methyl esters
FAO	Food and Agriculture Organisation
F:C ratio	forage: concentrate ratio
GC	gas chromatography
GHG	green house gas
Kt	kilotonne
LCFA	long chain fatty acid
MAD fiber	modified acid detergent fiber
MCFAs	medium chain fatty acids
ME	metabolisable energy
OM	organic matter
PUFA	polyunsaturated fatty acid

Abreviations and Acronyms continued

NAEI	National Atmospheric Emissions Inventory
N	nitrogen
NDF	neutral detergent fiber
SF_6	sulphur hexaflouride
SCFA	short chain fatty acid
TMR	total mixed ration
UK	United Kingdom
UNFCCC	United Nations Framework Convetion on Climate Change
VFAs	volatile fatty acids
WSC	water soluble carbohydrate

CHAPTER 1. Introduction

1.0 Introduction

Methane is produced during normal rumen metabolism as a by-product of feed fermentation (Moss *et al*., 2000). However its production is undesirable as it represents a loss of energy to the animal (Popova *et al*., 2011) and is a potent greenhouse gas (Zhang *et al*., 2008). Methane was therefore listed as a target for emission reduction because when compared to the other major greenhouse gasses, carbon dioxide and nitrous oxide which have 100 and 120 years atmospheric half-life respectively, methane has a very short atmospheric half-life of only 12-15 years (Moss *et al*., 2000). Additionally methane has a high global warming potential (GWP) some 21 times more effective in trapping heat than carbon dioxide (Intergovernmental Panel on Climate Change, 2007) over a 100 yr. period. According to the UK greenhouse gas (GHG) inventory, 2010 report, the United Kingdom agricultural sector was responsible for 7.1% of the total national greenhouse gas (GHG) emissions in 2010 of which 33% of the emissions were from livestock production. Table 1 shows methane emissions by different livestock categories in England in 2010.

Table 1. Emissions of methane from agricultural livestock sources in England in 2010

Livestock Category	$KtCO_2$ emissions
Rumen fermentation	
Cattle	6, 674
Sheep	1, 383
Goats	8.2
Pigs	114
Horses	81
Poultry	-
Deer	3.8
Subtotal	8, 264
Manure management	
Cattle	1,079
Sheep	41
Goats	0.21
Pigs	416
Horses	6.3
Poultry	201
Deer	-
Subtotal	1, 743

Source: GHG inventories for England, Scotland, and Wales and Northern Ireland-2010 report

It is clear from Table 1 that in 2010, cattle rumen fermentation was the biggest contributor of methane followed by sheep. Methane from manure management is considerably less but again cattle contribute the most. According to Odegard and van der Voet (2014), by the year 2050, the global demand for beef and milk will more than double resulting in the number of animals being expected to increase to cope with the demand. As a consequence, the environmental impact of methane production associated with livestock expansion will worsen if corrective measures are not taken now (Moss *et al*., 2000; Reynolds *et al*., 2011).

1.1 Mitigation of methane emissions policy background

The UK is party to the United Nations Framework Convention on Climate Change (UNFCCC), a treaty constituted in 1994. The treaty requires member states to correctly report greenhouse gas emissions and to give information about compliance, best practices and policies they have adopted to mitigate climate change. The principle objective of the formation of the treaty is to stabilise the GHG emissions in the atmosphere. The UNFCC treaty is not legally binding so the Kyoto protocol was ratified in 2005 which became legally binding; it commits member states to set individual targets for GHG emission reductions (Monteny *et al*., 2006). The first commitment period for the Kyoto protocol was the period 2008 to 2012.

Following ratification of the Kyoto protocol, the UK Climate Change Act (2008) was passed which committed the UK to a legally binding long term frame work of cutting carbon emissions by reducing the GHG emissions by 12.5% below the 1990 levels between the periods 2008-2012 (Lovett *et al*., 2003). Under this act, the UK has a target of lowering the GHG emissions by 80% against the 1990 baseline by the year 2050 with a minimum of 34% reductions to be achieved by the year 2020. Fig 1 shows the trend in total methane emissions within the UK during the period 1990 to 2011.

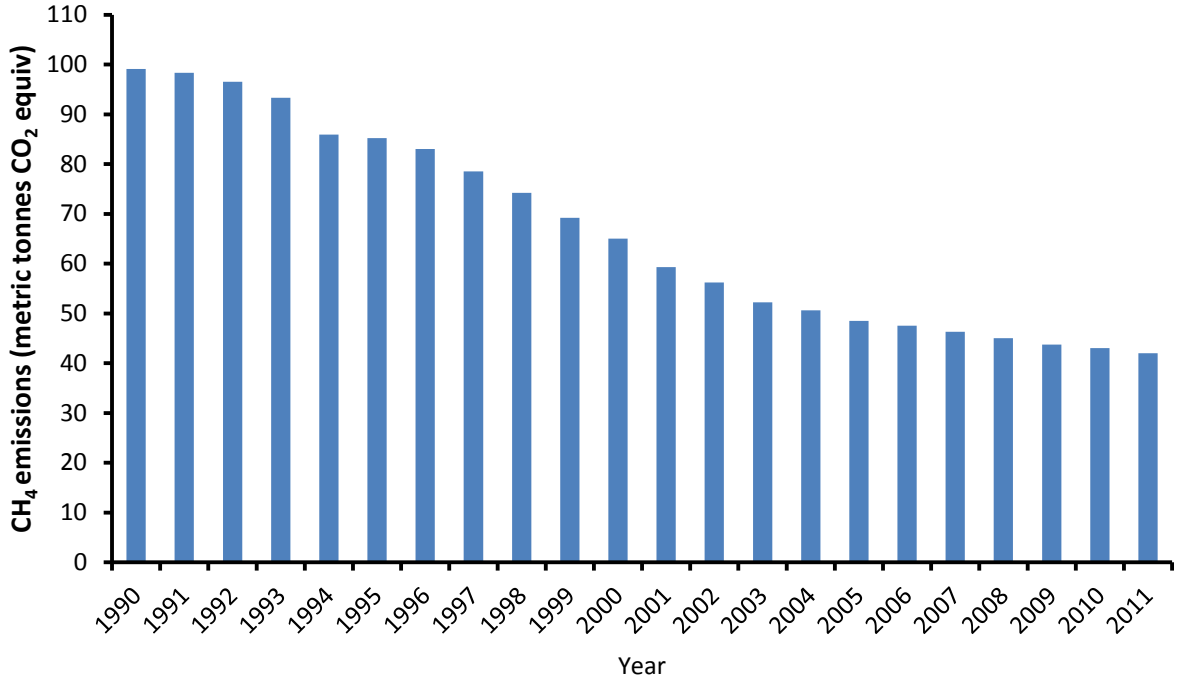


Figure 1. Changes in calculated total methane emissions (Mt, CO_2 equiv) from 1990 to 2011 within the UK
Source UNFCCC, 2011 report, 1990-2011 methane inventory emissions
Source: National Atmospheric emission inventory website: http://naei.defra.gov.uk/overview/ghg-overview

As shown in Fig 1, in 1990 methane emissions were 99.1 (metric tonnes of CO_2 equivalent) while in 2011, emissions had reduced to 42 (metric tonnes of CO_2 equivalent). This indicated that the total methane emissions had declined by 58% when compared to the 1990 levels (UNFCC report, 2011). The main sources of methane are coal mines, agriculture, waste disposal and gas leakage from distribution systems (Knapp *et al*., 2014). According to the UNFCC report, (2013) all the sources of methane in the UK had reported reductions in methane emissions since 1990 with the agricultural sector recording 20.6% reduction between 1990 and 2011. The decline in agricultural methane emissions was attributed to a combination of reduced livestock numbers and improvements in diet quality (Milne *et al*., 2014; UNFCC report 2013). In order to meet the emission reduction targets for 2050, further improvements are required including manipulation of feed.

Methane has a high energy value, heat of combustion of 55 MJ/kg (Eckard *et al*., 2010). The loss in energy through methane production indicates a large inefficiency in the production system, therefore knowledge of how feed influences rumen methane

production can help identify mitigation strategies both at a national and farm level (McGinn *et al*., 2004). Reducing methane losses by improvements in feed conversion efficiency may result in increased milk production with the consequence that reduction strategies can bring about more rapid economic and environmental benefits (Mao *et al*., 2010). According to Martin *et al.,* (2009) dietary manipulation and improved production efficiency appear to be the most viable options to reduce methane production from ruminants. Studies conducted to date indicate that a variety of dietary manipulations can have a significant impact in reducing the emissions.

CHAPTER 2 Literature Review

2.1. Stoichiometry of methane production in the rumen

Understanding how various biochemical processes lead to production of methane in the rumen is important for identification of mitigation strategies (Ominski and Wittenberg, 2004). Rumen microorganisms are adapted to live in an environment with a pH of 5.5 to 7 with a temperature range of 39°C to 40°C (Hungate, 1966). Production of methane starts with the action of different ruminal microorganisms, bacteria, protozoa and fungi when they hydrolyse and ferment complex feed components such as proteins and polysaccharides into simple products including amino acids, sugars and alcohols (Moss *et al*., 2000). The products are further fermented to volatile fatty acids (VFAs), hydrogen (H_2) and carbon dioxide (CO_2) (Iqbal *et al*., 2009) which are products of rumen fermentation.

Proportions of the 3 main VFAs produced, propionate, acetate and butyrate vary with the composition of the basal diet and once produced, get absorbed across the ruminal wall into the blood system for subsequent metabolism (Moss *et al*., 2002). Ruminants derive 70% of the metabolisable energy from VFAs (Tagang *et al*., 2010). According to Kumar *et al*., (2013), the rumen microbial population comprises of $10^{10\text{-}11}$ bacteria, $10^{8\text{-}9}$ methanogens, 10^6 ciliate protozoa and 10^6 fungi/ml. Methane (CH_4) is produced by the methanogens, the main organisms being *Methanobacterium ruminatium* (Hungate, 1966) which act at the terminal stages of fermentation and reduce CO_2 to CH_4 (Popova *et al*., 2011). *Entodinium* spp make up approximately 95% of the total protozoa count (Hristov *et al*., 2009). *M ruminantium*, are also the dominant methanogen species in the rumen (Mao *et al*., 2010). Two types exist, one which lives in close association with the protozoa and the other is free living. Usually supplements or additives that target protozoa also result in elimination of the methanogens that are associated with the protozoa and may or may not affect the free living methanogens.

The main substrates for methane production are CO_2 and H_2, although others can include acetate and formate (Janssen and Kirs, 2008). *Methanobacterium* species are known to effectively utilise hydrogen in methane formation (Hungate, 1966). Equations (i) to (iii) show the biochemical pathways that lead to production of CO_2 and H_2. Equation (iv) shows the terminal stage of metabolic reaction where the methanogens act to produce methane.

i) $C_6H_{12}O_6 + 2H_2O \rightarrow 2C_2H_4O_2$ (acetate) + $2CO_2$ + 8H

ii) $C_6H_{12}O_6 + 4H \rightarrow 2\ C_3H_6O_2$ (propionate) + $2H_2O$

iii) $C_6H_{12}O_6 \rightarrow C_4H_8O_2$ (butyrate) + $2CO_2$ + 4H

iv) $CO_2 + 4H_2 \rightarrow CH_4 + 2H_2O$

v) 4 Formate $\rightarrow CH_4 + 3CO_2 + 2H_2O$

vi) Acetate $\rightarrow CH_4 + CO$ (Moss *et al*., 2000; Martin *et al*., 2010).

The patterns of fermentation dictate the concentrations of hydrogen produced. With fibrous diets, the simple sugars are fermented via the pyruvate pathway and are converted into acetate, CO_2 and H_2 (Dohme *et al*., 2001). From equations (i) and (iii) it is apparent that microbes that produce acetate and butyrate also produce hydrogen and once produced, hydrogen is immediately utilised by methanogenic archaea to produce methane (Moss *et al*., 2000; Martin *et al*., 2010). Methanogenic removal of hydrogen allows the rumen to function optimally while its accumulation slows ruminal fermentation processes (Janssen and Kirs, 2008). Therefore one of the objectives of dietary manipulation is to re-channel the hydrogen produced during normal ruminal fermentation from methane production to either go to propionate production or re-channel it to be used by alternative hydrogen acceptors (McGinn *et al*., 2004).

2.1.1 Effect of dietary manipulation on ruminal microbe populations and methane production

A number of studies have examined how diet impacts on microbial populations in the rumen. From these studies, it is clear that to effectively reduce methane production, diets usually have an effect on either protozoa or on the methanogens or both. The effects of the diets on ruminal microbial populations vary from diet to diet (Mao *et al.*, 2010). Table 2 shows how various dietary manipulations impact on rumen microorganisms.

Table 2. Examples of how dietary manipulation impact on ruminal microbial populations

	Basal diet TMR		
Hassanat *et al*., (2013)			
Starch level in TMR (60:40)	17%	22.8%	30%
Total protozoa (× 10^5/mL)	4.85^b	4.75^b	3.35^a
Entodiniomorphs(× 10^5/mL)	4.83^b	4.68^b	3.29^a
Holotrichs (× 10^3/mL)	1.94^b	4.03^a	3.8^a
CH_4, g/d	440^a	483^a	434^{ab}
CH_4, g/kg DM intake	20.3^b	20.7^b	17.7^a

	Basal diet (TMR)		
Hristov *et al*., (2009)			
Oil addition to TMR	CT(stearic acid)	Lauric acid	Coconut oil
Total protozoa, x10^4/ml	138.2^a	27.2^b	22.9^b
Entodiniomorphs, x10^4/ml	132.6^a	25.8^b	20.9^b
CH_4 g/h	6.5^a	7.1^a	2.5^b

	Rye + conc (3:2)			
Mao *et al*., (2010)	CT	Tea saponins	Soybean oil	TS + soybean oil
*Methanogens	0.34^a	0.36^a	0.20^b	0.24^b
*Protozoa	9.71^a	5.72^b	4.71^b	5.42^b
*Fungi, X 10^{-2}	5.43	4.03	3.49	4.20
*R. flavefaciens, ×10^{-1}	1.43^a	0.70^{ab}	0.28^b	0.76^{ab}
CH_4, L/kg DM intake	26.2^a	19.0^c	22.6^b	21.2^b

[a,b] Within each row, means with different superscripts significantly differ; TS= tea saponins, CT= control

* presented as percent of total microbial population

As seen in Table 2, Hassanat *et al*., (2013) showed that varying starch levels in the total mixed ration (TMR) fed to cows has different impacts on ruminal microbes depending on starch levels in the diets. At 30% starch supplementation, there were significant reductions in methane output as a result of reductions in total protozoa counts, particularly the *Entodiniomorphs spp* were significantly reduced. At 17 and 22.8% starch supplementation, methane output was unchanged and at the same time there was no effect on the total protozoa count. Though *Holotrich spp* were significantly lowered at 17% starch level, this did not have an effect on methane output. What was established in this experiment was that *Holotrich spp* are less important in methane mitigation and that high levels of starch in the diet deplete *Entodiniomorphs spp*. According to Williams (1986), both *Entodiniomorphs* and *Holotrich* spp are ruminal protozoa spp but the *Entodiniomorphs* spp are the most abundant. This is the reason 100% starch supplementation is considered a possible defaunating strategy. In a study by Mao *al*., (2010), a basal diet of wild ryegrass and concentrate was supplemented with either tea saponins, soybean oil, or a combination of tea saponins plus soybean oil. In the rumen, methanogen populations were reduced only by the soybean oil treatment while the saponins treatments had no effect. However protozoa numbers were reduced by both the saponins and the soybean oil treatments. When the methane output was examined, it was observed that all the 3 supplements significantly reduced methane output when compared to the control despite each one of them showing varied effects on protozoa and methanogens numbers. A study by Hristov *et al*., (2009) used coconut oil, lauric acid and stearic acid to determine the impact on ruminal microbial populations and methane production. Coconut oil and lauric acid reduced total protozoa counts by 80% while stearic acid was ineffective. Methane output was reduced only by coconut oil and unchanged by lauric and stearic acid supplementation. What was established in the study

was that though lauric acid reduced the *Entodiniomorphs spp,* methane output was not reduced most probably because lauric acid had no effect on the methanogens. According to Hristov *et al*., (2009) *Entodiniomorphs* are the most common protozoa species in the rumen and account for 80% of the total protozoa count. It is therefore expected that an effective defaunating agent would act on these species of protozoa.

2.2 Dietary mitigation strategies that reduce methane output in ruminants

Manipulation of the ruminant diet can have a great impact on methane output (Moss *et al*., 2000). Changes in forage to concentrate ratio, dietary fibre content, physical and chemical characteristics of feed, feeding level, supplementation with additives such as fats and plant based compounds such as saponins, tannins and essential oils and antibiotics such as monensin have all been reported to alter ruminal fermentation patterns and influence methane output (Lovett *et al*., 2003; Reynolds *et al*., 2011). Figure 2 shows a summarised overview of the different dietary manipulations that can be used to reduce methane production.

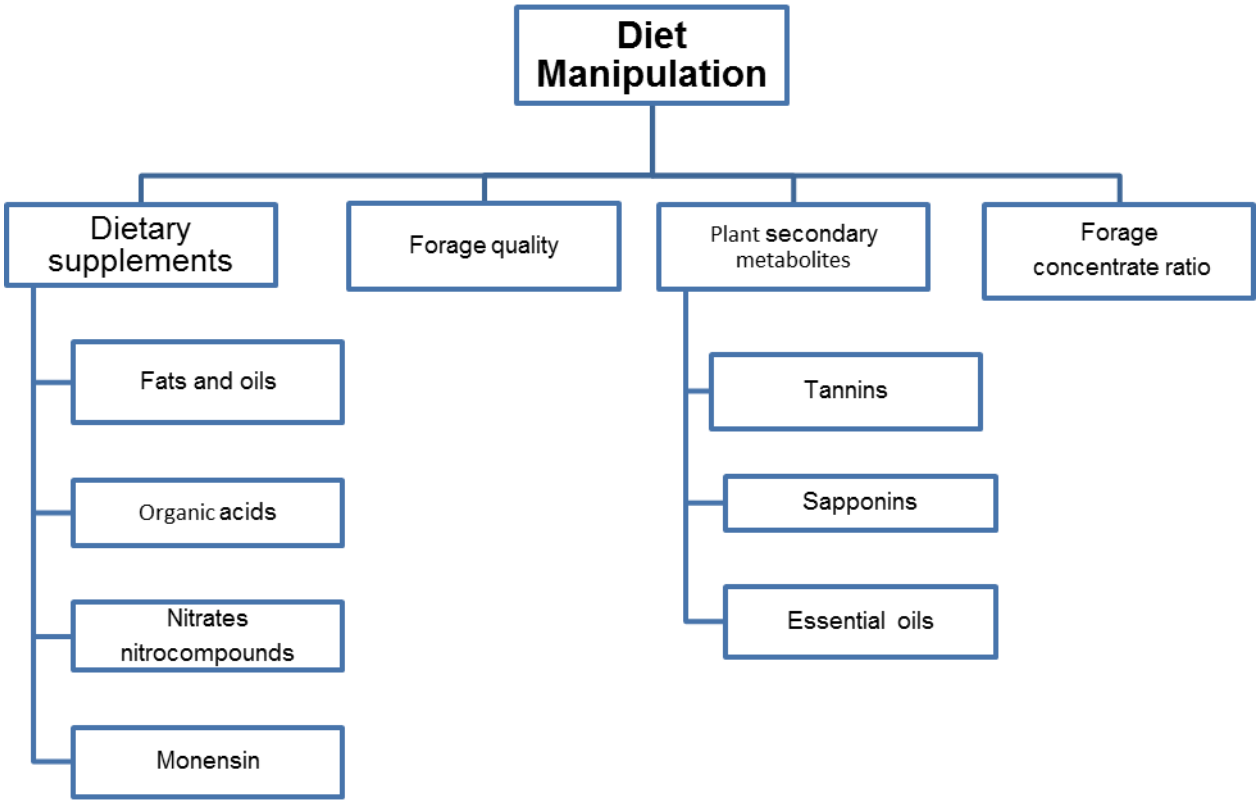


Figure 2. Summary of various dietary manipulations that can be used to mitigate methane production (Source: Eckard *et al*., 2010).

2.3 Use of fat supplementation to mitigate methane output

Oil supplementation has been identified as one of the most promising ways of reducing methane output from ruminants (Dohme *et al*., 2001; Reynolds *et al*., 2011). Most studies have confirmed the potential of oils in reducing methane production (Martin *et al*., 2008) both *in vivo* and *in vitro*, but the effects on methane output vary with the presentation of the oil (Martin *et al*., 2008), level of the oil, fatty acid profile of the oil and composition of the basal diet (Dong *et al*., 1997; Machmuller *et al*., 2001).

2.3.1 Mode of action of fats

Supplemental oils reduce methane production by multiple modes of action. In the rumen, fats are hydrolysed to fatty acids by microbial lipases (Toral *et al*., 2009) and it is the fatty acids that exert their effects on the ruminal environment either through bio-hydrogenation (Mao *et al*., 2010), reduction of organic matter fermentation (Martin *et al*.,

2010) or by a direct toxic effect on protozoa and methanogens (Mao *et al*., 2010). Oils and fats reduce protozoa numbers and consequently the methanogens that live on the surface of protozoa die off and this results in a reduction in methane production because interspecies hydrogen transfer between protozoa and methanogens is reduced or terminated (Lovett *et al*., 2003). Protozoa are involved in fibre fermentation in the rumen so elimination of protozoa tends to slow down fermentation (Lovett *et al*., 2003). The presence of oil in the rumen also serves as an alternative hydrogen sink, via hydrogenation of the oil.

2.3.2 Influence of fats on methane production

Most studies on the effects of supplemental fats on methane production have been conducted using medium chain fatty acids (MCFAs), the C_8-C_{16}, long chain fatty acids (C_{18}) and omega 3 fatty acids (Fieves *et al*., 2003). Table 3a and b summarise some previous studies on effects of various fats on methane production.

Table 3a. Summary of *in vitro* studies on effects of oil supplementation on methane production

Study	**Basal Diet**	**Oil Supplement**	**level of supply**	**% methane reduction**
Dohme *et al*., (2000)	Forage:conc 50:50	coconut oil	53 g/kg DM	21% mmol/d
		palm kernel oil	‘’	34% mmol/d
		palm oil	‘’	20% mmol/d
Dong *et al*., (1997)	100% grass hay	canola oil	10% wt/wt	26% mmol/g DM
	‘’	cod liver oil	‘’	29% mmol/g DM
	‘’	coconut oil	‘’	59% mmol/g DM
Dong *et al*., (1997)	90% wheat, 10% grass hay	canola oil	10% wt/wt	31% mmol/g DM
		cod liver oil	‘’	47% mmol/g DM
		coconut oil	‘’	85% mmol/g DM
Fievez *et al*., (2003)	hay/conc (65/35)	eicosapentanoic acid	75, 100 & 125 mg	74% mmol/mol
		docosapentanoic acid	75, 100 & 125 mg	36% mmol/mol
		soybean oil	‘’	56% mmol/mol
Machmuller *et al*., (2001)	High conc or high forage diet	coconut oil	0.58 g/d	62% on conc diet (mmol/d)
	High conc or high forage diet	lauric acid	0.74 g/d	78% on av on both diets (mmol/d)
Zhang *et al*., (2008)	wild rye and corn meal (1:1)	stearic acid	35 g/kg DM	4% mmols
	wild rye and corn meal (1:1)	oleic acid	‘’	9% mmols
	wild rye and corn meal (1:1)	linoleic acid	‘’	3% mmols
	wild rye and corn meal (1:1)	linolenic acid	‘’	5% mmols
	wild rye and corn meal (1:1)	stearic acid	70 g/kg DM	4% mmols
		oleic acid	‘’	16% mmols
		linoleic acid	‘’	42% mmols
		linolenic acid	‘’	62% mmols

Table 3b. Summary of *in vivo* studies on effect of oil supplementation on methane production

Study	**Basal Diet**	**Oil Supplement**	**level of supply**	**% methane reduction**
Beauchemin *et al*., (2009)	45% barley & 33% barley grain	crushed sunflower seeds	3.1-4.2% DM	10% on DM intake
Beauchemin & McGinn (2006)	75% barley silage, 25% barley	canola oil	4.60% DM	32% g/d, 21 % GE intake
Chung *et al*., (2011)	barley silage	ground linseed	150 g/kg DM	33% g/kg DM intake
	grass hay	ground linseed	150 g/kg DM	no effect
Eugene *et al*., (2011)		Forage / starch + extru. linseed oil		20% L/kg DM intake
Martin *et al*., (2008)	59% corn silage, 35% conc	crude linseed	5.7% DM	12% g/d, 15%GE intake
		extruded linseed	‘’	38 % g/d, 28% GE intake
		linseed oil	‘’	64% g/d, 55% GE intake
Machmuller *et al*., (2000)	maize silage, grass hay & conc	coconut oil	6% DM	26 % GE intake
		rapeseed oil	‘’	19 % GE intake
		sunflower oil	‘’	27% GE intake
		linseed oil	‘’	10% GE intake
Machmuller *et al*., (2001)	60:40 forage:conc ratio	coconut oil	60 g/kg DM	no effect
Mao *et al*., (2010)	60 % forage, 40% conc	soybean oil	30 g/kg DM	13% (daily)
Moate *et al*., (2011)	TMR (60:30, F:C)	brewers grain	51 g/kg DM	5% g/kg DM intake
		hominy meal +canola	52 g/kg DM	4.8% g/kg DM intake
		hominy meal	65 g/kg DM	11.6 % g/kg DM intake
Odongo *et al*., (2007)	TMR	myristic acid	5%	36% L/d

Significant reductions in methane output have been observed with fats that contain medium chain fatty acids (MCFA; Soliva *et al*., 2004). Most MCFAs have been found to effectively reduce methane emissions by as much as 50% both *in vitro* and *in vivo* as can be seen in Tables 3a and 3b. For example, in an *in vitro* study by Dong *et al*., (1997) coconut oil supplementation at 10% wt/wt DM reduced methane by 59% on a hay based diet and by 85% on wheat concentrate based diet, while in a study by Machmuller *et al*., (2001) addition of coconut oil and lauric acid at 0.58 g/d and 0.74 g/d in a Rusitec fermenter reduced methane production by 62 and 74 % respectively. The effect of MCFAs on methane output is attributed to their potent effect on protozoa. Protozoa produce hydrogen that is utilised by methanogenic bacteria to synthesise methane (Zhang *et al*., 2008). Therefore reductions in protozoa numbers tend to reduce methane production. For example, Hristov *et al*., (2009) observed an 80% reduction in protozoa numbers with coconut oil supplementation which subsequently resulted in significant reductions in methane production. However, even among the fatty acids, the effects on methane output have been extremely variable. For example Dohme *et al*., (2001) examined seven MCFAs with C_8 – C_{14} fatty acids and compared them with long chain fatty acids C_{16}, C_{18} and $C_{18:2n\text{-}6}$ on their effects on methane output in dairy animals and observed that only $C_{12:0}$, $C_{14:0}$ and $C_{18:2n\text{-}6}$ reduced methane output by 18% (C_{12}, C_{14}) and 25% ($C_{18:2n\text{-}6}$), while the rest were ineffective. In the same study, C_8 and C_{10} increased methanogen numbers while $C_{12:0}$ only reduced protozoa numbers.

A study by Soliva *et al*., (2004) has shown that oils that have a combination of MCFAs tend to have a greater inhibitory effect on methane production and proposed that when fatty acids interact, the synergistic effect on methane production is usually greater than when any of them are used separately. For example, Soliva *et al*., (2004) demonstrated a synergy between lauric and myristic acids when used at ratios of 4:1 and 4:2 respectively,

which produced 70 and 60 % reductions in methane output, but when used separately, myristic acid had no effect and lauric acid produced only a 45 % reduction in methane output. Odongo *et al*., (2007) also observed only a 36% reduction in methane output with myristic acid.

The long chain FAs of the C_{18} group includes oleic, linoleic and α-linolenic acid. Common sources are rapeseed, sunflower, and linseed oil respectively (Ueda *et al*., 2003). Like the MCFAs, Zhang *et al*., (2008) observed that among the C_{18} FAs, effects on ruminal microorganisms differ between individual FAs. Both linoleic and α-linolenic acid reduce protozoal numbers, but greater effects are observed with α-linolenic acid (Zhang *et al*., 2008). With the C_{18} group of FAs, the degree of unsaturation was observed to influence methane output (Zhang *et al*., 2008). In Table 3b, this was evident when the FAs were added to the basal diet at the same level of 70 g/kg, α-linolenic acid suppressed methane production more than stearic, oleic or linoleic acid. The variable effects of oils on rumen fermentation characteristics can be attributed to differences in FA composition of the oil, the level of supplementation and composition of the basal diet (Toral *et al*., 2009).

Studies on the very long chain omega 3 fatty acids eicosapentanoic acid and decosapentanoic acid are limited. The few studies that have been conducted (Boeckaert *et al*., 2006; Fievez *et al*., 2003) show that very long chain omega 3 fatty acids have strong methane inhibition effects ranging from 60-80%, and inhibition is linked to the amount of unesterified DPA. For example Fievez *et al*., (2003) observed that when used at increasing concentrations of 0.5 to 5 mg/l, *in vitro* methane suppression by eicosapentanoic acid and decosapentanoic acid was found to be proportional to the degree of unsaturation and to the amount of PUFA added in the incubation media.

Finally, Table 3a and 3b show that effects of oils on methane production are also influenced by the basal diet. Only a few studies for example, Machmuller *et al*., (2001) and Chung *et al*., (2011) examined the interaction between supplemental oils and basal diets in influencing methane output. Machmuller *et al*., (2001) examined the effects of coconut oil and lauric acid on a high forage and a high concentrate diet in a Rusitec study. Coconut oil reduced methane production by 62% (mmol/d) on a high concentrate diet but had no effect on a high forage concentrate diet. On the other hand, lauric acid reduced methane production by an average of 78 % (mmol/d) on both the high forage and high concentrate diet. In the same study, when coconut oil was tested *in vivo* by supplementing sheep fed a diet with 60:40 forage to concentrate ratio at 60 g/kg DM, it did not have any effect on methane production. Chung *et al*., (2011) also examined the effects of linseed oil on methane production on two basal diets. Dairy cows were fed either a barley silage diet or a hay based diet were supplemented with ground linseed at 150 g/kg DM. Cows fed the silage based diet produced 33% (g/kg DM intake) lower methane production when compared to the un-supplemented control while methane production in cows fed a hay based diet was unaffected.

2.3.3 Challenges of fat supplementation

Fat supplementation of ruminant diets is associated with a number of challenges. Dietary fat supplementation of more than 5-6 % is often accompanied by reductions in DM intake and milk production due to reduced fibre fermentation (Boadi *et al*., 2004). Fibrolytic bacteria in particular are very sensitive to inhibition by dietary fats (Hristov *et al*., 2009). Beauchemin and McGinn (2006a) observed a 15 % depression total tract digestibility when canola oil was supplemented to the diet at 4.6% DM in beef cattle. However the effects of oils on fibre digestibility are not conclusive. For example, when using the Rusitec technique, Soliva *at al*., (2004) reported inhibition of fibre degradation only in the

first 10 days of the 25 day experimental period, suggesting the negative effect may be temporal. Cieslak *et al*., (2006) also reported no effect on fibre degradability when plant oils where used at 7% supplementation. Toral *et al*., (2009) observed a lack of effect on neutral detergent fibre (NDF) degradability when sheep on a high concentrate diet were supplemented with a combination of fish oil and sunflower oil at 30 g/kg DM, and suggested that microorganisms may have adapted to the oil in the feed resulting in normal NDF digestibility to occur.

To date only a few studies have evaluated the persistence of effects of different supplemental fats on methane production. Table 4 shows details of some previous studies that were conducted to assess persistency of the effects of oils on methane production. Grainger *et al*., (2010) reported increasing reductions in daily methane outputs of 13% and 23% in weeks 3 and 12 respectively following whole cotton seed supplementation of dairy animals at 3.5% DM. When methane production was expressed per kg DM intake, methane production was reduced by 5% in week 3 and 15% in week 12. Similarly, Jordan *et al*., (2006) also observed methane reduction of 19% (L/d and kg/DM intake) during the period 14-18 and 70-74 days of the experiment when beef heifers where supplemented with refined coconut oil at 250 g/d (8% DM intake). In a study by Moate *et al*., (2011), supplementation of cold pressed canola meal at 52 g/kg resulted in methane suppressing effects to last for 7 weeks.

Table 4. Studies examining persistency of effects of oil supplementation on methane production

		Methane production g/d		Methane production g/kg DM intake	
[1]Grainger *et al*., (2010)	**Sampling**	**CTR**	**Diet**	**CTR**	**Diet**
	Week 3	592[a]	512[b]	35.4[a]	33.6[b]
	Week 6	600[a]	505[b]	36.3[a]	32.8[b]
	Week 10	600[a]	505[b]	36.2[a]	32.8[b]
	Week 12	625[a]	480[b]	37.2[a]	31.8[b]
[2]Moate *et al*., (2011)	Week 3	500[a]	461[b]	25[a]	23.3[ab]
	Week 7	500[a]	460[b]	25[a]	22.9[b]
	Week 10	500[a]	467[ab]	25[a]	23.4[ab]

[a,b] within each row for a particular unit of measure, means with different superscripts significantly differ

[1]CTR diet contained wheat grain and cotton seed meal (92% and 8% respectively; 2% total fat content), Diet contained wheat grain and whole cotton seed (52% and 48% respectively; 5.3% total fat content).

[2]Basal diet was a TMR composed of alfalfa hay, ryegrass silage, wheat grain and canola meal, diet composed of hay, ryegrass silage, wheat grain and cold pressed canola meal with total fat content of the diet at 5.2%.

2.4 Use of concentrates to mitigate methane output

According to Johnson *et al*., (1994), the quality of feed an animal consumes determines the quantities of methane produced. Concentrates are considered high quality feeds and are commonly used in dairy farming and in beef intensive feedlot systems (Beauchimin and McGinn, 2005) primarily to improve productivity. However recent studies provide evidence that concentrate addition to ruminant diets may help to reduce methane emissions.

2.4.1 Effects of dietary starch concentrate on methane production

The nature and fermentation characteristics of carbohydrates found in concentrates influence the proportion of volatile fatty acids (VFA) produced and consequently the amount of methane produced (Boadi *et al*., 2004). With starch addition to the diet, propionate as opposed to acetate production is favoured which leads to less hydrogen available for methane formation (Moss *et al*., 2000). The VFAs produced also lower the ruminal pH and subsequently negatively affect methane production (McGeough *et al*.,

2010; Beauchemin and McGinn, 2005) as a lower pH inhibits the growth of methanogens and protozoa (Boadi *et al*., 2004), while structural carbohydrates reduce fermentation rates and promote production of acetate. A number of studies have reported an increased feed intake with concentrate supplementation which is accompanied with either reductions (Beauchemin *et al*., 2009), no change (Beauchemin and McGinn, 2006) or increase in daily methane output (Beauchemin and Mc Ginn 2006; Lovett *et al*., 2005). Several factors contribute to the variations in methane output with concentrate supplementation, with the two most important factors being the forage to concentrate proportion and the composition of the concentrates in the diet (Eugene *et al*., 2011; Benchaar *et al*., 2001).

2.4.2 Concentrate proportion and methane production

Several studies have reported reduced methane output with increased concentrate supplementation particularly when results are expressed as a proportion of gross energy (GE) intake. For example, Lovett *et al.,* (2003) observed that decreasing the forage to concentrate ratio from 2:3 to 1:9 reduced methane output by 33% of GE intake. In a study by Aguerre *et al*., (2011), a progressive increase in F:C ratio in the diet of cows from 47:53, 54:46, 61:39 and 68:32 did not have any effect on DM intake but progressive increase in methane production was observed with values of 26, 28, 29 and 32 g/kg DM intake methane respectively. Changes in methane production were due to a progressive increase in NDF fraction of the diet when the forage levels were gradually increased. Some studies have also reported either no changes or an increase in methane production with increased concentrate supplementation. For example, in a study by Beauchemin and McGinn (2006), cows fed a high forage diet (comprising of 70% barley silage and 30% barley based concentrate) had a 1.3 kg/d lower DM intake when compared to those fed a high concentrate diet (comprised of 70 % maize based concentrate and 30% barley

silages) but methane production when expressed as g/d, g/kg DM intake, % of GE intake was similar between the two groups. The results of the studies conducted by Beauchemin and McGinn, (2006) show that to effectively mitigate methane output with concentrates, the most effective strategy would be to feed animals above energy maintenance requirements, as doing so results in an improvement in feed conversion efficiency and greatly reduces the proportion of GE lost as methane. This is clearly demonstrated in a study by McGeough *et al*., (2010) in Table 5

Table 5. Differences in methane output produced by whole crop wheat silage differing in grain: forage ratio, grass silage (GS) and *ad libitum* concentrate (ALC) supplementation in beef cattle

	Whole crop wheat silage					
Diet type	I	II	II	IV		
Grain: forage ratio	(11:89)	(21:79)	(31:69)	(47:53)	GS	ALC
Total DM intake, kg/d	10.6	11.4	11.4	11.0	9.20	10.9
CH_4 g/d	295	315	322	273	312	180
CH_4 g/kg DM intake	30.1	27.5	28	25.9	35.6	15.3
CH_4 % GE intake	8.9	8.24	8.52	6.79	9.72	3.71
CH_4 g/kg carcass gain	534	432	412	325	443	182

GS=grass silage, ALC= *ad libitum* concentrate feeding (McGeough *et al*., 2010)

It is clear from Table 5 that greater reductions in methane output were observed with *ad libitum* concentrate feeding regardless of the unit of expression. The DM intake did not differ much among the treatments yet methane emissions were lowest in the *ad libitum* concentrate treatment. The study demonstrated the effectiveness of *ad libitum* concentrate feeding in reducing methane emissions, whereas grass silage produced the highest methane emissions in terms of DM intake and GE loss. Additionally, increasing the grain proportion within whole crop wheat silage produced a quadratic response in methane output (g/d). Therefore it is evident that increasing concentrate content of the wheat silage increased the starch content of the diet which in turn reduced methane emissions relative to DM intake and carcass gain. Reductions in methane output with whole crop wheat silage are associated with the presence of starch in the silage which

favours propionate production in the rumen (Lovett *et al*., 2003). According to Benchaar *et al*., (2014), an increase in the grain proportion in the diet increases the starch levels and this greatly affects how much methane is produced. This was clearly illustrated in a study by Hassanat *et al*., (2013) who examined the effects of replacing alfalfa silage with corn silage in the TMR on methane production. In their study, corn silage in the TMR was replaced at 3 levels of 0, 50 and 100% formulated to provide 17, 22.8 and 30% starch content in the TMR respectively (Hassanat *et al*., 2013). A quadratic response in methane output was observed with production of 20.3, 20.7 and 17.7 g/kg DM intake methane production respectively. Methane output was significantly reduced only with the diet that supplied 30% starch.

Methane losses of 3.7% GE intake observed by McGeough *et al*., (2010) with *ad libitum* concentrate feeding is consistent with findings from many previous studies (Lovett *et al*., 2003; Whitelaw *et al*., 1984) where it was observed that diets comprising 90% concentrates when given at *ad libitum* levels produced consistent results of 2-4% of GE as methane losses regardless of the grain source in the concentrate portion of the diet. One of the reasons a*d libitum* concentrate reduces methane production is because it results in defaunation of animals. For example Whitelaw *et al.,* (1984) observed marked differences in methane output between defaunated and faunated cattle (4% vs 8.1% of GE intake respectively).

2.4.3 The influence of concentrate and forage composition on methane output

Composition of the concentrate also influences methane production (Lovett *et al*., 2005) Methane emissions from carbohydrate rich diets tend to be highly variable depending not only on the level of inclusion but also on the composition of the carbohydrate (Hindrichsen *et al*., 2005). For example, replacing a fibrous concentrate of sugar beet pulp

with a barley concentrate did not reduce DM intake, but methane output was reduced by 24% of GE intake in a simulation study by Benchaar *et al*., (2001). Variations in methane output were due to differences in the starch sources. In a study by Benchaar *et al*., (2014), replacing barley silage portion of the TMR with maize silage increased DM intake of the cows and reduced methane production when expressed as g/kg DM intake and GE intake. Results of the study by Benchaar *et al*., (2014) are shown in Fig 2

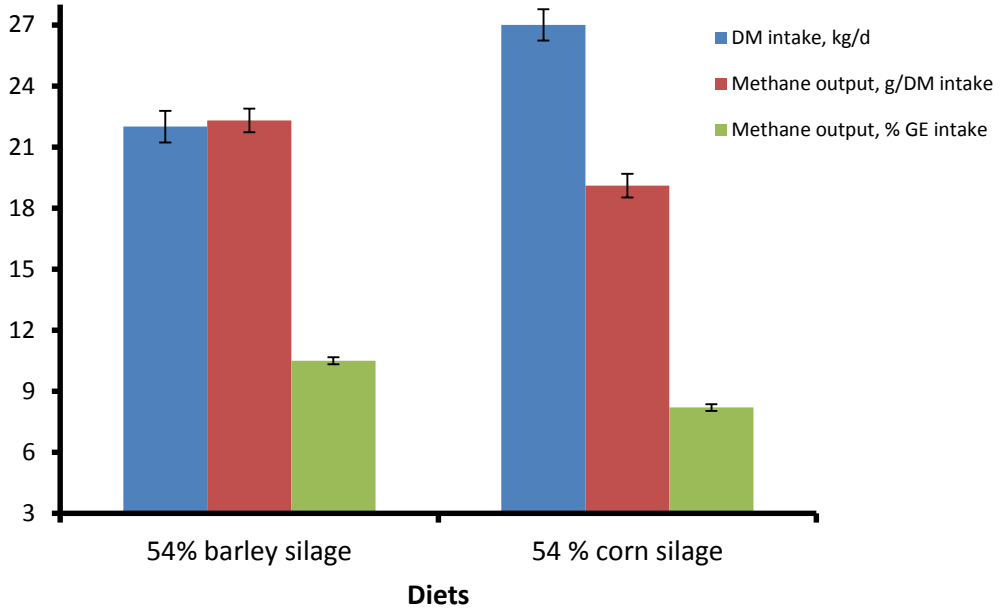


Figure 2. Effect of starch source in the silage on DM intake and methane production (Benchaar *et al*., 2014)

It is clear from Fig 2 that maize based silages when supplied at the same levels as barley silages reduce methane production when compared to barley silage regardless of the unit the results are expressed. Previous studies have shown that corn silage when supplied at the same level as barley silage tend to have higher starch levels (Beauchemin and McGinn, 2005). The high starch content in corn silage may have lowered the ruminal pH more than barley silage making the rumen environment unfavourable to the methanogens which are bacteria responsible for methane production. In another study

by Hart *et al*., (2012), cows were supplemented with the same amounts of concentrates that differed in starch levels of either 58 or 320 g/kg DM. The cows that received high starch concentrates produced 9.5% lower methane output (% GE intake) when compared to those that received the low starch concentrates.

2.4.4 Impact of concentrate supplementation on production parameters.

Several areas of research (McGeough *et al*., 2010) have demonstrated that increasing the grain content of the concentrate portion of the diet has economic benefits as evidenced by increased rate of carcass gains, increase in milk yields and milk constituents (Lovett *et al*., 2005) and shorter time to market. This is because with concentrate supplementation, gross energy losses are low and the retained energy is used to enhance productivity. A study by Fitzgerald and Murphy (1999) demonstrated that increasing concentrate supplementation from 4 to 8 kg/d in cows that were fed either grass silage or low starch maize increased fat, protein and lactose contents of milk and also the milk yield. Benchaar *et al*., (2013) fed cows a TMR with increasing levels of distillers' grain. The proportion of the grain in the diet ranged from 0-30% DM of total TMR ration. Results showed that DM intake, milk production and daily weight gains increased linearly with increasing proportion of distillers' grain in the diet. Methane output as a unit of gross energy intake also decreased linearly with increasing grain content (Benchaar *et al*., 2014).

2.4.5 Challenges of concentrate supplementation

Ominski and Wittenberg (2004) noted that grain supplementation has a limited effect on methane output when good quality forage is used. For example, Table 6 compares methane outputs from 4 previous studies. McCaughey *et al*., (1997) observed methane losses of 4.4% of the GE intake in grazing cattle while Lovett *et al*., (2003) observed similar amounts with 90% concentrate supplementation. A comparative study by O'Neill *et al*.,

(2011) observed that the methane output of cows on grass was low at 5.7 % GE intake compared to 6.5 % GE intake emitted by cows fed a TMR that was composed of 35% maize silage and 45% maize concentrate. This showed that it is possible for cows on pasture to emit lower methane outputs when compared to those fed TMR which are considered high quality feeds. In a study by Beauchemin and McGinn (2006b), methane losses of 6% GE intake were reported in heifers which remained unchanged even when high forage diets comprising 70 % whole barley silage were replaced with a high concentrate diet comprising 70% corn based concentrate.

Table 6. Effects of basal diet on methane production

Study	Basal diets	Total DM intake (kg/d)	CH_4 output % GE intake
McCaughey *et al*., (1997)	alfalfa/grass Pasture	14.9	4.0
Lovett *et al*., (2003)	Forage: conc		
	65:35	6.9	6.1
	40:60	8.4	6.6
	10:90	8.2	4.4
O' Neill *et al*., (2011)	Rye grass	14.3	5.7
	TMR	19.7	6.5
Beauchemin and McGinn (2006b)	70% barley silage	21.6	6.0
	70% corn based concentrate	20.0	6.0

TMR= total mixed ration; Conc= concentrate

The other challenge with concentrate supplementation is that the feeding management practised by farmers tends to influence methane output. Hindrichsen *et al*., (2006) simulated a real life scenario in which four feeding regimes commonly practised on dairy farms were compared for their milk production and methane outputs. Table 7 shows the results of the study by Hindrichsen *et al*., (2006).

Table 7. Impact of feeding management on methane output of dairy cows (Hindrichsen *et al.*, 2006)

Production level	Low	Medium	Medium	High
Milk/day	10kg	20kg	20kg	30kg
Diet type	1	2	3	4
DM intake kg/d	13.5[b]	19.6[a]	14.3[b]	20.9[a]
Milk yield	11.3[c]	21.9[b]	25.3[ab]	31.7[a]
CH_4 g/d/cow	323[b]	414[a]	369[ab]	414[a]
CH_4 g/kg DMI	23.9[ab]	21.2[b]	25.9[a]	21.1[b]
CH_4 g/kg milk	35.8[a]	19.6[b]	18.9[b]	14.4[b]
CH_4 % GE intake	7.14[ab]	6.31[b]	7.4[a]	6.14[b]

[a,b] With each row, means with different superscripts significantly differ
Diet 1= low quality hay & grass silage 1.5:1 ratio,
Diet 2 = mixture of diet 1 and conc in the ratio 1:1
Diet 3 = mixture of maize silage and grass silage in the ratio 1:1.
Diet 4 was a mixture of Diet 3 with concentrate in the ratio 1:1

Low yielding cows producing about 10 kg milk/d/cow or less were fed diet 1 composed of only low quality hay and grass silage in the ratio 1.5:1; the medium milk producers, that were producing on average 20 kg milk/d/cow were divided into 2 groups and were fed diets 2 or 3 which were composed of a mixture of diet 1 and concentrate in the ratio 1:1 for diet 2 or diet 3 which was a forage only diet composed of maize and grass silage in the ratio 1:1. The high producers were grouped as cows producing on average 30 kg milk/d/cow. This group were fed diet 4 which was a mixture of diet 3 and concentrate in the ratio 1:1. Milk and methane output results are presented in Table 7 which shows that DM intake, milk production and methane were all predicted to be influenced by the quality of the feed given to the cows. Dry matter intake was low with the forage based diets, diets 1 and diets 3, which had no concentrate portion in them. Methane output (g/kg DM intake) reduced with increased DM intake, and when expressed as a unit of milk yield. Methane output was highest with diet 1 which was an all forage diet of poor quality, while diets 2, 3 and 4 produced similar amounts of methane. Diet 3 was an all forage diet composed of maize and grass silage in the ratio 1:1; the maize in the diet was

predicted to increase the starch supply to the cows which resulted in a lower methane output. Hindrichsen *et al*., (2006) established that increasing productivity by using high quality feeds has the potential to reduce methane output when methane output is expressed per unit of animal product. The results of Hindrichsen *et al*., (2006) agree with Reynolds *et al.,* (2011), who stated that methane output in dairy cows when expressed per unit of milk yield reduces with an increase in milk yield and that considerable increases in methane output are observed when milk yield is below 20 L/d.

2.5 Use of organic acids to mitigate methane output

There is renewed interest in the use of organic acids as methane inhibitors following the ban of antibiotic use as growth promoters (Boadi *et al*., 2004). Organic acids, also called propionate enhancers, are intermediates of ruminal carbohydrate digestion particularly the propionic acid pathway (Castillo *et al*., 2004).

2.5.1 Mode of action of organic acids in reducing methane production

Commonly studied natural organic acids include fumarate, malate and aspartic acids. Their use in ruminant diets stimulates production of propionic acid which is a hydrogen competitor for methane (Jouany and Morgavi, 2007). Fumaric acid is reduced by H_2 or 2H to succinate, which is then converted to propionic acid (Wood *et al*., 2009). Organic acids stimulate growth of *Selenomonas ruminantium* bacteria (Khampa and Wanapat, (2007). *S. ruminantium* bacteria ferments soluble carbohydrates and utilises lactate as an energy source, therefore organic acids stimulate lactate utilisation by *S ruminantium* (Khampa and Wanapat, 2007; Castillo *et al*., 2004). As a consequence, organic acids help to regulate the pH of the rumen and therefore prevent lactic acidosis (Castillo *et al*., 2004).

2.5.2 Previous studies on organic acids and their limitations

Previous studies (Jouany and Morgavi, 2007) have indicated that high volumes of organic acids are needed to reduce methane production and such high volumes of acids would predispose animals to a risk of acidosis. For example to reduce methane production by 10%, 2.9 kg/d of sodium fumarate was required (Jouany and Morgavi, 2007). Foley *et al*., (2009) and Beauchemin and McGinn, (2006a) reported a lack of effect on methane production when dairy cows were supplemented with malate and fumaric acid at 480 g/d and 175 g/d respectively. Due to these limitations, there have been few studies on the use of organic acids as feed supplements. According to Shibata and Terada (2010), organic acids need to be tested under various feeding conditions to understand how they work. However recent studies by Wallace *et al*., (2006) and Wood *et al*., (2009) used encapsulated fumarate at 100 g/kg feed *in vitro* and in growing lambs respectively and reported reduced methane production by 75% without any negative effects on ruminal pH. The studies by Wallace *et al*., (2006) and Wood *et al*., (2009) offer hope of the ability of fumaric acid to reduce methane production without risks of acidosis.

Since the European Union banned the use of ionophores such as monensin and lasalocid as animal feed additives, efforts are being made to find alternatives (Jouany and Morgavi, 2007). According to Castillo *et al*., (2004), there is great potential for organic acids to replace monensin as ruminant feed additives owing to the advantages that organic acids have which are, the ability to regulate rumen pH, the potential to lower methane production and the safety to use in meat animals without worrying about residues. However one major limitation to the use of organic acids is the cost, as currently organic acids are still very expensive. Persistence of the effects of organic acids *in vivo* is also another aspect that has never been assessed to date (Jouany and Morgavi, 2007). Organic

acids also remain to be tested in *vivo* on a range of diets to assess their potential as feed additives (Boadi *et al*., 2004).

2.6 Use of nitrates, nitro compounds and sulphates in mitigating methane production

2.6.1 Mode of action of nitrates in reducing methane production

Nitrate and sulphate salts in the rumen act as strong sinks for hydrogen produced during ruminal fermentation (Nolan *et al*., 2010). With nitrate supplementation, reduction of nitrate to ammonia becomes more favourable in the rumen than reduction of CO_2 to CH_4 (VanZijderveld *et al*., 2010).

$$NO_3 + 2H \rightarrow H_2O + NO_2$$

$$NO_2 + 6H \rightarrow H_2O + NH_3$$

According to VanZijderveld *et al*., (2010), every mole of nitrate that is reduced results in reduction of methane production by one mole. In other words, according to VanZijderveld *et al*., (2010), for every 100 g of nitrate that is fed to animals, there is a 25.8 g reduction in methane production. Studies on nitrate and sulphate supplementation in ruminants are few (Bozic *et al*., 2009; Van Zijderveld *et al*., 2010; Zhang and Young, 2011) but have produced encouraging results of methane reductions of between 32-98.5% as shown in Table 8 which is a summary of previous studies on nitrate, sulphates and nitro-compounds.

Table 8. Effects of nitrates, sulphates and nitro-compound supplementation on methane production *in vitro* and *in vivo*

Study	Basal diet	Additive	% methane reduced
In vitro studies			Compared to control
Bozic *et al*., (2009)			
1. nitro-ethane	Alfalfa	1 mg/ml	92% (µmol/ml)
2. sodium nitrate	Alfalfa	1 mg/ml	63% (µmol/ml)
Anderson *et al*., (2008)			
Nitrocompounds	ryegrass	12 (µmol)	
1. 2-nitro-1-propanol			41% (µmol/ml)
2. 3-nitro-1-propionic acid			97% (µmol/ml)
3. Nitroethane			97% (µmol/ml)
4. 2-nitroethanol			97% (µmol/ml)
Zhang and Yang (2011)			
Nitrocompounds	Chinese rye grass: maize (4:1)	0,5,10,15 milimolar	79.4-98.5%
1. Nitroethane		0,5,10,15 milimolar	67, 75, 83% (mol/100 mol)
2. 2-nitroethanol		0,5,10,15 milimolar	80, 90, 62% (mol/100 mol)
3. 2-nitro-1-propanol		0,5,10,15 milimolar	68, 68, 81% (mol/100 mol)
In vivo studies			
van Zijderveld *et al*., (2011)			
Nitrate source	TMR (F:C 66:34)	21 g/kg DM	16% g/kg DM and GEI
Van Zijderverld *et al*., (2010)			
1. nitrate	Maize silage	2.6% of DM	32% L/d
2. Sulphate	Maize silage	2.6% of DM	16% L/d
3. Nitrate and sulphate	Maize silage	2.6% of DM	47% L/d
Nolan *et al*., (2010)			
1. nitrates	chaffed oaten hay	4% DM of KNO_3	23% L/kg DM intake

TMR = total mixed ration; F: C = forage to conc ratio

As seen in Table 8, animal based studies are few. For example, in a study by Van Zijderveld *et al*., (2010), supplementation of lambs with either a nitrate, sulphate or a combination of the two salts at 2.6% DM resulted in methane reductions (L/d) of 32, 16 and 47% respectively. At a ruminal microbe level, it was observed that methanogen numbers were greatly reduced with supplementation. In a more recent study, Nolan *et*

al., (2010) supplemented sheep with 4% DM of potassium nitrate (KNO_3) and reported a 23% reduction in methane output when expressed as L/kg DM intake. In all the studies with nitrate supplementation, DM intake was unaffected by supplementation which is a good attribute for a supplement.

Nitro-compounds have also been assessed as potential supplements for methane mitigation. Studies by Bozic *et al*., (2009), Anderson *et al*., (2008) and Zhang and Yang, (2011) demonstrated the potential of the nitro-compounds, nitro-ethane, 2-nitroethanol and 2-nitro-1-propanol to reduce methane production by 80% *in vitro* with no adverse effects on digestibility. However, the nitro-compounds are yet to be tested *in vivo* for their ability to reduce methane output.

Persistence of the effect of nitrates, nitro-compounds and sulphates on methane production still remains to be tested. Only one study by Van Zijderveld *et al*., (2011) assessed the persistence of nitrate supplementation on methane production in dairy cows. Cows were supplemented with 21 g of nitrate/kg of DM and methane production was reduced by 16% when expressed as either g/kg DM intake or per unit of gross energy, and effects persisted for a total of 89 days without negative effects on milk production.

2.6.2 Limitations of nitrate, nitro-compounds and sulphate supplementation

Nitrates and sulphates in ruminant diets need gradual introduction for animals to get adapted. According to Van-Zijderveld *et al*., (2010), if animals are un-adapted, consumption of nitrates results in accumulation of nitrites in the rumen. The nitrites are intermediate products which get absorbed across the ruminal wall and result in the formation of methemoglobinemia, a condition which restricts oxygen supply to the tissues and in severe cases can be fatal (Nolan *et al*., 2010). However from the previous

studies conducted, it can be established that nitrates do not compromise DM intake and do not present a health risk if managed correctly.

2.7 Use of secondary plant metabolites to mitigate methane production in ruminants

The interest in plant and secondary plant metabolites such as tannins, saponins and essential oils has grown following the ban of antibiotic as additives in animal production within the European Union (Jouany and Morgavi, 2007). In 2006, a large EU sponsored project called 'RUMEN UP' examined plant materials and assessed how they can be used to mitigate methane production in ruminants. The project was helpful in identifying plant species that have the potential to be used as dietary supplements in ruminants (Wallace, 2004) and brought to light several ways by which dietary manipulation and improved productivity can benefit both the animals and the environment. Fig 3 explains how dietary manipulation and improved productivity can benefit both the animals and the environment.

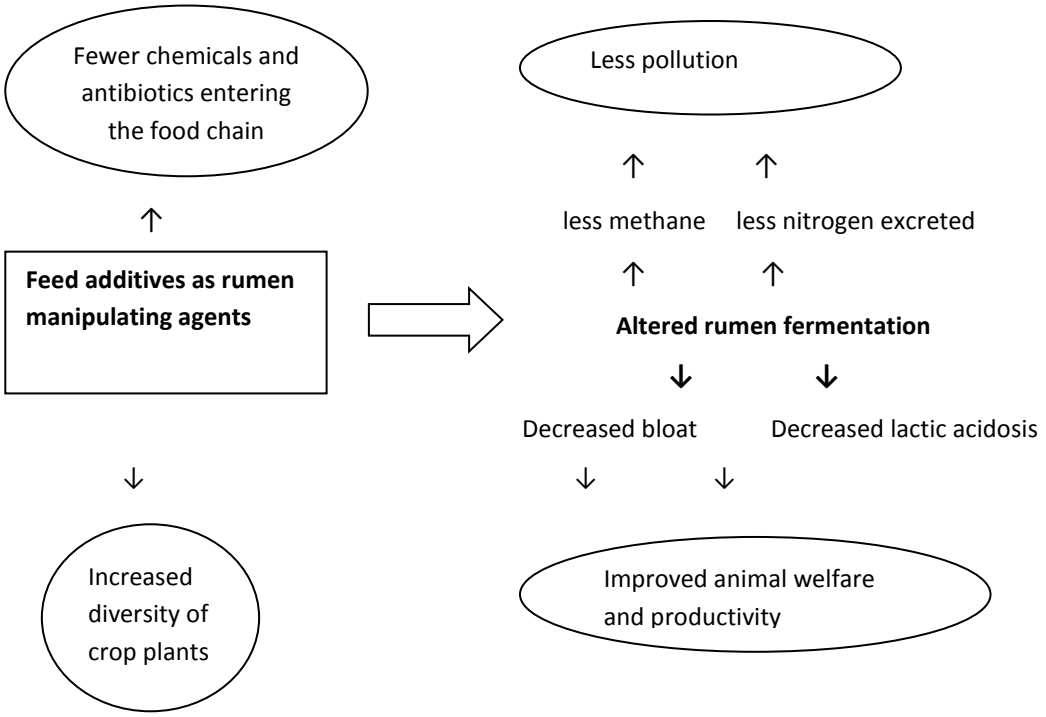


Figure 3. Beneficial effects of dietary manipulation on ruminant performance, welfare and the environment (Wallace, 2004).

As seen in Fig 3, increased use of natural products like essential oils (Jouany and Morgavi, 2007) saponins (Holtshausen *et al*., 2009), may result in less chemicals and antibiotics entering the food chain and there will be increased diversity of plants. Feed supplements such as essential oils and yeasts can increase ruminal pH preventing sharp declines in pH which occur due to sudden introduction of high amounts of concentrates thus help to prevent bloat and subsequent laminitis which compromises animal welfare (Wallace, 2004). Saponins and essential oils may reduce nitrogen and methane losses to the environment causing less environmental pollution (Jouany and Morgav, 2007).

2.7.1 Use of essential oils to mitigate methane production

Essential oils are secondary plant metabolites which reduce methane production by their toxic effect on ruminal bacteria, but optimal doses vary from compound to compound. Some authors (Jouany and Morgavi, 2007) have indicated optimal doses are influenced by the pH and diet type. There is however a considerable number of essential oils and very little is known about how they work. A study by Agarwal *et al*., (2009) showed that some essential oils like peppermint exert their effect by inhibiting ruminal bacteria, fungal organisms and methanogens.

Currently there are very few studies that have examined the effects of essential oils on methane production and most studies have been *in vitro*. A review by Benchaar and Greathead (2011) and a study by Agarwal *et al*., (2009) indicated that the challenge still remains of identifying essential oils that are able to modify rumen fermentation towards less methane output without compromising feed digestibility and animal productivity. A study by Macheboeuf *et al*., (2008) showed that the effects on methane output depend on the type of essential oil and dosage used. For example, Macheboeuf *et al*., (2008) observed 98% methane inhibition with carvacrol supplemented at 400 mg/40 ml of

rumen fluid. Agarwal *et al*., (2009) observed 20, 46 and 76% methane inhibition when peppermint *Mentha piperita was* supplemented at 0.3, 1 and 2 µl /ml of incubation media respectively. On the other hand, supplementation of an essential oil, Crina ruminants® at 1 g/d had no effect on daily methane emissions of beef heifers (Beauchemin and McGinn, 2006a) probably due to the low dose given. This is the major limitation in the use of essential oils as the effective dosage for methane mitigation varies from oil to oil and is also greatly influenced by the environmental conditions under which the plants are grown. Another limitation is the negative effect on fibre digestibility; for example a study by Beauchemin and McGinn, (2006a) in which only 1 g/d/heifer of Crina ruminants® essential oil was used reported that DM digestibility was reduced by 7%.

2.7.2 Use of saponins to mitigate methane production

Saponins are glycosides which have a sugar moiety linked to either triterpene (30 carbon atoms) or a steroidal aglycone (like cholesterol and phytosterols) (Wallace, 2004). Saponins exert their effects by being toxic to protozoa (Mao *et al*., 2010; Hart *et al*., 2008). Some authors have suggested that saponins may also have a direct effect on the methanogens (Patra and Saxena, 2010). Effects of saponins on methane production have been variable (Xu *et al*., 2010), being dependent on the type of basal diet, with positive results exerted when high concentrate based diets have been used (Patra and Saxena, 2010). Like other supplements, effects also depend on the concentration of the saponins (Hart *et al*., 2008). Effects are also optimal when ruminal pH is around 5.5 (Jouany and Morgavi, 2007). Other studies (Benchaar *et al*., 2008) have suggested that high dosage levels are needed to have an effect on protozoa.

Some studies (Holtshausen *et al*., 2009) have observed that a reduction in methane production due to saponins was due to the negative effects of saponins on NDF digestibility. For example, in a study by Holtshausen *et al*., (2009) *Yucca schidigera* and

Quillaja saponaria saponins supplemented to dairy cows at increasing doses of 15, 30 and 45 g/kg DM reduced NDF digestibility in a dose dependant manner which correlated with the reduction in methane production, and when a lower dosage of 10 g/kg DM was used, whole tract digestibility and methane production were unaffected by supplementation.

Intra ruminal infusions of *Yucca schidigera* and *Quillaja saponaria* given at 14 ml twice daily in adult sheep failed to reduce methane production (Pen *et al*., 2007). Xu *et al*., (2010) also studied the effects of *Yucca schidigera* saponins on a wide range of basal diets ranging from forage only to high concentrate diets in a 24 h *in vitro* study, and reported that the saponin lowered methane production in all the basal diets without any adverse effects on fibre digestibility, although further studies with animals were required. The study contradicts the assertion by Patra and Saxena, (2010) that saponins lower methane production only when used on high concentrate diets. On a 40% concentrate diet tea saponins supplementation at 3 g/d were able to reduce methane production by 28% (L/kg DM intake) in adult sheep (Mao *et al*., 2010).

2.7.3 Use of tannins to mitigate methane production

Tannins are water soluble polyphenolic polymers of two types, hydrolysable and condensed (Rochfort *et al*., 2008; Patra and Saxena, 2010). Tannins inhibit growth of methanogens and reduce protozoa numbers (Tan *et al*., 2011). Supplementation of tannins to ruminant diets should only be at 5% of the total diet as high inclusion rates negatively affect intake and digestion (Patra and Saxena, 2010). Two benefits of tannins are that they reduce incidence of bloat when certain legumes such as Lucerne and white clover are consumed, by forming complexes with dietary proteins and thus protect proteins from microbial ruminal degradation and so increase the flow of proteins to the duodenum which is beneficial to animals particularly high producing dairy cows (Patra and Saxena, 2010; Sinclair *et al*., 2009).

Previous studies have shown that effective tannin concentrations needed to reduce methane production vary with the basal diet used. A study by DeOliveira *et al*., (2007) found that sorghum containing 0.2 g/kg DM of condensed tannins when supplemented with concentrates reduced methane emissions in beef cattle while a higher concentration of 1 g/kg DM of condensed tannins when supplemented with urea was not effective. Supplementation of quebracho (91% tannin level) was not effective in reducing methane output in the study of Beauchemin *et al*., (2007a). Under grazing conditions, *Lotus corniculatus* and Sulla and the herb Chicory are reported to be effective in reducing methane emissions (Ramírez-Restrepo and Barry, 2005). Extracts of condessed tannins and saponins are commercially available but are still very expensive (Eckard *et al*., 2010) which may limit uptake by farmers. The major concern with tannins and saponin supplementation is the adaptability of the ruminal organisms to the compounds, although this has never been tested *in vivo* (Hart *et al*., 2008; Patra and Saxena, 2010).

2.8 Use of ionophores to mitigate methane production

Ionophores have previously been used in dairy animals in the European Union as feed additives to improve nitrogen and energy efficiency (Martineau *et al*., 2007)**.** The common ionophores used were monensin and lasalocid. In 2006, under regulation EC 1831/2003, the European Union affected a ban on ionophores and to date, ionophores are not used as feed additives (Jouany and Morgav, 2007). According to RUMA (Responsible Use of Medicines in Agriculture, 2005) guidelines, in the UK, monensin has been licensed to be used in growing cattle but not in milking and dry cows. However, in most countries including USA, Australia and Canada, some ionophores have been approved for use in food animals (Odongo *et al*., 2007). Canada and USA approved monensin for use in lactating dairy cows for the purpose of improving milk production and controlling subclinical ketosis (Erasmus *et al*., 2008).

2.8.1 Mode of action of ionophores

Ionophores are believed to produce positive effects to both the animals and the environment by altering rumen fermentation (Guan *et al*., 2006). Of particular importance is their effect on energy balance which subsequently affects methane production (RUMA, 2005). The mode of action of ionophores like monensin is that they promote proliferation of gram negative bacteria in the rumen by altering the ion transport system across the membrane of gram positive bacteria which then result in cell death (Odongo *et al*., 2007). According to Ipharraguerre and Clark, (2003), gram negative bacteria eg *Selemonas*, acetic acid bacteria and *E coli*, result in greater production of propionate and succinate thus helping to reduce methane production, with the gained energy being channelled to increase productivity. Furthermore lactate producing bacteria like *Streptococcus bovis* are very sensitive to ionophores, so in some cases the ionophores are used in preventing lactic acidosis (Odongo *et al*., 2007).

2.8.3 Effects of monensin supplementation on methane production and productivity

Monensin has been widely used as a feed additive to improve feed conversion efficiency and to control bloat (Grainger *et al*., 2008) but very few studies have focused specifically on the use of monensin to mitigate methane production (Grainger *et al*., 2010b). To date effects of monensin on animal performance are not clearly established. For example, contrary to the expectations of improving milk production in dairy animals, a number of studies (Grainger *et al*., 2008; Grainger *et al*., 2010b) have reported a lack of effect. When supplied at the recommended supplementary levels of 24 mg/kg DM intake and above (Alzahal *et al*., 2008) milk production did not improve. Only a few studies Arieli *et al*., (2008) and Phipps *et al*., (2000) have shown that milk production is improved when cows

are supplemented with monensin in early lactation. The disparities in results led Erasmus *et al*., (2008) to suggest that effects of monensin on milk production may be diet related.

Results of effects of monensin on methane production are also conflicting. McGinn *et al*., (2004) and Odongo *et al*., (2007) reported a 7% reduction in methane output (g/d) and persistent effects after 6 months of supplementation. In another study, Guan *et al*., (2006) supplemented steers with either monensin at 33 mg/kg DM or by a two week rotation of two ionophores, monensin and lasalocid with lasalocid supplied at 36 mg/kg DM, and observed that methane output was reduced by 25% (L/kg DM intake and % GE intake) at 2 and 4 weeks of supplementation, but effects were not sustained, and by 8 weeks of supplementation, methane production was unaffected. From this finding Guan *et al*., (2006) established that effects of ionophores on methane production may be temporal. On the other hand, Grainger *et al*., (2010b) supplemented grazing cows with a high dosage of monensin at the rate of 471 mg/cow/d added to the concentrate portion of the diet and reported no effect on methane production, milk production or DM intake. The persistence of effects was tested and results after 12 weeks of supplementation remained unchanged. According to Grainger *et al*., (2010b), the result could have been influenced by the way monensin was delivered which was by top dressing of the basal diet causing a single dose supply, while most previous studies supplied monensin as a rumen controlled release capsule. Grainger *et al*., (2010b) therefore concluded that monensin supplementation cannot be used as a strategy for reducing methane production in grazing cows. In another grazing trial by Grainger *et al*., (2008), cows were given a monensin releasing capsule with a release rate of 240 mg/d or 14.5 mg/kg DM intake, and it was observed that methane production and milk production were unchanged with monensin supplementation.

2.9 Grazing ruminants to reduce methane output and impact on productivity

Allowing ruminants to graze may improve their welfare (Charlton *et al*., 2011) and pastures are considered a cheap source of nutrients (Bargo *et al*., 2003). Currently there has been renewed interest to understand effects of grazing on productivity and methane production. However animals on pasture tend to have low productivity and according to Bargo *et al*., (2003), the low productivity of ruminants on pasture is attributed to the low DM intake which cannot support high yielding cows. The management practices adopted by farmers may also have an impact on milk yield and possibly on methane output (Hindrichsen *et al*., 2006). One factor that may influence yield is the pasture allowance. Table 9 shows results of a study by O'Neill *et al*., (2012) which examined the effect of grazing cows on pastures with varying pasture allowance on productivity and methane production.

Table 9. Effect of pasture allowance with or without supplementation on milk yield and methane production (O'Neal *et al*., 2012)

	Low PA + PMR	Low PA	High PA
Pasture allowance, kg DM/cow	15	15	19
Grass DM intake, kg/d	12.5[a]	13.9[b]	14.9[b]
PMR DM intake, kg/d	3.9	0	0
Total DM intake, kg/d	16.5[a]	13.9[b]	14.9[b]
Milk yield, kg/d	17[a]	13.1[b]	14.6[c]
CH_4 output, g/d	406[a]	349[b]	384[c]
CH_4 output, g/kg DM intake	25	25	26
CH_4 output, g/kg milk yield	23.9	24.9	24.5

[a,b] Within a row, means with different superscripts significantly differ
Low PA= low pasture allowance, High PA= high pasture allowance, PMR= Partial mixed ration

As shown in Table 9, cows were allocated to grazing fields that had either low or high pasture allowance of 15 and 19 kg DM/cow respectively. Cows on low pasture allowance were grazed with or without 4 kg/cow/d of partial mixed ration (PMR), while those on high pasture allowance were un-supplemented. Milk yield and daily methane output (g/d) increased with an increase in total DM intake. However when methane output was

expressed as g/kg DM intake and g/kg milk yield, no differences were found among the three treatments. Pasture allowance had no effect on methane output. What was established by O'Neill *et al*., (2012) was that pasture allowance with or without PMR supplementation had no effect on methane production when expressed as unit of DM intake or g/kg milk yield.

Another study by O'Neill *et al*., (2011) compared the productivity of cows under two management systems; a grazing system and total TMR based system. One group of cows was kept indoors and had access to TMR throughout the study period while the other was grazed on pastures on a rotational basis with a pre-grazing herbage mass of 1400 kg DM/ha. The TMR fed cows performed better in terms of DM intakes (19.7 vs. 14.3 kg/cow/d), and milk yields (29.5 vs. 21.1 kg/cow/d). Subsequently TMR fed cows produced higher methane emissions (397 vs. 251 g/d and 20.3 vs. 18.1 g/kg DM intake and 6.5 vs. 5.7 % GE intake) regardless of the unit of expression when compared to the grass fed group. The methane results further emphasis the point that methane output is driven by DM intake, as increasing DM intakes increases intake of fermentable material which result in increased volumes of methane output. Due to the difference in DM intake between treatments, it is not possible to determine whether grass has an inherent ability to reduce methane production. In the study by O'Neill *et al*., (2011), the crude protein content of grass was 240 g/kg DM while that of TMR was 160 g/kg DM and OM digestibility was also 98 g/kg higher in the grass than in the TMR which could have contributed to differences in methane production.

2.9.1 Influence of herbage mass on methane production

Good grazing management can help to mitigate methane production. One factor is the herbage mass. A study by Wims *et al*., (2010) examined the impact of varying herbage

mass on productivity and methane output in mid lactation dairy cows. Cows were allocated to two treatments of either low herbage mass of 1000 kg DM/ha or high herbage mass of 2 200 kg DM/ha.

Table 10. Effects of varying herbage mass on DM intake, milk yield and methane production in dairy cows (Wims *et al*., 2010)

	Low	High
Herbage mass, kg DM/ha	1000	2200
CP levels, g/kg dry matter	275	211
NDF levels, g/kg dry matter	479	497
Dry matter intake, kg/d	14.6	14.6
milk yield, kg/cow/d	18	17
CH_4 output, g/d	278[a]	320[b]
CH_4 output, g/kg DM intake	19.2[a]	22.3[b]
CH_4 output, g/kg milk yield	16.4[a]	19.9[b]
CH_4 output, % GE intake	6.4[a]	7.4[b]

[a,b] within a row, means with different superscripts significantly differ

From Table 10, it is clear that DM intake of the cows was unaffected by the herbage mass. Methane output regardless of the unit of expression was lower on the low herbage mass principally because the grass was of higher quality when compared to the high herbage mass. This is evidenced by the higher CP levels (275 g/kg DM) and low NDF levels associated with the low herbage mass when compared to 211 g/kg DM CP levels associated with the high herbage mass. According to Wims *et al*., (2010), high grazing intensity results in low herbage mass because grass tend to be less mature and more digestible and decreases methane production.

2.9.2 Increasing sugar content of grass and the impact on productivity and methane production

Research involving ways of improving the water soluble carbohydrate content of grass is receiving a lot of attention due to the potential positive impact it can have on reducing N losses into the environment (Ellis *et al*., 2012). Miller *et al*., (2001) demonstrated that feeding cows a high water soluble (WSC) grass improved utilisation of protein in the

rumen and consequently resulted in less N being released into the environment, and milk yield also increased by 2.7 kg/cow/d. Lee *et al*., (2001) examined the impact of increasing WSC content on live weight gain in growing lambs and observed that lambs that were grazed on pastures with 33 g/kg DM higher WSC content gained an average of 34 g/d extra weight. In another study, Trevaskis *et al*., (2004) allocated cows to two grazing times, in the morning or in the afternoon to optimise WSC concentration. The afternoon pastures were 52 g/kg DM higher in WSC concentrations than the morning pastures. Cows that grazed in the afternoon produced 2.1 kg/d more milk and had a higher milk protein yield and higher live weight change when compared to those that were grazed in the morning (Trevaskis *et al*., 2004).

The impact of WSC on methane production is unclear. Most studies have focused on improving WSC content of grasses with the subsequent aim of improving intake and productivity and reducing N losses into the environment. Taweel *et al*., (2005) hypothesised that an increase in WSC content of grass has the potential to shift fermentation patterns in the rumen towards propionate production and thus would result in less methane production. Ellis *et al*., (2012) used a mechanistic model to predict that CH_4 production tended to increase with an increase in WSC levels in grass. However when results were expressed as a unit of milk yield, variable results were observed and in some cases, reductions in methane output were observed. According to Molle *et al*., (2008), the WSC content of grass is normally in the range 50-200 g/kg DM depending on species and variety. New breeding techniques can produce grass varieties with WSC contents of between 200–400 g/kg DM (Lee *et al*., 2001). Only a few previous studies have examined the effect of grazing ruminants on pastures containing high WSC concentrations on methane production and results are not conclusive. For example, in a study by Kim *et al*., (2011), growing lambs fed grass containing a high WSC content of 42

g/kg DM higher than the control produced 17% (L/kg DM) and 25% (L/kg live-weight gain) lower methane production when compared to those fed a control diet. Studies conducted in dairy cows showed that high WSC concentrations may not be effective in reducing methane production. For example, in a study by Staerfl *et al*., (2012), methane production expressed as g/d and g/kg DM intake did not differ when two groups of dairy cows fed grass that differed in WSC concentrations by 90 g/kg DM.

2.9.3 Grazing and the impact on the fatty acid profile of cow's milk

It is widely known that pasture grass contains high levels of unsaturated fatty acid (PUFAs), particularly α-linolenic acid although the composition varies with harvest date (Atti *et al*., 2006). According to Clapham *et al*., (2005), the diet of ruminants is reflected in the fatty acid profile of the product so the diet of ruminants can be manipulated to produce milk and meat which is healthier for human beings. Table 11 shows the variations in fatty acid profile of perennial ryegrass at 3 different harvest dates.

Table 11. Concentration of fatty acids in perennial ryegrass at 3 harvest times (Clapham *et al*., 2005)

			mg/g of DM						
harvest	lauric	myristic	Palmitic	palmitoleic	stearic	oleic	linoleic	α-linolenic	Total
6 weeks	0.027	0.62	6.99	0.94	0.30	1.46	6.76	34.7	51.8
9 weeks	0.046	0.62	6.35	0.74	0.32	1.01	5.74	31.5	46.3
12 weeks	0.072	0.61	5.91	0.56	0.28	0.71	5.47	26.8	40.5

Harvest dates were calculated from the day of seeding

As can be seen from Table 11, α-linoleic acid contributes on average 60% of the total fatty acids in ryegrass. It is clear that FA composition decreases with increasing maturity of the grass. For example, the α-linolenic acid content was highest at 6 weeks after seeding with 34.7 mg/g DM, reduced to 31 mg/g DM at 9 weeks and reduced further to 26.8 mg/g DM by 12 weeks. When ruminants have access to fresh grass, there is increased availability of

polyunsaturated fatty acids from the grass that make up the FA composition of milk (Bauman and Griinari, 2003). For example, a study by Renna *et al*., (2012) examined the milk FA profile of goats when they were abruptly moved from an indoor hay and concentrate based diet to a total grazing system where they had access to fresh grass only. The α-linolenic acid composition in hay was 33% of the total FAs while the concentrate was composed of 57% linoleic acid FA and the fresh grass had 63% of α-linolenic acid. Grazing caused the short and medium chain fatty acids in milk to decrease in concentration by 17 and 33% respectively while $C_{18:2}$ *cis*-9, *trans*-11, C18:2 *trans*-7, *cis*-9 and $C_{18:2}$ *trans*-8, *cis*-10 CLA increased by an average of 260% and α-linolenic acid concentration increased by 93%. In another study by Atti *et al*., (2006), cows that were grazed on either green barley grass or rye grass were compared to those confined to a feedlot. The milk fatty acid profile of the three groups was such that no change was observed in the short chain fatty acid profile ($C_{4:0}$ to $C_{10:0}$). The medium chain fatty acid $C_{12:0}$ and $C_{14:0}$ concentrations were higher in the feedlot group when compared to concentrations in those that were grazed. The long chain fatty acids $C_{18:0}$, CLA's and α-linolenic acid were increased in the grazing groups when compared to the feedlot group. The *cis*-9, *trans*-11 $C_{18:2}$ content was 2.4 g/kg in the feedlot group compared to 7.3 and 10.3 g/kg in the two grazing groups, while α-linolenic acid concentrations were increased from 2.7 g/kg in the feedlot cattle to 4.7 and 4.4 g/kg in the grazing groups. Fig 4 shows the metabolic pathways of the C_{18} fatty acids in the rumen. Unsaturated fatty acids in grass undergo extensive hydrogenation in the rumen to form C_{18} FAs and their intermediate products (Gomez-Cortes *et al*., 2009). The conjugated linoleic acids are transported to the mammary gland where they inhibit *de novo* synthesis of short and medium chain fatty acids, $C_{8:0}$ to $C_{16:0}$ (Bauman and Griinari 2003).

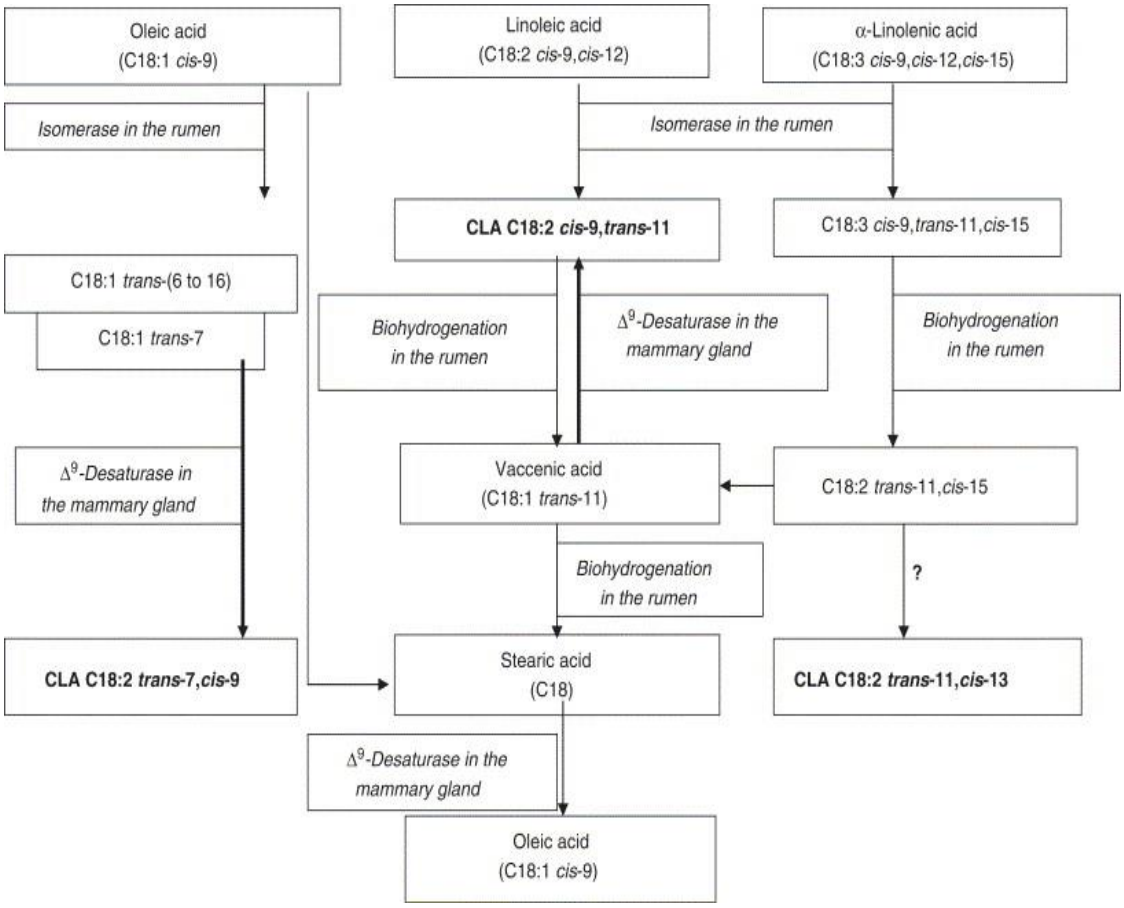


Figure 4. Impact of diet on milk fatty acid profiles (Collomb *et al*., 2006)

According to Kay *et al*., (2004), concentrate diets and plant oils such as sunflower oil that have high concentrations of linoleic acids produce *cis*-9, *trans*-11 CLA, *trans*-10, *cis*-12 CLA and vaccenic acid as intermediate products of ruminal bio-hydrogenation while bio-hydrogenation of α-linolenic acid from pasture produces vaccenic acid as the main product. The important source of cis-9, *trans*-11 CLA in grazing cows is via desaturation of vaccenic acid in the mammary gland through the action of Δ^9 desaturase.

2.10 Other methane mitigation measures that can be applied at farm level

Other mitigation measures that can be applied on a dairy farm in order to reduce methane production include; genetic selection for high milk production, improved fertility rates and health, lower culling rates, reduced age at first calving which can subsequently

reduce on the replacement rates and eventually lead to reduced methane production (Wall and Moran, 2010; Knapp *et al*., 2014). According to Knapp *et al*., (2014), within the United Kingdom, replacement stocks account for about 25% of the whole herd. Therefore, reductions in culling from 35% to 30%, coupled with reduction in age at first calving from 26 to 24 month has potential to reduce herd methane emissions by 4.6%. Similarly, Wall *et al*., (2010) using a prediction model showed that increasing number of lactations in a dairy herd has potential to reduce methane emissions by 4.4%. Beauchemin *et al*., (2011) did a whole life cycle assessment study using a whole farm model. The study was conducted over a period of 8 yrs. The impact of increased longevity and improved reproductive performance on greenhouse gas emissions was assessed. Increasing longevity of cow herds by a year and increasing calf survival rates from 85 to 90%, increased CH_4 emissions because more calves were born and an increase in weaning rates also increased CH_4 emissions, but in both cases GHG intensity was lowered by 1% and 4% respectively. Another study by Vellinga *et al*., (2011) using a dairy farm simulation model, analysed data from 70 dairy farmers in order to assess impact of various farm mitigation strategies on methane production. It was observed that reducing the dairy cow replacement rate by 5 to 9% reduced GHG emissions by 20-30 g CO_2 per kg of milk.

Increasing heat detection rates among dairy cows can also reduce methane emissions at farm level. A Defra study (Chadwick *et al*., 2007) on the implications of mitigation measures on long term national methane emissions highlighted a number of stratergies using a model and reported that among other measures, an increase in heat detection rates was able to reduce methane emissions by 7%. Similarly, a simulation study by Del Prado *et al*., (2010), observed that increased cattle fertility rates through genetic improvement was able to reduce GHG emissions per litre of milk by 5%. Garnsworthy

(2004) using a model also predicted that when dairy cow fertility rates are improved to 1995 levels, methane production would reduce by 11%.

It may also possible to select for animals that have a high feed efficiency. A review study by Waghorn and Hegarty, (2011) highlighted the importance of improving feed conversion efficiency as it can lower total herd methane emissions as less feed is used to produce animal products and thus less amount of methane per animal product.

2.11 Measurement of methane emissions from ruminants

Approximately 87% of rumen methane emitted by ruminants is belched out, while 13% comes through the hindgut (Munoz *et al*., 2012). To reduce methane emissions from ruminants, it is important to accurately measure methane production under different management strategies. Two widely used methods of estimating methane output from ruminants are respiratory chambers and sulphur hexafluoride (SF_6) tracer techniques (Boadi and Whittenberg, 2002).

2.11.1 Respiratory chambers

Respiratory chambers measure the rate of methane emission from the animal which comes from both rumen and hind gut fermentation. The chamber technique has been the common technique to measure methane missions (Boadi *et al*., 2002). According to Boadi *et al*., (2002), animals have to be confined and the eructated gas and the gas produced by hindgut fermentation quantified and an indirect method is used to determine methane production. The respiratory chamber technique has its limitations. It requires large capital costs to set up and maintain and also requires prior training of the animals (Bhatta *et al*., 2007). The major limitations with the chamber technique are that for measurements to be taken, animal movements are restricted as they are put in a

confined environment which tends to affect feed intake and methane output (Beauchemin and McGinn, 2005; Johnson and Johnson, 1995). Beauchemin and McGinn, (2005) observed reductions of 22 and 31% in DM intake during the period feedlot cattle were in the chambers. Losses were attributed to the stress associated with sampling and the decreased energy expenditure. Beacheuchemin and McGinn, (2006) also observed 15-25% reductions in DM intake with beef cattle when using respiratory chambers.

2.11.2 The SF_6 tracer technique

The SF_6 tracer technique (Johnson *et al.,* 1994; McGinn *et al.*, 2006) measures respired and eructated methane but does not account for the losses through the rectum (Munoz *et al.,* 2012). The method involves placing a small permeation tube of known permeation rate of SF_6 in the rumen. Gas samples are then collected through a capillary tube connected to a collection canister on the neck or back of the animal and the methane levels are calculated using the known rate of SF_6 release (Johnson and Johnson, 1995). The technique allows animals to be housed or grazing in their normal environment and a large number of animals can be sampled at the same time (Boadi and Whittenberg, 2002). The SF_6 tracer technique is a relatively new procedure which has advantages when compared to the respiratory chamber technique, including being cheaper and easier to use. The SF_6 tracer technique was validated first by Johnson *et al*., (1994) and later by Boadi *et al*., (2002). Boadi *et al*., (2002) reported no difference in mean daily methane production (L/d) when the SF_6 tracer technique and the respiratory chamber technique were both tested on beef heifers.

Previous studies have indicated that the SF_6 tracer technique may have some limitations. A study by Munoz *et al*., (2012) compared the SF_6 tracer and the respiratory chamber techniques by measuring methane output of cows using both techniques simultaneously

and found that methane output was similar with both techniques. However, the correlation of the results obtained in the two techniques varied with the units of expression of methane output. When methane output was expressed as g/d there was a correlation of 0.69 and when expressed as g/kg DM intake, the correlation was only 0.64. A strong correlation of 0.88 was obtained when results were expressed as g/kg milk yield. According to Munoz *et al*., (2012), the variation in results was as a result of the reduction in the release rate of the SF_6 in the rumen over time. In the study, the SF_6 permeation tubes were recovered from the rumen of the cows and the post-experimental released rates were determined and compared with the pre-experimental SF_6 release rate. Results showed that release rates decreased to as low as 66% of the initial release rates. Due to this, calculated methane output increased with time. Pinares-Patino *et al*., (2011) also reported variation in methane output results when they compared the SF_6 tracer technique to the respiratory chamber technique. Emission rates of the SF_6 permeation tubes also decreased with time and resulted in overestimation of methane output results.

The other limitation with the SF_6 tracer technique is that SF_6 is released intermittently in the breath of an animal, and confined animals are the most affected (Pinares-Patino *et al*., (2011). The variations in SF_6 are noticed when hourly sampling of gas is done and less noticed when sampling is done in periods of 24 h. Finally, the SF_6 technique does not measure post-ruminal methane production which the chamber technique accounts for. McGinn *et al.* (2006) compared the chamber and SF_6 techniques on cattle kept in similar environments and found that they produced comparable results, however the SF_6 technique was found to underestimate methane emissions by 4%, thought to be the result of post ruminal fermentation.

2.10.3 Indirect measurement of methane production from milk fatty acid profiles

Several previous studies have attempted to establish a relationship between concentrations of FAs in milk and amounts of methane released by cows with the objective of being able to use milk FA concentration to predict methane output. Dijkstra *et al*., (2011) observed that milk FAs are potential indicators of amounts of methane produced by ruminants. Chilliard *et al*., (2009) produced predictive regression equations which can be used to determine amounts of methane output from FA concentrations in milk. The predictive equations failed to address concerns where supplementation changes milk FA profiles without any changes in methane output. Dijkstra *et al*., (2011) used data from 3 experiments and examined the relationship between milk FA concentrations with amounts of methane released. In their studies, four fatty acids, $C_{8:0}$, $C_{10:0}$, $C_{15:0}$ and $C_{16:0}$ were found to have positive correlation with methane output. In another study (Chilliard *et al*., 2009), the FAs $C_{8:0}$ and $C_{16:0}$ showed positive correlation with methane production with R^2 values of 0.81 and 0.82 respectively, while the FA $C_{18:0}$ showed negative correlation with methane production and had an R^2 value of 0.88. The equation by Chilliard *et al*., (2009) is as follows:

CH_4 output (g/d) = –100.8 (±22.0) × milk trans-16+*cis*-14 $C_{18:1}$ (% of total FA) + 6.78 (±1.75) × milk $C_{16:0}$ (% of total FA) + 13.1 (±3.86) × forage intake (kg of DM/d) – 80.1 (±60.9) (Chilliard *et al*., 2009)

Mohammed *et al*., (2011) supplemented dairy cows with crushed oil seeds of sunflower, flax or canola at 3.3% DM and used the feed intake data, milk FA concentrations and rumen fermentation parameters to formulate predictive equation for determining methane production. The best regression equation (R^2=0.90) that was produced incorporated concentrations of *cis*-9 $C_{17:1}$ and iso-$C_{16:0}$ and the total *entodiniomorphs* count. The equation was

CH_4 (g/d) = −910.8 (±156.7) × milk *cis*-9-$C_{17:1}$ + 331.2 (±88.8) × milk $C_{16:0}$ iso + 0.0001 (±0.00) × total *entodiniomorphs* count + 242.5 (±39.7).

A comparison of equations predicted by Chilliard *et al*., (2009) and Mohammed *et al*., (2011) show that both recognised concentrations of the FA $C_{16:0}$ to be correlated to daily methane production. The notable difference was that while Chilliard *et al*., (2009) used forage DM intake, whereas *et al*., (2011) used total *entodiniomorphs* count as part of the equation. Mohammed *et al*., (2011) was of the view that milk FAs concentrations cannot be used singly to determine methane production. Major challenge of the predictive equations is the lack applicability of the equations across a range of dietary conditions.

2.10.4 Summary of literature review

Methane emissions from ruminant animals are dependent on the quantities of feed consumed and the composition of the diet. Dietary manipulation of feed is therefore one of the most promising ways of reducing emissions and a number mitigation measures have been reviewed. From previous studies, oil supplementation and increasing concentrate levels have shown great potential to reduce methane emissions while having a positive impact on productivity which is desirable. Additionally the inclusion of grass which is high in WSC and α-linolenic acid offers the potential to reduce methane production although few studies have been reported in this area.

2.10.5 Hypothesis and Objectives of the current project

The hypothesis to be tested is that manipulation of the ruminant diet will result in reductions in methane emissions and improvement in animal performance. The aims and objectives of the study are to determine the effects of manipulating the diet including altering the concentrate starch levels, different starch sources, supplementation with oils and inclusion of grazed grass in the diet on rumen

fermentation and methane production *in vitro* and *in vivo.* The second objective was to determine the effect of feed manipulation on animal performance, milk fatty acid profile and to determine the relationship between fatty acid concentrations in milk with the amount of methane produced.

CHAPTER 3 General materials and methods

3.1 Proximate analysis of samples

3.1.1 Dry matter determination

Dry matter content of the basal diets and faecal samples was determined according to Association of Official Analytical Chemists (AOAC, 2000; 934.01). Subsamples were accurately weighed and then dried in an oven (Binder, Cole-Palmers, UK) at 105°C overnight. Samples were cooled in a desiccator and reweighed. Dry matter (DM) was calculated as follows

$$\text{DM (g/kg)} = \frac{\text{Weight (g) of sample after oven drying}}{\text{(Weight (g) of sample before oven drying)}} \times 1000 \qquad \text{Equation 3.1.1}$$

3.1.2 Organic matter and ash determination

Samples of dried feed and faecal samples were analysed according to Association of Official Analytical Chemists (AOAC, 2000) for ash (942.05). Approximately 5 g of previously dried samples was accurately weighed into labelled porcelain crucibles. Samples were ashed in a muffle furnace (Carbolite® AAF 1100, Hope Valley, UK) for 4 h at 550°C, cooled in a desiccator and reweighed. Ash content was calculated as:

$$\text{Ash content g/kg DM} = \left\{\frac{\text{Weight (g) of ash}}{\text{Weight (g) of dry sample before ashing}}\right\} \times 1000 \qquad \text{Equation 3.1.2}$$

Organic matter (OM, g/kg DM) was calculated as 1000 minus ash content (g/kg DM).

3.1.3 Crude protein (CP) determination

Samples were analysed according to Association of Official Analytical Chemists (AOAC, 2000) for crude protein (AOAC, 988.05) by use of a Leco FP 528 auto analyser (Leco Corp, Stockport, UK). Samples of dried feed were milled with a Delongh KG 79 (Freemans PLC,

Sheffield, UK) to pass through 1 mm mesh and 0.5 g of sample accurately weighed into aluminium foil and placed into the auto analyser. Crude protein (CP) levels in samples were calculated as

CP (g/kg DM) = Nitrogen content (g/kg DM) X 6.25 Equation 3.1.3

3.1.4 Neutral detergent fibre determination

Samples of dried feed were analysed for neutral detergent fibre (NDF) according to the method by Van Soest *et al*., (1991). The working standards were; neutral detergent solution prepared by adding 93 g of di-sodium ethylene diamine tetra acetic acid dehydrate (EDTA), 34 g sodium tetraborate ($Na_2B_40_7.10H_2O$), 150 g sodium dodecyl suphate (SDS), 50 ml of tri-ethylene glycol, 22.8 g anhydrous disodium hydrogen phosphate (Na_2HPO_4) to make 5 L solution with distilled water and pH adjusted to approximately 6.9-7.1. Alpha amylase solution was prepared by dissolving 2 g of α-amylase (α-1, 4-glucan 4-glucanohydrolase, Enzyme # 3.2.1.1, ~80EU/mg) from *Bacillus subtilis spp* in 90 ml distilled water followed by addition of 10 ml of tri-ethylene glycol. To determine NDF, 0.4 to 0.6 g each of previously dried samples was accurately weighed into ceramic crucibles. Crucibles were tightly fitted onto the Fibertech® 1020 hot and 1021 cold extractor (Foss UK Ltd, Cheshire, UK) making sure valves were in the closed position. Cold neutral detergent reagent (25 ml) and a few drops of Octanol, reagent grade (Sigma Aldrich, Dorset, UK) were added to each of the samples. The heat control knobs were turned to full and as the samples started boiling, heat was reduced. Samples were digested for 30 min after which the heat was switched off. Another 25 ml of cold neutral detergent reagent and 2 ml of α-amylase solution were added and samples brought to the boil and digested for a further 30 min. Samples were then filtered and washed with 20-30 ml of hot distilled water (80°C) to remove all neutral detergent reagent. A further 2 ml of

α-amylase solution and 25 ml of hot distilled water (80°C) were added to the samples and allowed to stand for 15 min before filtering and washing 3 times with hot distilled water. Crucibles were removed from the Fibertec® hot and cold extractor and dried overnight at 105°C. Crucibles were then cooled in a desiccator and weighed. The samples were then ashed in a muffle furnace (Carbolite® AAF 1100, Hope Valley, UK) at 550 °C or 4 h, cooled and reweighed. The weight of NDF in the sample was calculated as;

$$\text{NDF (g/kg DM)} = \frac{\{\text{Weight (g) of oven dried sample - Weight (g) of ash }\}}{\{\text{Weight (g) of sample}\}} \times 1000 \qquad \text{Equation 3.1.4}$$

3.1.5 Determination of whole tract digestibility by the acid insoluble ash method

Whole tract digestibility was determined according to the method described by Van Keulen and Young, (1977). Duplicate samples of 5 g of previously dried feed and faecal samples were weighed into ceramic crucibles. Samples were oven dried overnight at 105°C, reweighed and ashed in a muffle furnace (Carbolite® AAF 1100, Hope Valley, UK) for 4h at 550°C, cooled and reweighed. The ash residue was transferred into kjeldahl digestion tubes (Foss Tecator Digestor Unit, Hilleroed, Denmark) and 100 ml of 2M hydrochloric acid (Fisher Scientific Ltd, Leicestershire, UK) was added. Samples were boiled at 150°C for 5min on the digester unit. After cooling, the hydrolysate was filtered (Whatman® No 41 filter paper, Fisher Scientific Ltd, Leicestershire, UK) and washed with hot distilled water. The filter papers with ash residues were transferred back into the crucibles and ashed for 4h at 550°C. After cooling the crucibles were re-weighed and acid insoluble ash (AIA) calculated as:

$$\%\ \text{AIA} = \frac{\{\text{Weight (g) of crucible +ash- Weight (g) of crucible}\}}{\{\text{Weight (g) of dry sample}\}} \times 100 \qquad \text{Equation 3.1.5}$$

Digestibility (g/kg) of the dry matter of feed was calculated as:

$$\text{Digestion coefficient of DM} = 1000 - 1000 \times \frac{\text{g/kg DM indicator in feed}}{\text{(g/kg DM indicator in faecal)}} \qquad \text{Equation 3.1.6}$$

3.2 Milk sample analysis

3.2.1 Milk compositional analysis

Milk compositional analysis (protein, fat and lactose contents) was conducted using a Milkoscan Minor 78110 auto analyser (Foss Electric, Denmark) that had been calibrated using standard samples (Eurofins®, Wolverhampton, UK). Milk samples were prepared for composition analysis by gently shaking the samples and warming to 40°C for 15 min in a water bath (Clifton® Nickel Electro Ltd, Weston super mare, UK) prior to analysis.

3.2.2 Milk fatty acid profile determination

Milk fatty acid profile determination was conducted by first separating lipids from the milk by centrifugation, followed by trans-methylation process which produced methyl esters for GC analysis. Separation of the lipid layers in milk samples was done using method B as described by Feng *et al*., (2004). Milk samples from individual cows were bulked by correcting for am and pm yields to produce 30 ml from individual cows and placed into 50ml conical plastic tubes. The bulked milk samples were centrifuged (Beckman, Avanti™ 30 Centrifuge, Harbor Boulevard, California) at 17,800 X g for 30 min at 4°C. After centrifugation, milk samples were separated into 3 layers i.e., the top lipid layer, middle protein layer and the bottom water layer. An aliquot about 1g of the top lipid layer was transferred into clear labelled 2.5 ml eppendorf tubes (Fisher Scientific Ltd,

Leicestershire, UK) for subsequent methylation. Methylation of lipids was done according to the procedure by Christie (1982) with a few modifications according to a method by Chouinard *et al*., (1999). Two standard reagents were used for this procedure; methylation reagent and termination reagent. Methylation reagent was prepared by mixing 1.75 ml of methanol (Sigma Aldrich, Dorset, UK) with 0.4 ml of 30% sodium methoxide solution (Sigma Aldrich, Dorset, UK). Termination reagent was prepared by weighing 1g of oxalic acid into a 50 ml reagent bottle and 30 ml of diethyl ether added to the bottle and shaken. Approximately 40 mg of previously extracted lipid sample was weighed into labelled 10 ml extraction tubes. To this 2 ml of hexane and 40 µl of methyl acetate (Sigma Aldrich, Dorset, UK) was added. The tubes were vortexed (FB 15013 Topmix®, Fisher Scientific Ltd, Leicestershire, UK) for 30 sec and 40 µl of methylation reagent was added to each tube. Tubes were then tightly capped and vortexed for 2 min. Samples were left to stand for a further 8 min. Termination reagent (60 µl) was added and the tubes vortexed for another 30 s. Approximately 200 mg of calcium chloride was added to each sample, the tubes votexed again and left to stand for 1 h. The samples were then centrifuged (Rotina 46R, Hettich Lab tech, Tuttlingen, Germany) at 2600 X g for 30 min at 5°C. The solvent layer containing the methyl esters was transferred into labelled GC tubes and stored at -20°C for subsequent analysis.

Fatty acid analysis was conducted using a gas chromatograph (HP 6890, Germany) fitted with an automatic sampler (Agilent 6890 injector), integrator and FID detector (Agilent Inc. Wilmington, DE), equipped with a CP-Sil 88 fused silica capillary column 100 m x 0.25 mm (i.d), 0.2 µm film thickness column (Varian Inc., Walnut Creek, CA). Peaks were routinely identified by comparison of retention times with FAME standards (Sigma-Aldrich, Dorset, UK). Oven temperature was set at a maximum of 225°C, starting with a temperature of 70°C held for 2 min, followed by an increase of 8°C/ min to 110°C, then

increased by 5°C/min to 170°C and finally increased at 4°C/min to 225°C which was maintained until all peaks were analysed.

3.3 Determination of fatty acid content of TMR, grass and concentrate samples

The diets used in the two cow based studies and the grass samples in study 2 (Chapters 5 and 6) were analysed for fatty acid profiles by a method described by Sukhija and Palmquist (1988) using nonadecanoic ($C_{19:0}$) as an internal standard. The standard solutions used to methylate the samples were 10% methanolic HCl (20 ml acetyl chloride/ 100ml methanol) and 2 mg/ml nonadecanoic acid solution (200mg $C_{19:0}$/100 ml heptane). Dried feed and grass samples (0.5 g each) were accurately weighed into duplicate into 15 X 150 mm test tubes. The $C_{19:0}$ standard solution (2 ml) was added to each sample using a pipette, then 3 ml of 10% methanolic HCl was added. The test tubes were tightly capped, vortexed and heated in a 90°C water bath (Clifton, Nickel Electro^{TM}, UK) for 2 h while shaking very 30 min. After 2 h samples were left to cool and 1 ml heptane was added to each sample. Potassium carbonate (K_2CO_3, 10 ml) was slowly added to the test tubes and vortexed again. The test tubes were then centrifuged (Rotina 46R, Hettich Lab tech, Tuttlingen, Germany) for 5 min at 500 X g at 4°C to separate the layers. The organic solvent layers from each tube were transferred into 13 x 100 mm culture tubes, and approximately 1g of sodium sulphate and 0.5 g of activated charcoal added. The solvent layers were transferred into labelled GC tubes and stored at -20°C for subsequent GC analysis. The GC was programmed as described in section 3.2.2

FA were quantified from the chromatograms by removing the standard (C_{19})

$$\text{True \% fatty acid} = \frac{\{\text{Fatty acid \% in data X100}\}}{\{100 - \%\ C_{19}\ \text{in data}\}} \qquad \text{Equation 3.2.1}$$

The FAs (mg per 100g of feed) was calculated as:

Weight of total FA (mg/100 g feed) = $\frac{100}{\%C_{19}}$ X $\frac{\text{Weight } C_{19} \text{ (mg)}}{\text{(Weight of sample)}}$ Equation 3.2.2

3.4 Determination of starch content in feed samples

Starch content of feed samples was determined using a Megazyme starch assay kit (Megazyme International, Ireland). The starch kit had the following standards; Thermostable α-amylase, amyloglucosidase, GOPOD reagent buffer, GOPOD reagent enzymes, D-glucose standard solution and standardised regular maize starch control. Feed samples for analysis were milled to pass through a 0.5 mm screen (Endecotts Ltd, London, UK). Approximately 100 mg of sample was accurately weighed into glass test tubes (16 x 120 mm). The test tubes were tapped to ensure that the entire sample dropped to the bottom of the tube. To each sample, 0.2 ml of aqueous ethanol (80 % v/v) was added to wet the sample and aid dispersion. Samples were then stirred on a vortex mixer. Immediately 3 ml of thermostable α-amylase (contents of bottle 1 diluted 1:30 in Reagent 1; 100 mM sodium acetate buffer, pH 5.0) were added to the tubes and the samples boiled for 6 min. (with vigorous stirring after 2, 4 and 6 min). Amyloglucosidase (0.1 ml) was added, samples vortexed and incubated in a water bath (Clifton®, Nickel Electro, Manchester, UK) set at 50°C for 30 min. After incubation, the volume of the samples was adjusted to 10 ml with distilled water and the tubes centrifuged (Rotina 46R, Hettich, Tuttlingen, Germany) at 3,000 X g for 10 min. An aliquot of 1.0 ml from each sample was diluted to 10 ml with distilled water. Duplicate aliquots (0.1 ml) of the diluted solution were transferred to glass test tubes (16 x 100 mm) and 3.0 ml of GOPOD reagent added to the samples, D-glucose controls and reagent blanks. Tubes were then incubated in a water bath (Clifton®Nickel Electro, UK) at 50°C for 20 min. After 20 min, absorbance was read on a Jenway 6305 spectrophotometer (Bibby Scientific Ltd, Dunmow, UK) set at 610 nm wavelength and readings compared against reagent blanks. The starch content of

the samples was generated by the Mega-Calc® automatically by supplying absorbance and dilution values in the spread sheet using the following formular

Starch (g/kg) = (ΔE x (F/W) x 90) x 100 Equation 3.4.1

Starch (g/kg, dry weight basis) = starch (g/kg) x (100/100 – moisture content g/kg)

Equation 3.4.2

Where ΔE =absorbance read against reagent blank, F = (µg glucose)/absorbance 100 µg glucose; W= weight (mg) of sample, 90= adjustment from free glucose to anhydrous glucose

3.5 Determination of water soluble carbohydrate content of grass samples

Water soluble carbohydrate concentration in grass samples was determined as described by Thomas (1977). The working standard solution was anthrone reagent which was prepared by slowly adding (with constant stirring) 380ml of concentrated sulphuric acid (Fisher Scientific®, Loughborough, UK) to 165 ml distilled water followed by the addition of 0.5 g of thiourea and 0.5 g of anthrone. The solution was stirred until dissolved and left to cool before storage in a tightly stoppered bottle in a fridge at 4°C and was used within four days of preparation. Glucose working standards were prepared by dissolving pure glucose in distilled water to provide four different concentrations of 0.04, 0.08, 0.16 and 0.2 mg/ml glucose. Grass samples were collected by cutting the top 2/3 of the grazing horizon using a pair of scissors. A total of four am and four pm samples were collected. Samples were frozen and kept at -18°C until analysis. Before analysis, samples were freeze dried (Edwards 4K Modulyo freeze dryer, UK) at –50°C for 5 d and milled to pass through a 1 mm sieve (Endecotts Ltd, London, UK). The samples were pooled according to the am and pm sampling times. The milled samples (0.2 g each) were accurately weighed into 250

ml Duran bottles and 200 ml of distilled water added to each sample bottle and capped. The contents were then shaken on a digital laboratory shaker (HS 501 digital, IKA® labortechnik, Staufen, Germany) for 1 h and filtered through a Whatman No 1 filter paper (Fisher Scientific Ltd, Loughborough, UK). Approximately 50 ml of extract from each sample was retained for the determination of water soluble carbohydrate concentration. From each sample 2 ml was pipetted into labelled 50 ml culture tubes and then stood in ice water for 10 min. Anthrone reagent (10 ml) was slowly added down the side of each tube making sure a layer was formed under each sample. The tubes were stoppered and vortexed. The tubes were heated for exactly 20 min using a hot plate (VWR® 375 Hot plate stirrer, Henry Troemner LLC, USA) set at 300°C. After 20 min of boiling, samples were removed from the hotplate and immediately placed on ice for a few seconds to reduce the temperature. After cooling, absorbance reading was read using a Jenway 6305 spectrophotometer (Bibby Scientific Ltd, Essex, UK) set at 620 nm. A straight line plot was drawn using absorbance readings of the four standard glucose concentrations. A regression equation was derived from the plot and was used to calculate concentrations of water soluble carbohydrate in the grass samples as follows:

$$\mathbf{Y}=0.0049\,\mathbf{X}+0.0074\ (R^2=0.9993) \qquad \text{Equation 3.5}$$

Where **Y** values are the concentration of WSC (g/kg DM) in samples and **X** values the absorbance readings of the test samples

3.6 Determination of metabolisable energy content of grass using modified acid detergent (MAD) fibre

Metabolisable energy (ME) content of fresh grass was determined according to Givens *et al*., (1990). Metabolisable energy (ME) content of grass samples cut at 9am and 4pm during the grazing trial (Chapter 6) was determined indirectly using the MAD fibre

method. The working standard used was Cetyltrimethylammoniumbromide (CTAB)-acid solution prepared by dissolving 10g of CTAB in 1L of 0.5M H_2SO_4. Dried and milled grass samples (1g) were accurately weighed into clean filter crucibles (W_o) and 100ml of CTAB-acid solution added. Samples were then boiled gently for 2 h on a Fibertech® 1020 hot and 1021 cold extractor (Foss UK Ltd, Cheshire, UK). Samples were then filtered and residues washed with 3 x 50ml of hot distilled water. Samples were then dried in an oven at 105°C overnight. Crucibles with samples were again weighed (W_1) before ashing for 6h at 500°C. After ashing samples were cooled and re-weighed (W_2). The MAD fibre (g/kg DM) was calculated as follows:

$$\text{MAD fibre (g/kg DM)} = \frac{(W_1 - W_2)}{(W_o)} \times 1000$$

Metablisable energy (ME) was then calculated using the following equation

ME (fresh grass) MJ/kg DM =16.20 - 0.0185*MAD fibre (g/kg DM) Givens *et al.*, (1990)

3.7 Determination of forage DM intake of grazing cows using the *n*-alkane technique

Pasture DM intake of cows was measured indirectly using the *n*-alkane method as described by Meyes *et al.,* (1986). Samples of 0.1 g of dried faeces and 0.2 g of grass and TMR samples were accurately weighed (Mettler Toledo, XS205 dual range Leicester, UK) in duplicate into 4-ml glass screw-cap GC vials. To this was added 0.11 g of *n*-docosane (C_{22}) solution and *n*-tetratriacontane (C_{34}) in n-decane (0.3 mg/g) as internal standards. To the faecal samples, 1.5 ml of ethanolic KOH (1M) was added and 0.2 ml of ethanolic KOH added to the forage samples. The vials were capped and shaken gently, and the samples heated for 16 h at 90°C on a dry heat block heater (Dri-Block®, Cambridge, UK). After 16 h, the temperature was reduced to 60°C and 1.5 or 2 ml of heptane was added to the faecal and forage samples, respectively. Samples were gently shaken and 0.4 ml and 0.6 ml of water was added to the faecal and forage samples respectively. The tubes were then

shaken vigorously and let to stand for about 5 min. After separation into 2 liquid layers, the non-aqueous layer (top) was transferred into a second 4 ml GC vial using a Pasteur pipette. Another aliquot of 1.5 or 2ml of heptane was then added to the sample tubes and the extraction method repeated with the aqueous layer being transferred to the same vial. All the extracts collected in the second vial were then dried on a dry block heater fitted with a sample concentrator blowing air into the individual vials. The extracts were then re-dissolved in 0.3 ml heptane with warming and samples were applied gently to small columns containing 1 ml of silica gel bed. The hydrocarbons were eluted into the third 4ml GC vial by addition of 2 x 1.5 ml of *n*-heptane to the column. Heptane was removed by evaporation to dryness on a dry block heater. The extracts were again re-dissolved with warming (60°C) into GC auto sampler vials and capped for gas chromatography. Alkane analysis was done on a gas chromatograph model Phillips PU 4500 (Phillips, Surrey, UK) apparatus equipped with a flame ionisation detector and a 30 m X 0.32 mm i.d, 0.25 µm fused silica capillary column (Restek Corporation, Bellefonte, USA). Oven temperature was programmed 190°C for 3min; 6°C/min to 316°C. The carrier gas was helium with a flow rate of 9 ml/3ml *n*-dodecane and immediately transferred to the min.

Herbage DM intake for individual animals in each period was calculated using C_{32} as a dosed alkane and C_{33} as a natural herbage alkane as described by Mayes *et al*., (1986) and shown in the equation below:

$$\text{Herbage intake (kg DM/d)} = \frac{F_i/F_j*\{D_j + I_c*C_j\} - I_c*C_i}{H_i - \{F_i/F_j* H_j\}}$$

H_i, C_i, F_i = concentrations (mg/kgDM) of the natural odd chain alkane in herbage, TMR and faeces.

H_j, C_j, F_j = concentrations (mg/kgDM) of the even chained alkane in herbage, TMR and faeces.

I_c = intake of TMR (kg DM/d)

D_j = amount of alkane j dosed (mg/d)

3.8 Determination of methane concentrations in gas samples

Gas samples for methane analysis were collected from the first experiment which was an *in vitro* experiment and from the two *in vivo* trials using dairy cows. Gas samples from the *in vitro* and *in vivo* experiment were prepared differently.

3.8.1 Determination of methane concentration in gas samples from *in vitro* study

Gas samples collected every 12 h from each treatment in the *in vitro* study were collected in labelled Tedlar® gas sampling bags (Sigmal Aldrich, Dorset, UK). Analysis of gas samples was done according to a method described by Purcell *et al*., (2011). Prior to analysis, the gas chromatograph was calibrated using standard gas which contained 99% pure methane gas (Puris®, Sigma Aldrich, Dorset, UK). This was done by manually injecting the gas chromatograph with 10 ml of the standard methane gas which was diluted with air to make 25%, 50%, 75% and 100% methane concentration. Area units were recorded on the GC for each level of methane concentration. A straight line plot of percent methane concentration vs. area units was plotted. A regression equation was derived from the results of the standard gas and this was used to determine the concentration of methane gas in the test samples.

$\mathbf{Y}$=0.00002$\mathbf{x}$ (R^2=0.9985) Equation 3.8.1

Where Y= % methane level of the gas sample x= area units of test sample on the GC

Methane volume (mls) at each time period was then determined by multiplying the percent methane and gas (ml) released during *in vitro* fermentation at each time period. Gas samples from the treatments were analysed by manually injecting duplicate samples of 10 ml each using Leur-lock® syringes (Fisher Scientific, Loughborough, UK) and area readings taken. The gas chromatograph (GC) settings were GC model (7890A Agilent technologies, Buckinghamshire, UK) equipped with a 80/100 mesh Porapak N column 1.8 m long, 2.1 mm i.d (Sulpeco, Bellafonte, USA) and flame ionisation detector (FID). Temperatures settings were 170, 200 and 300°C in column, injector and detector respectively. The carrier gas (N_2) flow, H_2 flow and air flow were adjusted to 34 ml/min, 30 ml/min and were 400 ml/min respectively.

3.8.2 Determination of methane concentration in gas samples from the *in vivo* studies

Prior to the start of the two *in vivo* experiments, brass permeation tubes (bolus) weighing approximately 50g and containing SF_6 (Agri Food and Bioscience Institute, Hillsborough, UK) were inserted in the rumen of individual cows using a balling gun (Nasco®, Wimsconsin, USA). The SF_6 permeation rates of the individual boluses were determined three weeks before inserting in the cows. The permeation tubes were kept in an incubator set at 39^{o}C. The weights of the permeation tubes were recorded three times/week in order to determine the daily change in weight. Data of the individual boluses were fitted in a regression equations in excel (R^2>0.99) and permeation rate per day for each bolus was determined. The canisters used in the experiment were designed to half fill over a 24-h period were evacuated to -97 kPa and strapped on the backs of individual cows. Representative breath samples from each cow were collected into the pre-evacuated canisters by means of Teflon tubing fitted to a halter. Canisters were changed every day after morning milking at around 10:00h. Canisters containing air samples were taken to the laboratory and pressurised with nitrogen gas (N_2) to 17 kPa.

Subsamples of air were taken from each canister using 50 ml Leur-lock® syringes (Fisher Scientific, Loughborough, UK). Concentrations of SF_6 and CH_4 in respired air and ambient air samples were determined by gas chromatograph model 7890A (Agilent Technologies, California, USA) fitted with an electron capture detector (350°C) to determine SF_6 and flame ionisation detector (300°C) to determine CH_4 concentrations. The GC was fitted with 2 columns, a 1.8 m 80/100 mesh Porapak N column (Sulpeco, Bellafonte, USA) and a 3 m 40/60 mesh molecular sieve 5A column (Resteck corporation, Bellefonte, USA). Injector temperature was set at 100°C. Nitrogen was used as carrier gas at a flow rate of 60 ml/min. Chromatographic analyses were performed after calibration with standard gases (Scott-Marrin Inc, Riverside, USA) for SF_6 and CH_4. Standard concentrations were 357 ppmv±1% CH_4 and 1036 pptv ± 5% SF_6. Standards were run at the beginning of each day and after every 10 samples. The CH_4 and SF_6 concentrations in the samples were determined from the peak areas and amounts relative to the known standards. Daily CH_4 production by each animal was calculated using the known permeation rate of SF_6 for each tube as follows

$$CH_4\,(g/d) = SF_6 \text{ permeation rate } (g/d) \times \frac{(CH_4)}{(SF_6)} \qquad \text{Equation 3.8.2}$$

(Johnson and Johnson, 1995)

CHAPTER 4. Effects of starch source and oil source on fermentation characteristics and methane production *in vitro*

4.1 Introduction

An *in vitro* study is a cheaper and quicker method of determining rumen fermentation characteristics and methane production of various ruminant feeds (Purcell *et al*., 2011). Previous studies have shown that *in vitro* fermentation and methane production can be altered by addition of supplements to a basal diet (Getachew *et al*., 2005). Different supplements such as fats, essential oils and saponins have been evaluated and each one can alter fermentation characteristics in different ways (Castro-Montoya *et al*., 2012).

Carvacrol is an essential oil classified as a phenolic monoterpenoid which has antimicrobial properties (Busquet *et al*., 2006). The chemical structure is 2-methyl-5-isopropyl-1-phenol (Macheboeuf *et al*., 2008) as shown in Fig 5.

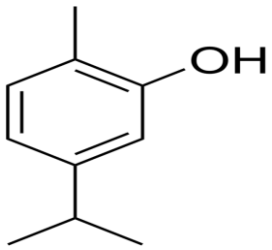


Figure 5. Chemical structure of carvacrol

Carvacrol is found in high concentrations in oregano and thymol essential oils (Benchaar and Greathead, 2011). Concentrations in oregano are on average 600 mg/g (Castillejos *et al*., 2008). The phenolic group helps carvacrol to carry out its antimicrobial activities (Macheboeuf *et al*., 2008). Carvacrol inhibits both gram positive and gram negative bacteria (Benchaar and Greathead, 2011). According to Macheboeuf *et al*., (2008), carvacrol works by increasing permeability of cells leading to loss of fluids and eventual

cell death. A review by Patra and Saxena (2010) indicated that carvacrol can inhibit methane production by 13-98% depending on the dosage used.

Linseed oil is a vegetable oil that is rich in $C_{18:3n-3}$ (Zhang *et al*., 2008). Linseed oil reduces methane production by either a reduction in organic matter fermentation (Chung *et al*., (2011), hydrogenation or by direct toxic effect on the methanogens and protozoa (Vargas *et al*., 2011). Effects of linseed oil on methane production vary with the level of supplementation (Zhang *et al*., 2008). For example, addition of 50 g/kg of linseed oil to a TMR in a rusitec technique reduced methane production by 25% (mmol/g) when compared to the un-supplemented control. In a study by Zhang *et al*., (2008) supplementation of linolenic acid at 35 and 70g/kg DM reduced methane production by 45% and 62% respectively. At higher levels of supplementation, linseed oil tends to reduce DM fermentability (Eugene *et al*., 2011).

Fish oil mediates effects on methane production through two FAs; eicosapentanoic acid (EPA) and docosahexanoic acid (DHA) whose concentrations vary according to the type of fish oil (Castro-Montoya *et al*., 2012). Reduction in methane production with fish oil supplementation is thought to be due to a direct toxic effect on methanogens (Fieves *et al*., 2007). Reductions of up to 80 % in methane production have been observed in studies by Fieves *et al*., (2003) and Fieves *et al*., (2007).

Various *in vitro* studies have been conducted to determine effects of oil supplementation on methane production but few (Lovett *et al*., 2003) have compared the effect of starch source and oil source on fermentation characteristics and methane production *in vitro.* According to Castro-Montoya *et al*., (2012), the effectiveness of a supplement to alter fermentation characteristics depends on the basal diet. For example, Castro-Montoya *et al*., (2012) compared effects of oil sources on 3 basal diets, concentrate, maize silage and

grass silage and established that the effects of the oil sources varied with the basal diet, and that interactions were observed between the basal diet and oil sources which also influenced methane production.

4.2. Hypothesis, aims and objectives

The hypothesis to be tested was that starch source and oil source can alter *in vitro* rumen fermentation characteristics and reduce methane production. The objectives of the experiment were to determine the effects of starch source and oil source and the interaction between the two on *in vitro* gas fermentation kinetics, methane production, rumen fluid pH and NDF digestibility.

4.3. Materials and Methods

4.3.1 Experimental design, basal diets and treatments

The study was conducted using an *in vitro* batch culture technique as described by Sinclair *et al*., (2005). The experimental design was a 3x3x2 factorial design with a control. The 3 starch sources; wheat (W), barley (B) and maize (M) and 3 oil sources; carvacrol (Cv), linseed oil (LO) and fish oil (FO) were included at two dosage levels of either 4% or 8% of DM. The starch sources were mixed with dried grass (Emerald green feeds®, Lincoln, UK) to provide approximately 25 % starch level in the basal diet. Each of the basal diets had 6 treatments which were the three oil sources at 2 dosage levels. Therefore, the treatments for each of the basal diets were Cv1 (carvacrol supplied at 4% of DM), Cv2 (carvacrol supplied at 8% of DM), LO1 (linseed oil supplied at 4% of DM), LO2 (linseed oil supplied at 8% of DM), FO1 (fish oil supplied at 4% of DM), FO2 (fish oil supplied at 8% of DM). The CT diets were the W, B, and M that had no added oil supplements. The basal diets were milled to pass through a 2 mm screen. The dietary combinations were chosen to represent a range of forage and starch source

combinations used in dairy rations in the United Kingdom. The experiment was replicated 3 times, one week apart, to provide 3 replicates per treatment. Chemical composition of the basal diets is presented in Table 12.

Table 12. Chemical composition of the basal diets, grass and wheat (W), grass and barley (B), grass and maize (M) used in the *in vitro* study

	W	B	M
Dry matter, g/kg DM	932	931	939
Organic matter, g/kg DM	919	917	914
Crude protein, g/kg DM	155	150	158
Ash, g/kg DM	76	77	81
NDF, g/kg DM	337	336	344
Starch, g/kg DM	240	230	270

4.3.2 Oil sources and inclusion levels

The three oil sources used in the experiment were menhaden fish oil, linseed oil and carvacrol all purchased from Sigma Aldrich (UK) and were of 98-99% purity. The densities were 0.93 g/ml, 0.93 g/ml and 0.976 g/ml for menhaden fish oil, linseed oil and carvacrol (488 mg/L of rumen fluid for carvacrol), respectively. Either 0.1 ml or 0.2 ml of the oil sources were used to make 4 or 8% DM oil supplementation, respectively. Menhaden fish oil and linseed oil were in form of triglycerides and their FA composition is presented in Table 13.

Table 13. Fatty acid composition of Menhaden fish oil (FO), Linseed oil (LO) and Carvacrol (Cv) used in the *in vitro* study

	FO	LO	Cv*
FA g/100g			
$C_{14:0}$	8.27	-	-
$C_{16:0}$	19.04	6.00	-
$C_{16:1}$	9.64	-	-
$C_{18:0}$	3.51	2.50	-
$C_{18:1}$	9.05	19.0	-
$C_{18:2n\text{-}6}$	2.27	24.1	-
$C_{18:3n\text{-}3}$	1.35	47.4	-
$C_{20:0}$	2.94	0.50	-
$C_{20:1}$	2.34	-	-
$C_{20:5n\text{-}3}$	11.54	-	-
$C_{22:6n\text{-}3}$	6.20	-	-
0thers	23	0.50	-

*Cv is an essential oil with no fatty acid content

4.3.3 Donor animals and Inocula

Two male rumen cannulated sheep of average weight 65±3.2 kg live weight were fed a vitamin and mineral free diet for 15 days prior to rumen fluid collection. Animals were cared for according to the Harper Adams University policy on Research Ethics. The animals were fed in one meal of 2.8 kg/d per animal of a commercial coarse diet (S.C Feeds Ltd, Stafford, UK; Table 14) at 8:00 h, and both animals had unlimited access to water and grass hay throughout the experimental period.

Table 14. Ingredient composition (g/kg DM) of the commercial diet fed to sheep

Ingredient	Amount
Barley cooked/rolled	200
Peas micronized	50
Maize micronized	50
Soybeans micronized	50
Wheat feed	60
Soypass	25
Soya hipro	50
Sugar beet	150
Molasses	80
Protein pellets[1]	250
Oat feed	35

[1]Contained (50% rape meal, 25% sunflower meal,20% palm kernel, 5% molasses)

4.3.4 Rumen fluid and media solution

On the first day of each experimental period, rumen fluid was collected from the cannulated sheep 4 h after morning feeding into a previously warmed Erlenmeyer flask. The collected rumen fluid was strained using four layers of cheese cloth. Culture fluid was prepared according to the procedure of Theodorou *et al*., (1994) by mixing 1.5 L of strained rumen fluid with 6.0 L media solution at 39°C under continuous flushing with CO_2. The media solution was prepared 24 hours before the experiment and autoclaved at 121 °C for 15 minutes to remove dissolved gases. All chemicals used in constituting the media solution were purchased from Sigma Aldrich®, UK. The pH readings of the medium solution were taken before and after 48 h of *in vitro* incubation at 39°C using a pH meter (Mettler Tolledo, Manchester, UK). Tables 15 and 16 show the media composition.

Table 15. Solution composition of the media used in the *in vitro* study

Ingredient	Amount (ml)
Microminerals solution	0.1
Buffer solution	200
Macromineral solution	200
Reducing solution	40.0
Indicator solution	1.0
Deionised water	559

Table 16. Chemical composition of the individual solutions that made the media solution

	Amount
Micro-mineral solution (g/100ml)	
Calcium chloride ($CaCl_2.H_2O$)	13.2
Manganese chloride ($MnCl_2.6H_2O$)	10
Cobalt chloride ($CoCl_2.6H_2O$)	1
Iron Chloride ($Fe\ Cl_3.\ 6H_2O$)	8
Buffer Solution g/1000ml	
Ammonium hydrogen carbonate (NH_4CO_3)	4
Sodium hydrogen carbonate $NaHCO_3$	35
Macro-mineral Solution (g/ 1000ml)	
Di- sodium hydrogen orthophosphate ($Na_2HPO_4.12H_2O$	9.45
Potassium di-hydrogen orthophosphate (KH_2PO_4)	6.2
Magnesium sulphate 7-hydrate ($MgSO_4.\ 7H_2O$)	0.6
Reducing Solution (g/100ml)	
Cystein HCl	0.625
Anaerobic indicator (g/100ml	
Resazurin	0.1

4.3.5 *In vitro* gas production

The *in vitro* gas production technique was based on the procedure of Theodorou *et al*., (1994) using 21 X 307 ml Doran fermentation bottles as modified by Sinclair *et al.,* (2005). The experimental design had 21 fermentation bottles with each of the 3 basal diets, W, B, M which were also referred to as the CTR diets. Each of the starch sources had 7 treatments each comprising Cv1, Cv2, LO1, LO2, FO1, FO2 and CTR (no additive). Table 17 shows the experimental design used.

Table 17. Experimental design of the *in vitro* study

Starch source		W	B	M
Oil source	Dosage			
Cv	1	X	X	X
	2	X	X	X
LO	1	X	X	X
	2	X	X	X
FO	1	X	X	X
	2	X	X	X
CTR	No additive	W	B	M

Dosage 1= added at 4% DM; Dosage 2= added at 8% DM;

Approximately 2.5 g DM of each basal diet was weighed into the Duran fermentation bottles. In addition, 3 blanks containing only rumen fluid and buffer were included. Either 0.1 ml or 0.2 ml of the oil sources Cv, LO or FO were dispensed into the bottles. To each of the bottles, 200 ml of the freshly prepared buffer solution was dispensed, the bottles tightly capped, gently shaken and incubated for a total of 48 h at 39°C. Head space pressure readings were taken every 3 hours at 0, 3, 6, 9, 12, 15, 18, 21 and 24 h on the first day and every 6 hours on the second day of incubation at 30, 36, 42 and 48 h using a pressure transducer (Tracker 220, Bailey and Mackey Ltd, Birmingham, UK). Gas produced during incubation was collected into labelled gas bags. Gas collected every 12 h was pooled and put into separate bags labelled 0-12h, 12-24h, 24-36h, 36-48h for subsequent methane analysis. After 48 h of incubation, bottles were uncapped, the contents swirled on ice to stop fermentation and the pH measured immediately using a pH meter (Mettler Tolledo, Manchester, UK). The fermentation contents in each bottle were filtered through 50 ml sintered glass crucibles (porosity P1) using a pump and residues dried at 60°C for 48 h. The fermentation contents were used for the determination of NDF. Collected gas samples were subsampled for subsequent methane analysis by GC.

4.4 Laboratory analyses

Samples of the basal diets were analysed for DM, OM, CP, NDF and ash as described in Sections 3.1.1 to 3.1.4. Starch concentration in the basal diets were determined using Megazyme starch essay kit (Megazyme International Ireland Ltd, Wicklow, Ireland) as described as in Section 3.4. Methane concentration in the gas samples collected from the *in vitro* study were analysed by GC (7890A Agilent technologies, Buckingham, UK) as described in Section 3.8.1.

NDF digestibility was calculated using the equation of Hall and Mertens, (2003) as follows;

$$\text{NDF dig (g/kg DM)} = \frac{\text{NDF in sample - NDF post fermentation)}}{\text{(NDF sample)}} \times 1000$$

Gas pressure data were converted to gas volume (ml) using the equation of Purcell *et al*., (2011) as:

$$\text{Gas production (ml)} = \frac{(Vh)}{(Pa)} \times Pt.$$

Where Vh equals head space volume (107.55ml), Pa equals the atmospheric pressure (101.4 kPa) and Pt the pressure transducer reading (kPa).

Gas samples from the treatments collected during the 4 incubation periods 0-12 h, 12-24h, 24-36 h and 36-48 h were injected manually into the GC as described in Section 3.8.1. The volume of methane gas (ml) in the sample was calculated according to Kumar *et al*., (2013) using the equation

Methane production (ml) = Total gas (ml) X methane content (%) in treatment.

4.3.5 Statistical Analyses

Data were subjected to a general analysis of variance as a 3x3x2 factorial design with a control (CT) using GenStat model, 13th edition (VSN International Ltd, 2010). The starch sources (S) were wheat, barley and maize. The oil sources (O) were Cv, LO or FO which were added at two levels of 4% DM (1) or 8% DM (2). The CTR diets were the starch sources W, B and M without any added oil. Effects of starch source (S), oil source (O), dosage (D), time of incubation (T), and the interactions of starch source and oil source (SxO) on gas production, methane output, pH, NDF were analysed. Significant differences

between individual means were identified using the Tukey's multiple range test. Mean differences were considered significant at $P<0.05$ and trends were identified at $P<0.1$.

4.6 Results

4.6.1 Chemical composition

The chemical composition of the 3 basal diets used in the study were similar, but NDF content was slightly higher in M with a concentration of 344 g/kg DM when compared to concentration of 337 and 336 g/kg DM found in W and B, respectively (Table 12). Linseed oil and fish oil differed in FA composition (Table 13). Carvacrol is an essential oil comprised of 98% carvacrol with no FA content. Linseed oil was composed of 47% $C_{18:3n-3}$, 24% $C_{18:2n-6}$ and 19% $C_{18:1}$ while menhaden fish oil was composed of 12% eicosapentanoic acid (EPA), 6% docosahexanoic acid (DHA), 2% linoleic acid, 19 % palmitic acid, 10% palmitoleic acid and 9% oleic acid.

4.6.2 Cumulative and rate of gas production

The three starch sources, wheat (W), barley (B) and maize (M) exhibited differences in cumulative and rates of gas production ($P<0.001$; Figs 6 and 7). Cumulative gas production was higher in W, intermediate in B and lowest in M with mean values of 175, 158 and 120 ml/g DM, respectively. Rate of gas production followed a similar trend with mean values of 9.2, 8.1 and 5.5 ml/g DM/h in W, B and M respectively. Cumulative gas production also varied with oil source, dosage and time of incubation ($P<0.001$; Fig 6). There were interactions between starch source and oil source (SXO), starch source and dosage (SXD), starch source and time (SXT), starch source, oil source and dosage, (SXOxD) ($P<0.001$; Tables 18 and 19).

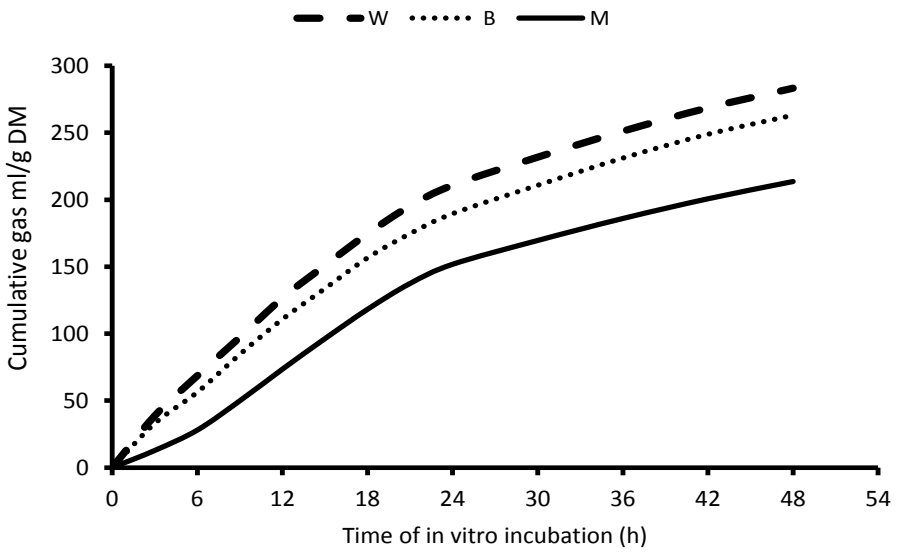


Figure 6. Cumulative gas production (ml/g DM), of the three starch sources, W, B and M during the 48h of *in vitro* incubation at 39°C.

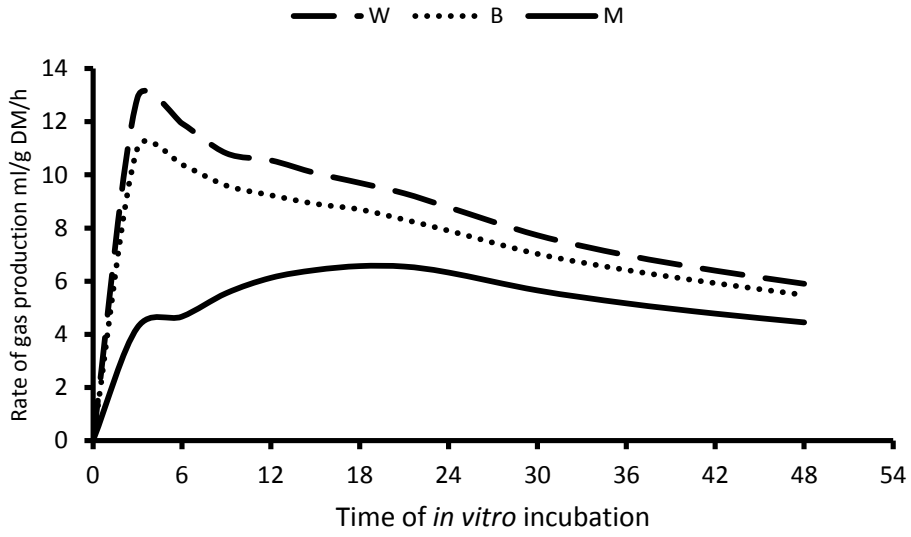


Figure 7. Rate of gas production (ml/g DM/h) of the three starch sources, W, B and M during the 48 h of *in vitro* incubation at 39°C

Among the oil sources, Cv suppressed cumulative and rate of gas production more than LO and FO particularly at the highest rate of inclusion. When compared to the CTR diets, Cv suppressed cumulative gas production by 21, 30 and 28% in W, B and M based diets

respectively (Tables 18 and 19). Rate of gas production (ml/g DM/h) followed a similar trend, it was highest in W, medium in B and lowest in M based diet at 7.2, 5.2 and 4.1 ml/g DM/h respectively.

Effects of LO on cumulative gas production varied with basal diet and dosage used. When compared to the respective CTR diets, LO1 did not have any effect on cumulative gas production in W and B based diets, but increased gas production by 40% in the M based diets. At a higher dosage, LO2 increased gas production in W, B and M based diets by 8, 19 and 40% respectively when compared to the respective CTR diets.

Fish oil at a lower dosage (FO1) increased gas production by 18 % in the W and M based diets and had no effect in B based diets when compared to the respective CTR diets. At a higher dosage, FO2 had no effect on gas production in W and B based diet and increased gas production by 61% in M based diets (Tables 18 and 19).

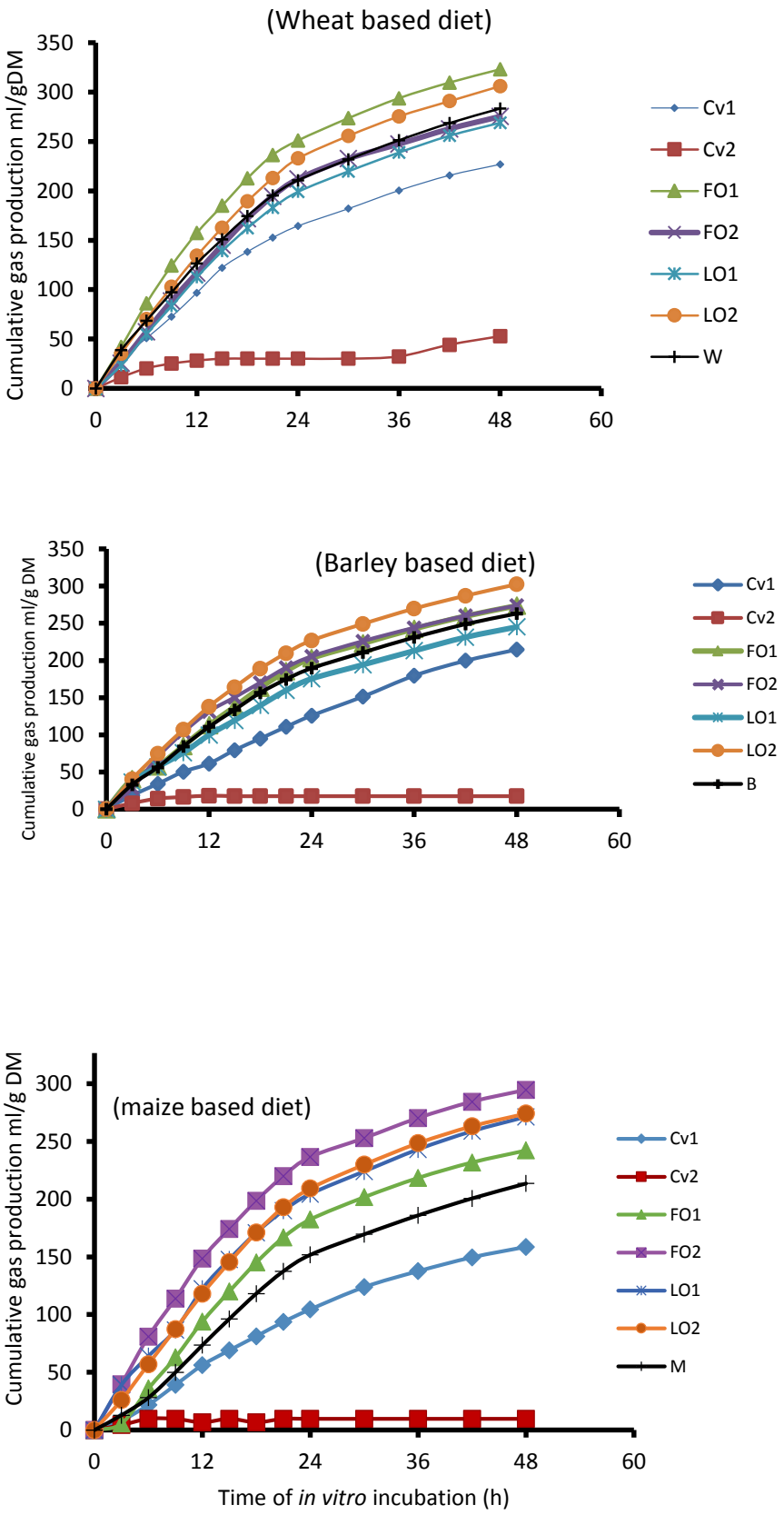


Figure 8 . Cumulative gas production (ml/g DM) of the treatments, Cv, LO and FO at the two dose levels, 1 and 2 on Wheat (W), Barley (B) and Maize (M) based diets during 48 h of *in vitro* incubation at 39°C

Table 18. Effect of starch and oil source on fermentation characteristics following *in vitro* incubation in buffered rumen fluid at 39°C

Starch source	W							B							M						
Oil source	CTR	Cv1	Cv2	LO1	LO2	FO1	FO2	CTR	Cv1	Cv2	LO1	LO2	FO1	FO2	CTR	Cv1	Cv2	LO1	LO2	FO1	FO2
Cum gas, ml/g DM	175	138	30.4	162	189	208	169	158	110	16.5	146	188	165	171	120	86.9	8.70	169	169	142	193
Rate, gas production	9.2	7.2	1.9	8.0	9.7	11.0	8.5	8.1	5.2	1.2	7.8	9.9	8.3	8.9	5.5	4.1	0.6	8.9	8.5	6.6	10.3
NDF dig, g/kg	744	553	572	724	710	708	717	708	529	182	752	701	696	690	733	587	320	739	771	825	719
Initial pH.	6.93	6.93	6.93	6.93	6.93	6.93	6.93	6.93	6.93	6.93	6.93	6.93	6.93	6.93	6.93	6.93	6.93	6.93	6.93	6.93	6.93
Final pH.	5.76	6.35	6.77	5.82	5.77	5.78	5.83	5.77	6.44	7.00	5.70	5.73	5.84	5.77	5.69	6.44	7.0	5.72	5.74	5.84	5.80
pH. Change	1.17	0.58	0.16	1.11	1.16	1.15	1.10	1.16	0.49	0.07	1.23	1.20	1.09	1.16	1.24	0.49	0.07	1.21	1.19	1.09	1.13

Rate of gas production; ml/g DM/h; W, B or M are wheat, barley or maize based treatments with no added oil

Table 19. SED and *P*-values of main effects and interactions of starch, oil and dosage on fermentation characteristics of carvacrol, linseed oil and fish oil following 48 h of *in vitro* incubation at 39°C

	SED					*P*- values				
Variables	S	SXO	SXD	SXOXD	SxT	S	SxO	SxD	SXT	SxOxD
Cum gas, ml/g DM	6.06	6.87	7.93	7.93	27.45	<0.001	<0.001	<0.001	<0.001	<0.001
Rate of gas prod.	0.500	0.500	0.500	0.500	1.731	<0.001	<0.001	<0.001	<0.001	<0.001
NDF dig, g/kg DM	28.9	32.7	37.8	37.8	NA	0.531	<0.001	<0.001	NA	<0.001
Final pH.	0.077	0.087	0.100	0.100	NA	0.583	<0.001	<0.001	NA	<0.001
pH change	0.080	0.091	0.105	0.105	NA	0.593	<0.001	0.047	NA	0.005

S= starch source; O= oil source; D= dosage; T=time of incubation; NA= not applicable; SED= standard error of difference of the mean
Rate of gas production, ml/g DM/h

Table 20. Effect of starch and oil source on *in vitro* methane production (ml/g DM) at 0-12, 12-24, 24-36 and 36-48 h incubation in buffered rumen fluid at 39°C

Starch source	W							B							M						
Oil source	CTR	Cv1	Cv2	LO1	LO2	FO1	FO2	CTR	Cv1	Cv2	LO1	LO2	FO1	FO2	CTR	Cv1	Cv2	LO1	LO2	FO1	FO2
Time of incubation																					
0-12 h	4.5	3.3	0.2	7.2	10.8	11.6	7.1	6.7	0.78	0.1	4.2	9.8	9.8	10.7	2.5	2.7	0.0	10.3	8.3	2.0	10.6
12-24 h	11.7	6.4	0.2	15.5	16.0	20.2	20.1	14.4	2.2	0.1	14.9	16.5	21.3	15.2	14.2	5.2	0.0	20.8	15.4	12.9	16.8
24-36 h	16.1	8.3	0.2	18.2	19.9	25.0	20.8	18.8	2.9	0.1	20.5	20.3	24.3	20.1	16.0	7.3	0.0	20.7	18.9	19.1	20.2
36-48 h	6.8	1.3	0.1	6.0	5.6	5.7	5.1	5.7	0.5	0.0	6.7	5.5	6.0	4.9	5.5	1.3	0.0	4.8	4.0	3.6	3.2
Total CH_4 output	39.0	18.4	0.7	45.0	50.3	62.5	43.1	45.5	6.4	0.2	35.6	49.9	50.1	45.0	34.9	16.5	0.0	48.4	44.2	36.4	47.5

CH_4 production, ml/g DM

Table 21. SED and *P*-values of main effects and interactions of starch, oil and dosage on methane production following 48 h of *in vitro* incubation at 39°C

	SED				*P*- values			
Variable	S	SXO	SXD	SXOXD	S	SxO	SxD	SxOxD
0-12 h	1.474	1.671	1.930	1.930	0.153	<0.001	0.138	<0.001
12-24 h	2.407	2.729	3.151	3.151	0.422	<0.001	0.266	0.102
24-36 h	2.628	2.980	3.441	3.441	0.658	<0.001	0.109	0.279
36-48 h	0.515	0.584	0.675	0.675	0.286	<0.001	0.006	0.891
Total CH_4 output	8.03	9.10	10.5	10.5	0.496	<0.001	0.354	0.186

S= starch source; O= oil source; D = dosage; SED= standard error of the difference of the mean; CH_4 production, ml/g DM

4.6.3 Methane production

In the current study, methane production (ml/g DM) was expressed as methane output produced per 12 h of incubation at four time periods, 0-12, 12-24, 24-36 and 36-48 h and as total methane output produced during the whole 48 h of *in vitro* incubation (Tables 20 and 21). Methane production varied with the time of *in vitro* incubation. Interactions between starch source and oil source (SXO) were observed at all time periods (P<0.001), while a dosage effect (SXD) and (SX0XD) was only observed at 36-48 h and at 0-12h of *in vitro* incubation respectively.

Generally, highest amounts of methane output (ml/g DM) were produced during the periods 24-36 h of *in vitro* incubation in all treatments, while the least amounts were produced during the last 12 h between 36-48 h of *in vitro* incubation. The 3 oil sources, Cv, LO and FO behaved differently in the basal diets and effects varied with the time of *in vitro* incubation.

At 0-12 h of *in vitro* incubation, Cv reduced methane production only in B diet by an average of 94% and was ineffective in M and W. LO had no effect on methane production in B but increased methane production in M and W by 278 and 96 % respectively. FO increased methane production in B, M and W by 54, 155 and 100% respectively

At 12-24 h of *in vitro* incubation, Cv reduced methane production in all the starch sources W, B and M by 92, 82 and 72 % respectively while LO had no effect on methane production in all the 3 starch sources. FO increased methane production by 73% in W and had no effect in B and M.

Between 24-36 h of *in vitro* incubation, Cv reduced methane production in all the starch sources by 74, 92 and 77% in W, B and M respectively. FO only increased methane

production in W based diet by 42% and had no effect in B and M. LO completely had no effect in all the 3 starch sources.

At 36-48 h of *in vitro* incubation, Cv reduced methane production in the 3 starch sources by 96, 88 and 90 % in B, M and W based diets. FO only reduced methane production by 38% and 21% in M and W based diets respectively and was ineffective in B based diets. LO only reduced methane production by 20% in M based diet and was ineffective in B and W.

A dosage effect ($P<0.05$) was observed only during 36-48 h of *in vitro* incubation. All the 3 oil sources Cv, LO and FO. Cv reduced methane production by 91, 76 and 81% in B, M and W respectively at a lower dosage, while at a higher dosage fermentation was inhibited. FO also reduced methane production by 34 and 16% in M and W based diets respectively at a lower dosage while at a higher dosage methane production was reduced by 43 and 26% in M and W. There was no dosage effect observed with LO.

When methane output was expressed as total methane production, there was no starch source and no dosage effect on methane production ($P>0.1$), but an interaction SXO ($P<0.001$; Tables 20 and 21) was observed. When compared to the CTR, only Cv1 reduced total methane production in all the 3 basal diets. Reductions of 53, 86 and 53% were observed in W, B and M based diets respectively. At a higher dosage, Cv halted fermentation. When compared to the CTR, LO did not have any effect on methane production in all the three basal diets. On the other hand, FO at a lower dosage (FO1) stimulated a 60 % increase in methane production in the W based diets, but did not have any effect on methane production in B and M based diets (Tables 20 and 21).

4.6.4 pH of incubation media

All treatments had the same initial pH of 6.93 (Tables 18 and 19). After 48 h of *in vitro* incubation in buffered rumen fluid at 39°C, the final pH differed in the treatments (P<0.001; Tables 18 and 19). There was no starch source effect (P>0.1) observed on final pH or on pH change. The M diet showed a numerically higher final pH of 5.69 when compared to 5.76 and 5.77 of the W and B based diets respectively. The interaction (P<0.001) between starch source and oil source (SXO) and starch source, oil source and dosage (SXOXD) showed that the oil sources behaved differently in the starch based diets and effects varied with dosage. Cv1 lowered the pH of the media from 6.93 to an average of 6.40, which is a reduction of 0.54 pH units. When compared to the CTR diet, pH change was lower in the Cv1 based diet while a higher dosage (Cv2) only reduced pH by 0.1-0.3 pH units (Table 18). The final pH in the Cv treatments ranged from 6.35 to 7.0. All the LO and FO treatments reduced the final pH of the media by an average of 1.1 pH units, with the pH in the LO and FO treatments ranging from 5.70 to 5.84. When compared to the CTR diets, pH change in the LO and FO based diets did not differ from that of the CTR diets.

4.6.5 *In vitro* NDF digestibility

Neutral detergent fibre (NDF) digestibility (g/kg) in the treatments ranged from 182 g/kg in Cv supplemented diets to 825 g/kg in the FO supplemented diets (P<0.001; Tables 18 and 19). The NDF digestibility of the startch sources did not differ (P>0.1) from each other. W, B and M control diets had digestibilities of 744, 708 and 733 g/kg DM respectively. Interactions (P<0.001) were observed between SXO, SXD, SXOXD. Generally, Cv suppressed NDF digestibility more than LO and FO. Cv1 suppressed NDF digestibility to the same extent in all 3 basal diets producing a mean value of 550 g/kg. When compared

to the CTR diets, reductions in NDF digestibility with Cv1 supplementation were 26, 25 and 20 % in W, B and M based diets respectively. At the higher dosage, Cv did not have further effect on digestibility in the wheat based diets, while in B and M based diets NDF digestibility was reduced to 182 and 320 g/kg respectively.

Linseed and fish oil supplementation resulted in a similar NDF digestibility of approximately 700 g/kg which did not differ from that of the CTR diets. The only exception was with FO1 which increased digestibility by 13% when compared to the CTR in the maize based diets while at the higher dosage, NDF digestibility was unaffected by supplementation.

4.7 Discussion

In the current study, the hypothesis that was tested was that starch source and oil source are able to alter *in vitro* fermentation and result in changes in gas production and rate of fermentation, pH, NDF digestibility and subsequently reduce methane production. The second hypothesis that was tested was that there would be interactions between starch sources and oil source that would affect fermentation characteristics. In the study, changes in fermentation characteristics were observed with changes in cumulative and rates of gas production, the pH of the incubation media was lowered by the oil sources and NDF digestibility was reduced by carvacrol. Methane production was also reduced by the oil sources at different incubation times thus the hypothesis is accepted.

4.7.1 Cumulative and rate of gas production

In the current study, cumulative and rate gas production varied with the basal diet with W producing the highest cumulative and rate of gas production, B was intermediate and M was lowest. This agrees with findings from previous studies. For example, Chaves *et*

al., (2009) observed that barley underwent rapid fermentation when compared to maize. A review by Reynolds (2006) also stated that the starch sources wheat and barley undergo rapid degradation in the rumen when compared to maize.

The interaction between SXO, SXD and SXOXD resulted in the oil sources producing effects that varied with the basal diets. Cv1 decreased cumulative and rate of gas production in all the 3 basal diets when compared to the CTR diets. In contrast, effects of LO and FO varied in the 3 basal diets. LO1 increased gas production by 40% in the M based diet but had no effect in the W or B based diets, while a higher dosage increased gas production by 8, 19 and 40% in the wheat, barley and maize based diets respectively when compared to the CTR diets. Similarly, FO1 had varied effects in the 3 basal diets, a lower dosage increased gas production by 18% in wheat and maize based diets and higher dosage increased gas production by 61% in the maize based diets.

In the current study, Cv1 reduced mean gas production by 21, 30 and 28 % in the W, B and M based respectively diets when compared to the CTR. This agrees with findings reported in previous studies which show that carvacrol reduces gas production in a dose dependent manner. For example, in a study by Macheboeuf *et al*., (2008), *in vitro* supplementation of carvarcol at 1.5, 2, 3 and 5 mmol/L reduced gas production by 19, 39, 56 and 75 % respectively when compared to the un-supplemented control. Similarly, in the current study a dose response with carvacrol supplementation was observed, at 4% DM supplementation, carvacrol suppressed gas production by 21, 30 and 28% in the W, B and M based diets while at a higher dosage of 8% DM, fermentation was completely inhibited. In a study by Araujo *et al*., (2011), the addition of carvacrol at 667 mg/L to a concentrate/hay diet in an *in vitro* study reduced gas production by 66 %.

Previous studies have also reported varied effects of linseed and fish oils on gas production. For example, Zhang *et al*., (2008) reported 21 and 27 % suppression in gas production when α-linolenic acid (the main FA in linseed oil) was supplemented at 35 and 70 g/kg DM. In contrast, Vargas *et al*., (2011) reported that linseed oil supplemented at 5% DM did not have any effect on gas production when using a Rusitec system. In another study by Patra and Yu, (2013), supplementation of fish oil at 3.1 and 6.2 ml/L in an *in vitro* study did not have any effect on gas production. Reasons were not given.

4.7.2 Methane production

In the current study, methane production (ml/g DM) was examined at four different time periods; 0-12 h, 12-24 h, 24-36 h and 36-48 h of *in vitro* incubation in buffered rumen fluid at 39°C. It was observed that from 0-36 h, there was a progressive increase in methane production with incubation time. Highest amounts of methane were produced between 24-36 h of *in vitro* incubation in all the treatments. This may be due to fibrous material which produces a high amount of methane being degraded during this time. Getachew *et al*., (2005) observed that the highest amount of methane (ranging from 20-23 ml/g DM) was produced during 6-24 h of an *in vitro* incubation.

In the current study, carvacrol had no effect on methane production in the W and M diets in the first 12 h of incubation, but reduced methane production at all the other time periods when compared to the CT diets. Very few previous studies have examined the effects of carvacrol on methane production *in vitro*. Results obtained from those studies that have been conducted have indicated that carvacrol greatly suppresses methane production. For example, Macheboeuf *et al*., (2008) examined carvacrol supplementation at 0, 1.5, 2, 3, 4 and 5 mmol/L *in vitro* on a mixed basal diet composed of corn grain, soybean meal and hay, and reported a dose dependent response with methane

production reduced by a range of 13-95%. A similar finding was reported in the current study, Cv supplementation at 4% DM suppressed methane production by 52-80% while at 8% supplementation microbial fermentation was completely inhibited. In a study by Araujo *et al*., (2011), addition of carvacrol at 667 mg/L to a concentrate/hay diet in an *in vitro* study reduced methane production by 95 %. A more recent study by Hristov *et al*., (2013) tested 3 levels of oregano at 250, 500 and 750 g/d in dairy cows and reported decreased methane production by 9, 36 and 25% (g/kg DM intake) respectively when compared to the un-supplemented control.

In the current study, linseed oil (LO) generally either did not affect or increased methane production depending on the time of incubation. Reductions in methane production were only observed during the period 36-48 h in the M diet, with a value of 20 % when compared to the CTR. Few studies have compared the effects of oil source on methane production using a range of basal diets. Most previous studies have used a single basal diet. For example in a study by Vargas *et al*., (2011), linseed oil supplied at 5 g/kg DM to a TMR in a rusitec fermentation reduced methane production (mmol/g fermented OM) by 28% when compared to the un-supplemented control. Zhang *et al*., (2008) used a mixture of cornmeal and wild rye meal and supplemented with α-linolenic acid at 0, 35, and 70 g/kg DM and reported that methane production (mmols) was reduced by 46 and 62% respectively.

In the current study, when compared to the un-supplemented CT diets, fish oil either increased methane production or had no effect on methane production. The only exception was a 35 and 42 % reduction at low and high dosage respectively in the M diet at 36-48 h when compared to the CTR. Total methane production with fish oil supplementation also did not differ from that of the CT diets with the exception of a 60 % increase in methane production in the W diet. Very few previous studies have examined

effects of fish oil on methane production *in vitro*. The effects of fish oil supplementation on methane production are influenced by the composition of the oil. This was clearly demonstrated by Fieves *et al*., (2003) who used two types of fish oil that differed in FA composition. Fish oil A was composed of 19% EPA and 12% DHA, while fish oil B was composed of 6% EPA and 7% DHA. Fish oil A reduced methane production by a maximum of 75% while fish oil B suppressed methane production by a maximum of only 37%. In the current study menhaden fish oil was composed of 11.5 % EPA and 6% DHA, a similar composition to fish oil B which suppressed methane production by 37 % in the study of Fieves *et al*., (2003). In contrast to this, other studies have reported no differences in methane production when fish oil was compared with other oils. For example, Patra and Yu (2013) compared the effects of fish oil to that of coconut oil at 3.1 and 6.2 ml/L *in vitro* and reported that both fish oil and coconut oil reduced methane production to the same extent (9%) when supplied at 3.1 ml/L and no further decrease in methane production was observed at the 6.2 ml/L dosage.

Effects of fish oil may also be influenced by the composition of the basal diet. This was demonstrated by Castro-Montoya *et al*., (2012) when they compared the effects of fish oil on methane production on 3 basal diets; a standard concentrate diet, grass silage and maize silage. Fish oil supplemented at 100-200 g/kg produced a high amount of methane on standard concentrates and grass silage with amounts averaging 40 mmol/mol while only 30 mmol/mol of methane was produced on a maize silage diet. There was an interaction between fish oil and the basal diets which resulted in a higher suppression of methane production in the maize silage than the other two basal diets.

4.7.3 *In vitro* pH

The final pH of the CTR diets though statistically similar, numerically had different values. W and B had a slightly higher final pH of 5.77 while that of M was lower at 5.69. A previous study by Chaves *et al*., (2009) that reported that barley when compared to maize undergoes rapid fermentation in the rumen and consequently reduces the pH more than the maize. This contradicts the finding in the current study. Change in the pH of the fermentation contents in the current study was mediated by the oil source and dosage, and no starch source effect was observed. Carvacrol supplementation reduced the pH of the media by 0.5 units while linseed oil and fish oil supplementation reduced the pH by a larger margin of 1.1 pH units. When compared to the CTR diets, carvacrol effectively reduced pH of the media at the two dose levels while effects of linseed oil and fish oil did not differ from the CTR diets.

Previous studies show that effects of carvacrol on pH have been consistent. For example, in a study by Macheboeuf *et al*., (2008), *in vitro* supplementation of carvacrol at 0, 1.5, 2, 3, and 5mmol/L resulted in the pH of the media of 6.1, 6.3, 6.5, 6.90 and 7.0 respectively. In another *in vitro* study by Busquet *et al*., (2006), the addition of carvacrol at 0, 3 and 30 mg/L of fermentation fluid did not change the pH of the media which remained at pH 5.9, while a high dosage of 300 and 3 000 mg/L increased the pH to 6.3 and 7.2 respectively. It was concluded by Busquet *et al*., (2006) that although carvacrol altered the pH of the media with increasing dosage, the pH remained consistently high at around pH 6, a finding in agreement with that of the current study. In another study by Chaves *et al*., (2009), carvacrol supplemented at 0.2 g/kg DM to either barley or corn based diets did not change the pH of the media when compared to their respective CTR. The pH remained at 6.23 and 6.09 for barley and corn based diets respectively.

In the current study, the pH of the incubation media was reduced from 6.93 to an average of 6.35 at a lower dosage while at a higher dosage, fermentation was halted and pH in the fermentation vessels increased to 7.0. It can therefore be concluded that carvacrol mediates its effects by decreasing pH of the media. At a higher inclusion level, carvacrol was toxic and pH increased to 7.

Previous findings on the effects of linseed oil or α-linolenic acid on pH have been variable. For example, in a study by Zhang *et al*., (2008), α-linolenic acid supplementation at 35 and 70 g/kg increased the pH of the media from 6.37 to pH 6.52 and 6.56 respectively. In a Rusitec technique used by Vargas *et al*., (2011), addition of linseed oil to a TMR at 50 g/kg did not change the pH of the media which remained at pH 6.67.

The effects of fish oil on pH have also been variable. In an *in vitro* study by Patra and Yu, (2013), the addition of fish oil to a hay diet at 3.1 and 6.2 ml/L did not alter methane production and *in vitro* pH remained unchanged at 6.21. In contrast, in a study by Fievez *et al*., (2003), the two types of fish oils used reduced methane production by 75 and 37% while the pH of the media remained unchanged with supplementation, within the range at 5.70 to 5.78 and 5.77 to 5.88. In the current study, fish oil reduced pH of the media from 6.9 to a range of 5.78-5.84, a similar change observed in the CTR diet.

Methane production *in vitro* or *in vivo* is also influenced by the pH of the rumen environment (Bhata *et al*., 2006). A low pH suppresses methane production because cellulolytic and methanogenic bacterial activity is inhibited in such environments (Moss *et al*., 2000; Chung *et al*., 2011). This was demonstrated by Bhata *et al*., (2006), when a basal diet comprising hay, corn and soybean meal was incubated in a Rusitec fermenter, DM digestibility reduced from 60 to 42 % and a reduction in methane production of 59 % was observed when pH of the fermentor was reduced from pH 7 to pH 6.

In the current study, *in vitro* final pH environment ranged from 6.44-6.77 in the carvacrol supplemented treatments and 5.72-5.84 in the LO and fish oil supplemented treatments. Although methane production was lowest in the carvacrol treatment, principally it was due to a toxic effect on microbial metabolism.

4.7.4 NDF digestibility

Oil supplementation has been known to induce reductions, have no effects or even increase fibre digestibility (Sinclair *et al*., 2005). In the current study, when compared to the CTR, Cv1 reduced NDF digestibility in all the 3 basal diets, linseed oil did not have any effect in all the basal diets while fish oil increased NDF digestibility by 13% only in the maize based diets and did not have any effect in any other diets. The digestibility of NDF in the carvacrol based diets ranged from 182-572 g/kg which is lower when compared to that observed in LO and FO based diets that had a digestibility of above 690 g/kg. The lack of effect of linseed oil and fish oil on NDF digestibility agrees with earlier work of Beauchemin *et al*., (2009) and Cieslak *et al*., (2006) on supplementation of 4.2% crushed canola seed and 7 and 9% oilseed supplementation respectively in dairy cows and *in vitro*. Another study by Vargas *et al*., (2011) reported no change in NDF digestibility when linseed oil was supplemented at 5% DM to a TMR. The lack of effect of oils on NDF digestibility in the current study could have been either due to the oils not having an effect on the cellulolytic bacteria or that certain species of cellulolytic bacteria were inhibited and other species multiplied (Toral *et al*., 2009).

Some studies have reported reduced NDF digestibility due to oil supplementation. Beauchemin *et al*., (2008) reported a reduced DM fermentability with 3.2% sunflower and flaxseed supplementation, while Patra and Yu (2013) reported 10 and 15% reductions in NDF degradability when fish oil was supplied at 3.1 and 6.2 ml/L *in vitro.* Vafa *et al*.,

(2009) also reported an average of 3.3% reduction in DM digestibility with fish oil supplemented at 2, 4 and 6% DM on alfalfa hay diet.

An increase in NDF digestibility following oil supplementation has been reported in previous studies. In the current study, only FO1 increased NDF digestibility in the maize based diet while linseed oil had no effect in any of the 3 basal diets. For example, Ueda *et al*., (2003) observed a 3% increase in NDF total tract digestibility with 3% linseed oil supplementation when compared to the un-supplemented control diet.

The digestibility of NDF gives an estimate of how digestible feed is and may be closely related to methane formation (Boadi and Wittenberg, 2002; Dohme *et al.*, 2000). Reductions in NDF digestibility is also one of the ways in which oil supplementation mediate reductions in methane production (Johnson *et al*., 2002; Beauchemin *et al*., 2006). In the current study, Cv1 supplementation reduced methane output and this was accompanied with severe reductions in NDF digestibility. Many studies have also reported similar results. For example, in a study by Chung *et al*., (2011), supplementation of dairy cows on silage based diets with ground linseed at a rate 150 g/kg DM reduced NDF digestibility by 20% while methane production (g/kg DM intake) was reduced by 33% and was associated with a reduction in protozoa numbers.

4.8 Conclusions

The current study has shown that oil sources carvacrol, linseed oil and fish oil when added at the same level of supplementation, differed in fermentation characteristics. The three starch sources, wheat, barley and maize differed in fermentation characteristics, with wheat producing a high fermentation rate and maize producing a lower fermentation. Methane production among the starch sources varied with time of incubation. Within each treatment, methane production when expressed as ml/g DM

varied with time of incubation and the basal diet used. Carvacrol reduced methane production more than linseed oil and fish oil, but the effects were mediated by a severe reduction in NDF digestibility. Linseed oil and fish oil had no effect on total methane production when compared to the CTR diet, except at a lower dose of fish oil (FO1) which increased methane production by 60% in the wheat based diet.

CHAPTER 5: The effect of starch source and oil source on the performance, methane production and milk fatty acid profile of Holstein dairy cows

5.1 Introduction

Loss of energy in cattle through methane output has been a source of concern due to its impact on climate change (Moss *et al*., 2000). The energy losses through methane production when expressed on the basis of gross energy intake vary between 2-12% (Van-Ziljderveld *et al*., 2010). The dietary composition of cattle feed has a major influence on the amount of methane released from the rumen into the environment. A number of previous studies (e.g. Zhang *et al*., 2008; Beauchimin *et al*., 2009; Eugene *et al*., 2011) have indicated that lipid and starch supplementation of ruminant diets appear to be some of the most effective strategies to reduce ruminant methanogenesis.

Dietary fats mediate reduced methane output in two main ways; by reducing fibre fermentability and by being toxic to protozoa (Ivan *et al*., 2001) and methanogenic bacteria (Moss *et al*., 2000). Additional beneficial effects of fats are that they increase the energy density of ruminant diets and therefore enhance milk production and can be used to modify the milk fatty acid composition (Odongo *et al*., 2007). A study by Beauchemin *et al*., (2009) indicated that processed oilseeds composed of long chain FAs have considerable potential to reduce methane production of ruminants. Several studies have reported improved animal performance following oil addition to the diet which may in part be explained by the reduction in methane production which is channelled towards improving productivity (Jordan *et al*., 2006).

Oil supplementation of ruminant diets can also be employed to alter the milk fatty acid profile (Kazama *et al*., 2010). A study by Collomb *et al*., (2004) showed that sunflower oil,

being rich in linoleic acid ($C_{18:2n-6}$), can reduce the proportion of short chain fatty acids (C_6-C_{14}) and increase the C_{18} FAs, which are beneficial to human health. Studies by Colomb *et al*., (2004) and Kazama *et al*., (2010) have also shown that the monounsaturated fatty acid ($C_{18:1}$) content of milk can be increased by 50 to 80% by feeding lipids such as flax oil which is rich in 18-carbon fatty acid. Palmitic acid ($C_{16:0}$) content of milk fat can also be reduced by 20 to 40% by addition of mono or polyunsaturated fatty acids unless the supplemented lipid is rich in $C_{16:0}$ (Odongo *et al*., 2007). Sunflower oil was chosen based on the potential benefit of reducing methane production and ability to increase the long chain fatty acids in milk. A rumen protected fat, megalac was also chosen since it is has small or no effects on rumen metabolism and its fatty acid profile differs from that of sunflower oil.

Concentrate supplementation mediates reduced methane output by promoting ruminal propionate production and reducing ruminal pH, creating an unfavourable environment for methanogenic bacteria (Lovett *et al*., 2005). A number of previous studies (Lovett *et al*., 2005; McGeogh *et al*., 2010) have recorded reduced methane output with concentrate supplementation particularly if the concentrate contains starch levels of 30% or more (McGeough *et al*., 2010; Hart *et al*., 2012). With concentrate supplementation, retention time of feed in the rumen is reduced which also reduces the methane output (Moss *et al*. 2000). Considerable reductions in methane output are observed with *ad libitum* concentrate supplementation or with diets containing >80 concentrate supplementation which result in a decrease of 2-4% units of methane on a gross energy intake basis (Moss *et al*., 2000; McGeough *et al*., 2010).

To date there is limited information (Beauchimen *et al*., 2009) on the combined effects of oil supplementation and starch source on methane output for a range of dietary conditions. Only one previous study by Eugene *et al*., (2011) examined the combination of

oil and starch type on methane output of bulls using linseed oil supplemented at 1.2 % DM and starch supplemented at 33% DM. In the study by Eugene *et al*., (2011), methane production in the bulls when expressed as L/d and L/kg LW gain was reduced by 20 and 24 % respectively when compared those that were fed a high fibrous diet. The reduction in methane production was as a result of the reduced DM intake associated with oil supplementation.

The objective of the current study was to determine the impact of feeding cows with concentrates high in wheat or maize while supplementing with a fat source high in saturated or unsaturated fatty acids on the methane production, productivity, blood metabolites and milk fatty acids in dairy cows. The hypothesis that was tested was that a combination of starch source and oil source would reduce methane production and that the effects on methane production would be additive.

5.2 Materials and Method

The work described in this paper was conducted in accordance with the requirements of the UK Animals (Scientific Procedures) Act 1986 (Her Majesty's Stationery Office, 2000) and was approved by the Harper Adams University Ethics Committee.

5.2.1 Animals, experimental design and treatments

Sixteen multiparous Holstein cows that were approximately 60-100 days in lactation and yielding 38 (±3.9) kg of milk/d at the beginning of the study were used in a 4x4 Latin square design with a 2 X 2 factorial arrangement of treatments. Each period consisted of 23 days of adaptation to the diets followed by a 7 d sampling period. Based on recordings in the week prior to commencing the study, animals were blocked and allocated to one of four treatments according to calving date, milk composition, body condition score and

live weight. The four treatments were; Maize based concentrates and rumen protected fat (MP); Wheat based concentrate and rumen protected fat (WP); Maize based concentrate and sunflower oil (MS); Wheat based concentrate and sunflower oil (WS). All the cows received a basal total mixed ration (TMR) based on maize and grass silage (2:1 DM basis) with added rapeseed meal, distillers grains, urea and molasses as shown in Table 22. The rumen protected fat (Megalac®, Volac International Ltd, Lampeter, Wales) and sunflower oil (KTC edible Ltd, Wednesbury, UK) were added to the basal total mixed ration (TMR) to supply approximately 29 g/kg DM of Megalac® or 25 g/kg DM of sunflower oil to provide a similar supplementary fat level. Cows also received one of two concentrates in pellet form that were formulated to be isonitrogenous and isoenergetic and to contain approximately 290 g/ kg DM starch from either maize or wheat, respectively (Table 23). The concentrates were fed at 7.5 kg /cow/d *as fed* via out of parlour feeders in three equal meals of 2.5 kg separated by a minimum of 6 h between meals. The out of parlour feeders were calibrated to ± 0.1 kg at the beginning of each collection period.

Table 22. Diet composition (g/kg DM) and chemical composition (g/kg DM) for a basal total mixed ration that contained Megalac (Meg) or sunflower oil (Sun)

	Meg	Sun
Maize silage	645	648
Grass silage	275	276
Rapeseed meal	24	24
Maize distiller dark grains	24	24
Urea	3	3
Megalac	29	---
Sunflower oil	---	25
Chemical composition		
Dry matter, g/kg	323	317
Ash	56	50
Organic matter,	944	950
Crude protein	104	104
NDF	357	373
Starch	221	231
Fatty acids	66	63
Fatty acid, g/100g FA		
$C_{16:0}$	31	14
$C_{18:0}$	2.5	2.3
$C_{18:1n\text{-}9}$	31	31
$C_{18:2n\text{-}6}$	28	47
$C_{18:3n\text{-}3}$	4.7	4.6

Table 23. Ingredient composition (kg/t) and chemical composition of the wheat and maize based concentrates

	Wheat	Maize
Wheat	400	---
Maize	---	400
Maize distillers dark grains	121	121
Soybean meal	165	165
Rapeseed meal	150	150
Propass[1]	50	50
Vegetable oil	13	13
Molasses	68	68
Calcined magnesite	1	1
Limestone	20	20
Rock salt	5	5
Dicalcium phosphate	5	5
Minerals and vitamins[2]	3	3
Total	1000	1000
Chemical composition		
Dry matter	876	863
Crude protein	259	247
NDF	126	124
Ash	83	86
Starch	290	332
Fatty acids	49	46
Fatty acid, g/100g FA		
$C_{16:0}$	24	25
$C_{18:0}$	2.2	2.6
$C_{18:1n\text{-}9}$	31	33
$C_{18:2n\text{-}6}$	38	35
$C_{18:3n\text{-}6}$	0.5	0.7

[1]Contained protein 0.1%, ash 81.6%, sodium 23.9%, calcium 4.8%, phosphorus 0.1%, magnesium 5.9%, copper 4 mg/kg

[2] Contained calcium 21%, phosphorus P 3%, sodium 10%, sodium 5%, sodium selenite 30 mg/kg, selenium (from *saccharomyces cerevisiae*), 10 mg/kg, cobalt carbonate, 70 mg/kg, manganese 5000 mg/kg, zinc oxide 4000 mg/kg, zinc chelate 2000 mg/kg, copper sulphate 1 500 mg/kg, copper chelate 1000 mg/kg, vit A 500 000 iu/kg, vit D_3,100 000 iu/kg vit E 4000 iu/kg, vit B12 2 500 mcg/kg

The grass silage was from a first cut sward consisting predominantly of ryegrass and both the grass and maize silage were ensiled in a roofed, concrete clamp. The forages and straight feeds were mixed using a Keenan compact forage mixer (Richard Keenan & Co. Ltd, Carlow, Ireland) calibrated to ± 1 kg and fed through Insentec roughage intake feeders (RIC feeders) fitted with an automatic animal identification. The forage weighing

system was calibrated to ± 0.1 kg (Sinclair *et al*., 2005; Sinclair *et al*., 2007). Fresh feed was offered daily after morning milking at approximately 0900h at the rate of 1.05 of *ad libitum* intake, with refusals collected twice weekly on a Tuesday and Friday. Each of the experimental cows had a transponder fitted around their neck to allow access to the out of parlour feeders and forage bins.

5.2.2 Housing

Cows were housed in super comfort cubicles fitted with rubber mattresses and bedded with sawdust and lime twice weekly. The loafing area was scraped four times daily using automatic scrapers. All cows had continuous access to water throughout the experimental period.

5.2.3 Sampling procedure

Cows were weighed and condition scored (Lowman *et al*., 1976) after the morning milking in the week before the start of the first period and at the end of each period. The cows were milked twice daily at approximately 05:30 and 15:30 h with milk yield recorded at each milking. Milk samples were taken in duplicate on four occasions during the final 7 days of each period (Mon pm, Tue am, Thurs pm, Fri am). Milk samples were preserved with 2-bromo-2-nitropropane-1, 3-diol (Sigma Aldrich, Dorset, UK) and stored at 4°C for subsequent composition analysis to determine fat, protein and lactose contents and a second set of milk samples were immediately centrifuged by a method described by Feng *et al*., (2004), and the fat layer collected and stored at -20^{o}C prior to subsequent fatty acid analysis. Feed intakes were recorded daily and forage and concentrate samples collected twice during each sampling period and frozen at -20°C prior to subsequent analysis. Faecal samples were collected every day during the sampling period between 07 30h and 08 30h and between 14 30 and 15 00h. Fresh voided samples were collected either from

the floor of the housing unit or grabbed as cows stood up on being approached. Samples were frozen at -20°C prior to subsequent analysis. Blood samples from the jugular vein were collected in duplicate at 08:00 h and at 12:00 h on the 2nd day and at 10:00 h and 14:00 h on the 4th day of each sampling period. The blood samples were collected into sodium heparinised vacutainers for subsequent urea and β hydroxybutyrate (3-OHB) determination and into vacutainers containing potassium oxalate for glucose determination. Blood samples were immediately centrifuged (Avanti 30, Beckman, Beckman Laboratories, USA) at 3000 g for 15 min and the plasma collected and stored at -20°C prior to subsequent analysis of urea, 3-OHB and glucose.

Sampling of respired air from the cows was done using evacuated canisters which were being changed everyday at 10:00 hrs during the 5 day sampling period. From the respired air, rumen methane production was measured using the sulphur hexafluoride (SF_6) tracer technique according to the method described by Johnson and Johnson (1995) as described in Section 3.8.2. Calibrated brass permeation tubes weighing approximately 58 g were inserted into the rumen of each cow. The permeation rate of SF_6 from the tubes was determined prior to insertion in the rumen and averaged 5.5 ± 0.0005 mg/d. Respired gas from each animal was sampled into pre-evacuated (85 to 97 kPa) canisters as described by Johnson and Johnson (1995; Fig 9). The collection devices were changed every 24 h at approximately 1000 h. The canisters containing the respired gas samples were immediately transported to the laboratory and pressurised with N_2 gas to 17 kPa. Subsamples of eructated air were collected for subsequent analysis of SF_6 and CH_4 as described in section 3.8.2. Background concentrations of the gases were also measured by collecting air samples at ambient conditions around the dairy unit during the 5 d sampling period. This was done by placing the evacuated cylinders, one just outside the housing unit and the other one in the central part of the housing unit near the out of

parlour feeding area. The two cylinders were being changed everyday at same time as those attached on the animals.

Figure 9. Cow wearing a full gas collection pack

5.2.4. Laboratory analyses

The TMR and concentrate samples were freeze dried (Edwards freeze dryer Modulyo, Bristol, UK) at -50°C for 5 days and milled in a Delonghi KG79 grinder (Freemans PLC, Sheffield, UK) to pass through a 1 mm screen (Endecotts Ltd, London, UK). The milled samples were analysed in duplicate for DM, OM, N, according to the Association of Official Analytical Chemists (AOAC, 2000) for DM (934.01), CP (988.05) and ash (942.05) as described in sections 3.1.1 to 3.1.3. Neutral detergent fibre (NDF) was analysed according to Van Soest *et al*., (1991) as described in section 3.1.4. Milk compositional analysis was conducted using a Milkscan Minor 78110 (Foss Electric, Hillerod, Denmark) as described in section 3.2.1. Milk samples for fatty acid analysis were centrifuged by the method of Feng *et al*., (2004) as described in section 3.2.2. Methylation of the lipids collected was done according to the procedure by Christie (1982) with modifications

according to Chouinard *et al*., (1999) as described in section 3.2.2. Fatty acid content in TMR and concentrate samples were analysed according to Sukhija and Palmquist (1998) using nonadecaenoic acid ($C_{19:0}$) as an internal standard as described in section 3.3.

Samples of eructated air from the cows and the ambient air samples were analysed for concentrations of SF_6 and CH_4 by gas chromatography as described in section 3.8.2. Daily CH_4 production by each animal was calculated using the known permeation rate of SF_6 for each tube and the concentrations corrected for background levels of SF_6 and CH_4 in the breath samples using the equation:

$$CH_4\,(g/d) = SF_6 \text{ permeation rate } (g/d) \times \frac{[CH_4]}{[SF_6]}$$

Where CH_4 (g/d) is the emission rates of the individual cows, SF_6 permeation rate (g/d) is the known release rate of SF_6 before boluses were inserted in the rumen and $[CH_4]$ and $[SF_6]$ are sample concentrations corrected for background levels expressed in micrograms per cubic meter (Johnson and Johnson, 1995). Faecal samples were oven dried at 60°C for 48h, milled and acid insoluble ash determined using the method described by Van Keulen and Young (1977) using 2M hydrochloric acid as described in section 3.1.5. Concentrate and TMR samples were also analysed for acid insoluble ash and dry matter digestibility of the samples was determined as described in section 3.1.5. Plasma samples were analysed for glucose, 3-OHB and urea concentration using commercial diagnostic kits (Catalogue nos. GL 1611, RB 1007 and UR220 respectively, Randox Laboratories, London, UK) using a Cobas-Mira Plus auto-analyser (ABX diagnostics, Montpellier, France)

5.2.5 Statistical analyses

The mean values of the data from the individual cows i.e. methane output (g/d), feed intake data (kg/d), and milk performance (kg/d) was determined by calculating the

average values collected during the 5 day sampling period for each individual animal. Data was analysed as a Latin square design using Genstat 13.1 (VSN International Ltd, Hemel, Hempstead, UK) with a 2X2 factorial arranegement of treatments. The statistical model included main effects of starch source and oil source, starch sources being wheat or maize and oil sources were either Megalac or sunflower. The period effect was the row and cow effect taken as the column. Interaction between starch source and oil source was also determined. Plasma data calculated as mean values at each particular time period was analysed using repeated measures of analysis of variance. Multiple comparisons among treatment means were performed by the Tukey's method. Results were presented as treatment means with SED, and significance considered at $P<0.05$.

5.3 Results

5.3.1 Feed analysis

The two TMR rations had a similar DM, organic matter, crude protein, NDF, starch and total fatty acid content (Table 22). However, there were differences in the FA composition. The TMR containing Megalac was higher in $C_{16:0}$ (31 g/100g fatty acids) whereas the TMR containing sunflower oil was high in $C_{18:2n\text{-}6}$ (47 g/100g fatty acids). The two concentrates had a similar DM, crude protein, ash and total fatty acid content, and had a similar fatty acid profile (Table 23). The concentrates only differed in the starch content, wheat based concentrates supplied 290 g/kg DM starch, while the maize based concentrates supplied 332 g/kg DM starch. The oil intake was 2.0% of total DM intake for megalac based diets and 1.6 % of total DM intake for sunflower oil based diets.

5.3.2 Intakes and animal performance

Cows when fed the sunflower based diets (WS and MS) ate 2.5 kg/d (total DM) less than those fed the Megalac based diets ($P<0.001$; Table 24). Mean total DM intake was 18.5 kg

DM/cow/d for cows offered the sunflower based diets and 21 kg DM/d for those fed the Megalac based diets, representing a 12% reduction in feed intake with the sunflower oil based diets. There were no starch source effects on DM intake and no interaction between starch and oil source on DM intake was observed. There was no effect (P>0.05) of dietary treatment on daily milk yield, which averaged 33.2 kg/d, but milk fat levels were 3 g/kg lower (P<0.01) in cows when fed a sunflower oil based diet when compared to those fed a Megalac based diet, and also tended (P=0.051) to be lower when fed the maize compared to the wheat based concentrate. When corrected for fat content, average daily milk yield was 2.3 kg lower (P<0.05) in cows when fed sunflower oil based diets than those fed Megalac based diets. There was no effect (P>0.05) of dietary treatment on milk protein content or yield.

Nutrient intakes differed among the treatment groups. Cows fed the sunflower oil based diets had lower N intakes (0.20 vs. 0.24 kg/d; Table 24) when compared to those fed the Megalac based diets. Though all the cows were fed 7.5 kg/d each on *as fed basis,* of either wheat or maize based concentrates, it was observed that cows that received the wheat based concentrates had a higher N intake of 1.70 kg/d/cow when compared to the ones that received maize based concentrates with N intakes of 1.61 kg/d/cow (P<0.001). Similarly, total N intakes differed. Cows that were fed the wheat based diets had on average 1.93 kg/d of total N intake when compared to 1.83 kg/d N intake in cows fed the maize based diets (P<0.001). Total N intakes also varied with oil source (P<0.001). Intakes were lower when cows were fed sunflower oil based diets (1.85 kg/d) when compared to those fed Megalac based diets with total N intakes of 1.90 kg/d.

Starch intakes were 0.2 kg/d higher in cows fed the maize based concentrates when compared to intakes in cows fed the wheat based concentrates (P<0.05; Table 24). However total starch intakes were similar in all the treatment groups (P>0.1).

Neutral detergent fibre (NDF) intakes also varied among the treatment groups. NDF intakes were 0.02 kg/d higher in cows fed the wheat based diets when compared to those fed the maize based diets (P<0.001) and where also 0.60 kg higher in cows fed the Megalac based diets when compared to those fed the sunflower oil based diets. Total NDF intake followed a similar trend, and were 0.70 kg higher in cows fed Megalac based diets when compared to those fed the sunflower oil based diets.

There was also no effect (P>0.05) of starch or oil source on live weight change but final condition score tended (P=0.06) to be higher in cows when fed the wheat compared to the maize based concentrate. There was a starch source effect (P<0.05) on condition score change; cows lost on average 0.06 body condition score when receiving the wheat based concentrate and gained 0.01 condition when receiving the maize based concentrate.

Table 24. Intakes (kg/d) and performance in dairy cows fed concentrates high in wheat or maize and supplemented with either Megalac (Meg) or Sunflower oil (Sun)

Starch in concentrate	Wheat		Maize			*P*-values		
Oil	Meg	Sun	Meg	Sun	s.e.d	Starch (S)	Oil (O)	S x O
TMR DM intake, kg/d	14.7	12.0	14.4	12.2	0.544	0.872	<0.001	0.576
Total DM intake, kg/d	21.3	18.4	20.8	18.6	0.559	0.670	<0.001	0.324
N TMR intake, kg/d	0.25	0.20	0.24	0.20	0.007	0.724	<0.001	0.325
N conc intake, kg/d	1.70	1.70	1.61	1.61	0.0004	<0.001	0.913	0.691
Total N intake, kg/d	1.95	1.90	1.85	1.81	0.006	<0.001	<0.001	0.304
Conc starch intake, kg/d	1.90	1.90	2.01	2.17	0.070	0.012	0.256	0.252
TMR starch intake, kg/d	3.26	2.73	2.99	2.79	0.111	0.329	0.002	0.136
Total starch intake, kg/d	5.16	4.63	4.99	4.96	0.164	0.650	0.092	0.133
Conc NDF intake, kg/d	0.83	0.83	0.81	0.81	0.0002	<0.001	0.915	0.692
TMR NDF intake, kg/d	5.27	4.41	5.07	4.50	0.145	0.721	<0.001	0.317
Total NDF intake, kg/d	6.10	5.24	5.88	5.31	0.145	0.637	<0.001	0.316
Milk yield, kg/d	32.6	32.9	33.6	33.6	0.761	0.111	0.793	0.760
Fat corr. milk yield, kg/d	30.5	27.9	28.9	27.0	1.460	0.248	0.037	0.776
Milk fat, g/kg	37.4	34.3	35.1	32.3	0.151	0.051	0.008	0.881
Fat yield, kg/d	1.22	1.12	1.16	1.08	0.058	0.249	0.037	0.774
Milk protein, g/kg	32.1	32.3	32.5	32.1	0.034	0.840	0.612	0.214
Protein yield, kg/d	1.04	1.05	1.08	1.07	0.027	0.134	0.997	0.671
Final Lwt, kg/d	668	670	673	671	5.320	0.406	0.977	0.608
Lwt change, kg/d	0.32	0.30	0.47	0.20	0.176	0.827	0.245	0.312
Final condition score	2.65	2.63	2.54	2.56	0.062	0.057	0.945	0.704
Condition score change	-0.08	-0.05	0.09	0.10	0.090	0.019	0.798	0.830

Lwt = live weight. Concentrate DM intake was on average 6.5 kg/d/cow

5.3.3 Methane production

Cows when fed the maize based diets produced 18.5 g/d less methane output when compared to those that fed wheat based diets (362 vs. 380.5 g/d respectively) representing a 5.8% difference in methane output between the two starch sources (P<0.05; Table 25). Oil source had no effect (P=0.485) on daily methane output and there was no interaction observed between oil and starch source (P=0.456). When methane output was expressed per unit of DM intake (g/kg DM intake), oil source had an effect (P<0.001) with cows fed diets containing Megalac producing 2.3 g/kg DM less methane when compared to those fed diets containing sunflower oil, representing a 12.8% increase in methane output with the sunflower oil based diets. When methane output was expressed per unit of milk yield (g/kg of milk), cows fed the maize based diets

produced a lower methane output (11.3 g/kg milk yield) than those fed the wheat based diets (12.3 g/kg milk; *P*=0.023), representing an 8.1% difference in methane output between the two starch sources. There was no interaction observed between the oil source and starch source on methane output when expressed as g/day, g/kg DM intake or g/kg milk (Table 25).

Table 25. Methane production in dairy cows fed concentrates high in wheat or maize and supplemented with a saturated fat source (Megalac: Meg) or sunflower oil (Sun)

Starch in conc	Wheat		Maize			*P*-values		
Oil	Meg	Sun	Meg	Sun	s.e.d	Starch (S)	Oil (O)	S x O
CH_4, g/d	386	375	362	362	11.3	0.027	0.485	0.456
CH_4, g/kg DM intake	18.3	20.7	17.7	19.8	0.690	0.127	<0.001	0.744
CH_4, g/kg milk yield	12.7	11.8	11.3	11.3	0.549	0.023	0.231	0.267
CH_4, g/kg fat yield[1]	13.7	14.3	13.1	14.8	1.080	0.928	0.130	0.479

[1]fat corrected yield

5.3.4 Blood metabolites

There was a starch source effect on plasma urea concentrations (*P*<0.001) with cows fed the maize based concentrates having 0.5 mmols/L lower concentration than those fed the wheat based concentrate (Table 26). This difference was evident at all sampling times (Fig 10). There was a trend (*P*<0.10) for cows fed the maize based concentrate to have lower plasma concentrations of 3-OHB (Fig 11).

Table 26. Plasma metabolites in dairy cows fed concentrates high in wheat or maize and supplemented with saturated fat source (Megalac: Meg) or sunflower oil (Sun)

Starch in conc		Wheat		Maize		*P*-values		
Oil	Meg	Sun	Meg	Sun	s.e.d	Starch (S)	Oil (O)	SXO
Urea, mmol/L	4.42	4.390	3.81	4.00	0.138	<0.001	0.390	0.276
3-OHB, mmol/L	0.589	0.569	0.534	0.557	0.0281	0.097	0.959	0.281

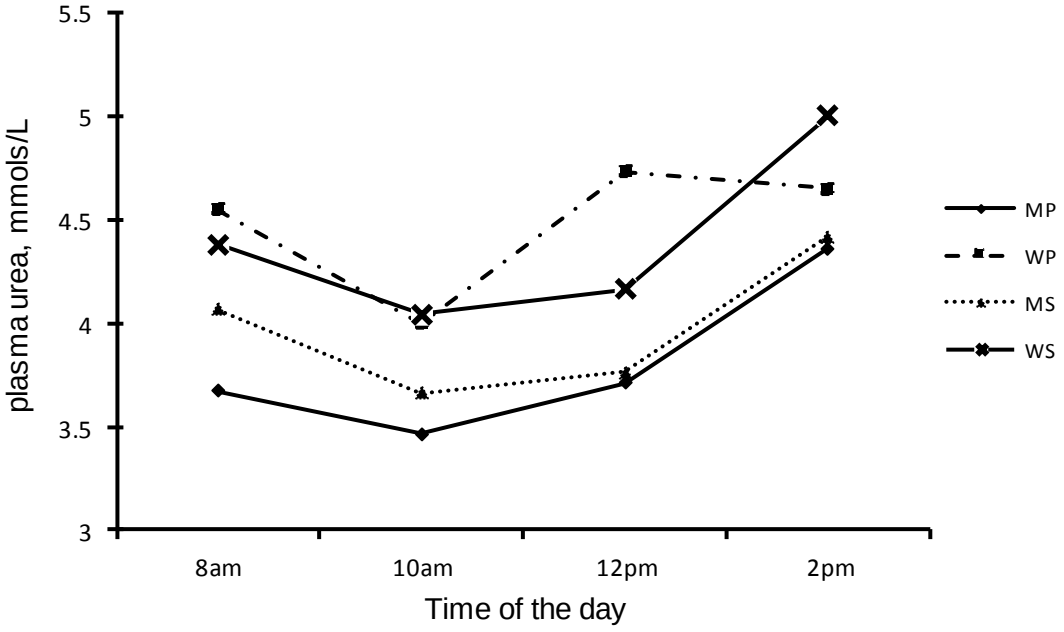


Figure 10. Mean plasma urea concentrations in dairy cows fed concentrates high in wheat (W) or maize (M) and supplemented with a saturated fat source (Megalac:P) or sunflower oil (S)

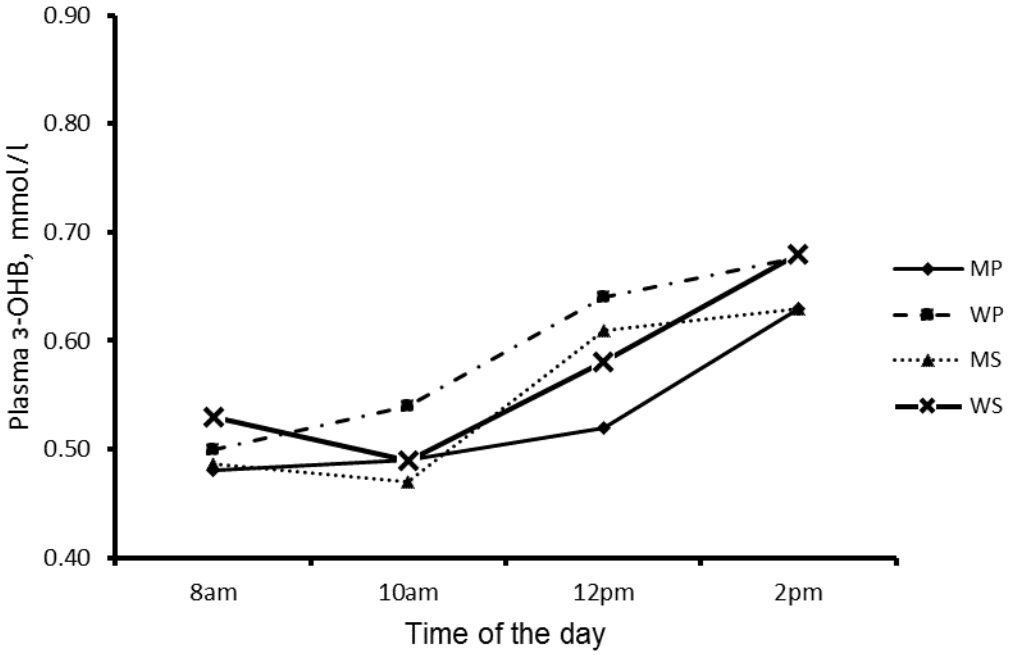


Figure 11. Mean plasma 3-OHB concentrations in dairy cows fed concentrates high in wheat (W) or maize (M) and supplemented with a saturated fat source (Megalac:P) or sunflower oil (S).

5.3.5 Milk fatty acid profile

There was no effect of starch or oil source on the milk fatty acid content of the short chain fatty acids (C_4-C_{10}; Table 27). Among the medium chain FAs, $C_{16:0}$ was lower by 3.8 g/100g in cows when fed the sunflower oil based diets (P<0.001) which was a reduction of 12.4%. The long chain FA $C_{18:0}$ was increased by 1 g/100g in cows when fed sunflower oil compared to Megalac (P<0.001) which was a 9.8% increase. Cows fed sunflower oil based diets (WS or MS) also had increased (P<0.05) concentrations of the *trans*-fatty acids *trans*-9 $C_{18:1}$, *trans*-11 $C_{18:1}$, *cis*-9, *trans*-11 CLA and *trans*-10, *cis*-12 CLA by 0.08 g/100g, 1.3 g/100g, 0.25 g/100g and 0.01 g/100g, thus representing increases of 8, 46, 25 and 8.3% respectively. Feeding the maize based concentrates also increased (P<0.05) the concentrations of all the *trans*-fatty acids. There was no effect (P>0.005) of sunflower oil on *cis*-9, *cis*-12 $C_{18:2}$ or *cis*-9, *cis*-12, *cis*-15 $C_{18:3}$. There was an interaction between starch and oil source (P<0.05) due to increased $C_{18:0}$ concentrations in cows fed the wheat based concentrates and sunflower oil but $C_{18:0}$ concentrations decreased in cows fed the maize based concentrates and sunflower oil. Feeding sunflower oil decreased (P<0.001) milk saturated fatty acids and increased (P<0.001) monounsaturated fatty acids and polyunsaturated fatty acids (P<0.05).

Table 27. Milk fatty acid profile in dairy cows fed concentrates high in wheat or maize and supplemented with a saturated fat source (Megalac: Meg) or sunflower oil (Sun)

	Wheat		Maize			*P* - values		
	Meg	Sun	Meg	Sun	s.e.d	Starch (S)	Oil (O)	SXO
Milk FAs (g/100g)								
$C_{4:0}$	2.50	2.34	2.45	2.40	0.070	0.968	0.037	0.284
$C_{6:0}$	1.42	1.34	1.35	1.35	0.052	0.473	0.235	0.265
$C_{8:0}$	0.70	0.66	0.62	0.63	0.055	0.172	0.667	0.474
$C_{10:0}$	1.96	1.91	1.80	1.88	0.084	0.130	0.773	0.258
$C_{12:0}$	2.60	2.58	2.42	2.51	0.088	0.063	0.560	0.417
$C_{14:0}$	9.71	9.94	9.56	9.66	0.178	0.105	0.206	0.617
$C_{15:0}$	0.78	0.81	0.77	0.77	0.024	0.099	0.578	0.349
$C_{16:0}$	30.9	27.2	30.4	26.4	0.505	0.103	<0.001	0.696
cis-9 $C_{16:1}$	1.48	1.43	1.54	1.43	0.093	0.736	0.234	0.664
$C_{17:0}$	0.39	0.40	0.38	0.40	0.007	<.001	0.062	0.722
$C_{18:0}$	10.38	11.4	9.97	11.0	0.245	0.963	<0.001	0.021
cis-9 $C_{18:1}$	24.8	25.7	25.6	26.1	0.536	0.125	0.083	0.565
trans-11 $C_{18:1}$	2.67	3.81	3.02	4.37	0.223	0.006	<0.001	0.516
trans-9 $C_{18:1}$	0.95	1.08	1.05	1.09	0.034	0.034	0.002	0.066
$C_{18:2n\text{-}6}$	2.39	2.43	2.53	2.45	0.061	0.058	0.631	0.226
cis 9,*trans* 11CLA	0.69	0.96	0.81	1.04	0.055	0.019	<0.001	0.584
*trans*11,cis12CLA	0.12	0.13	0.12	0.13	0.007	0.911	0.048	0.751
$C_{18:3n\text{-}3}$	0.50	0.50	0.45	0.50	0.021	0.054	0.114	0.055
ΣSFA	61.7	58.8	60.2	58.1	0.796	0.069	<0.001	0.456
ΣMUFA	31.2	33.9	32.6	34.5	0.780	0.067	<0.001	0.449
ΣPUFA	3.50	3.65	3.62	3.72	0.077	0.079	0.028	0.666

ΣSFA=sum of saturated fatty acids; $C_{4:0}$; $C_{6:0}$; $C_{8:0}$; $C_{10:0}$; $C_{12:0}$; $C_{14:0}$; $C_{16:0}$
ΣMUFA=sum of monounsaturated fatty acids; cis-9 $C_{18:1}$; trans-11 $C_{18:1}$; trans-9 $C_{18:1}$
ΣPUFA= sum of polyunsaturated fatty acids; $C_{18:2n\text{-}6}$; *cis*-9, *trans*-11 CLA; *trans*-11, *cis*-12 CLA; $C_{18:3n\text{-}3}$.

5.3.6 Whole tract digestibility

The DM digestibility of all the diets was high and was not affected by dietary treatment, although there was a trend (*P*=0.07) to be lower in cows when fed the maize compared to the wheat based concentrates (Table 28).

Table 28. Whole tract digestibility in dairy cows fed concentrates high in wheat or maize and supplemented with a saturated fat source (Megalac: Meg) or sunflower oil (Sun)

Starch in conc	Wheat		Maize			*P*-values		
Oil	Meg	Sun	Meg	Sun	s.e.d	Starch (S)	Oil (O)	SXO
DM, g/kg	0.77	0.74	0.75	0.74	0.009	0.067	0.256	0.505

5.4. Discussion

The current study examined the effect of starch source and oil source on methane production, productivity and milk FA profile of Holstein dairy cows. Megalac was used as an inert source of fat high in palmitic acid ($C_{16:0}$) as it was assumed to have minimal effect on methanogenesis (Rabiee *et al*., 2012). Sunflower oil was added to the diets at 1.6% DM basis in order to avoid negative effects on digestibility and feed intake, and maize starch was compared against wheat starch as it has a slower rate of release in the rumen and a lower rumen degradability (Moss *et al*., 2000; Chaves *et al*., 2009; Reynolds 2006).

5.4.1 Intakes and animal performance

Dry matter (DM) intake influences methane output in ruminants (Moss *et al*., 2000; Reynolds *et al*., 2011). In the current study, the addition of sunflower oil reduced total DM intake by 2.5 kg/cow/d or by 12%. A reduction in DM intake following oil addition to ruminant's diet has been reported in several previous studies. For example McGinn *et al*., (2004) reported a 6.6% reduction in DM intake following a 5% sunflower oil supplementation. In the current study both the sunflower oil and Megalac based diets were high forage diets with a similar composition. Both diets were composed of maize and grass silage in the ratio 3:1 with the silages making up 90% of the total TMR ration. Similar to Jordan *et al*., (2006), the reduction in DM intake with sunflower oil may have been as a result of reduced palatability. According to VanZijderveld *et al*. (2010), fats and oils indirectly lower methane outputs by reducing DM intake which occurs due to a reduction in DM digestibility of feed in the rumen, although in the current study, DM digestibility was unaffected by oil source. In a study by Petit *et al*., (2004), cows on a TMR ration when supplemented at 3.6 % DM with either Megalac or whole sunflower seeds had a similar DM intake which averaged 21 kg/d. Petit *et al*., (2004), however used whole sunflower seeds, unlike in the current study where refined sunflower oil was used.

Nutrient intake varied with dietary treatment. In the current study, though CP contents of the Megalac and sunflower based diets were similar at 104 g/kg DM, the N intake were higher in cows fed the megalac based diets at 0.24 kg/d when compared to intakes of 0.20 kg/d observed in cows fed the sunflower based diets. The higher intakes were as a result of the high TMR DM intakes observed in cows fed on this diet. Total N intakes followed a similar trend. On the other hand, N intakes of cows fed the wheat based concentrates was 0.09 kg/d higher than those fed the maize because the wheat based concentrates had a higher CP content of 259 g/kg DM when compared to 247 g/kg DM found in the maize based concentrates.

In the current study, cows fed the maize based concentrates gained 0.1 condition score while those that fed the wheat based concentrates lost 0.06. The increase in condition score with maize based concentrates can be attributed to the reduction in daily methane output associated with this diet in association with the greater predicted content of by-pass starch (Doreau *et al*., 2011).

5.4.2. Milk yield and components

In the current study, milk yield averaged 33.2 kg/d and was unaffected by dietary treatment but fat corrected yield was 2.2 kg/d lower when sunflower based diets were fed. The result is similar to findings by Beauchemin *et al*., (2009) who reported no change in milk yield following supplementation of dairy cows with sunflower seeds at 3.7% DM. However, some previous studies have reported either reduced or improved milk yields with sunflower oil supplementation. For example in a study by Johnson *et al*., (2002) both milk and 3.5% fat corrected milk (FCM) yields were greater when cows were fed the oilseeds. In a study by Petit *et al*., (2004), a 3.6 % supplementation of either Megalac or whole sunflower seeds resulted in cows fed the Megalac based diets producing 5.6 kg/d

more milk than those fed the sunflower seed based diets. According to Petit *et al*., (2004), cows on the Megalac based diet had a 4.4 Mcal/d higher digestible energy intake which could have influenced the increase in milk yield. In the current study, the lower fat corrected yield reported when cows were fed sunflower based diets could have been due to the lower DM intake on this diet.

It is well established that milk fat content can be modified by fat supplementation (Chilliard *et al*., 2009). In the current study, both milk fat content (g/kg) and fat yield (kg/d) were reduced by 3 g/kg and 0.09 kg/d respectively with sunflower oil addition. The *trans*-fatty acids absorbed as a result of ruminal bio-hydrogenation are thought to be responsible for depressed milk fats (Bauman and Griinari, 2001). A meta-analysis by Rabiee *et al*., (2012) associated milk fat depression to an increase in *trans*-10, *cis* 12 CLA concentrations in milk fat. In the current study, cows fed sunflower oil based diets had an 8% higher concentration of *trans*-10, cis 12 CLA in the milk fat when compared to those fed Megalac based diets. The high *trans*-10, cis 12 CLA concentration may have contributed to the low milk fat content reported in the current study. Consistent with findings in the current study, some previous studies have also reported reduced milk fat content following oil supplementation. For example, in a study by Rego *et al*., (2009), supplementation of grazing cows with 0.5 kg/d of sunflower oil reduced the milk fat content by 0.5% and the fat yield by 0.12 kg/d when compared to the un-supplemented control. In another study, Petit *et al*., (2004) observed no changes in milk fat concentration, but a 0.17 kg/d lower fat yield was reported in cows supplemented with 3.6% whole sunflower seeds when compared to those fed a Megalac based diet.

On the other hand, it is possible that oil supplementation may fail to have an effect on milk fat yield and milk fat concentration. According to Chilliard *et al*., (2009), this may happen when the supplemented fats have a slow release of FAs in the rumen, a common

phenomenon with rumen inert fats and whole oil seeds, and consequently results in low concentrations of *trans*-fatty acids. For example, in a study by Beauchemin *et al*., (2009), supplementation with sunflower seeds at 3.7% had no effect on any of the milk component concentrations.

5.4.3 Methane production

In the current study, daily methane output was 18.5 g lower in cows receiving the maize compared to those that received the wheat based concentrates. Similarly, methane output when expressed per kg of milk yield was 1 g/kg lower in cows fed the maize based concentrates. A literature search found that no previous study has been conducted to compare the effects of feeding dairy cows maize or wheat based concentrates on methane production. However, a few review studies have highlighted that maize is more effective in reducing methane production when compared to wheat. For example, a review by Doreau *et al*., (2011) stated that compared to wheat, maize degradation in the rumen is low and hence maize escapes into the small intestine where it is digested to a greater extent. Some studies have examined the effects of maize and barley based silages on methane production. For example, in a study by Benchaar *et al*., (2014), cows when fed TMR composed of 54% barley silage produced 6.6 % GE intake as methane output when compared to 5.1% of GE intake produced when cows were fed a TMR diet composed of 54% maize silage. In the current study, though wheat and maize based concentrates were supplied at the same level, the maize based concentrates contained a higher starch content of 332 g/kg compared to 290 g/kg in the wheat based concentrates. Similarly starch intakes were 0.2 kg/d/cow higher in cows fed maize based diets when compared to those fed the wheat based diets. The higher starch content in the maize based concentrates therefore were responsible for the lower methane output. The total starch intake (kg/d) was similar in all the cows and only showed a tendency to be higher

in cows fed the Megalac based diet probably due to the high total DM intake observed on this diet.

In the current study, methane output when expressed per kg of DM intake was influenced by oil source, with cows fed the sunflower oil based diets producing 2.3 g/kg DM higher methane output when compared to those fed the Megalac based diets. The increase in methane output observed with the sunflower oil based diet was unexpected considering that DM intake was reduced on this treatment and the expectation was that methane output would also be reduced. In previous studies, reductions in methane output were observed when high concentrations of sunflower oil were used. For example, McGinn *et al*., (2004) used 5% sunflower oil supplementation and observed a 21 % reduction in methane output (g/d and GE intake). Similarly, Beauchemin *et al*., (2007b) reported a reduction of 15% of GE intake in methane output following 3.4% sunflower oil supplementation. Machmuller *et al*., (2000) also reported a 27% reduction in methane output (g/kg live weight) when lambs receiving a maize silage diet were supplemented with sunflower seeds at 60 g/kg DM. A study by Beauchemin *et al*., (2009) reported a 10% reduction in daily methane production (g/d) in dairy cows when crushed sunflower seeds were supplied to a TMR (composed of silage and barley grain) at 3.3% DM. However when methane output was expressed on the basis of DM intake and GE intake, no changes in methane output were observed.

According to Beachemin *et al*., (2009), the effects of oils on methane production vary with presentation of the oil and the composition of the basal diet. In the case of sunflower oil, this has not been fully investigated. Only one previous study examined how the presentation of sunflower oil affects methane production, and results showed that whole sunflower seeds are more effective in reducing methane output when compared to refined sunflower oil. For example, in a study by Beauchemin *et al*., (2007b), heifers were

fed whole crop barley silage supplemented with either sunflower oil or sunflower seeds at 59 g/kg DM. Heifers fed sunflower oil based diets produced 12% and 16 % less methane when expressed on the basis of DM intake and GE intake respectively, while heifers fed sunflower seed based diets produced 23% (DM intake) and 25% (GE intake) reductions in methane output when compared with the un-supplemented controls. It is clear from this study that sunflower oil supplemented at the same level had a considerably less effect on methane production. Sunflower seeds were more effective in reducing methane production due to the reduction in fibre fermentation which consequently resulted in reduced feed intake and thus reduced methane production.

5.4.4 Milk fatty acid profile

The FA profile of the oils used in the current study were reflected in the milk FA profile, a finding which is consistent with other studies by Collomb *et al*., (2004) and Van-Zijderveld *et al*., (2011). According to Odongo *et al*., (2007) and Halmemies-Beauchet-Filleau *et al*. (2011), the effects of seed oils on milk FA profiles tend to be variable and depend greatly on the FA profile of the oil and type of basal diet. A review by Woods and Fearon, (2009) noted that processed oilseeds such as sunflower are easily digested by animals and this enhances their effect on milk FA profile. Sunflower oil is rich in linoleic acid ($C_{18:2n\text{-}6}$) which makes up approximately 70% of the FA content, while Megalac is composed of 48% palmitic acid ($C_{16:0}$) and 36% oleic acid ($C_{18:1}$) fatty acids, the profiles which are expected to reflect in the milk FA profile of cows fed the respective diets (Beauchemin *et al*., 2009; Petit *et al*., 2004).

In the current study, sunflower oil did not have any effect on the C_4-C_{14} FAs while palmitic acid ($C_{16:0}$) was reduced by 12.7%. The reduction or no change in short and medium chain FAs in milk fat following sunflower oil supplementation of dairy cows has been reported

in previous studies. For example, in a study by Rego *et al*., (2009), supplementation of grazing cows with 0.5 kg/d of sunflower oil reduced $C_{4:0}$, $C_{6:0}$, $C_{8:0}$, $C_{12:0}$, $C_{14:0}$ and $C_{16:0}$ by 26, 38, 45, 46, 35 and 25% respectively when compared to the un-supplemented control. In another study by Martinez-Marin *et al*., (2012), supplementation of dairy goats with sunflower oil at increasing doses of 0, 30, 48, 66 g/d did not change the concentrations of C_4-C_{14} milk FAs, while $C_{16:0}$ was reduced by 7, 20 and 35% when sunflower oil was supplied at 30, 48 and 66 g/d respectively. Similarly, in a study by Petit *et al*., (2004), cows on a TMR ration and supplemented with either Megalac or whole sunflower seeds at 3.6% did not differ in concentrations of $C_{10:0}$, $C_{12:0}$ and $C_{14:0}$ in the milk fat, but $C_{16:0}$ concentration was 36% higher in the Megalac supplemented cows.

Sunflower oil supplementation also increases the long chain fatty acid (LCFA) profile of milk fat. A previous study by Halmemies-Beauchet-Filleau *et al*., (2011) showed that feeding oil seeds to ruminants reduced the C_4-C_{16} milk FAs and increased the C_{18} FA profile which is consistent with the current finding. Previous studies also show that supplementation with sunflower oil increased the long chain FA profile of milk. For example, in a study by Rego *et al*., (2009), 0.5 kg/d of sunflower oil supplementation increased the $C_{18:0}$ and *cis*-9 $C_{18:1}$ level by 18 and 34% respectively. In a study by Petit *et al*., (2004), supplementation of cows with whole sunflower seeds at 3.6% increased $C_{18:0}$ and *cis*-9 $C_{18:1}$ concentration in milk by 54 and 20 % respectively.

Sunflower oil supplementation also increases concentrations of *trans*-fatty acids in milk fat. In the current study, feeding sunflower oil increased the trans-fatty acids *trans*-9 $C_{18:1}$, *trans*-11 $C_{18:1,}$ *cis*-9, *trans*-11 CLA and *trans*-10, *cis* 12 CLA being increased by 8, 46, 25 and 8.3% respectively. Increased concentrations of *trans*-fatty acids following supplementation with sunflower oil was expected and is consistent with that reported by Collomb *et al*., (2004), who attributed it to ruminal bio-hydrogenation of the $C_{18:2n-6}$ FA in

the rumen. According to Machmuller *et al*., (2000), hydrogenation of $C_{18:2n-6}$ and $C_{18:3n-3}$ in the rumen lies in the range between 0.83 and 0.89 and may explain why in the current study *trans* fatty acid concentrations increased in the milk FA profile. Halmemies-Beauchet-Filleau *et al*., (2011) also reported a 42% increase in *trans*-9 $C_{18:1}$ with sunflower oil supplementation. In a study by Toral *et al*., (2010), addition of 20 g/kg DM of sunflower oil to the TMR increased concentrations of *trans*-fats, *trans*-9 $C_{18:1}$, *trans*-11 $C_{18:1}$ and *cis*-9, *trans*-11 $C_{18:2}$ by 107, 270 and 260% respectively. Rego *et al*., (2009) also reported an increase in concentration of *trans*-9 $C_{18:1}$, *trans*-11 $C_{18:1,}$ and cis-9, *trans*-11 CLA by 140, 23 and 35% respectively while *trans*-10, *cis*-12 CLA decreased by 75%. In another study by Martinez-Marin *et al*., (2012), dairy goats supplemented with refined sunflower oil at increasing dosage of 0, 30, 48 and 66 g/d had milk *trans*-fatty acids *trans*-9 $C_{18:1}$, *trans*-11 $C_{18:1}$, and *cis*-9 *trans*-11 CLA increased in a dose dependent manner. *Trans*-9 $C_{18:1}$ increased linearly to a maximum of 200%, *trans*-11 $C_{18:1}$ increased to 635% and *cis*-9 *trans*-11 CLA to 354%. Another study by Petit *et al*., (2004) reported increased concentration of the milk fat *trans*-fatty acid *trans*-9 $C_{18:1}$ by 160% when whole sunflower seeds where supplemented at 3.6% DM compared to milk fat of cows supplemented with Megalac at the same level.

A review by Niwinska *et al*., (2011) examined factors that influence increased concentrations in milk of *cis*-9, *trans*-11 $C_{18:2}$ and *trans*-11 $C_{18:1}$ also referred to as rumenic acid and vaccenic acid respectively. The two *trans*-fatty acids are formed alongside each other in the rumen as a result of bio-hydrogenation of polyunsaturated fatty acids linoleic or α-linolenic acids. While *trans*-11 $C_{18:1}$ is only synthesised in the rumen, *cis*-9, *trans*-11 $C_{18:2}$ can be synthesised within the mammary gland by Δ^9 desaturation of *trans*-11 $C_{18:1}$. Niwinska *et al*., (2011) also pointed out that in the presence of linoleic acid, ruminal bacteria produce *trans*-11 $C_{18:1}$ while others act on *trans*-11 $C_{18:1}$ and bio-hydrogenate it to

stearic acid ($C_{18:0}$). This explains the increased concentration of $C_{18:0}$ and *trans*-11 $C_{18:1}$ in milk fat of cows that were fed the sunflower oil based diets compared to those that were fed the Megalac based diets in the current study. It is therefore highly likely from the current study that considerable ruminal bio-hydrogenation of linoleic acid in sunflower oil led to an increased concentration of $C_{18:0}$ and an increased concentration of the *trans*-fatty acids *trans*-11 $C_{18:1}$ and *cis*-9, *trans*-11 $C_{18:2}$ consistent with the finding of many previous studies.

In the current study, concentrations of $C_{18:2n\text{-}6}$ and $C_{18:3n\text{-}3}$ remained unchanged with sunflower oil supplementation. Similarly, Martinez-Marin *et al*., (2012) observed no changes in milk fat $C_{18:2n\text{-}6}$ and $C_{18:3n\text{-}3}$ concentrations of dairy goats when supplementation of sunflower oil was increased in a dose dependent manner from 0, 30, 48 and 66 g/d. This contrasts findings with other studies. For example, Rego *et al*., (2009) reported a 12% increase in $C_{18:2n\text{-}6}$ concentration and a 30% decrease in $C_{18:3n\text{-}3}$ concentration following sunflower oil supplementation. According to Kazama *et al*., (2010), an increased concentration of $C_{18:2n\text{-}6}$ in milk occurs when dietary sources of $C_{18:2n\text{-}6}$ escape ruminal fermentation.

5.4.5 Plasma metabolites

Plasma β hydroxybutyrate (3-OHB) levels can be used to assess ketosis, with normal levels in cows expected to be below 1.0 mmol/L (McNamara *et al*., 2003; Zhang *et al*., 2011). In the current study 3-OHB varied with time and was in the range 0.49 and 0.70 mmol/L, a normal range observed in healthy animals. The highest levels of 3-0HB of 0.6 and 0.7 mmol/L were observed at 12 pm and at 2 pm respectively. Plasma glucose levels ranged between 3.54 and 3.68 and were within normal range, and Chimonyo *et al*., (2002) cited 2.5 mmol/L as the minimum plasma glucose level expected in normally fed cattle. It was

observed that blood glucose levels were inversely proportional to the 3-OHB level, i.e. when blood glucose levels increased, there was a proportionate decrease in 3-OHB levels. High urea levels in plasma are indicative of a high dietary protein intake or excessive mobilisation of muscle (Chimonyo *et al*., 2002) with normal levels in cattle of 3.4-7.3 mmol/L (McGeough *et al*., 2011). McNamara *et al*., (2003) observed that blood urea level varied with type of diet being higher in a high concentrate diet (5.4 mmol/l) than in a low concentrate diet (5.2 mmol/l). McGeogh *et al*., (2010) observed urea levels of 8.73 and 6.54 mmol/L in plasma of cows fed diets comprising 11:89 and 21:79 concentrate to forage ratios respectively which reduced to 4.2 mmol/L when concentrate levels were increased to 31:69 and 47:53. The plasma urea levels in the current study fall within this range, and this shows that the diets used in the study had adequate levels of protein. However, cows fed the maize based concentrates had 0.5 mmols/L lower plasma urea concentrations when compared to those fed the wheat based concentrates. The lower plasma urea levels associated with the maize based diets could have been due to an increase in tissue N retention as a result of maize starch escaping rumen digestion thus being a readily available in form of glucose or energy for tissue synthesis as protein or fat (Reynold *et al*., 2001). Contrary to the findings in the current study, Johnson *et al*., (2002) reported increased N concentrations in serum by 9 and 15% when plant oils were supplemented at 4.0 and 5.6% DM respectively. The increase was attributed to an increased N absorbtion from the rumen.

5.4.6 DM digestibility

In the current study, DM digestibility was unaffected by dietary treatment, therefore the reduction in total DM intake associated with sunflower oil based diets was probably due to reduced palatability. Similar to the finding in the current study, Beauchemin *et al*., (2007) reported no changes in DM digestibility which remained at 60% when heifers on a

whole crop barley silage where supplemented with sunflower oil at 5.9 % DM. The DM digestibility in the current study was 74% with sunflower oil supplementation and higher than that reported by Beauchemin *et al*., (2007). Difference in digestibility was probably due to differences in basal diets used. In the current study, a TMR basal diet with 2:1 ration of maize silage: grass silage was used while a ratio of 2:1 ratio of barley silage: barley grain was used in a study by Beauchemin *et al*., (2007). According to Woods and Fearon, (2009), processing of oil seeds increases their digestibility therefore refined oils tend to have higher digestibility than oil seeds. In the same study by Beauchemin *et al*., (2007), DM digestibility was unaffected by sunflower oil supplementation but was reduced by 6.6% by sunflower seeds when supplemented at the same level. Similarly, in the current study sunflower oil supplementation had no effect on DM digestibility.

Several studies have also reported changes in diet digestibility following sunflower oil supplementation. For example, Machmuller *et al*., (2000), observed a 21% reduction in NDF digestibility with sunflower seed supplementation at 6% DM. Beauchemin *et al*., (2009) also observed a 20% reduction in DM digestibility when crushed sunflower seeds were supplemented at 3.7%. Similarly Beauchemin *et al*., (2007) observed a 7% reduction in DM digestibility when sunflower seed were supplemented at 59 g/kg. However, sunflower oil supplied at the same level did not have any effect on DM digestibility suggesting that the presentation of the oil may have an impact on DM digestibility.

According to Chung *et al*., (2011), the effect of oils on whole tract digestibility is influenced by the type of forage in the basal diet. Chung *et al*., (2011) demonstrated that when a hay based or a barley silage based diet were supplemented with the same level of ground linseed at 150 g/kg DM, DM digestibility was reduced by 7% with the barley silage based diets and only by 3% with the hay based diets. In the current study, the Megalac and sunflower based diets had similar composition of maize and grass silage in the ratio

2:1 and the NDF content similar. The high DM digestibility of 74 to 77% onserved in the current study was unexpected and higher than that observed by Beachemin *et al*., (2007) and most previous studies. Some previous studies have also reported a lack of an effect of sunflower on digestibility. For example, in a study by Petit *et al*., (2004), Megalac and whole sunflower seeds when supplemented to dairy cows at 3.8% DM did not alter DM digestibility, which averaged 65 %.

5.6 Conclusions

Sunflower oil was not effective at reducing methane production in cows, but did alter the milk FA profile of the cows by increasing the polyunsaturated fatty acid content and reducing the palmitic acid content. Maize based concentrates were effective in reducing methane output when expressed as g/d or g/kg milk yield and improved the energy balance of the cows as evidenced by the positive condition score change and reduced plasma 3-OHB concentrations. From the current study, the starch and oil source acted independently, with few interactions between starch X oil observed on methane production or milk fatty acid profile. From the current study we accept the hypothesis that maize based concentrates when compared to wheat based concentrates reduce methane production and that oil source particularly sunflower oil supplementation of TMR has the potential to alter milk FA profile of dairy animals.

CHAPTER 6: The effect of time of grazing with or without TMR supplementation on performance, methane production and milk fatty acid profile of Holstein dairy cows

6.1 Introduction

Grazing can be applied in ruminant production as a management programme to reduce methane production and improve welfare of the animals (O'Neill *et al*., 2011; Chapinal *et al*., 2010). In grazing systems, methane production can be reduced when pasture is of high quality (less mature) which makes it to be highly digestible and consequently results in less methane being produced in the rumen (O'Neill *et al*., 2011). Ruminant grazing has the advantage of being a less expensive way of utilising fibrous material to produce meat and milk (Buddle *et al*., 2011; Taweel *et al*., 2006). The impact of grazing on the welfare of the ruminant is well established (Chapinal *et al*., 2010) and allowing cows to pasture is beneficial for the welfare of the cow as pasture is considered a natural environment (Charlton *et al*., 2011). Given a choice and under favourable weather conditions, cows prefer to be at pasture than indoors, and according to Legrand *et al*., (2009), incidences of mastitis and lameness are reduced when cows are at pasture. Hernandez-Mendo *et al*., (2007) demonstrated that when lame cows were allowed to graze, the gait improved and cows recovered within a short period of time and the lying times of the cows also increased. However, maintaining productivity at pasture remains a challenge in grazing systems (Buddle *et al*., 2011). In contrast to the welfare benefits of grazing, when given only access to pasture, cows fail to maintain productivity because dry matter intake tends to be low (Charlton *et al*., 2011). For example, in the study by Hernandez-Mendo *et al*., (2007), cows that were grazed compared to those fed indoors lost 2.6 kg/week of body

weight while cows housed indoors gained 0.5 kg/week. Milk production of cows at pasture also decreased at a rate of 1 kg/week when compared to the cows fed indoors.

A number of previous studies have also examined the impact of time of grazing on intake and productivity, and variable results have been recorded although the impact on methane production has not been assessed. For example, in a study by Chapinal *et al*., (2010), allowing cows to be at pasture overnight did not reduce the DM intake or milk production when compared to those that were kept indoors throughout the study. In another study by Soriano *et al*., (2001), dairy cows were grazed for 8 hours either during the day or at night and compared against those kept indoors and fed a TMR ration throughout the study. Dry matter intake was highest in the cows fed TMR only and lowest in the cows grazed at night. Milk production differed among the 3 groups, was highest in the cows kept indoors and averaged 29 kg/d, and was 1.5 kg/d lower in cows grazed during the day and 1 kg/d lower in cows grazed during the night when compared to the amount produced by the indoor cows. The impact of time of grazing on methane production was not determined.

Grass contains water soluble carbohydrates (WSCs) whose concentrations vary during the day (Tresvaskis *et al*., 2004; Taweel *et al*., 2006). Concentrations of WSCs tend to be high in the evening and at night and are low in the mornings and during the day. Previous studies (Staerfl *et al*., 2012; Taweel *et al*., 2006) have established that N (nitrogen) losses to the environment can be reduced when cows have access to high sugar grasses because the high levels of WSC improve grass N utilisation in the rumen. High levels of WSC concentrations in fresh grass have also been shown to improve productivity of animals. For example, Trevaskis *et al*., (2004) grazed cows either in the morning or in the afternoon, with morning and afternoon pasture differing in WSC concentration by 52 g/kg DM. Intake of DM did not differ between the two groups, but the cows grazed in the

afternoon produced 2.1 litres/cow/d more milk, 0.56 g more milk protein/cow/d and gained 0.36 kg live weight/cow/d more than those grazed in the morning. Kim *et al*., (2011) reported 20% higher live weight gains when growing lambs were fed a mix of grass varieties with high WSC concentration. However, some studies have reported no effect of WSC on productivity. For example, in a study by Taweel *et al*., (2005), cows that were fed fresh grass with 24-31 g/kg DM higher WSC concentrations did not show any improvement in milk yield, milk composition or DM intake possibly because the WSC concentration difference was not high enough to elicit a change in productivity.

A number of previous studies on WSCs have focused on reducing nitrogen (N) losses to the environment and improving animal performance, while studies which link WSC to reduced methane production are scant. According to Buddle *et al*., (2011), the effects of high sugar grasses on methane production are not yet established. Ellis *et al.,* (2012) used data from previous studies and predicted that grasses with a high WSC may produce high daily methane outputs when results are expressed as g/d and when expressed as a unit of DM intake, but when results are expressed as a unit of milk yield, methane output would be lower. Of the few studies that have examined the effect of WSC on methane production, results have not been conclusive. For example, in a study by Staerfl *et al*., (2012), dairy cows fed dried ryegrass with 90 g/kg DM higher WSC concentration compared to those fed ordinary grass did not reduce methane production when results were expressed as g/d, g/kg DM intake or g/kg milk. In contrast, Kim *et al*., (2011) fed growing lambs grass that differed in WSC concentration by 42 g/kg DM and observed that methane production when expressed as g/kg DM intake was 17% (L/kg DM) and 25% (L/kg live weight gain) lower in the lambs that were fed grass high in WSC concentration.

Pasture is also a rich source of polyunsaturated fatty acids (Gomez-cortes *et al*., 2009) particularly α-linolenic acid ($C_{18:3n-3}$) which is considered to be in the range 40- 50% of

total fatty acids. According to Chilliard *et al*., (2001), α-linolenic acid undergoes considerable hydrogenation in the rumen and so very little appears in the milk fatty acid profile. Hydrogenation of $C_{18:3n-3}$ in the rumen may help to reduce methane output in ruminants. For example, Zhang *et al*., (2008) used a mixture of cornmeal and wild rye meal and supplemented the diet with α-linolenic acid at 35 and 70 g/kg DM and reported that methane production (mmols) was reduced by 46 and 62% respectively when compared to the un-supplemented control.

A number of previous studies have examined the impact of grazing in general on milk fatty acid profile. According to Halmemies-Beauchet-Filleau *et al*., (2013), milk from cows fed fresh pasture has a high content of PUFA and low concentrations of SFAs when compared to cows fed conserved forages. Gomez-Cortes *et al*., (2009) also noted that one way of reducing the amounts of saturated fats and increasing levels of unsaturated fats in animal products is by allowing ruminants to graze fresh pastures.

The hypothesis that was tested in the current study was that allowing cows' access to pasture in the evening/night when grass WSC concentrations are at their highest would result in lower methane output, improve milk production and productivity and improve fatty acid profile of the milk, and that grazing would reduce methane production when compared to indoor housing. The objectives of the study were to determine the effects of timing of pasture access with or without TMR supplementation on the productivity, grass intake, methane production and milk fatty acid profile in high yielding dairy cows.

6.2 Materials and methods

6.2.1 Animals, experimental design and treatments

The study was conducted in the summer of 2012 over a period of 4 months from May to August. Sixty early lactation Holstein dairy cows (71 ± 9.2 days into lactation) and yielding

39.3 ± 0.72 kg/d of milk were used. Cows were allocated to one of two periods each of 35 d duration. In each period, thirty cows were randomly allocated to one of five treatment groups of six cows each, based on their milk yield, live weight and milk fat content measured in the week prior to allocation. Treatments groups were; **CT** Cows kept indoors all the time and fed *ad libitum* TMR; **DGT** cows turned out to pasture after morning milking between 06:00h and 15:00 h with access to TMR at pasture; **DG** cows turned out to pasture after morning milking between 06:00 h and 15:00 h but without access to TMR at pasture, **NGT** cows turned out to pasture after afternoon milking between 16:00 h and 05:00 h milking, with access to TMR at pasture, **NG** cows turned out to pasture after afternoon milking between 16:00h and 05:00 h milking, but without access to TMR at pasture. CT cows were continually housed (no access to pasture) and had *ad libitum* access to a total mixed ration (TMR) composed of grass silage, maize silage and straight feeds, formulated to produce approximately 40 kg of milk per d according to Thomas, 2004 (Table 29).

Table 29. Composition of the total mixed ration (TMR) offered to dairy cows that were continuously housed (CT) or grazed during the day (DGT) or night (NGT)

Ingredient	g/kg DM
Maize silage	409
Grass silage	112
Alkagrain	103
Soyabean meal	76
Rapeseed meal	76
Wheat distillers dark grains	76
Soya hulls	67
Molasses	35
Chopped straw	21
Protected fat (Megalac)	13
Minerals	6
Limestone flour	4
Acid buff[1]	2

[1]AB vista, Wiltshire UK

TMR supplied at pasture had same composition as the one provided to the CT group. While indoors, TMR was accessed via electronic roughage intake bins (RIC bins; Insentec,

Marknesse, Netherlands; Chapinal *et al*., 2007; Fig 12), with fresh feed allocated daily at approximately 08:00h to provide 105% of *ad libitum* intake. After every morning milking, cows in treatment groups DGT and DG were taken out to pasture where they were able to loaf and graze from 06:00 h to 15:00 h. While at pasture treatment DGT had *ad libitum* access to the same TMR as the housed animals, available via individual Calan gates feeding system (American Calan, Inc., Northwood NH; Fig 13) placed in the grazing area and offered fresh daily at approximately 0800h. Treatments groups NGT and NG were kept indoors following morning milking, where they had *ad libitum* access to the TMR via the RIC bins. Following afternoon milking, treatment groups NGT and NG had access to pasture, with NGT group having *ad libitum* access to the TMR via the Calan gate feeding system while DG and DGT were indoors.

Figure 12. Cow feeding from the Ric bin when indoors

6.2.2 Indoor housing

When housed, cows were in the same portion of a cubicle building containing super comfort cubicles fitted with rubber mattresses. The cubicles were scraped using automatic scrapers four times daily and limed twice weekly. When grazing, cows were in

the same area of a field consisting predominately of perennial rye grass (*Lolilum perenne*). All cows had continual access to water when indoors and at pasture.

6.2.3 Grazing management

The grazing area was located at Harper Adams University. The experimental grazing area of 1.75 ha was divided into 4 paddocks. Each paddock was further sub-divided into two sub-paddocks. Cows were grazed in a rotational system and were allocated fresh grass daily after the morning and afternoon milking, coinciding with the time treatment groups were swapped. Fresh grass was provided by moving a temporary electric fence with each paddock. The excess herbage cover following grazing by the study cows was grazed down to approximately 1500 kg DM/ha using low yielding and dry cows.

6.3 Measurements

6.3.1 Pre-grazing herbage

Allocation of the cows to the grazing area was determined from pasture mass, estimated daily prior to grazing using a rising plate meter (Jenquip, Feilding, New Zealand) as described by Earle and McGowan, (1979). Thirty random heights were recorded in each paddock, by walking the field in a zig-zag pattern. The animals were rotationally grazed and received fresh grass daily after the morning and afternoon milking. Cows entered the paddocks at a grass cover of 2750 (+/- 250) kg DM/ha and received a herbage allowance of approximately 10 kg DM/d. Herbage cover and allowance was managed by altering the area available to the cows and using low yielding cows to graze excess growth followed by mechanical topping. Herbage samples were collected during the final 5 day of each period. These comprised of grass samples obtained by cutting by scissors random samples (*n*=minimum of 30) of the grazing horizon approximately 4 cm above ground level at 09:00 h and 16:00 h. Samples were stored at -20°C prior to subsequent analysis.

6.3.2 TMR and grass dry matter intakes

In the final 12 d of each period, all cows received 56 g/cow/d, of an alkane (dotriacontane; C_{32}) which was added to the total mixed ration (TMR). Feed, grass and faecal samples for analysis were collected in the final 5 d of each period. Faecal samples from each cow were collected four times a day at 04 30h, 10 00h, 14 30 h and 18 00 h during the final 5 d of each period and frozen at -20°C prior to analysis. The intake of TMR when cows were indoors was measured automatically using the electronic feed bins (Sinclair *et al*., 2005), while TMR intakes at pasture were measured using individual Calan bins placed in the grazing area. Pasture intake for each cow was calculated from the concentrations of a naturally occurring odd-chain tritriacontane (C_{33}) and the dosed even-chain dotriacontane (C_{32}) *n*-alkane in the TMR, grass and faeces as described in section 3.7 using the equation of Mayes *et al*., (1986).

Figure 13. Calan gate feeding system in the field for TMR

Figure 14. Cows grazing in the field

6.3.4 Animal performance

Milk yield was recorded at each milking during the final 7 days of each period, and sub-samples taken on four occasions, twice at 06:00 and twice at 15:00 h for subsequent analysis of fat, protein and lactose. Additional milk samples were taken on 2 occasions on day 4 for subsequent milk fatty acid profile determination. Individual cow live-weight and body condition scores were recorded at the start and end of each 5 week treatment period.

6.3.5 Methane production

Enteric methane production was measured using the SF_6 tracer technique using a method described by Johnson and Johnson (1995). Twenty-one days prior to the beginning of the study, each cow received a bolus each releasing known amount of sulphur hexafluoride (SF_6) which was inserted in the rumen. The boluses were inserted using a balling gun. Methane production was measured over a 5 d period in the week prior to allocation to treatments and during the final week of each period. During the final 5 d of each period, all cows were fitted with head collars and back packs as described by Hart *et al*., (2009) (Fig 14) with the back pack cylinders replaced daily after the morning milking. The

canisters were then pressurised with 17 kPa of pure nitrogen gas (BOC gas, Worsley Manchester, UK) and the gas samples collected into 20 ml leur lock syringes for laboratory analysis as described in Section 3.8.2.

6.4 Laboratory analyses

Grass and TMR samples collected during the 5 d sampling period were freeze dried (Edwards freeze dryer Modulyo, Bristol, UK) at -50°C for 5 days and milled (Delonghi KG 79, UK) to pass through a 1mm screen. Milled samples of grass and TMR were analysed in duplicate for DM, OM, CP, NDF and ash as described in Sections 3.1.1 to 3.1.4. Milk samples for compositional analyses were analysed for fat, protein and lactose content using a Milkoscan Minor 78110 auto analyser (Foss electric, Hillerod, Denmark) as described in section 3.2.1. Milk samples for FA determination were centrifuged as described by Feng *et al*., (2004) with the lipid layer methylated according to Christie (1982) with modifications as described by Chouinard *et al*., (1999) as described in sections 3.2.1 and 3.2.2. The FA content of grass and TMR samples were analysed as described by Sukhija and Palmquist (1988) using nonadecanoic acid ($C_{19:0}$) as an internal standard as described in section 3.3. Water soluble carbohydrate concentration and metabolisable energy content of the grass samples were determined as described as in sections 3.5 and 3.6 respectively. Grass intake was estimated by the *n*-alkane method of Mayes *et al*., (1986) as described in section 3.7. Respired gas samples from individual cows were analysed for methane concentrations as described in section 3.8.2.

6.5 Statistical analyses

Mean values from the individual cows of milk yield, milk components, methane outputs, and milk fatty acid concentrations were evaluated by analysis of variance as a 2 x 2 factorial design with a control. Treatment degrees of freedom were spilt into main effects

of grazing time (morning vs. evening) and access to TMR (with or without). The factorial design was compared to the control as Indoor vs outdoor (CT vs. all outdoor groups). Interaction between grazing time (morning vs. evening) and TMR access (with or without) was also assessed. All analyses were conducted using Genstat 13.

6.6 Results

6.6.1 Chemical analyses

Grass samples collected at 1600 h had 111 g/kg DM higher WSC concentration and 125 g/kg DM lower NDF content compared to samples collected at 0900 h (Table 30). The CP content of grass samples cut at 0900h and 1600h were similar and averaged 222 g/kg DM, while that of the TMR was 183 g/kg DM. Metabolisable energy content for am and pm grass samples were 12 and 12.5 MJ/kg DM respectively. The fatty acid content of the TMR and grass samples differed slightly, with the TMR having higher concentrations of $C_{16:0}$, *cis*-9 $C_{18:1}$ and $C_{18:2n\text{-}6}$ while grass samples had the highest concentrations of $C_{18:3n\text{-}3}$, averaging 45 g/100g.

Table 30. Chemical composition (g/kg DM) of the total mixed ration (TMR) and grazed grass (sampled at 0900h and 1600h) offered to dairy cows that were continuously housed or grazed during the day or night

	TMR	Grass-am	Grass-pm
DM, g/kg	413	225	217
ME, MJ/kg DM	---	12	12.5
OM	924	908	914
CP	183	223	221
NDF	374	479	354
WSC	---	157	268
Fatty acids, g/100g	34.2	35.8	30.3
FA g/100g FAME			
$C_{16:0}$	28.2	15.9	16.4
$C_{18:0}$	2.3	0.7	0.9
$C_{18:1n\text{-}9}$	25.4	1.5	1.0
$C_{18:2n\text{-}6}$	30.2	9.8	9.2
$C_{18:3n\text{-}3}$	7.4	49.2	40.2

FAME = fatty acid methyl esters

6.6.2 Intake

Cows in CT or DGT had the highest total DM intake of approximately 26 kg DM/d while total DM intake in all the other grazing groups were considerably lower(P<0.005; Table 31). Among the grazing groups, daytime grazing (DGT and DG) increased total DM intake by 1.8 kg DM/cow/d compared to night time grazing (NGT and GT).

Grass intake was affected by the grazing time (P<0.05), with cows grazed in the afternoon (NG) having a 1.8 kg/d higher grass intake compared to those grazed in the morning (DG) with no TMR supplementation. The interaction between grazing time and TMR supplementation (P<0.005) reduced grass intake in the cows grazed in the afternoon (NGT) by 1.9 kg/d while it did not have any effect on the grass intake of the cows grazed in the morning (DGT).

6.6.3 Milk yield and composition and animal performance

Milk yield was highest in cows receiving CT or DGT (38.6 and 38.0 kg/d respectively) and lowest in NG at 33.6 kg/d (P<0.05; Table 31). Among the grazing groups, day time grazing increased milk yield by 1.9 kg/d (P<0.05) and TMR supplementation at pasture increased milk yield by 2.5 kg/d (P=0.005). Milk fat content and fat yields were unaffected by dietary treatment. Protein yield (kg/d) was highest in cows receiving CT or DGT with a mean of 1.28 kg/d, and was on average 0.15 kg/d lower in all the other grazing groups (P<0.005). Among the grazing groups, TMR supplementation increased protein yield by 0.15 kg/d during the day and 0.08 kg/d at night. Protein concentration (g/kg) showed a tendency to be increased (P<0.1) by 1 g/kg with night time grazing when compared to day time grazing. Lactose yield (kg/d) was highest in cows receiving CT or DGT (approximately 1.70 kg/d; P<0.05) and considerably lower in all the other grazing groups. TMR

supplementation during the day increased lactose yield by 0.14 kg/d while night time grazing with TMR supplementation increased yield by 0.13 kg/d.

Cows receiving CT or NGT gained 1.1 kg/d of live weight, while DGT cows only gained 0.45 kg/d. Without TMR supplementation, DG and NG lost 0.1 kg/d of live weight. A similar trend was observed with condition score; access to TMR at pasture increased condition score and condition score change (P<0.05), with cows receiving DGT or NGT cows gaining approximately 0.16 condition score, compared to a change of 0.04 and -0.01 by the DG and NG cows respectively.

Table 31. Effect of grazing during the day with access to total mixed ration (TMR: DGT) or without access (DG), or at night with access to TMR (NGT) or without access (NG) compared to continuous housing (CT) on intake and performance in high yielding dairy cows

	CT	DGT	DG	NGT	NG	s.e.d.	In vs. Out[1]	Grazing time[2]	TMR[3]	Int[4]
Grass intake, kg/d	0.00	1.10	0.80	0.70	2.60	0.450	------	0.044	0.015	0.002
TMR intake, kg/d	26.2	25.8	22.0	24.3	18.4	0.810	<0.001	<0.001	<0.001	0.059
Total DM intake, kg	26.2	26.9	22.8	25.0	21.1	0.940	0.004	0.011	<0.001	0.817
Milk yield, kg/d	38.6	38.0	35.3	35.9	33.6	1.21	0.003	0.033	0.005	0.838
Fat, g/kg	37.0	37.9	35.4	35.8	37.6	2.68	0.876	0.973	0.866	0.264
Fat, kg/d	1.42	1.45	1.23	1.27	1.27	0.097	0.126	0.337	0.119	0.123
Protein, g/kg	34.1	32.8	31.5	33.2	33.4	0.950	0.063	0.085	0.426	0.275
Protein, kg/d	1.30	1.25	1.10	1.18	1.12	0.046	<0.001	0.572	0.002	0.175
Lactose, g/kg	44.1	44.3	44.1	44.3	43.4	0.790	0.855	0.523	0.321	0.581
Lactose, kg/d	1.70	1.69	1.55	1.59	1.46	0.065	0.014	0.045	0.004	0.973
Lwt, kg[5]	692	698	677	690	651	14.9	0.481	0.292	0.064	0.567
Lwt change, kg/d	1.10	0.45	-0.11	1.10	-0.10	0.437	0.041	0.311	0.006	0.296
Condition score	2.54	2.63	2.44	2.48	2.38	0.010	0.458	0.141	0.042	0.494
CS change	0.15	0.17	0.04	0.15	-0.01	0.085	0.393	0.538	0.023	0.842

[1]Inside cows vs. all cows at grass, [2]Grazing at night vs. daytime, [3]Grazing with or without access to TMR, [4]Interaction between grazing time and TMR provision, [5]Lwt= live weight, CS= condition score

6.6.4 Methane production

When compared to the CT group, three grazing groups (DG, NGT, NG) produced significantly lower daily methane outputs (P<0.005; Table 32), while daily methane output by DGT was not different from CT. When expressed as g/kg milk yield, cows receiving CT produced 2 g/kg more methane when compared to the mean production of all the grazing groups (14.9 vs. 12.9 g/kg milk yield). Similarly, when expressed as g/kg fat

corrected yield, methane production was 3.2 g/kg higher in the CT group when compared to the mean production in the grazing groups (18 vs. 14.8 g/kg fat corrected yield; P<0.05). Methane production when expressed as g/d, g/kg milk yield or g/kg fat corrected yield was not affected by the grazing time or access to TMR at pasture, and no interaction was observed between grazing time and TMR provision.

Table 32. Effect of grazing during the day with access to a total mixed ration (TMR: DGT) or without access (DG), or at night with access to a TMR (NGT) or without access (NG) compared to continuous housing (CT) on the methane production of high yielding dairy cows

	CT	DGT	DG	NGT	NG	s.e.d.	In vs. Out[1]	Grazing time[2]	TMR[3]	Int[4]
CH_4, g/d[5]	524	474	447	458	425	28.4	0.003	0.363	0.148	0.883
CH_4, g/kg DMI	20.1	17.2	19.4	18.6	20.6	0.461	0.019	0.036	<0.001	0.675
CH_4, g/kg milk[5]	14.6	12.8	12.7	13.0	13.0	0.906	0.023	0.732	0.963	0.977
CH_4, g/kg FC yield	18.0	14.8	14.5	14.9	14.8	2.00	0.048	0.896	0.906	0.969

[1]Inside cows vs. all cows at grass, [2]Night vs. daytime, [3]With or without TMR, [4]Interaction between grazing time and TMR provision, [5]Based on 8 cows per treatment, DMI= dry matter intake

6.6.5 Milk fatty acid profile

The short chain fatty acid (C_8) concentrations in milk were not affected by dietary treatment (*P*>0.1; Table 33). Grazing without TMR supplementation reduced (*P*<0.05) the concentrations of the medium chain fatty acids $C_{10:0,}$ $C_{12:0}$ and $C_{14:0}$ by 20, 20 and 10% respectively when compared to concentrations in the supplemented grazed cows. Cows receiving CT or DGT had the highest concentrations of $C_{15:0}$, while in all the grazing groups, concentrations were lower (*P*<0.005) and TMR supplementations at pasture increased $C_{15:0}$ concentrations in milk. The highest concentration of *cis*-9 $C_{18:1}$ was in the milk FA of the cows grazed without TMR supplementation (*P*<0.001); cows receiving DG had a 21% higher concentration of *cis*-9 $C_{18:1}$ than those receiving DGT, while the concentration in cows receiving NG was 1.4 g/100g or 6% higher than those receiving NGT. The time of grazing affected $C_{18:2n\text{-}6}$ concentrations in milk (*P*<0.001), with cows grazed in the afternoon (NGT and NG) having mean concentrations of 2.6 g/100g, while

cows grazed during the day (DGT and DG) had mean concentration of 2.3 g/100g. The cows receiving CT had the lowest concentration of $C_{18:3n-3}$ at 0.40 g/100g in milk and grazing increased concentrations in all the other treatment groups with levels ranging from 0.42 g/100g to 0.52 g/100g. Interactions were observed between time of grazing and TMR provision with cows offered TMR having low concentration of *cis*-9 $C_{18:1}$ and $C_{18:2n-6}$ during the day and higher concentrations during the night. Grazing tended (P=0.007) to decrease the SFA content of milk, but had no effect (P>0.1) on the MUFA content. The provision of TMR at grass increased the SFA content, particularly during day time. Provision of TMR at grass also decreased milk FA content of MUFA (P<0.001). Access to pasture also increased milk FA content of PUFA compared to continuously housed cows (P<0.01).

Table 33. Effect of grazing during the day with access to a total mixed ration (TMR: DGT) or without access (DG), or at night with access to a TMR (NGT) or without access (NG), compared to continuous housing (CT) on milk fatty acid profile of high yielding dairy cows

	CT	DGT	DG	NGT	NG	s.e.d.	In vs. Out[1]	Grazing time[2]	TMR[3]	Int[4]
g/100g										
$C_{8:0}$	0.95	1.00	0.96	0.92	0.86	0.104	0.922	0.194	0.438	0.870
$C_{10:0}$	2.56	2.78	2.09	2.35	2.21	0.243	0.313	0.374	0.020	0.115
$C_{12:0}$	3.11	3.46	2.50	3.03	2.68	0.255	0.344	0.498	<0.001	0.101
$C_{14:0}$	10.5	10.9	9.27	10.0	9.65	0.439	0.127	0.435	0.002	0.052
$C_{14:1c9}$	1.15	1.25	1.27	0.91	1.07	0.233	0.897	0.108	0.588	0.645
$C_{15:0}$	1.02	1.07	0.77	0.89	0.76	0.068	0.009	0.054	<0.001	0.088
$C_{16:0}$	31.0	31.5	29.0	29.0	29.5	0.830	0.070	0.089	0.092	0.014
$C_{16:1c9}$	1.53	1.47	1.45	1.35	1.62	0.146	0.636	0.806	0.251	0.167
$C_{17:0}$	0.44	0.50	0.48	0.49	0.47	0.035	0.112	0.675	0.431	0.999
$C_{18:0}$	8.01	8.01	8.38	8.82	8.11	0.397	0.314	0.350	0.540	0.062
Cis-9 $C_{18:1}$	22.7	21.5	26.0	23.8	25.2	0.950	0.060	0.288	<0.001	0.027
$C_{18:2n\text{-}6}$	2.45	2.25	2.37	2.72	2.50	0.119	0.912	<0.001	0.538	0.049
trans-11$C_{18:1}$	0.62	0.63	0.78	0.67	0.68	0.170	0.552	0.627	0.565	0.538
*CLA	0.31	0.34	0.35	0.29	0.38	0.124	0.778	0.864	0.508	0.596
$C_{18:3n\text{-}3}$	0.40	0.42	0.51	0.52	0.51	0.056	0.042	0.223	0.334	0.255
$C_{20:5n\text{-}3}$	0.45	0.40	0.65	0.66	0.61	0.149	0.134	0.274	0.333	0.056
ΣSFA	61.4	62.6	56.7	58.9	57.9	1.620	0.069	0.283	0.004	0.037
ΣMUFA	26.1	25.1	29.6	26.9	28.7	1.100	0.111	0.554	<0.001	0.083
ΣPUFA	3.60	3.41	3.83	4.15	3.99	0.226	0.182	0.007	0.424	0.074

[1]Inside cows vs. all cows at grass, [2]Night vs. daytime, [3]With or without TMR, [4]Interaction between grazing time and TMR provision; *CLA= *cis*-9, *trans*-11 CLA

6.7 Discussion

6.7.1 Diet chemical composition

The current study established that WSC concentrations of *Lolium perenne* rye grass fluctuate throughout the day, with higher concentrations in the evening/night and lower concentrations in the morning/day time with a difference of 111 g/kg DM (157 vs. 268 g/kg DM) in WSC concentrations between the morning and afternoon samples. Neutral detergent fibre (NDF) concentrations in grass were observed to be inversely proportional to the WSC concentration, with samples cut at 9am having higher levels (479 g/kg DM)

when compared to the samples cut at 4 pm (354 g/kg DM). According to Fulkerson and Donaghy (2001) and Trevaskis *et al.* (2001), WSC concentrations in ryegrass peak at 2–4h before sunset. Lee *et al*., (2002) also reported that the high levels of WSC in grass were combined with low levels of NDF, a similar finding to what was observed in the current study. The combination of high WSC content and low fibre content is assumed to be responsible for the high DM intakes associated with consumption of grass containing high amounts of WSCs (Ellis *et al*., 2012). It was therefore anticipated that grass DM intake would be higher in cows grazing at night.

6.7.2 Intake and animal performance

In the current study, total DM intake was highest in cows receiving CT or DGT, and among the grazing groups, intake increased with TMR supplementation. In terms of performance, live weight change was highest in cows receiving CT, and when cows were at pasture, live weight change increased with TMR supplementation. At pasture, the cows that were not supplemented (DG and NG) lost 0.1 kg/d of live weight, whereas all other groups gained condition. Similar results have been observed in previous studies. For example, in a study by O'Neill *et al*., (2011), cows fed TMR gained a body weight change of 0.5 kg/d and 0.4 of body condition score while those that were at pasture lost 0.5 kg/d body weight and 0.3 units of body condition score. Schroeder *et al*., (2003) also observed that when cows were fed a TMR, they gained a body weight of 23 kg and a condition score of 0.1 units over a period of 5 weeks when compared to those that were grazed and supplemented with concentrates and lost 6 kg body weight and 0.2 units of body condition score within the same period.

6.7.3 Milk yield and milk components

Cows receiving CT or DGT produced the highest milk yield and among the grazing groups, milk yield increased with TMR supplementation at pasture and when grazed during the day than at night. Therefore milk yield in the current study increased with an increase in total DM intake. In the current study, TMR intake and milk yield increased when cows were grazed during the day when compared to those that were grazed at night. The finding supports previous studies that have established that milk yields when cows are at pasture are driven by DM intake. In the current study, cows fed TMR at pasture produced 2.5 kg/d higher milk yield compared to those that had no access to TMR. Similar findings have been reported in previous studies. For example, in a study by Lovett *et al*., (2005), increase in concentrate supplementation at pasture from 1 to 6 kg/cow/d increased milk yield by 5.2 kg/cow/d. Similarly, Bargo *et al*., (2003) reviewed the literature on grazing and supplementation and its impact on milk production and reported that milk production of high yielding dairy cows increased linearly with an increase in the amount of concentrate supplementation. The results in the current study are also similar to those observed by O'Neill *et al*., (2011) who reported a higher milk yield of 29.5 kg/d produced by cows when fed a TMR compared to 21.1 kg/d milk yield produced by cows that were on pasture. In the same study by O'Neill *et al*., (2011), DM intakes correlated with milk yields such that milk yields increased with an increase in DM intake with intakes of TMR and pasture fed cows being 27 and 19.6 kg/cow/d respectively.

In the current study, milk protein and milk lactose yields increased with an increase in milk yield with concentrations being unaffected by dietary treatment. Therefore yields were highest in CT and DGT cows and also with TMR supplementation at pasture. Previous studies have also reported higher milk protein, fat and lactose yield with increased milk yield. For example, Atti *et al*., (2006) reported a 47 and 57% increase in

milk fat and milk protein yield respectively in ewes grazed on barley or ryegrass pastures compared to those kept in a feedlot which were associated with a higher milk yield. O'Neal *et al.*, (2011) also reported a 34% higher fat and protein yield and a higher milk yield in TMR fed cows when compared to grass fed cows. Lovett *et al*., (2005) also reported an increase in both milk yield and fat and protein yield by concentrate supplementation at pasture.

In the current study, milk protein concentration (g/kg) showed a tendency to increase with grazing time and with being indoors. Protein concentration was highest in cows that received CT and among the grazing groups, night time grazing increased protein concentration by 1.1 g/kg, perhaps as a result of the high WSC concentration in the grass at the time of grazing. This agrees with reports from some previous studies and according to Taweel *et al*., (2005), high WSC concentrations in grass stimulates an increase in propionate production in the rumen and supplies more energy to the cows. With increased energy, milk protein production is increased and plasma proteins are not oxidised. Taweel *et al*., (2006) indicated that an increase in WSC content in grass produces significant changes in productivity and milk production, but only when metabolisable protein in cows was deficient. In such situations, WSCs become beneficial because it stimulates an increase in microbial protein which in turn improves productivity.

In the current study, the high WSC content of grass in the evening did not have any impact on milk yield. Findings in the current study are similar to those of Taweel *et al*., (2005) who fed two groups of cows with grass that varied in WSC by a range of 24-31 g/kg DM and observed that the differences in WSC concentration were too small to stimulate a difference in DM intake, milk yield or concentrations of any of the milk components. In the current study despite having a difference in WSC concentrations in grass of 111 g/kg DM, DM intake was similar at both grazing times. Reasons for this result are not clear.

However, according to Taweel *et al*., (2005), high concentration of WSC in the diet can lower the pH of the rumen and when this happens, fibre degradability is reduced, retention time is increased and consequently DM intake is also reduced. This may have occurred in the current study, but ruminal pH was not measured.

6.7.4 Methane production

Daily methane production was highest in cows receiving CT or DGT which had the highest total DM intakes. Several studies have reported links between daily methane production and DM intake. For example Dini *et al*., (2012) compared methane production of cows grazed on either high legume pastures or on high ryegrass pastures and observed that methane production did not differ between the two groups because DM intakes were similar. This agrees with Buddle *et al*., (2011) who stated that daily methane output is driven by DM intake. In another study by O'Neal *et al*., (2012), cows grazed pastures that differed in herbage allowance and observed that daily methane production was directly influenced by the total DM intake. In another study (Lovett *et al*., 2005), daily methane output increased with an increase in fibrous concentrate supplementation at pasture, but when methane output was expressed per unit of fat corrected yield, methane output was actually observed to be lower.

When methane production is expressed per unit of animal product, variable results have been observed in previous studies. According to Wims *et al*., (2010), under grazing conditions, other factors besides DM intake play a role in determining methane production. In the current study, methane production per unit of milk yield and per unit of fat corrected yield was lower in all the grazing groups when compared to continuously housed cows. This was due to the grass being of better quality when compared to the TMR. The fresh grass in the current study had a high CP content of 220 g/kg DM and a

high amount of linolenic acid ($C_{18:3n-3}$) averaging 45 g/100g compared to the TMR which had a CP content of 180 g/kg DM and 7 g/100g $C_{18:3n-3}$. Similar result was reported by O'Neal *et al*., (2011) who observed that cows grazed perennial ryegrass pastures produced 12% less methane per unit of DM intake and 13 % less methane expressed as GE intake when compared to cows fed TMR due to the ryegrass being of better quality with a higher CP content and a higher OM digestibility than the TMR. In a study by Wims *et al*., (2010) cows grazed on either low herbage or high herbage pastures had similar DM intakes but methane output when expressed per unit of milk yield and per unit of grass DM intake was higher in cows grazed on high herbage mass pastures because the grass was of poor quality and had a low CP content and a lower OM digestibility.

High WSC concentrations in pm grass did not have any effect on methane production in the current study despite concentrations being 111 g/kg DM higher than in am grass samples. Similar findings were reported by Staerfl *et al*., (2012) who found no difference in methane production when expressed as g/d, g/kg DM intake or g/kg milk yield when cows were fed perennial ryegrass grass that had 90 g/kg DM higher WSC concentration compared to the control group. However one previous study reported a positive effect. Kim *et al*., (2011) fed growing lambs grass that differed in WSC concentration by 42 g/kg DM and observed that methane production when expressed as g/kg DM intake was 17% (L/kg DM) and 25% (L/kg live weight gain) lower in the lambs that were fed grass high in WSC concentration. Kim *et al*., (2011) used growing lambs whereas in our study dairy cows were used, the species differences could have contributed to the difference because ruminal microbial populations tend to vary with species. According to Staerfl *et al*., (2012) high WSC concentrations in grass tend to replace either the CP or NDF content or both. In the current study, pm grass with high WSC concentration had a low concentration of NDF when compared to the am grass.

6.7.5 Milk fatty acid profile

In the current study, dietary treatment did not have any effect on the SCFA, $C_{8:0}$ concentrations in milk, while grazing with no supplementation decreased milk MCFAs concentrations of $C_{10:0}$, $C_{12:0}$ and $C_{14:0}$. This was similar to what was reported in previous studies. For example, Gomez-Cortes *et al*., (2009), subjected cows to feeding regimes of either total grazing, grazing with oat grain supplementation or only fed TMR and observed that cows that were only grazed had reductions in milk fat concentrations of $C_{10:0}$ and $C_{12:0}$ of 6 g/100g and 3.4 g/100g respectively representing 50% and 29% reductions when compared to those fed the TMR. Halmemies-Beauchet-Filleau *et al*., (2013) also observed that the milk profile of cows fed fresh pasture had 31 and 19% lower concentrations of $C_{12:0}$, and $C_{14:0}$ respectively when compared to those fed only a hay based diet. Wales *et al*., (2009) tested the impact of increasing grain supplementation from 0 to 3 to 6 kg /cow/d at pasture on the milk FA profile in dairy cows and observed that $C_{10:0}$ and $C_{12:0}$ and $C_{14:0}$ gradually increased in concentration and concluded that there was a proportionate increase in the concentrations of MCFAs in relation to the amount of concentrate consumed. Grazing causes reductions in SCFA and MCFA concentration in milk fat because the long chain FAs decrease *de novo* synthesis of short and medium chain fatty acids in the mammary gland (AbuGhazaleh, 2008). According to Gomez-Cortez *et al*., (2009) feeding pasture to ruminants results in ruminal bio-hydrogenation with production of C_{18} intermediate products such as CLA's which can reduce *de novo* synthesis of milk fat.

It is well established that pasture feeding increases *cis*-9 $C_{18:1}$ concentration in milk (Chilliard *et al*., 2007). Similarly, in the current study, *cis*-9 $C_{18:1}$ increased by 21 and 6 % in DG and NG cows when compared to DGT and NGT respectively. Grazed pastures had a low *cis*-9 $C_{18:1}$ concentration of 1 g/100g, but milk fat of cows that grazed pastures

without TMR supplementation had high concentrations averaging 26 g/100g. According to Gomez-Cortes *et al*., (2009) and Chilliard *et al*., (2007), the increased concentration in milk occurs first as result of hydrogenation of $C_{18:3n-3}$ to $C_{18:0}$ in the rumen, and a further desaturation via Δ^9 desaturase in the mammary gland. Previous studies have also reported increased concentrations of the C_{18} fatty acids with grazing. For example, in a study, by Gomez-Cortes *et al*., (2009), pasture fed cows had an 86% increase in *cis*-9 $C_{18:1}$ when compared to TMR fed cows. Halmemies-Beauchet-Filleau *et al*., (2013) also compared the milk FA profiles of cows grazed on fresh pastures to those fed a hay based diet and reported that grazing increased $C_{18:0}$ and *cis*-9 $C_{18:1}$ concentration in milk by 35 and 37% respectively.

When cows are grazed, the impact on milk concentration of $C_{18:2n-6}$ tends to vary from one study to another whereas most consistently reporting an increase in $C_{18:3n-3}$ concentrations. A review by Dewhurst *et al*., (2006) established that fresh forage increases concentrations of $C_{18:3n-3}$ in milk while concentrations of other FAs are not very consistent. Halmemies-Beauchet-Filleau *et al*., (2013) also stated that with pasture feeding, concentrations of $C_{18:2n-6}$ tend to be inconsistent. In the current study, $C_{18:2n-6}$ concentrations in milk were affected by the time of grazing, with concentrations being low during the day and high during the night when compared to concentrations found in cows that were kept indoors throughout the study. It is not clear what could have caused the variability in milk concentrations of $C_{18:2n-6}$ with time of grazing as grass concentrations were similar at both sampling times. Previous studies have also reported varying results of either no change, reductions or increase in concentration of $C_{18:2n-6}$ when cows are grazed. For example, Atti *et al*., (2006), compared the milk FA profiles of goats grazed on either barley or ryegrass pastures to those fed only a concentrate ration in the feedlot and reported no changes in the $C_{18:2n-6}$ concentrations in the milk across the

treatment groups. In another study by Renna *et al*., (2012), goats were abruptly turned to pasture and there was a progressive increase in $C_{18:2n\text{-}6}$ concentrations in milk from day 1 day to day 3, with a maximum increase of 22% observed on day 3 and thereafter a progressive decline in concentration was recorded, with the highest decline of 32% recorded on day 18 of pasture feeding. Reasons for the variations in $C_{18:2n\text{-}6}$ concentrations were not clear. Halmemies-Beauchet-Filleau *et al*., (2013) reported increased milk concentrations of both $C_{18:2n\text{-}6}$ and $C_{18:3n\text{-}3}$ by 14 and 24 % respectively in cows grazed fresh pastures compared to those fed a hay based diet. In another study by Rego *et al*., (2008), a partial supplementation of grazing cows with 6 kg/cow/d of grass silage or soya bean meal did not change FA profile of milk. Milk *cis*-9, *trans*-11 CLA, vaccenic acid (*trans*-11 $C_{18:1}$), and α-linolenic acid ($C_{18:3n\text{-}3}$) concentrations were unaffected by supplementation at pasture (Rego *et al*., 2008). Finally, Couvreur *et al*., (2006) reported that cows fed a diet with increasing proportion of 0, 30, 60 and 100 % of fresh grass caused a linear increase in proportions of $C_{18:3n\text{-}3}$ from 0.22g/100g to 0.7 g/100g and proportions of *cis*-9 $C_{18:1}$ from 19.4 to 21.1g/100g.

Trans-fatty acids are intermediate products of PUFA bio-hydrogenation in the rumen and grazing cows tend to have high concentrations in milk fat (Gomez-Cortes *et al*., 2009). In the current study, the two main *trans*-fatty acids, *trans*-11 $C_{18:1}$ and c*is-9*, *trans*-11 CLA did not show any notable increases following dietary manipulation. Only numerical increases were observed such that cows that were grazed with no supplementation at pasture had a slight increase of *trans*-11 $C_{18:1}$ and c*is-9*, *trans*-11 CLA in their milk fat. This finding contrasts with Rego *et al*., (2008) who stated that grazing cows have persistently high concentrations of *cis*-9, t*rans*-11 CLA, *trans*-11 $C_{18:1}$, and $C_{18:3n\text{-}3}$ in their milk. The milk fatty acid *cis*-9 *trans*-11CLA has been extensively studied (Bauman and Griinari, 2001; Kay *et al*., 2004; AbuGhazaleh, 2008) because of its anti-carcinogenic benefits in human

health. According to AbuGhazaleh, (2008), c*is-9*, *trans*-11 CLA is formed either from ruminal bio-hydrogenation of linoleic acid or synthesised from trans-11 $C_{18:1}$ in the mammary gland. Kay *et al*., (2004) established that about 90% of *cis*-9 *trans*-11CLA found in milk of cows fed fresh pasture is synthesised in the mammary gland by Δ^9 desaturase using *trans*-11 $C_{18:1}$ as the precursor. In the current study, high concentrations of *trans*-11 $C_{18:1}$ were found in cows receiving DG or NG that only had access to grazing with no TMR supplementation (av. 0.73 g/100g) while cows receiving CT had 0.62 g/100g of *trans*-11 $C_{18:1}$ concentrations. Some studies have also reported reduced CLA concentrations when cows are supplemented at grass. For example, in a study by Wales *et al*., (2009), increasing grain supplementation at pasture from 3 to 6 kg/cow/d did not have any effect on trans-11 $C_{18:1}$, whereas *cis*-9, *trans*-11CLA concentration was decreased by 13%.

In the current study, there was no treatment effect on eicosapentanoic acid (EPA), $C_{20:5n-3}$ concentrations, but the interaction between grazing time and TMR showed a tendency to reduce concentration when cows were grazed during the day, and increased concentrations when cows were grazed at night. According to Renna *et al*., (2012), EPA concentrations in milk are as a result of desaturation and elongation of $C_{18:3n-3}$.

6.7.6 Conclusions

Grazing cows with or without TMR supplementation reduced methane production when expressed as g/kg milk yield and g/kg fat corrected yield. Having a high concentration of WSC in grass did not reduce methane production but increased protein concentration of milk. Grazing altered the milk fatty acid profile, with cows that had only pasture having high concentrations of *cis*-9 $C_{18:1}$ and $C_{18:3n-3}$ and lowered concentrations of $C_{6:0}$, $C_{10:0}$, $C_{12:0}$, and $C_{14:0.}$ Grazing at night increased milk PUFA content, whereas provision of TMR at pasture increased the SFA content, and decreased the MUFA content. The beneficial

effect of grazing cows during the day with access to TMR on methane production without compromising performance warrants further investigation.

CHAPTER 7 General discussion

The overall hypothesis that was tested in the thesis was that a range of dietary manipulations can alter rumen fermentation and result in reduced methane output and at the same time increase or have no effect on productivity and improve the FA profile of milk.

7.1 Effect of dietary manipulation on fermentation characteristics and methane production

The objective of the first *in vitro* study was to determine the effect of starch and oil source on fermentation characteristics and methane production. The starch sources that were tested were wheat, barley and maize added to grass to supply 25 % starch to the whole diet, while the oil sources carvacrol, linseed oil and fish oil were assessed at two dosage levels of 4 and 8% of total DM. The three starch sources were reported to differ in fermentation characteristics and methane production. Wheat based diets produced the highest cumulative and rates of gas production and methane output and maize based diets produced the least amounts.

The *in vitro* experiment also showed that of the three oil sources supplied at the same level, carvacrol was the most effective in reducing methane output. Linseed oil and fish oil showed dose dependent but non-significant effects on gas production and methane output when compared to the control. The *in vitro* experiment also demonstrated that methane production varied with time of incubation and oil source. Linseed oil (LO) and fish oil (FO) differed with each other in effects on methane production. LO was unsuccessful in reducing methane production at all time periods of *in vitro* incubation

while FO reduced methane production by 35% and 42% at 36-48 h of *in vitro* incubation when added at 4 and 8 % DM respectively.

Another factor that was apparent from the *in vitro* study was that even when oil sources are supplied at the same dosage levels, effects on fermentation and methane production varied among the oil sources. For example, carvacrol when supplied at 8% completely inhibited microbial fermentation and the dose was toxic to the microbes. To date, no essential oil dosage level has been identified as effective for methane mitigation. Reasons for this could be that essential oils are too varied in composition, and even with exactly the same essential oil, effects tend to vary depending on the growing conditions of the plant, time of harvest etc (Benchaar *et al*., 2008).

The digestibility of NDF of the basal diets showed that carvacrol supplied at 4% DM to the basal diets suppressed digestibility to an average of 53%, which was similar across the basal diets. At 8% supplementation, carvacrol further inhibited fibre digestion. On the other hand linseed oil (LO) and fish oil (FO) had no effect on NDF digestibility when compared to the control.

The results of the *in vitro* study helped in designing the experiment for the first cow study. The *in vivo* experiment was a 4X4 Latin square with two starch sources wheat and maize, and two oil sources Megalac and sunflower oil. The aim of the experiment was to test the combination of starch and oil source on methane production, productivity and milk fatty acid profile. Starch sources were fed as wheat and maize based commercial feeds. The wheat based concentrates supplied 290 g/kg and maize based concentrates supplied 330 g/kg starch levels to the cows. Megalac is a commercial product used by farmers and was chosen since it assumed to have inert properties (composed of calcium salts of palm fatty acids), and therefore has minimal effects on rumen fermentation

(Rabiee *et al*., 2012). Megalac was composed of 48% palmitic acid and 36% oleic acid, while sunflower oil was composed of 48% linoleic acid. The study demonstrated that wheat and maize when supplied at the same level have different effects on methane production and productivity. Maize is slowly fermented in the rumen (Chaves *et al*., 2009) therefore produces less methane while wheat has a fast fermentation rate (Doreau *et al*., 2011; Reynolds 2006) and therefore produced higher amounts of methane. The experiment demonstrated that maize based concentrates were more effective in reducing methane output when results were expressed as g/d and g/kg milk compared to the wheat based concentrates. Results of starch sources on methane production verified the findings of the *in vitro* study. When methane results were expressed as g/kg DM intake, an oil source effect was observed in which sunflower oil increased methane output when compared to the Megalac based diets principally due to a reduction in DM intake on this treatment. The reason for the higher methane outputs could have been due to the rumen microorganisms becoming acclimatised to the oil since it was supplied at a very low dose. The higher methane output with sunflower oil when compared to Megalac suggests that sunflower oil stimulated increased methane output.

The interaction between starch source and oil source on methane production was also investigated in study 2. From the results it was established that there was no interaction between starch and oil source on methane production when results were expressed as g/d, g/kg DM intake, g/kg milk yield and g/kg fat corrected yield. It is possible that results of starch and oil source interaction may differ when other basal diets are fed (Beauchemin *et al*., 2009; Chung *et al*., 2011) and this needs investigation.

The final experiment investigated the impact of time of grazing and TMR supplementation of high yielding dairy cows on methane production and productivity. Results of the study were that all the groups of cows that were allowed access to pasture produced a lower

methane output when results were expressed as g/kg milk yield compared to those fed only the TMR. Within the grazing groups, time of grazing did not have an impact on methane production. The reduction in methane output with grazing could have been due to the higher α-linolenic acid levels in grass, which are in the range 450-500 g/kg of the total fat acids in fresh grass compared to preserved forages in TMR which tend to have low concentrations of α-linolenic acid.

Most previous studies have reported low methane outputs when grazing ruminants. For example in a study by O'Neal *et al*., (2011), cows that were grazed perennial ryegrass pastures produced 37 and 11 % less methane production when expressed on a g/d or g/kg DM intake respectively when compared to those that were fed a TMR. The low methane production with grass was attributed to the high organic matter digestibility and high CP concentrations when compared to TMR. The dietary concentration of α-linolenic acid has also been linked with production of low methane output in ruminants and this is a potential area of investigation. For example, Martin *et al*., (2009) supplemented dairy cows fed a hay based diet with extruded linseeds at 3 dose levels of 2, 4 and 6% DM supplementation. Methane production was reduced by 15, 19 and 40% respectively. In another study by Zhang *et al*., (2008), supplementation with α-linolenic acid at 35 and 70 g/kg DM reduced methane production by 46 and 62 % respectively when compared to the un-supplemented control. In the current study, it is suggested that α-linolenic acid could have played a role in reducing the methane output. The NDF levels in am grass samples were higher than those found in the TMR, so it is probable the low methane outputs in the grass fed cows could have been as a result of the higher α-linolenic acid concentration rather than an effect of fibre.

7.2 Effects of dietary manipulation on productivity

The effects of dietary manipulation on productivity were also tested in the two cow based studies. Farmers can easily adopt dietary recommendations only if the changes are able to either improve productivity or have no effect on productivity but at the same time have an added advantage of reducing methane output. A comparison of the two cows studies show that in the first study with two starch and oil sources it was observed that the maize based diets increased the condition score change of the cows suggesting a positive energy balance. In contrast, cows that consumed wheat based concentrates were in negative energy balance and had a negative condition score change. These effects were due to maize based concentrates supplying a high level of starch to the cows. Starch intake in the cows fed maize based concentrates was 0.2 kg/d higher than those fed the wheat based concentrates. When compared to wheat, maize is slowly fermented in the rumen, so the starch from maize escapes fermentation and flows to the small intestine where it is absorbed and goes to the liver and is later utilised as a source of glucose for the cow (Reynolds, 2006). The higher glucose supply may therefore be the reason productivity was higher with the cows fed the maize based concentrates.

In the second grazing study, milk yield was higher in the cows that were housed indoors and lower in all the grazing groups except those that were grazed during the day with access to TMR. The differences in milk production were associated with changes in DM intake. Milk yield increased with TMR supplementation at pasture hence justifying that DM intake is a major factor that limits productivity at pasture. In the grazing trial, afternoon and morning pastures differed in WSC concentrations, with afternoon pastures having a higher level than the morning pasture. This was however, unable to improve milk yield or condition score change. The higher level of WSC in grass did result in a higher protein concentration level in milk. Increased WSC concentrations also did not reduce

methane output. Previous studies that examined effects of high WSC concentration on methane production are few and results are inconclusive. For example, Staerfl *et al*., (2012) fed cows dried ryegrass which was 90 g/kg DM higher in WSC concentrations compared to the control and observed that this had no effect on methane production. While in a study by Kim *et al*., (2011), growing lambs fed grass which was 42 g/kg DM higher in WSC concentrations produced 17 % (L/kg DM) and 25% (L/kg live weight gain) lower methane production when compared to the control. The study by Kim *et al*., (2011) used growing lambs whose ruminal microorganisms at species level may have been different from those of dairy cows. In the current project, the study was carried out in dairy cows and may therefore explain the differences in results with those of Kim *et al*., (2011).

What was established from the two cow studies was that altering the starch source particularly the inclusion of maize in the diet of cows reduces methane output and increases productivity i.e. results in an increase in milk yield and condition score change, while increasing WSC concentration in grass has no impact on methane production and productivity but higher WSC concentration in grass increased milk protein concentration. The impact of WSC on methane output is a new area of research and effects on methane production still remain inconclusive. This also requires further investigation.

7.3 Effects of oil sources on milk fatty acid profile

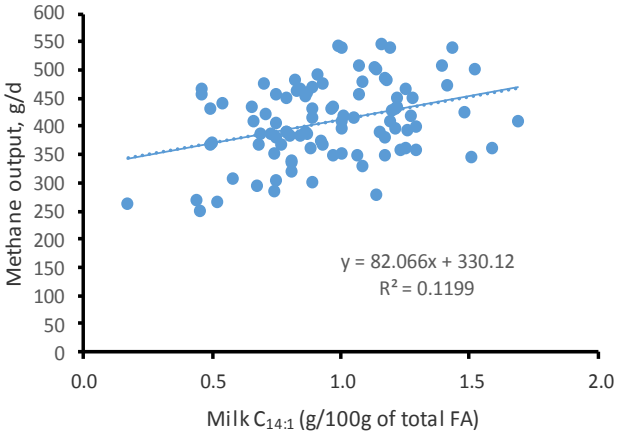
Effects of dietary manipulation on milk FA profile was tested in the two cow based studies. In both studies, dietary FA profiles were reflected in the milk FA profiles of the cows. Cows that were fed sunflower oil based diets in the first cow study and the cows that had access to pasture in the grazing study had higher levels of C_{18} FA concentrations in milk, where as the cows fed megalac based diet in the first trial and those housed

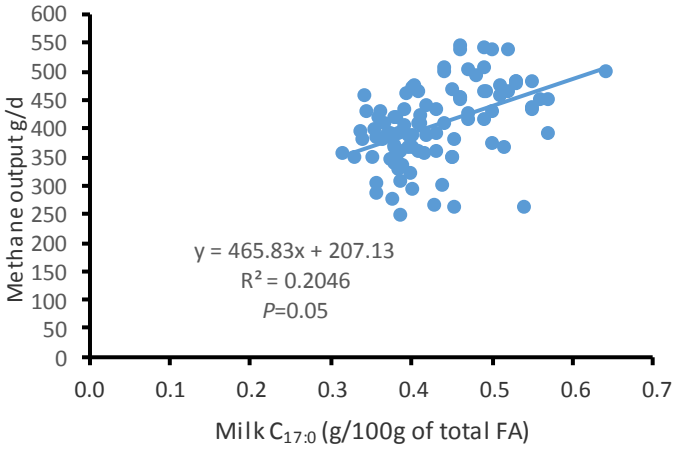
indoors through out in the second trial both had higher concentrations of C_{16} fatty acids. The greater concentration of C_{18} FAs is attributed to the greater intakes of either linoleic acid from sunflower based diets or α-linolenic acid from the fresh pastures. This is consistent with what has been reported in many previous studies in which oils composed of long chain fatty acids when added to ruminant diets tend to reflect their FA profile in the milk (Martinez-Marin *et al*., 2012). Bio-hydrogenation effects in the rumen tend to reduce longer PUFAs chains to SFAs (Sinclair *et al*., 2007, Chikunya *et al*., 2005). Martinez-Marin *et al*., (2012) supplemented dairy goats with refined sunflower oil at increasing dosage of 0, 30, 48 and 66 g/d and reported an increase in milk *trans*-fatty acids *trans*-9 $C_{18:1}$, *trans*-11 $C_{18:1}$, and *cis*-9 *trans*-11 CLA concentrations which increased in a dose dependent manner. *Trans*-9 $C_{18:1}$ increased linearly to a maximum of 200%, *trans*-11 $C_{18:1}$ increased to 635% and *cis*-9 *trans*-11 CLA to 354%.

The first cow study used Megalac and sunflower oil sources in the diet while the second cow study was a comparison between cows housed indoors and those that had access to fresh pasture. Fresh pasture is rich in α-linolenic acid and sunflower oil used in the first cow study is rich in linoleic acid. In both studies, the short chains FAs were unchanged with dietary manipulation with major changes being in the medium and long chain fatty acids. Cows fed sunflower oil had higher contents of *trans*-9 $C_{18:1}$, *trans*-11 $C_{18:1}$, *cis*-9, *trans*-11 CLA and *trans*-10, *cis* 12 CLA in the milk fat, while grazing with no TMR supplementation increased concentrations of *cis*-9 $C_{18:1}$, *trans*-11 $C_{18:1}$ and *cis*-9, *trans*-11 CLA in the milk FA profile. The results obtained were in agreement with a number of other studies. For example in a study by Renna *et al*., (2012), when goats were moved from indoor diet to total grazing, there was a gradual increase in the milk FA concentrations of $C_{18:0}$, *trans-11* $C_{18:1}$ and *α*-linolenic acids and other long chain FA.

7.4 Predicting methane output from milk fatty acid profiles

Previous research has attempted to predict methane production from milk fatty acid profiles. According to Mohammed *et al*., (2011), milk fatty acid have a common pathway with short chain fatty acids propionate, butyrate and acetate that are produced during rumen fermentation, therefore the milk fatty may be used to be predict methane production. In the current study, results of dry matter intake and milk fatty acid profiles from the two cow studies were used to predict methane output. The data set was drawn from the two different cow studies described in chapters 5 and 6 containing a total of 97 individual observations. The test variables included total DM intake (kg/d) from the individual cows and the individual milk FA profile (g/100g of total). In the two studies variables used were similar but feeding conditions in the two studies differed. In the first study, the cows were fed a TMR basal *ad libitum* supplemented with either Megalac or sunflower oil and also had access to 7.5 kg/d/cow *as fed* of either wheat or maize based concentrates. The second cow study was a grazing trial where cows had access to TMR while housed indoors and had access to pasture either at night or during the day. The dependent variable are the methane (CH_4, g/d) results in the experiments and the independent variables were total DM intake, kg/d and the milk fatty acids $C_{14:1}$, $C_{17:0}$, $C_{18:0}$, $C_{18:1n9t}$ and $C_{18:2n6}$. The independent variables were chosen based on the significance of the *P*-value of each regression coefficient (Figs 15a and 15b). The FA $C_{16:0}$ was not included because its *P*-value was not significant. Analysis of data was done using multivariate regression in Excel 2013 Microsoft software.





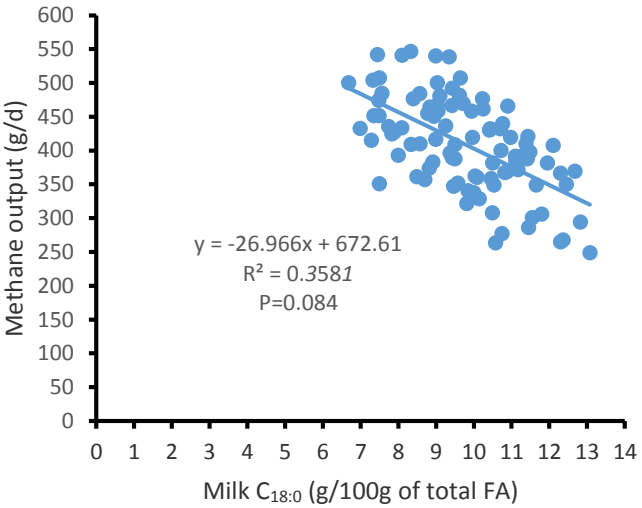


Figure 15a.Correlation between selected milk FA concentrations (g/100g) and daily methane output (g/d).

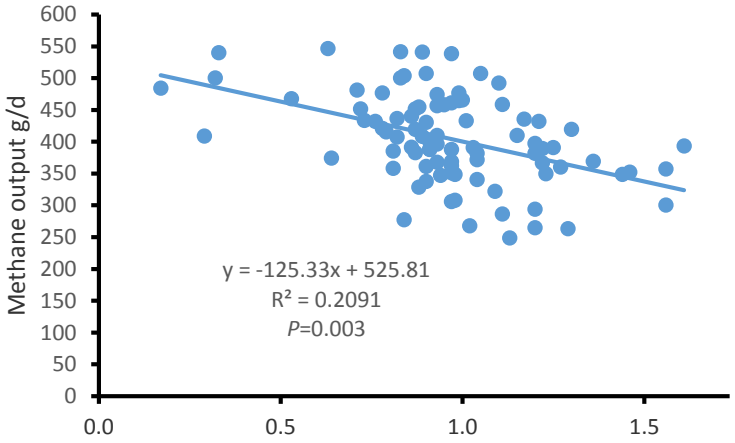


Figure 15b. Correlation between selected milk FA concetrations (g/100g of total FA) and daily methane output (g/d).

Table 34. Anova table showing multivariate regression analysis using data from the two cow studies

	df	*SS*	*MS*	*F*	*Significance F*
Regression	6	259360.3	43226.72	20.05172	9.44097E-15
Residual	90	194018.5	2155.762		
Total	96	453378.9			

Table 35. Regression analysis of parameters with methane output (g/d) using data from two cow studies

Variables	*Coefficients*	*P-value*	*n*
Intercept	142.13	0.173	
Total DM intake	6.61	0.000071	97
$C_{14:1}$	54.70	0.007	97
$C_{17:0}$	166.68	0.052	97
$C_{18:0}$	-7.79	0.084	97
trans-9 $C_{18:1}$	-45.79	0.003	97
$C_{18:2n-6}$	47.27	0.008	97

From the above, the derived regression equation is

CH_4 (g/d) = 142.14+6.61*(Total DM intake, kg/d) + 54.7*($C_{14:1}$) + 166.7*($C_{17:0}$) + (-7.8*$C_{18:0}$) + (-45.8*trans-9 $C_{18:1}$) + 47.3*($C_{18:2n-6}$).

Where milk fatty acids are represented as g/100g of total FA

In the current study the correlation (R^2= 0.54) of methane production and the test variables was not very strong. This could have been due to the varying dietary conditions in the two experiments.

The previous study by Mohammed *et al*., (2011) came up with the following predictive equation.

CH4 (g/d) = −910.8 (±156.7) × milk *cis*-9-$C_{17:1}$ + 331.2 (±88.8) × milk $C_{16:0}$ iso + 0.0001 (±0.00) × total entodiniomorphs + 242.5 (±39.7). (R^2 =90)

The derieved equation differs from that of Mohammed *et al*., (2011) in that the measured fatty acids are different from the ones measured in the current study and total endodiniomorphs were not measured in the current study. The equation by Chillard *et al*., (2009) also differs from the derived equation in that the isomers *trans*-16 $C_{18:1}$ and *cis*-14 $C_{18:1}$ were not measured in the current study and the FA $C_{16:0}$ was found not to have a significant *P*-value. However, the evaluation does give hope of the potential to predict methane production from milk fatty acid concentrations under varying feeding conditions. In summary, the equation obtained cannot be tested using data obtained in previous studies because different fatty acid profiles were used.

In summary, prediction of methane production from milk fatty acid profile and intake parameters is possible. The major limitation is that prediction equations prove difficult to be tested on previous studies because of varying dietary conditions and secondly, the milk fatty acid profiles chosen or used in one prediction equation may differ from those used in other studies. All in all, with refinement, milk fatty acid profile may be good predictors of methane production.

7.5 Conclusions

Results obtained in the thesis support the hypothesis that was being tested of the ability of various dietary manipulations to alter fermentation characteristics, reduce methane production and alter milk FA profile. A range of dietary manipulations including addition of carvacrol, supplementation with maize based concentrates and grazing were effective strategies to reduce methane output. Addition of sunflower oil to the diet and an increase in WSC concentration in grass were not effective in reducing methane production. Milk FA profile was altered by sunflower oil addition and by grazing the cows i.e $C_{16:0}$ was reduced. The long chain PUFA and *trans*-fatty acids were increased.

7.6 Perspective

The project has established that it is possible to mitigate methane production in ruminants through a range of dietary manipulations. What are needed are dietary manipulations that can reduce methane production without having negative effects on productivity. According to Beauchemin *et al*., (2008), farmers are most likely to adopt dietary recommendations that have a positive impact on productivity. Adoption of dietary strategies with negative effects on productivity especially a reduction in milk yields are unlikely to be embraced by farmers. The first cow study where sunflower oil was used resulted in cows having a low DM intake, but the sunflower oil changed the milk FA profile by reducing the $C_{16:0}$ and increasing C_{18} FAs which are desirable for human health. From the study, the results can help farmers have options for the best management practice to adopt. When choosing between two starch sources, wheat or maize based concentrates; maize is more effective at reducing methane production and at the same time increases productivity. From the grazing trial, grazing as a whole reduces methane production. What farmers want are grazing options that do not compromise productivity.

Results of the grazing trial show that grazing during the day with supplementation produces similar levels of production i.e. milk yield, body condition score and live weight change as those cows kept indoors throughout but effectively reduces methane production when results are expressed as g/kg milk yield and g/kg fat corrected yield. Grazing cows also has an additional advantage of proving *α*-linolenic acid in fresh pasture effectively increased its concentration in milk which improves the health quality of milk for human consumption. Lastly grazing in considered a cheap strategy of feeding ruminants (Beauchemin *et al*., 2008).

7.6.1 Cost implications of methane mitigation strategies

Grain or concentrate feeding of ruminants though effective in reducing methane production are produced at great cost. According to Boadi *et al*., (2011) grain production is associated with use of fertilisers and transport costs which tend to increase cost of the grains. Therefore the need to reduce methane production should be weighed against the cost of the high quality feed. However grain or concentrate supplementation of dairy cows has shown to be effective in increasing milk production (Vellinga *et al*., 2011). Increase in milk production is a positive attribute which is desirable as methane emmisions per unit of milk yield tend to reduce.

The cost of the oil supplements also has to be evaluated. Literature shows that processed oils like sunflower oil tend to be more expensive when compared to whole seeds (Beauchemin *et al.*, 2007) because of added processing cost. Main advantage is that they improve the FA profile of the milk by increasing the C_{18} fatty acids and reduce the medium chain FAS.

The long chain FAs eg *cis*-9, *trans*-11 conjugated linoleic acid (CLA), *cis*-9 18:1, and 18:3 *n*-3 have a potential beneficial to human health (Hristov *et al*., 2009). According to Del

prado *et al*., (2010), the high cost of the oils can be offset by the niche consumer population who opt to go for animal products like milk and meat that have a health benefit attached to the product and are willing to pay a premium price for such products. Another advantage of supplementing cows with refined oils is that they tend not to affect whole tract digestibility (Beauchemin *et al*., 2008).

7.6.2 Need for whole system approach to methane mitigation strategies

Before implementing methane mitigation strategies, consideration should be given to the impact of the strategies on the whole system. According to Eckard *et al*., (2010) a whole system assessment ensures that emission reduction strategies in one sector do not result in an increase in emissions in another part of the production system. For, example increase in concentrates or starch sources to dairy rations reduces methane production in cattle, but the production of grains involves clearing of land which tend to increase the GHG emissions through an increase in transport and processing emissions (Beauchemin *et al*., 2008). Therefore option of feeding grains should be balanced with the effect of grain production on the environment. According to Vellinga *et al*., (2011), a number of mitigation options may be carried out on one farm. Example, reducing replacement rates and improving fertility rate can be applied on the same farm whilst dietary changes are also implemented. A DEFRA modelling study (Chadwick *et al*., 2007) observed that at UK national level, a 30% increase in milk production per cow coupled with a reduction dairy cow numbers while maintaining a high milk production is able to reduce methane emissions at national level by 24%. All in all, cost effectiveness of the mitigation strategies is what will influence farmers to adopt the recommendations (Vellinga *et al*., 2011; Beauchemin *et al*., 2007).

CHAPTER 8 References

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