Institut für Tierwissenschaften
Rheinische Friedrich-Wilhelms-Universität Bonn

Effects of medium-chain fatty acids and ration type on *in vitro* ruminal methane production

Inaugural-Dissertation

zur

Erlangung des Grades

Doktor der Agrarwissenschaften

(Dr. agr.)

der

Landwirtschaftlichen Fakultät

der

Rheinischen Friedrich-Wilhelms-Universität Bonn

vorgelegt im Oktober 2014

von

M. Sc. Taufiq Wisnu Priambodo

aus

Jakarta, Indonesien

Referent:	**Prof. Dr. Karl-Heinz Südekum**
Korreferent:	**PD Dr. Joachim Clemens**
Tag der mündlichen Prüfung:	**23.01.2015**
Erscheinnungsjahr:	**2015**

SUMMARY

Several studies *in vitro* and *in vivo* have pointed out the effectiveness of medium-chain fatty acids (MCFA) in suppressing ruminal methane production. Yet no study has elucidated the effect of MCFA type and concentration as well as its forms (single and combined) in different diets on methane production. The objective of this study was to determine the type, concentration and proper form of MCFA application, with regard to the negative impact on the process of fermentation in the rumen.

A systematic and comprehensive *in vitro* (Hohenheim gas test, HGT) experiment was designed and conducted to determine how different types of MCFA at different level of concentrations, in different formats and mixed in different diet, affect rumen methane formation. To complement the results produced by previous studies, C_8, C_{10}, C_{12} and C_{14} were included in the experiment. Each MCFA was mixed into 3 different diets, with the forage (F): concentrate ratios of 25:75 (25F); 50:50 (50F) and 75:25 (75F), to obtain MCFA concentrations of 1, 3, and 5% (w/w) in each diet. At 5% concentration, the effect of single and combined forms of MCFA on methane formation was also compared and assessed. Total gas production, feed digestibility, ammonium production, short-chain fatty acids production and protozoa numbers were also observed to analyze side effects of MCFA supplementation on ruminal nutrient turnover.

The first part of the study was conducted to assess the effects of different chain (carbon) lengths of MCFA as well as differences in the concentration level of each MCFA within different diets on ruminal methane production and other variables. Methane formation suppression was observed ($P \leq 0.05$) at treatments which included the combination between C_{10} and C_{12} in diet 50F. The MCFA concentration increments significantly decreased methane formation following a linear trend.

The second part of the study focused on determining effects of MCFA forms on ruminal methane production. The effectiveness of single form of MCFA was compared with the combination form of two MCFA, in uniform concentration (5% w/w). Both MCFA forms suppressed methane production to a highest degree in combination with diet 50F, with C_{12} being the most effective, irrespective the arrangement of MCFA application (single vs. combined). MCFA inclusion into different diets lowered short-chain fatty acids production, and was accompanied by the decrease of acetate:propionate ratio and protozoa cells reduction.

ZUSAMMENFASSUNG

Zahlreiche Studien haben bereits sowohl *in vitro* als auch *in vivo* die Wirksamkeit von mittelkettigen Fettsäuren (medium-chain fatty acids, MCFA) zur Unterdrückung der ruminalen Methanproduktion gezeigt. Bisher gibt es keine Studien, in denen der Einfluss des MCFA-Typs, der MCFA-Konzentration – einzeln oder in Kombination – und unterschiedlicher Rationen auf die Methanproduktion in einem systematischen Ansatz untersucht wurde. Das Ziel dieser Studie war es deshalb, auch im Hinblick auf mögliche negative Effekte der MCFA auf den Fermentationsprozess im Pansen, den Typ, die Konzentration und die geeignete Form (einzeln, kombiniert) für die MCFA-Anwendung zu bestimmen.

Ein systematischer und umfassender *in vitro*-Versuch (Methode Hohenheimer Futterwerttest) wurde durchgeführt, um zu bestimmen, wie unterschiedliche MCFA-Typen in unterschiedlichen Konzentrationen, unterschiedlichen Formen und in unterschiedlichen Rationen die Methanbildung im Pansen beeinflussen. Um die lückenhaften Befunde vorhergehender Studien zu ergänzen, wurden C8, C10, C12 und C14 in den Versuch einbezogen. Jede MCFA wurde in drei verschiedene Rationen mit Grobfutter (Forage, F):Konzentratfutter-Verhältnissen von 25:75 (25F), 50:50 (50F) und 75:25 (75F) in Konzentrationen von 1, 3 und 5 % (w/w) eingemischt. Bei der 5 %-Stufe wurde auch der Effekt einzelner und kombinierter MCFA auf die Methanbildung verglichen und bewertet. Ebenfalls ermittelt wurden die Gesamtgasbildung, die Verdaulichkeit der Ration, die Produktion an Ammonium und kurzkettigen Fettsäuren sowie die Anzahl an Protozoen, um weitere Effekte der MCFA-Supplementierung auf den ruminalen Nährstoffumsatz zu analysieren.

Der erste Teil der Studie wurde durchgeführt, um die Effekte unterschiedlicher (Kohlenstoff-)Kettenlängen der MCFA und Unterschiede in der Konzentration jeder MCFA in den unterschiedlichen Rationen auf die ruminale Methanproduktion und andere Variablen zu beurteilen. Eine deutliche Abnahme ($P \leq 0{,}05$) der Methanbildung wurde nur mit der Ration 50F in Behandlungen, die eine Kombination von C10 und C12 enthielten, beobachtet.

Der zweite Teil der Studie fokussierte auf die Wirksamkeit einzelner MCFA verglichen mit der Wirksamkeit der Kombination von jeweils zwei MCFAbei einem Gehalt 5 % in der Ration. Erneut war die Reduktion am deutlichsten bei der Ration 50F, und immer dann wenn C12 verwendet wurde, einzeln oder in Kombination mit anderen MCFA. Die Einbeziehung von MCFA in verschiedene Rationen erniedrigte die Produktion kurzkettiger Fettsäuren und dies war von einem engeren Verhältniss von Acetat zu Propionat und der Abnahme der Protozoenzahl begleitet.

Table of Contents

Figures

General Introduction

Tables

Chapter 1. General introduction

Chapter 2. The effect of chain length and concentration of MCFA

Chapter 3. MCFA fed individually or in two component mixtures

Abbreviations

DMI	Dry matter intake
FADH	Flavin adenine dinucleotide
GHG	Greenhouse gases
GWP	Global warming potential
HGT	Hohenheim gas test
MCFA	Medium-chain fatty acids
$NADH/NAD^+$	Nicotineamide adenine dinucleotide
NADPH	Nicotinamide adenine dinucleotide phosphate
NDF	Neutral detergent fiber
RUSITEC	Rumen simulation technique
SCFA	Short-chain fatty acids

CHAPTER 1. GENERAL INTRODUCTION

CHAPTER 1. GENERAL INTRODUCTION

Methane

1. Chemistry

Methane (CH_4 ; marsh gas or methyl hydrate) is the simplest and most abundant hydrocarbon in the earth's atmosphere. It is a colourless gas with a molecular weight of 16.0425 g/mol. Being permanently gaseous, methane cannot be liquefied by pressure alone. It is very stable and only begins to decompose into elements at 785°C (Crabtree, 1995; Houweling, 2000). Methane is a readily combustible gas (33.3 kJ/L at 20°C and 760 mm Hg pressure; Roderick et al. 1992),and being the simplest alkane ,it has bond angles of 109.5°. Presence of CH_4 in the air in concentrations ranging between 5 and 15% is reported to be explosive (Yusuf et al. 2012).

Table 1.1 Physical properties of methane

Property	Value
Melting point	-182.6°C
Boiling point	-161.6°C
Density at boiling point	0.4240
Critical temperature	-82°C
Critical pressure	45.8 atm
First and second ionization potential	13.16 eV and 19.42 eV
Viscosity (gas, 35°C)	1.12×10^{-4} g cm^{-1} s^{-1}
ΔH°	-17.899 kcal/mol
Structure	
C – H bond length	1.1068 Å
H – H distance	1.8118 Å

Source: Crabtree (1995).

Methane absorbs and emits long wave radiation at wavelengths λ = 3.31 μm and 7.66 μm respectively. The energies of photons at these wavelengths correspond to energy differences between different vibrational states of the CH_4 molecules (Houweling 2000).

2. Methane and Climate Change

Greenhouse gases (GHG) play an important role in regulating atmospheric temperature. An increase of the GHG concentrations in the atmosphere significantly increases temperatures and contributes to global warming. Greenhouse gases such as carbon dioxide (CO_2), nitrogen dioxide (NO_2), methane (CH_4) and ozone (O_3) contribute to climate change and global warming through their absorption of infrared radiation in the atmosphere. Methane is reported to contribute 22% of all the long-lived and mixed GHG, while NO_2 contributes 6% (Hook et al. 2010; Mirzaei-Aghsaghali and Maheri-Sis 2011).

A number of roles of methane in atmospheric chemistry and climate have been identified, including effects on tropospheric ozone, hydroxyl radicals and carbon monoxide concentrations. The atmospheric methane eventually reaches the stratosphere and reacts with free radicals to form CH_3^+, which then participates in a number of complex reactions leading to production and destruction of ozone (Cicerone and Oremland 1988; Barber 2007).

Johnson and Johnson (1995) demonstrated, using measurements of methane trapped in polar ice, that its atmospheric concentrations remained relatively stable at approximately 750 ppb until nearly 100 years ago when concentrations began to rise to the present level of approximately 1800 ppb. The more than 500 Tg (1 Tg = 1 million metric tons) of methane that enters the atmosphere annually exceeds its atmospheric and terrestrial oxidation. At this rate, methane is expected to contribute between 15 to 17% of the global warming over the next 50 years. In 1750 the concentration of CH_4 in the atmosphere was 676 - 716 ppb, and rose to 1745 ppb in 1998 and 1800 ppb in 2008 (Yusuf et al. 2012).Recently, global concentration of methane was estimated at 1774 ± 1.8 ppb with total increase of 11 ppb since 1998 (Hook et al. 2010). Mirzaei-Aghsaghali and Maheri-Sis (2011) noted that global surface temperatures are predicted to increase by up to 6°C during the 21st century.

The global warming potential (GWP) of CH_4 is 21- to 25-fold greater than that of CO_2 (Boadi et al. 2004; Hook et al. 2010). Lashof and Ahuja (1990) reported that CH_4 and NO_2, grouped as trace gases, have the ability to absorb infra-red radiation more strongly than CO_2, although they are present at concentrations that are two to six orders of magnitude lower than that of CO_2. Methane and NO_2 are responsible for 43% of the increase in radiative forcing from 1980 to 1990.

Domesticated ruminants account for as much as 80 Tg/year of methane emission (Table 1.2), and among ruminants, beef cattle are the largest contributors (Table 1.3). Domestic ruminants are reported to be responsible for 25% of total anthropogenic CH_4 emission (Machmueller et al. 2003).

Enteric fermentation in the large intestine of ruminants has been estimated to account for 13% of total enteric methane emissions (van Zijderveld 2011).

Table 1.2 Estimates of source of atmospheric CH_4

Source	**CH_4 emission** (10^{12} g/year)
Ruminants	80
Termites	10
Rice paddies	110
Swamps and marshes	115
Landfills	40
Lake and oceans	15
Other natural	30
Coal mining	35
Natural gas flaring	45
Biomass burning	55
Total	535
Total biogenic	400
Total anthropogenic	365

Source: van Zijderveld (2011).

Table 1.3 The global annual methane contribution estimation from domesticated animals

Animal	**CH_4 emission** (Tg/year)
Dairy cattle	18.9
Beef cattle	55.9
Sheep and goats	9.5
Buffalo	6.2 - 8.1
Camels	0.9 – 1.1
Pigs (hindgut)	0.9 – 1.0
Horses (hindgut)	1.7

Source: Hook et al. (2010)

Table 1.4 Estimated annual enteric CH_4 emissions from the main domesticated livestock

	CH_4 emission (kg CH_4 animal $^{-1}$ year $^{-1}$)	**Assumed average body weight** (BW, kg)	**CH_4 emission** (g kg BW^{-1} year $^{-1}$)
Ruminants			
Dairy cows	90	600	150
Beef cattle	65	400	163
Sheep	8	50	160
Goats	8	50	160
Non-ruminants			
Swine	1	80	13
Poultry	<0.1	2	-
Horses	18	600	30

Source: van Zijderveld (2011).

Methanogens and Methanogenesis

Methanogens and Methanogenesis

The domain Archaea is home to many microbes that were previously misclassified as bacteria owing to their prokaryotic morphology. Archaea are clearly monophypetic and their status is underpinned by unique features such as a distinctive cell membrane containing isoprene side chains that are ether-linked to glycerol3. (Allers and Mevarech 2005).

Methanoarchaea grow and synthesize their biomass from CO_2, H_2, N_2 or NH_4 and inorganic salts. The genome size of a Methanoarchaea is less than 40% of the size of the *Escherichia coli* genome. Within this small genome the complete autonomous and autotrophic information is encoded (Reeve et al. 1997).

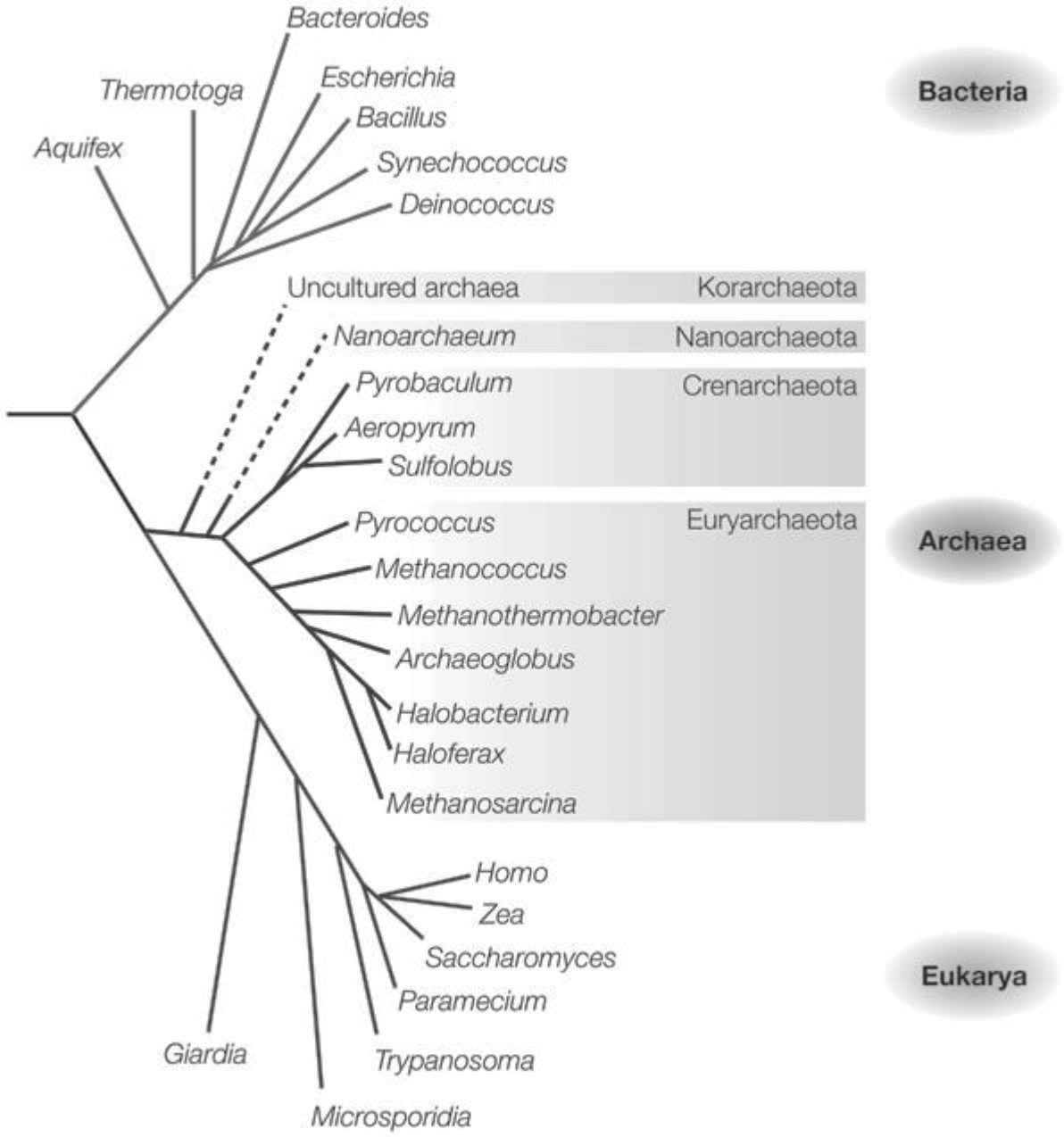

Figure 1. Archaeal taxonomy (Allers and Mevarech 2005).

Methanoarchaea can be found in a wide range of salinities from fresh water to hyper saline water. Some species typically require at least 1 mmol L^{-1} Na^{+}, while others grow at salt concentrations as high as 3 mol L^{-1}. Most Methanoarchaea have pH optima near neutrality, with the exception of *Methanobacterium subterraneum* and *Methanobolus taylorii* which have capability to grow in an environment with pH as high as 9.0 (Barber 2007).

The symbiotic relationship between methanogens and protozoa may generate 37% of rumen CH_4 emissions (Finlay et al. 1994). Methanogens are hydrophobic and therefore stick to feed particles as well as onto the surface of protozoa. About 0.2 of the total ruminal methanogenic population are to be found on the surface of protozoa (Lovett et al. 2003; Boadi et al. 2004).

The uniqueness of methanogens includes three unique coenzymes: methanofuran, tetrahydromethanopterin, deazaflavin F_{420}, which are involved in electron transfer in place of ferredoxin; coenzyme M, involved in methyl group transfer; and Factor B, involved in enzymatic formation of CH_4 from methyl coenzyme M. Furthermore, methanogens lack muramic acid in the cell wall and have isoprenoids ether-linked to glycerol or carbohydrates in their cell membrane, which makes them distinctly different from bacteria. Methanogens typically grow at slightly acidic to slightly alkaline pH (between 6 and 8) and survive only in environments with a redox potential below -300 mV (Garcia et al. 2000; Boadi et al. 2004).

Methanogenesis or methane production occurs in a wide variety of anaerobic environments such as fresh water and marine sediments, anoxic waters, sludge digesters and the intestinal tract of animals, especially ruminants (Roderick et al. 1992). Biological methanogenesis is an important component of the carbon cycle in a variety of anaerobic habitats, such as marshes, lake muds, rice paddies, marine sediment, geothermal habitats and animal gastrointestinal tracts (Zinder 1992).

There are four steps in the process of organic substrate degradation by Archaea and their symbionts in natural anaerobic habitats (Garcia et al. 2000):

1. Hydrolysis of polymers by hydrolytic microorganisms.
2. Acidogenesis from simple organic compounds by fermentative bacteria
3. Acetogenesis from metabolites of fermentations by homoacetogenic or syntropic bacteria
4. Methanogenesis by methagenic A rchaea

Production of methane by methanogens requires a food chain of at least three interacting metabolic groups of obligatory anaerobic microbes (Figure 2).

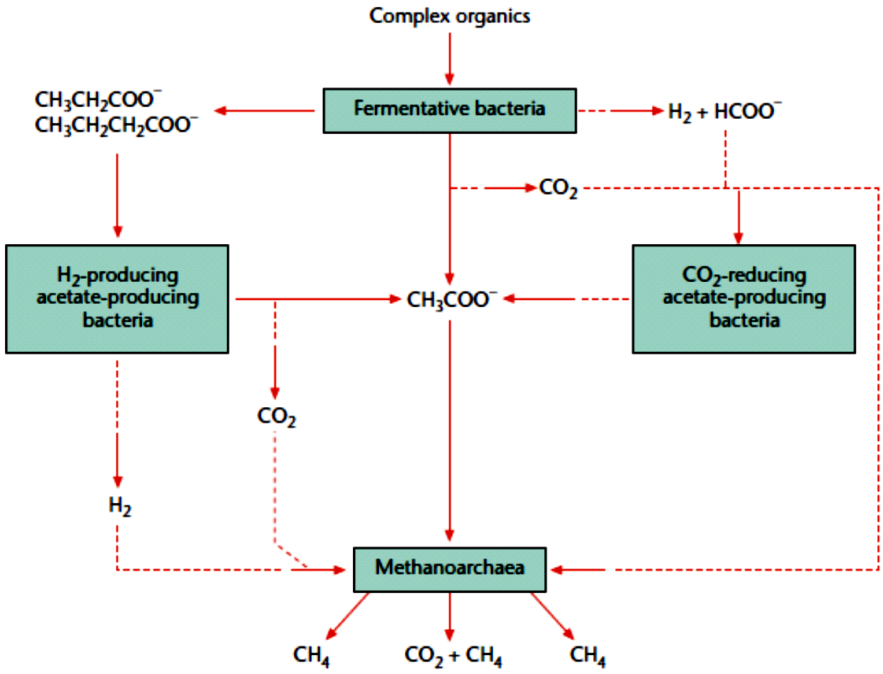

Figure 2. Microbial food chain in anaerobic environments (Barber 2007).

In ruminants, methane is predominantly produced in the rumen (83 - 93%) and to a small extent in the large intestine (13% on average). Rumen CH_4 is primarily emitted from the animal by eructation (Boadi et al. 2004; Hook et al. 2010; Bell and Eckard, 2012). It is a natural end-product of rumen fermentation that plays a fundamental role in the efficacy of feed digestion by rumen microbes. Methanogens utilize H_2 to generate energy for growth by reducing CO_2 into CH_4 according to the following reaction equation (Boadi et al. 2004; Jouany 2008):

$CO_2 + 4H_2 \rightarrow CH_4 + 2H_2O \quad \Delta G_O' = -135.6$ kJ

Formate can also be used as primary substrate for methane production:

$4HCOOH \rightarrow 3CO_2 + CH_4 + 2H_2O$

Other substrates that are used by methanogens during the generation of CH_4 include acetate (*Methanosarcina* and *Methanosaeta*), methanol, methylamines, dimethyl sulphide and some alcohols (Boadi et al. 2004).

Through interspecies hydrogen transfer, fermentative bacteria (H_2-producing bacteria) and methanogens (H_2-consuming microbes), maintain the ruminal H_2 concentration below 1 kPa. Thus re-oxidation of NADH to NAD^+ can occur and the fermentation process can continue (Jouany 2008).

Without the removal of hydrogen, re-oxidation of reduced co-factors (NADH, NADPH and FADH) would be inhibited by the accumulated hydrogen and the production of short-chain fatty acids (SCFA) would be inhibited. Soon after production, hydrogen is used by methanogenic Archaea to reduce CO_2 into CH_4. In the absence of methanogens, organic matter cannot be degraded as effectively in the gut (Boadi et al. 2004; Martin et al. 2009; van Zijderveld 2011).

Another stoichiometric equation proposed by Jouany (1995) describes precisely typical yield proportions of the end products of rumen fermentation (molar basis):

50 glucose equivalents = 59 acetate (C2) + 23 propionate (C3) + 9 butyrate (C4) + 24 CH_4 + 53 CO_2 + 230 ATP

The equation above indicates the methane production is related to hydrogen-producing fermentative reactions and negatively related to hydrogen-using fermentative reaction. If the ratio of acetate to propionate is greater than 0.5, then hydrogen would be available to form methane. The excess of hydrogen, after that used by methanogens will lead to the formation of ethanol and lactate, which inhibits microbial growth, forage digestion and SCFA production (Bell and Eckard 2012). During the production of acetate and butyrate from hexose fermentation, hydrogen is released, whereas propionate production results in the net uptake of hydrogen (van Zijderveld 2011):

$C_6H_{12}O_6 + 2H_2O \rightarrow 2C_2H_3O_2 + 2CO_2 + 4H_2 + 2H^+$ (Acetate/C2)

$C_6H_{12}O_6 + 2H_2 \rightarrow 2C_3H_5O_2 + 2H^+ + 2H_2O$ (Propionate/C3)

$C_6H_{12}O_6 \rightarrow C_4H_7O_2 + H^+ + 2H_2 + 2CO_2$ (Butyrate/C4)

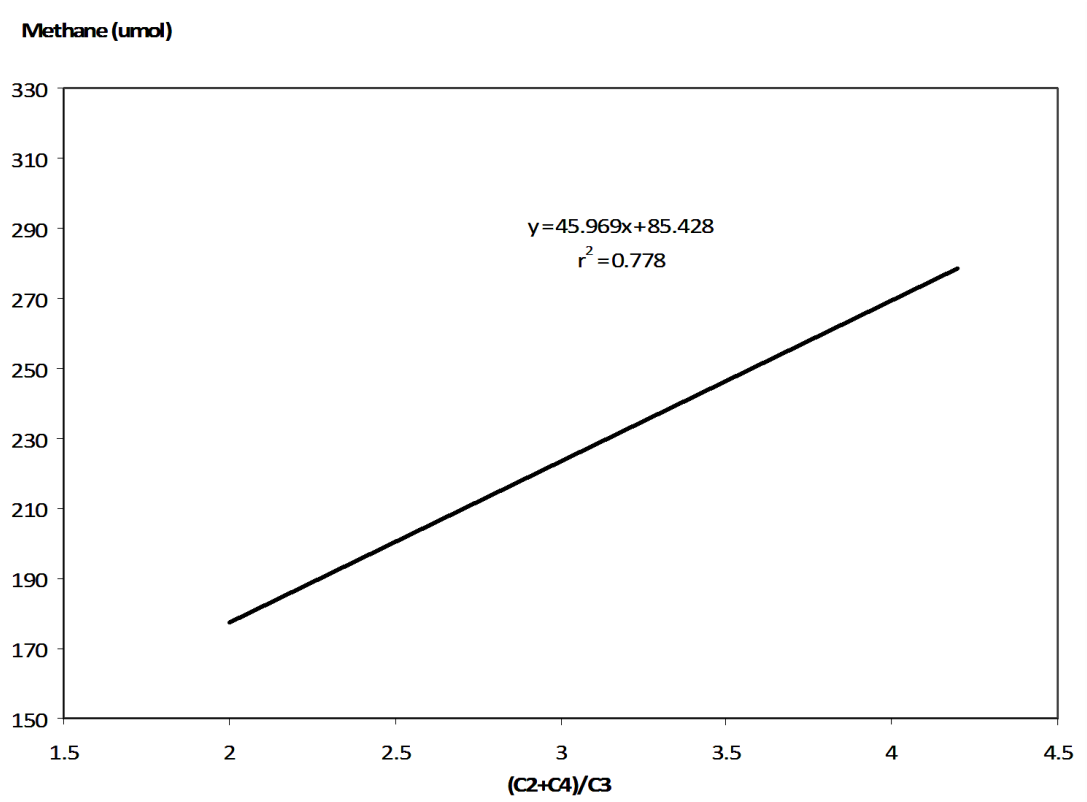

Figure 3. Relationship between (C2 + C4)/C3 ratio and methane production (Moss et al. 2000).

Among methanogens, only *Methanobrevibacter ruminantium* and *Methanosarcina barkeri* were found in the rumen at densities greater than 10^6/mL, and are thus assumed to play a major role in ruminal methanogenesis (Boadi et al. 2004).

The interaction of methanogens with bacteria through interspecies H_2 transfer in the fermentation process allows methanogens to gain energy for their own growth, preventing the accumulation of H_2 which benefits the growth of H_2-producing bacteria allowing further degradation of fibrous feed material (Boadi et al. 2004). Methanogens, therefore, occupy the terminal position of anaerobic food chains (Roderick et al. 1992; Garcia et al. 2000).

Fermentation and VFA production by the microbes in the intestinal tract of animals is accompanied by the production of methane. The molar percentage of SCFA produced during enteric fermentation influences the production of CH_4. It is explained stoichiometrically as follow:

CH_4 = 0.45 acetate (C_2) – 0.275 propionate (C_3) + 0.40 butyrate (C_4).

Acetate and butyrate production results in CH_4 production, while propionate formation serves as a competitive pathway for H_2 utilization in the rumen (Moss et al. 2000; Boadi et al. 2004; van Zijderveld 2011).

Methane Mitigation

Decreasing enteric CH_4 emission from ruminants without altering animal production is desirable both as a strategy to reduce global greenhouse gas emissions and as a means of improving feed conversion efficiency (Martin et al. 2009). Existing dietary mitigation strategies include the addition of ionophores and fats, use of high-quality forages and increased use of grains, methanogen control through vaccination, the use of bacteriocins and probiotics (acetogens and live yeasts), as well as plant extracts (Boadi et al. 2004; Martin et al. 2009).

Fatty Acids

Fat supplementation is one of the most promising dietary strategies to mitigate CH_4 production, although it also affects milk production and composition (Grainger and Bauchemin 2011). Supplementation of coconut oil, which is classified as a rumen-defaunating agent and which is even more potent than linseed oil, at a rate of 3.5 and 7% is reported to suppress CH_4 production by 28 and 73% respectively (Machmueller et al. 1998). Machmüller et al. 1998 also recorded a decrease of ciliate protozoa population from 1.7 x 10^5/ml at the beginning of the experiment to 1.0 x 10^5/ml and 0.3 x 10^5/ml, respectively, in response to the addition of 3.5 and 7% coconut oil concentrations.

The application of crushed sunflower seed, flaxseed and canola seed in lactating dairy cow feeds decreased CH_4 production and population of protozoa (Table 1.4).

Table 1.5 Effect of fat source on ruminal fermentation

Item	**Control**	**Sunflower**	**Flaxseed**	**Canola**
Digestible dry matter intake (kg/d)	26.6	29.7	24.2	22.3
Methane (g/animal ·d)	293	264	241	265
Protozoa, (· 10^6/mL)	8.28	5.16	6.35	5.23

Source: Beauchemin et al. (2009)

Goel et al. (2009) investigated the inhibitory effects of capric acid on methane production *in vitro*. The application of 4 and 6% capric acid significantly suppressed methane production by 33 and 85%,

respectively. The addition of both dosages of capric acid, however, decreased total VFA production by23 %.

Van Zijderveld et al. (2011) demonstrated that a mixture of capric (C10) and caprylic acid (C8) did not affect methane production in lactating dairy cows. In single *in vitro* incubations, capric as well as lauric (C12) and myristic acids (C14) have demonstrated a particular action against rumen protozoa (Matsumoto et al. 1991; Machmüller and Kreuzer 1999). In addition, a diet containing 10% coconut oil almost completely eliminated methanogens in a Rusitec setup (Machmüller and Kreuzer 1999). The application of coconut oil, cod liver oil and canola oil depressed CH_4 production and methanogenic population regardless of basal diet (Dong et al. 1997).

Dong et al (1997) concluded that though the reduction of methane production is not directly related to the number of double bonds, the depression of methane production increases with the degree of fatty acid unsaturation. The order of inhibitory effects of long chain fatty acids to the growth of pure culture of *Methanobacterium ruminantium* was: $C_{18:1} > C_{14} > C_{12} > C_{16} > C_{18}$. Soliva et al. (2004) described the toxicity level being between C_{12} and C_{14}; although C_{14} mainly inhibited the growth of methanogens and not as toxic to them as C_{12} is when supplemented at the same concentration.

Medium-chain FA (MCFA; C_8 - C_{16}) has been shown to suppress CH_4 production (Dohme et al. 2000). An application of MCFAs, namely lauric (C_{12}) and myristic (C_{14}) acids given as single FA decreased CH_4 production and the combined supplementation of both demonstrated greater depressive effects on CH_4 production in ruminants (Dohme et al. 2001). Another study conducted by Soliva et al. (2004) described the substantial effect of the application of C_{12} alone. At the same dosage (30 mg MCFA per incubation unit) C_{12} alone reduced the emission of CH_4 by 74% compared to the unsupplemented control, much more than C_{12}:C_{14} ratios of 1:2 (57%) and 2:1 (27%). A single application of C_{12} (32.5 mg) interrupted the recovery of hydrogen in the rumen to as low as 61% while at the same dosage no effect was observed on ruminal pH, SCFA concentration and ciliate protozoa counts.

Soliva et al. (2004) concluded that the decrease in methanogen counts depended primarily on the total amount of MCFA and less on the type of MCFA supplied. An addition of a mixture of 10 mg C_{12} and 10 mg C_{14} will have similar depressing effect on methanogenic counts as 20 mg of C_{12} alone.

The addition of excessive fat (more than 5 - 6% of the ration DM) depresses fibre degradation in the rumen and reduces acetate production and milk fat content (Boadi et al. 2004). The efficacy of C14 in diminishing CH_4 formation was about the same as that of C_{12} after 22 days of supplementation to

sheep *in vivo* (Machmueller et al. 2003) and a 10day *in vitro* experiment using a concentrate-based diet (Dohme et al. 2001). The palatability of C_{12} is less when compared to C_{14} due its particular odour and soapy taste (Külling et al. 2001).

Diet Influence on Methanogenesis

The energy losses attributable to methane range from 2% to 7% of gross energy (GE), depending on animal category and dietary conditions. Losses are lowest with cereal-rich or highly digestible grass diets, and highest with high-fibre diets with low digestibility. Starch digestion in the rumen stimulates propionate production whereas fibre digestion increases acetate production (Jouany 2008). In another study, Mc Geough et al. (2010) demonstrated that methane output per kilogram of DMI is reduced when grain content in whole-crop wheat was increased.

The correlation between methane emission per unit digested DM and plant fibre content is positive and significant. Therefore, replacing C4 plants with C3 plants is recommended in order to abate ruminants' methane production (Ulyatt et al. 2002). The administration of diet composed primarily of concentrates has been demonstrated to increase levels of intake and reduce ruminal pH and as methanogens are acutely pH sensitive, this will reduce CH_4 production (Yan et al. 2000; Lovett et al. 2003; Mirzaei-Aghsaghali and Maheri-Sis. 2011).

Increasing the level of concentrate in the diet leads to a reduction in CH_4 emissions as a proportion of energy intake or expressed by unit of animal product (milk and meat). A decrease in methane production (from 6 and 7% to 2 and 3% of GE intake) was observed when the concentrate portion in the diet increased from 30 - 40% to 80 - 90%, respectively (Martin et al. 2009). Benchaar et al. (2001) conducted an experiment to find out the effect of concentrate proportion on CH_4 production. The ratios of forage to concentrate investigated were 100:0, 80:20, 50:50 and 30:70 of a diet based on Lucerne hay supplemented with a concentrate mixture. Their results showed that an increase in dietary concentrate reduced ruminal passage rates of liquids and solids, increased ruminal starch degradation, decreased NDF, linearly depressed ruminal microbial efficiency, increased total SCFA and propionate production and declined CH_4 production.

Martin et al. (2009) explained that a shift of SCFA production from acetate to propionate occurs with development of starch-fermenting microbes, which result in a lower CH_4 production as the decline of relative proportion of ruminal hydrogen happens. High-concentrate diets also decrease the ruminal

pH. The low ruminal pH might inhibit the growth and/or activity of methanogens and cellulolytic bacteria.

Rumen fluid from cows fed a 90% concentrate diet had lower pH values (6.22 vs. 6.86), higher SCFA concentrations (85 vs. 68 mM) and lower acetate to propionate ratio (2.24 vs. 4.12) than rumen fluid from cows fed 100% forage. The CH_4 production by ruminants tends to increase with maturity of forage and legume forages yield lower CH_4 than grass forages. Friesian and Jersey dairy cows grazing a condensed tannin-containing legume (*Hydesarum coronarum*) emitted less CH_4 per unit of DMI (19.5 g/kg) than cows grazing perennial ryegrass pastures (24.6 g/kg) (Boadi et al. 2004).

A study by McCaughey et al. (1999) indicated that pasture quality improvement through addition of legumes (lucerne) to pasture mix may potentially reduce CH_4 production by close to 10%. Cows produce lower CH_4 when fed with lucerne-grass pastures (373.8 L/d) compared to cows on grass-only pastures (411.0 L/d). A similar result was obtained when results were expressed relative to body weight (0.74 vs. 0.81 L/kg body weight). Energy lost through eructation of cows grazing lucerne -grass pastures was also lower than that of cows grazing grass-only pastures (7.1 vs. 9.5% of GE intake).

Garcia-Martinez et al. (2005), examining different doses of fumaric acid in different rations, found that the application of fumaric acid resulted in a CH_4 decrease when combined with high-forage diet (80% forage), compared to medium- (50%) and low- (20%) forage diets. Due to their use of different diets and experimental conditions, there is needed to further investigate the discrepancies in the effects of fumaric acid on CH_4 production.

References

Barber, R.D. 2007. Methanogenesis: Ecology. In: Encyclopaedia for Life. John Wiley & Sons, Ltd: Chichester. DOI: 10.1002/9780470015902.a0000475.pub2

Beauchemin, K.A., McGinn, S.M., Benchaar, C., Holtshausen, L. 2009. Crushed sunflower, flax, or canola seeds in lactating dairy cow diets: Effects on methane production, rumen fermentation, and milk production. Journal of Dairy Science 92: 2118–2127.

Bell, M.J. and Eckard, R.J. 2012. Reducing Enteric Methane Losses from Ruminant Livestock – Its Measurement, Prediction and the Influence of Diet, Livestock Production, Khalid Javed (Ed.), ISBN: 978-953-51-0814-6, InTech, DOI: 10.5772/50394. Available from: http://www.intechopen.com/books/livestock-production/reducing-enteric-methane-losses-from-ruminant-livestock-its-measurement-prediction-and-the-influence

Benchaar, C., Pomar, C., Chiquette, J. 2001. Evaluation of dietary strategies to reduce methane production in ruminants: A modelling approach. Canadian Journal of Animal Science 81: 563-574.

Boadi, D., Benchaar, C., Chiquette, J., and Massé, D. 2004. Mitigation strategies to reduce enteric methane emissions from dairy cows: Update review. Canadian Journal of Animal Science 84: 319–335.

Cicerone, R.J. and Oremland, R.S. 1988. Biogeochemical aspects of atmospheric methane. Global Biogeochemical Cycles 2: 299-327.

Crabtree, R.H. 1995. Aspects of methane chemistry. Chemical Reviews 95: 987-1007.

Finlay, BJ., Esteban, G., Clarke, K.J., Williams, A.G., Embley, T.M., Hirt, R.P. 1994. Some rumen ciliates have endosymbiotic methanogens. Microbiology Letters 117: 157-162.

Garcia, J.-L., Patel, B.K.C., Olliver, B. 2000. Taxonomic, phylogenetic and ecological diversity of methanogenic archaea. Anaerobe 6: 205-226.

García-Martínez, R., Ranilla, M.J., Tejido, M.L., Carro, M.D. 2005. Effects of disodium fumarate on *in vitro* rumen microbial growth, methane production and fermentation of diets differing in their forage:concentrate ratio. British Journal of Nutrition 94: 71-77.

Goel, G., Arvidsson, K., Vlaeminck, B., Bruggeman, G., Deschepper, K., Fievez, V. 2009. Effects of capric acid on rumen methanogenesis and biohydrogenation of linoleic and α-linolenic acid. Animal 3: 810-816.

Grainger, C. and Beauchemin, K. A. 2011. Can enteric methane emissions from ruminants be lowered without lowering their production? Animal Feed Science and Technology 166-167: 308–320.

Hook, S.E., Wright, A-D.G., McBride, B.W. 2010. Methanogens: Methane producers of the rumen and mitigation strategies. Archaea 2010: 1-11.

Houweling, S. 2000. Global modelling of atmospheric methane sources and sinks, Universiteit Utrecht, Utrecht.

Johnson, K. A. and Johnson, D.E . 1995. Methane emission from cattle. Journal of Animal Science 73: 2483-2492.

Jouany, J.-P. 2008. Enteric methane production b ruminants and its control. In: Andrieu, S. and Wilde, D. (eds.) Gut efficiency; the key ingredient in ruminant production. Elevating animal production and health. p 35-55. Wageningen Academic Publishers, Wageningen.

Külling, D.R., Dohme, F., Menzi, H., Sutter, F., Lischer, P., Kreuzer, M. 2002. Methane emissions of differently fed dairy cows and corresponding methane and nitrogen emissions from their manure during storage. Environmental Monitoring and Assessment 79: 129-150.

Lashof, D.A. and Ahuja, D.R. 1990. Relative contribution of greenhouse gas emissions to global warming. Letters to Nature 344: 529-531.

Lovett, D., Lovell, S., Stack, L., Callan, J., Finlay, M., Conolly, J., O'Mara, F.P. 2003. Effect of forage/concentrate ratio and dietary coconut oil level on methane output and performance of finishing beef heifers. Livestock Production Science 84: 135-146.

Machmüller, A., Ossowski, D.A., Wannerm M., Kreuzer, M. 1998. Potential of various fatty feeds to reduce methane release from rumen fermentation *in vitro* (rusitec). Animal Feed Science and Technology 71: 117-130.

Machmüller, A. and Kreuzer, M. 1999. Methane suppression by coconut oil and associated effects on nutrient and energy balance in sheep. Canadian Journal of Animal Science 79: 65-72.

Machmüller, A., Soliva, C.R., Kreuzer, M. 2003. Methane-suppressing effect of myristic acid in sheep as affected by dietary calcium and forage proportion. British Journal of Nutrition 90: 529–540.

Martin, C., Morgavi, D.P., Doreau, M. 2009. Methane mitigation in ruminants: From microbe to the farm scale. Animal 4: 351-365.

Matsumoto, M., Kobayashi, T., Takenaka, A., Itabashi, H. 1991. Defaunation effects of medium-chain fatty acids and their derivates on goat rumen protozoa. Journal of General and Applied Microbiology 37: 439-445.

Mc Geough, E.J., O'Kiely, P., Hart, K.J., Moloney, A.P., Boland, T.M., Kenny, D.A. 2010. Methane emissions, feed intake, performance, digestibility, and rumen fermentation of finishing beef cattle offered whole-crop wheat silages differing in grain content. Journal of Animal Science 8: 2703-2716.

McCaughey, W.P., Wittenberg, K., Corrigan, D. 1999. Impact of pasture type on methane production by lactating beef cows. Canadian Journal of Animal Science 79: 221-226.

Mirzaeli-Aghsaghali, A. and Maheri-Sis, N. (2011). Factors Affecting Mitigation of Methane emission from Ruminants I: Feeding strategies. Asian Journal of Animal and Veterinary Advances 6(9): 888-908.

Moss, A.R., Jouany, J-P., Newbold, J. 2000. Methane production by ruminants: Its contribution to global warming. Annales de Zootechnie 49: 231-253.

Reeve, J.N., Nöling, J., Morgan, R.M., Smith, D.R. 1997. Methanogenesis: Genes, genomes, and who's on first? Journal of Bacteriology 179: 5975–5986.

Allers, T and Mevarech, M. 2005. Archaeal genetics-The third way. Nature 6:58 -73

van Zijderveld, S. 2011. Dietary strategies to reduce methane emissions from ruminants, Wageningen University, Wageningen.

Ulyatt, M.J., Lassey, K.R., Shelton, I.D., Walker, C.F. 2002. Methane emission form dairy cows and wether sheep fed subtropical grass-dominant pastures in midsummer in New Zealand. New Zealand Journal of Agricultural Research 45: 227-234.

van Zijderveld, S.M, Dijkstra, J., Perdok, H.B., Newbold, J.R., Gerrots, W.J.J. 2011. Dietary inclusion of diallyl disulphide, yucca powder, calcium fumarate, an extruded linseed product, or medium-chain fatty acids does not affect methane production in lactating dairy cows. Journal of dairy Science 94: 3094-3104.

Yan, T., Agnew, R.E., Gordon, F.J., Porter, M.G. 2000. Prediction of methane energy output in dairy and beef cattle offered grass silage-based diets. Livestock Production Science 64: 253-263.

Yusuf, R.O., Noor, Z.Z., Abba, A.H., Hassan, M.A.A., Din, M.F.M. 2012. Methane emission by sectors: A comprehensive review of emission sources and mitigation methods. Renewable and Sustainable Energy Reviews 16: 5059–5070.

CHAPTER 2. The effect of chain length and concentration of medium-chain fatty acids, and of ration composition, on mitigating methane emission: *in vitro* study

T.W. Priambodo[1], J. Hummel[2], and K.-H. Südekum[1]

[1] *Institute of Animal Science, University of Bonn*

[2] *Department of Animal Sciences, University of Göttingen*

To be submitted

Abstract

A modified Hohenheim gas test (HGT) was employed to determine the effectiveness of four medium-chain fatty acids (MCFA) in mitigating ruminal methane formation. The MCFA, namely $C_{8:0}$, $C_{10:0}$, $C_{12:0}$ and $C_{14:0}$ were included into diets consisting of (dry matter (DM) basis) 25, 50 and 75% grass silage (forage, F), at three different concentrations: 1, 3 and 5% of DM (w/w). The measurements encompassed: methane formation, total gas production, DM digestibility, and ammonium production. Diets were incubated for 6, 10 and 24h. A reduced methane production was obvious for 50F diets which contained the combination of $C_{10:0}$ and $C_{12:0}$. Increasing MCFA concentration linearly lowered ($P< 0.05$) methane formation. Already at the lowest inclusion level, MCFA reduced ($P<0.05$) total gas volume and DM digestibility, as well as ammonium concentration.

Key words: methane, medium-chain fatty acids, chain length, concentration, diet type

Introduction

Methane is an unavoidable by-product of enteric fermentation. As a green-house gas, it contributes to the carbon footprint of ruminant-derived food production, with a great warming effect of 25 times that of CO_2. It may account for 2 - 15% loss of the gross energy intake by ruminants (Johnson and Johnson 1995; Flachowsky and Lebzien 2012; FAO 2010; Chong et al. 2014).

Several studies have shown that fatty acids of different carbon chain lengths (Blaxter and Czerkawski 1966) and form, i.e. as pure substance (Odongo et al. 2007) or as a part of natural products (Dohme et al. 1999; Mc Ginn et al. 2004; Beauchemin and McGinn 2006; Martin et al. 2008; Jalč et al. 2009), have inhibitory effects against methanogens, rumen bacteria, protozoa and may lower methane emission by ruminants (Henderson 1973; Matsumoto et al 2001).

Medium-chain fatty acids (MCFA: $C_{8:0}$ - $C_{16:0}$) are among those fatty acids, which are reported to having the most extensive methane mitigating effects (Dohme et al. 2001; Machmüller 2002; Machmüller 2005; Panyakaew et al. 2013). Supplementation of lauric acid ($C_{12:0}$) as free acid (Machmüller et al. 2002; Soliva et al. 2004; Božic et al 2009) or complexed with cyclodextrin (Ajisaka et al. 2002) and in combination with myristic acid ($C_{14:0}$) significantly suppressed methane production and ciliate population (Soliva et al. 2004). Lauric acid has an inhibitory effect on Gram positive-rumen bacteria and, therefore, might have methane reducing potential if applied in high forage diets (Kobayashi 2010).

By taking into consideration ration ingredients, Machmüller et al. (2003) confirmed that $C_{14:0}$ is a potent methane inhibiting agent. Myristic acid suppressed methane concentration by 58% in a concentrate-based diet, while in a forage-based diet methane was only reduced by 22%, when compared to the unsupplemented diet. Oother investigations of effects of ration composition on methane inhibitory effects of MCFA were conducted by Dohme et al. (1999) and Krüling et al. (2001).

However, the influence of a range of MCFA, included singly or in combinations, at varying concentrations in diets ranging from low to high forage proportions, on ruminant methane emission has never been investigated. In this study, a systematic *in vitro* research was designed to evaluate and to compare effects of different MCFA chain-length ($C_{8:0}$, $C_{10:0}$, $C_{12:0}$ and $C_{14:0}$) in different diets (forage (grass silage) to concentrate: 75:25; 50:50 and 25:75), at 3 different MCFA concentrations (1, 3 and 5%) on methane concentration, total gas production, DM digestibility and ammonium

production, as well as a part of screening of potential methane inhibitors which furthermore provides a comprehensive data for further *in vivo* testing.

Material and methods

Diet preparation

Grass silage, maize grain and solvent-extracted soybean meal were finely ground (3- amd 1-mm sieve size) and used to formulate three mixed diets with ratios (dry matter (DM) basis) of silage (forage) to concentrate of 75:25 (75F), 50:50 (50F) and 25:75 (25F). Medium-chain fatty acids, namely $C_{8:0}$ (caprylic acid), $C_{10:0}$ (capric acid), $C_{12:0}$ and $C_{14:0}$ (SAFC® Supply Solution; Sigma-Aldrich, St. Louis, MO, USA) were included at concentrations of 1, 3 and 5% of DM.

Modified Hohenheim gas test (HGT)

In vitro gas production was determined according to Menke and Steingass (1988). Rumen fluids were collected from two ruminally fistulated German Blackheaded Mutton sheep, fed a mixed diet of 600 g grass hay and 600 g mixed concentrate per day. Feeds were offered in two equal meals at 07:00 and 19:00 h. Rumen fluid was collected prior to the morning feeding and strained through two layers of cheesecloth into pre-warmed and insulated flask. All laboratory handling of rumen fluid was carried out under a continuous flow of CO_2. Samples (200 ± 2 mg) were accurately weighed into a 100 ml glass syringe. The syringe pistons were lubricated with vaseline and inserted into the syringe. Four replicates of each treatment were incubated in two HGT runs which were carried out on different dates. Three blanks containing 30 ml of medium only were included in each run together with triplicates of a standard hay and standard concentrate obtained from the Institute of Animal Nutrition, University of Hohenheim, Germany. The syringes were placed in a rotor inside the incubator (39 °C) with about one rotation per minute. The cumulative gas production as well as CH_4 was read at the following times: 6, 10 or 24h. At reading, each syringe was taken out from the incubator and the incubator's door closed to avoid temperature decrease.

Methane measurement

Methane concentration relative to total gas volume (as %, v/v; range 0 to 40%)) was measured using an infrared analyzer (Advanced Gasmitter® Pronova Analysentechnik GmbH & Co. KG, Berlin,

Germany) at 6, 10 and 24 hours incubation time. Goel et al. (2008) and Jayanegara et al. (2009) applied the corresponding method for methane analysis in their experiments.

Methane measurement can be started as follows once the calibration process has been completed. The syringe is taken out from HGT incubator; gas volume of the particular syringe is read and noted; the syringe is cooled down and stored on ice for approximately 20 minutes. A cooled syringe is withdrawn from the ice, connected with the inlet of the analyzer and the gas volume is noted again. The gas from the syringe is gently injected into the device; injecting moisture or liquid must be avoided. Syringe is gently disconnected from the analyzer inlet; CH_4 value (in %) is noted. The volume of the syringe after injection is also noted. The remaining gas inside the analyzer is sucked out using a syringe which is plugged to the analyzer outlet port. The CH_4 -free condition inside the analyzer is indicated by 0% value; the analyzer is then ready for the subsequent CH_4 measurement.

To increase the volume of measurement an additional dilution step is required for syringes which contains less than 40 ml gas by adjusting the piston position back to 40 ml syringe volume to let normal air join and mix with the gas inside the syringe.

Chemical analysis

The DM of feedstuffs and mixed diets were determined by freeze-drying (silage only) and subsequent oven-drying at 105 °C overnight. Dried feedstuffs were successively ground in mills with 1 mm screens. All feedstuffs and mixed diets were analysed for ash, crude protein and detergent fibre fractions. Starch content was determined by enzymatic hydrolysis of starch to glucose (Brandt et al. 1987), employing the heat-stable α-amylase Termamyl 120 L (Novo Industrials, Bagsværd, Denmark). The N was determined using Dumas procedure and crude protein calculated as N × 6.25. The neutral detergent fibre (NDF) analysis was conducted according to Van Soest et al. (1991). Detergent fibre analyses were performed without the use of decalin. Sodium sulphite was omitted and triethylene glycol was used instead of 2-ethoxyethanol in the NDF procedure. The NDF values are expressed without residual ash and therefore designated NDFom. Ammonium was determined using the Kjeldahl method (without digestion step). Diet and feed composition data are presented in Table 1.

Statistical analysis

Data obtained from the experiments of three diet ratios were analyzed using the Generalized Linear Model (GLM) procedure in the SAS® software (9.2 versions) according to model as follow:

$$Y_{ijklm} = \mu + M_i + F_j + C_k + T_l + \varepsilon_{ijklm.}$$

where Y is value of observation, μ is population mean, M is treatment MCFA ($_i$= 1, 2, 3, 4; 1 = $C_{8:0}$, 2 = $C_{10:0}$, 3 = $C_{12:0}$, 4 = $C_{14:0}$), F is forage:concentrate ratio ($_j$ = 1, 2, 3; 1 = 75:25, 2 = 50:50, 3 = 25:75), C is MCFA concentration ($_k$ = 1, 2, 3; 1 = 1%, 2 = 3%, 3 = 5%), T is incubation time ($_m$ = 1, 2, 3; 1 = 6 h, 2 = 10 h, 3 = 24 h), and ε_{ijklm} is the residual error.

Orthogonal polynomial contrasts were used to investigate further the effect of MCFA chain length on CH_4 production within the same diet and MCFA concentration, as well as effect of different MCFA concentrations with in the same diet and incubation time. The overall least squares means were declared significant at $P < 0.05$ unless otherwise stated.

Results

Chain length

The effect of MCFA chain length on CH_4 concentration was investigated by comparing the MCFA effect with in the same diet, concentration and incubation time.

No effect of MCFA chain length on CH_4 production was observed for 25F and 75F diets. When included separately, an effect was only found at the highest MCFA inclusion level (5%), at 24h incubation, in diet 25F. The effect followed a linear and quadratic pattern, with $C_{10:0}$ and $C_{12:0}$ as the most effective agents in reducing CH_4 production. Both MCFA ($C_{10:0}$ and $C_{12:0}$) reduced CH_4 from 2.463 mmol g^{-1} at control to 1.791 and 1.707 mmol g^{-1}, respectively.

The inclusion of MCFA into the 50F diet generated the most obvious CH_4 reduction (Table 2). At 1% concentration, at 6 and 10 h incubation, a linear response to increasing MCFA chain length was observed. Lauric acid was the most potent agent in reducing CH_4 production, at both incubation times. Furthermore, the addition of 3% MCFA lowered CH_4 production at all three incubation times. The variation of chain length did not effect the CH_4 production at 6 h incubation time differently, although it reduced the CH_4 formation when compared to control (P = 0.02). At 10 h and 24 h incubations, the chain length of MCFA affected the CH_4 production diversely, a linear reduction was observed at 10 h and a quadratic response at 24 h incubation. Capric acid ($C_{10:0}$) obviously was the most effective MCFA in reducing CH_4 formation. Methane production was also lowered at the 5% inclusion level of MCFA. Increasing chain-length affected CH_4 production following a quadratic pattern at 6 h incubation, and both patterns (linear and quadratic) were seen at 10 h and 24 h

incubations. Methane production was reduced most at the inclusion of $C_{8:0}$ at 6 h incubation and $C_{12:0}$ at 10 and 24h incubation time.

MCFA concentration

Methane production

In the 25F diet, an effect of a gradual change of MCFA concentration on CH_4 production was observed for $C_{10:0}$ and $C_{12:0}$, at 24 h incubation. The effect followed a linear trend, as the highest MCFA concentration (5%) contributed most to the reduction of CH_4 formation.

Furthermore, MCFA concentration reduced CH_4 production in the 50F diet (Table 3). A linear trend of CH_4 reduction was found for different concentrations of $C_{10:0}$ and $C_{12:0}$ at 10 h and 24 h incubation. Subsequently, the inclusion of the highest concentration (5%) of $C_{12:0}$, suppressed CH_4 formation to 46% of the control at both incubation times (10 h and 24h).

The inclusion of MCFA into the 75F diet apparently did not effect CH_4 formation.

Gas Production and Digestibility

The MCFA inclusion in the different diets to some extent influenced total gas production. Relative to the control, total gas volume decreased in a range between 1 to 20%. With the 25F diet, the application of $C_{12:0}$ at 5% concentration and at 24 h incubation time resulted in 20% reduction of total gas. Furthermore, $C_{10:0}$ and $C_{12:0}$ were the MCFA which profoundly affected gas production in the 75F diet. The application of both MCFA reduced, at 5% concentration and 24 h incubation time, otal gas production by 16 and 12%, compared to control (Table 4).

The reduction of DM digestibility was only observed when MCFA were included in the 50F and 75F diets. Among MCFA, $C_{10:0}$ and $C_{12:0}$ were found significantly affecting DM digestibility at the 5% inclusion level. The digestibility decrease was 11 to 18%, relative to the control.

NH_3-N

The supplementation of different MCFA on diet ratio and different time of incubation did not effect the NH3-N production ($P < 0.05$). The exceptional case was at the combination of MCFA with the 50F

diet at 24 h incubation time. Three MCFA ($C_{8:0}$, $C_{10:0}$ and $C_{14:0}$) reduced the NH_3-N concentration in the range of 25 - 30% compared with the control ($p < 0.05$). Capric acid at 5% concentration most effectively lowered NH_3-N concentrations. The NH_3-N reduction of the different MCFA concentrations followed a linear pattern.

Discussion

Chain length effect

One of the main objectives of this study was to investigate the effect of MCFA chain length on methane mitigation, in combination with variation of the forage to concentrate ratio of mixed diets and at different incubation times. Medium chain fatty acids have been reported to have inhibitory activity against Gram- positive bacteria, even at low concentrations. Methanogens are Archaea with a cell wall which resembles Gram-positive bacteria. Therefore, methanogens can be expected to have sensitivity against MCFA which eventually influence methane formation (Koster and Cramer 1986). A relationship between MCFA chain length and toxicity has been described by Matsumoto et al. (1991), who indicated that $C_{10:0}$ and its derivates have a stronger toxicity than fatty acids having shorter and longer carbon chains. In another study, Dohme et al. (2008) have examined effects of seven saturated MCFA and long-chain FA ($C_{8:0}$; $C_{10:0}$; $C_{12:0}$; $C_{14:0}$; $C_{16:0}$ and $C_{18:0}$) and one unsaturated long-chain FA ($C_{18:2}$) on methane mitigation. Among MCFA, only $C_{12:0}$ and $C_{14:0}$ affected methane formation as did the long-chain $C_{18:2}$.

Zeitz et al. (2013) reported that $C_{10:0}$ had a similar anti-methanogenic activity as $C_{12:0}$, and was superior to $C_{14:0}$ and $C_{18:0}$. A longer carbon chain corresponds to increasing melting temperature, namelly 31, 45, 58 and 69°C for $C_{10:0}$, $C_{12:0}$, $C_{14:0}$ and $C_{18:0}$. Melting point influences the distribution of FA in the medium and FA-methanogen cell contact probability (Zeitz et al. 2013). Our data supports the previous studies, with $C_{12:0}$ appearing as the preferential methane inhibitor.

Several factors have been suggested to affecting MCFA activity in the rumen. Already Harfoot et al. (1974) underlined that the methane inhibition by MCFA might be attenuated as they may attach to rumen microbes or feed particles. Furthermore, Galbraith et al. (1971) concluded that the non-esterified FA has to be in solution and to remain sufficiently lipophilic to permit adsorption on the cell wall surface of the microbes. In our study, $C_{14:0}$ was the least active CH_4-mitigating agent, when compared to the other MCFA. This is not in line with previous results (Dohme et al. 2001; Machmüller et al. 2002; Machmüller et al. 2003), but confirms a study conducted by Soliva et al.

(2001) who reported that the addition of $C_{14:0}$ had no methane suppressing effect, even when supplemented at high doses. We assume that $C_{14:0}$ may intimately be affiliated with feed particles, which would reduce its affinity and toxicity to rumen microbes, thus resulting in little CH_4 lowering effect. Another explanation could be that cations (e.g., Ca^{2+}) can chelate MCFA, resulting in insoluble and inactive complexes (Machmüller et al. 2003). The formation of Ca soaps is long known to alter the MCFA effect on rumen fermentation (Jenkins and Palmquist 1982).

The current study revealed interactions between diet type and methane formation. Methane formation of the unsupplemented control of the diets exhibited a quadratic pattern, highest methane formation was found with of the 50F diet compared with 25F and 75F diets. The strongest effect of MCFA chain length on methane mitigation was observed when MCFA were included in the 50F diet, which supports previous *in vitro* findings (Dong et al. 1997, Machmüller et al. 2001).

Shifting diet type from forage dominated diet to concentrate dominated diet generally leads to lowered CH_4 production per unit fermented substrate (Hungate 1966). Increasing dietary concentrate appears to be an effective feeding strategy in decreasing rumen methagonesis (Machmüller et al. 2003). Furthermore, Lovett et al. (2003) found that reducing forage to concentrate ratio led to a methane reduction, following a quadratic pattern. However, when studying the relationship between diet compositions, intake level and methane production, Moe and Tyrrell (1980) revealed that the nature of the carbohydrate digested is less important at feed intake levels below 1.5 times maintenance. In another study Machmüller et al. (2003) provided 1.3 times maintenance energy requirements, which resulted in almost the same level of methane for different diets that was produced per unit apparently digested organic matter although the composition of the organic matter digested was different. Therefore, the methane lowering effect of increasing concentrate proportion can not be regarded as a comprehensive conclusion. Apart from the MCFA inclusion, the adjustment of forageinclusions, the adjustment of forage and concentrate ratios is an effective means to manipulate ruminal fermentation pathways and reducing enteric methane production (Lovett et al. 2003). The reducing effect of dietary oil on methane formation is greater on low forage diets, as the protozoan contribution to ruminal hydrogen level is greater in diets rich in starch (Lovett et al. 2003).

MCFA concentration effect

Methane production

The outcome of the current study confirms the finding of previous studies (Machmüller and Kreuzer 1999; Machmüller 2006; Soliva et al. 2004), where increasing levels of MCFA, amplified MCFA methane inhibiting effect. In an *in vivo* experiment, Machmüller and Kreuzer (1999) used three different diets with increasing proportions of coconut oil (0, 3.5 and 7%). The supplementation of coconut oil at 3.5 and 7% suppressed methane production by 28 and 73%, respectively, relative to the unsupplemented diet.

Our study also to some extent affirmed previous findings (Machmüller 2006) that supplementation of MCFA profoundly inhibit methane formation at concentrate-dominated diets compare to the forage-dominated diets. Forage-dominated diets are assumed to have larger feed particles to which MCFA can attach, hindering the association between MCFA and rumen microbes, which in turn weaken methane inhibitory effect of MCFA (Harfoot et al. 1974).

Based on our observations, in addition to $C_{12:0}$, $C_{10:0}$ was found to have a profound inhibitory effect on methane formation. This is, to a certain extent, in contrast with findings of several studies conducted by Machmüller (2006) and Dohme et al. (2008), who reported $C_{12:0}$ and $C_{14:0}$ being the methane inhibitor MCFA. Relatively short incubation time is assumed to influence the $C_{14:0}$ methane reduction efficacy in the current study. An effect of $C_{14:0}$ supplementation in concentrate-based diet decreased methane production by 58%, after a feeding period of 22 d (Soliva et al. 2004). In addition, using concentrate-based diet, Dohme et al. (2001) found that $C_{14:0}$ diminished methane formation to a similar extent as $C_{12:0}$ did, after 10 d incubation time using a rumen simulationtechnique (RUSITEC).

Gas production and digestibility

The anaerobic environment in the rumen provokes little energy utilization by resident microorganisms during the catabolism of saturated FA (Nagaraja et al. 1997), although fats are actively hydrolyzed and FA saturated in the rumen (Hawke and Robertson 1964). Therefore substituting fat for carbohydrate in diets for ruminants decreases the amount of ATP available for microbial growth (Firkins 1996). Often the inclusion of fats inhibit ruminal microbial activities and fibre digestion (Brooks et al. 1954; Henderson 1973; Jenkins 1993).In the work presented here, a

severe decrease of both, gas production and DM digestibility were observed at the inclusion of $C_{10:0}$ and $C_{12:0}$ in diet consisting of 50% grass silage (50F).

NH3-N

Hristov et al. 2004 mentioned that the supplementation of MCFA affect ruminal ammonia concentration. The ammonia concentration in the rumen is related mostly to total protozoa numbers and to protozoal and bacterial activities in the rumen. The supplementation of MCFA inhibited proteolysis and deamination, apart from decreasing protozoa and bacteria population activity. A similar finding reported by Machmüller et al. (2003) was that the supplementation of $C_{14:0}$ profoundly affected the NH_3-N concentration in forage- and concentrate-based diets, which conforms with our data. Particularly in the 50F diet NH_3-N was influenced by the supplementation of MCFA, notably $C_{8:0}$ and $C_{10:0}$.

Conclusions

The result of this comprehensive *in vitro* study pointed out that $C_{10:0}$ and $C_{12:0}$, at particular supplementation levels, and when included in a 50F diet had the most consistent effect on inhibiting ruminal methane formation. In accordance with previous studies which affirmed the efficacy of $C_{10:0}$ and $C_{12:0}$ as prospective agents reducing rumen methanogens (Henderson 1973) and ciliate population (Matsumoto et al.1991).

References

Ajisaka, N., Mohammed, N., Hara, K., Mikuni, K., Hara, K., Kumata, T., Kanda., and Itabashi, H. 2002. Effects of medium-chain fatty acid-cyclodextrin complexes on ruminal methane production *in vitro*. Animal Science Journal 73: 479–484.

Beauchemin, K.A., and McGinn, S.M. 2006. Methane emissions from beef cattle: effects of fumaric acid, essential oil, and canola oil. Journal of Animal Science 84:1489-1496.

Blaxter, K. L., and Czerkawski, J. 1966. Modifications of the methane production of the sheep by supplementation of its diet. Journal of the Science of Food and Agriculture 17: 417-420.

Božic, A. K., Anderson, R.C., Carstens, G.E., Ricke, S.C., Callaway, T.R., Yokoyama, M.T., Wang, J.K., Nisbet, D.J. 2009. Effects of the methane-inhibitors nitrate, nitroethane, lauric acid, lauricidin and the Hawaiian marine algae chaetoceros on ruminal fermentation *in vitro*. Bioresource Technology 100: 4017-4025.

Brandt, M., Schuldt, A., Mannerkorpi, P., Vearasilp, T. 1987. Zur enzymatischen Stärkebestimmung im Darminhalt und Kot von Kühen mit hitzestabiler Amylase. Archives of Animal Nutrition 37: 455.

Brooks, C.C., Garner, G.B., Gehrke, C.W., Muhrer, M.E., Pfander, W.H. 1954. The effect of added fat on the digestion of cellulose and protein by ovine rumen microorganisms. Journal of Animal Science 13:758-764.

Chong, L., Zhuping, Z., Tongjun, G., Yongming, L., Hongmin, D. 2014. Changes in methane emission, rumen fermentation, and methanogenic community in response to silage and dry cornstalk diets. Journal of Basic Microbiology 54: 521-530.

Crutzen, P. J., Aselmann, I., Seiler, W. 1986. Methane production by domestic animals, wild ruminants, other herbivorous fauna, and humans. Tellus 38B: 271-284.

Dohme, F., Machmüller, A., Estermann, B.L., Pfister, P., Wasserfallen, A., Kreuzer, M. 1999. The role of the rumen ciliate protozoa for methane suppression caused by coconut oil. Letters in Applied Microbiology 29: 187-192.

Dohme, F., Machmüller, A., Wasserfallen, A., Kreuzer, M. 2000. Comparative efficiency of various fats rich in medium-chain fatty acids to suppress ruminal methanogenesis as measured with rusitec. Canadian Journal of Animal Science 80: 473-482.

Dohme, F., Machmüler, A., Wasserfallen, A., Kreuzer, M. 2001. Ruminal methanogenesis as influenced by individual fatty acids supplemented to complete ruminant diets. Letters in Applied Microbiology 32: 47-51.

Dong, Y., Bae, H.D., McAllister, T.A., Mathison, G.W., Cheng, K., -J. 1997. Lipid induced depression of methane production and digestibility in the artificial rumen system (RUSITEC). Canadian Journal of Animal Science 77:269-278.

FAO. 2010. Greenhouse gas emission from dairy sector. A life cycle assessment. FAO, Rome: 1-98.

Flachowsky, G. and Lebzien,P. 2012. Effects of phytogenic substances on rumen fermentation and methane emissions: A proposal for a research process. Animal Feed Science and Technology 176: 70-77.

Goel, G., Makkar, H.P.S., Becker, K. 2008. Changes in microbial community structure, methanogenesis and rumen fermentation in response to saponin-rich fraction from different plant materials. Journal of Applied Microbiology 105:770-777.

Harfoot, D.C., Crouchman, M.L., Noble, R.C., Moore, J.H. 1974. Competition between foot particles and rumen bacteria in the up take of long chain fatty acids and triglycerides. Journal of Applied Bacteriology 37: 633-641.

Henderson, C. 1973. The effects of fatty acid on pure cultures of rumen bacteria. Journal of Agricultural Science 81:107-112.

Hristov, A.N., Ivan, M.,McAllister, T.A. 2004. *In vitro* effects of individual fatty acids on protozoa numbers and on fermentation products in ruminal fluid from cattle fed a high-concentrate, barley-based diet. Journal of Animal Science 82:2693-2704.

Hungate, 1966. The rumen and its microbes. Academic Press, London: 533.

Jalč, D., Čertík, M., Kundríková, K., Kubelková, P. 2009. Effect of microbial oil and fish oil on rumen fermentation and metabolism of fatty acids in artificial rumen. Czech Journal of Animal Science 54: 229-237.

Jayanegara, A., Togtokhbayar, N., Makkar, H.P.S., Becker, K. 2009. Tannins determined by various methods as predictors of methane production reduction potential of plants by an *in vitro* rumen fermentation system. Animal Feed Science and Technology 150:230-237.

Johnson, K.A, and Johnson, D.E. 1995. Methane emission from cattle. Journal of Animal Science 73:2483-2492.

Jenkins, T.C., and Palmquist, D.L. 1982. Effect of added fat and calcium on *in vitro* formation of soluble fatty acid soaps and cell wall digestibility. Journal of Animal Science 55:957-963.

Jenkins, T.C. 1993. Lipid metabolism in the rumen. Journal of Dairy Science 76:3851-3863.
Kobayashi, K. 2010. Abatement of methane production from ruminants: Trends in the manipulation of rumen fermentation. Asian-Australian Journal of Animal Science 23: 410-416.

Koster, I.W., and Cramer, A. 1987. Inhibition of methanogenesis from acetate in granular sludge by long-chain fatty acids, Applied and Environmental Microbiology 53:403-409.

Krülling, D. R., Dohme, F., Menzi, H., Sutter, F., Lischer, P., Kreuzer, M. 2002. Methane emissions of differently fed dairy cows and corresponding methane and nitrogen emissions from their manure during storage. Environmental Monitoring and Assessment 79: 129-150.

Lovett D., Lovell, S., Stack, L., Callan, J., Finlay, M., Conolly, J., O'Mara, F.P. 2003. Effect of forage/concentrate ratio and dietary coconut oil level on methane output and performance of finishing beef heifers. Livestock Production Science 84: 135–146.

Machmüller, A., Soliva, C.R., Kreuzer, M. 2003. Methane-suppressing effect of myristic acid in sheep as affected by dietary calcium and forage proportion. British Journal of Nutrition 90: 529-540.

Machmüller, A. 2006. Medium-chain fatty acids and their potential to reduce methanogenesis in domestic ruminants. Agriculture, Ecosystems and Environment 112: 107-114.

Matsumoto, M., Kobayashi, T., Takenaka, A., Itabashi, H. 1991. Defaunation effects of medium-chain fatty acids and their derivates on goat rumen protozoa. Journal of General and Applied Microbiology 37:439-445.

Martin, C., Rouel, J., Jouany, J.P., Doreau, M., and Chilliard, Y. 2008. Methane output and diet digestibility in response to feeding dairy cows crude linseed, extruded linseed, or linseed oil 86:2642-2650.

McGinn, S. M., Beauchemin, K.A., Coates, T., Colombatto, D. 2004. Methane emissions from beef cattle: Effects of monensin, sunflower oil, enzymes, yeast, and fumaric acid. Journal of Animal Science 82: 3346-3356.

Menke, K.H., Steingass, H., 1988. Estimation of the energetic feed value obtained from chemical analysis and gas production using rumen fluid. Animal Research and Development 28:7-55

Moe, P. W., & Tyrrell, H. F. 1980. Methane production in dairy cows. European Association of Animal Production Publication, 26:59.

Newbold, C. J., and L.M. Rode, L.M. 2006. Dietary additives to control methanogenesis in the rumen. International Congress Series 1293: 138–147.

Odongo, N. E., Or-Rashid, M.M., Kebreab, E., France, J., McBride, B.W. 2007. Effect of supplementing myristic acid in dairy cow rations on ruminal methanogenesis and fatty acid profile in milk. Journal of Dairy Science 90: 1851–1858.

Panyakaewa, P., Goel, G, Lourenc, M., Yuangklangb, C, Fieveza, V. 2013.Medium-chain fatty acids from coconut or krabok oil inhibit *in vitro* rumen methanogenesis and conversion of non-conjugated dienoic biohydrogenation intermediates. Animal Feed Science and Technology 180: 18–25.

Soliva, C. R., Meile, L., Hindrichsen, I.K., Kreuzer, M., Machmüler, A. 2004. Myristic acid supports the immediate inhibitory effect of lauric acid on ruminal methanogens and methane release. Anaerobe 10: 269–276.

Zeitz, J.O., Bucher, S., Zhou, X., Meile, L., Kreuzer, M., Soliva, C.R. 2013. Inhibitory effects of saturated fatty acids on methane production by methanogenic Archaea. Journal of Animal and Feed Sciences 22: 44-49.

Table 2.1. Chemical analysis of mixed diets and diet ingredients

Items	Diet			Feed		
(g kg^{-1} DM unless stated)	25F	50F	75F	Grass silage	Soybean meal	Maize grain
Dry matter (g kg^{-1})	897	897	892	899	904	914
Ash	66.1	79.2	83.3	106	80.9	21.6
Crude protein	183	194	165	106	515	144
Crude lipid	39.1	34.5	30.5	25.1	19.9	85.4
NDFom	289	349	465	603	-	-
ADFom	170	208	267	372	75.8	3.3
ADL	23.9	32	16	20.13	3.3	7.4
Starch (enzymatic)	31.85	24.63	6.92	1.17	1.98	30.66

Table 2.2. CH_4 formation (mmol g^{-1} digested DM) in diet 50F (50:50 forage to concentrate ratio)

IT (h)	MCFA conc. (%)	Treatments					SEM	F Anova	F Linear	F Quadratic	R^2	CV
		Control	$C_{8:0}$	$C_{10:0}$	$C_{12:0}$	$C_{14:0}$						
6	3	0.438	0.313	0.353	0.383	0.343	0.021	0.049	0.015	0.592	0.472	31.162
	5	0.438	0.285	0.37	0.383	0.435	0.028	0.193	0.319	0.075	0.317	22.371
10	3	1.275	1.217	0.85	0.905	0.967	0.085	0.095	0.038	0.171	0.485	21.012
	5	1.275	0.763	0.743	0.69	0.833	0.106	0.029	0.157	0.011	0.514	21.708
24	3	3.088	1.97	1.813	2.355	2.29	0.22	0.009	0.011	0.007	0.623	23.12
	5	3.088	2.25	1.887	1.863	2.475	0.225	0.006	0.027	0.001	0.617	17.727

IT=incubation time, MCFA= medium-chain fatty acid, SEM=standard error of the means, CV=coefficient variation, $P< 0.05$, n=4

Table 2.3. Regression equations of MCFA chain length effect on CH_4 concentration in diet 50F

IT (h)	MCFA conc. (%)	Regression equations	Intercept SE	Intercept P	x SE	x P
6	3	Y = 0.434 - 0.021 x	0.0253	< 0.0001	0.040379	0.04071
	5	Y = 0.435 - 0.036 x	0.02468	< 0.0001	0.00785	0.0003
10	3	Y = 1.286 - 0.034 x	0.118017	< 0.0001	0.0403	0.4071
	5	Y = 1.278 - 0.110 x	0.09514	< 0.0001	0.03244	0.004
24	3	Y = 3.082 - 0.264 x	0.24697	< 0.0001	0.08194	0.0053
	5	Y = 3.112 - 0.237 x	0.2162	< 0.0001	0.07027	0.0039

MCFA conc.= medium-chain fatty acid concentration, IT=incubation time, P< 0.05, n=4

Table 2.4. Effects of variation of MCFA concentration on selected variables

Grass silage	MCFA	IT (h)	MCFA concentration (%)				SEM	F Anova	F Linear	F Quadratic	R^2	CV
			0	1	3	5						
CH_4 formation (mmol/g digested feed)												
25	$C_{10:0}$	24	2.46	2.83	2.13	1.79	0.223	0.002	0.002	0.035	0.691	12.934
	$C_{12:0}$	24	2.46	2.59	1.55	1.51	0.290	0.005	0.002	0.71	0.642	21.353
50	$C_{10:0}$	10	1.28	1.34	0.85	0.64	0.168	0.004	0.001	0.288	0.653	23.857
		24	3.09	2.29	1.81	1.66	0.321	0.003	0.001	0.159	0.746	17.641
	$C_{12:0}$	10	1.28	0.62	0.72	0.69	0.152	0.012	0.012	0.031	0.615	29.267
		24	3.09	2.43	2.08	1.66	0.301	0.013	0.002	0.636	0.68	18.807
Gas production (ml/g DM)												
25	$C_{8:0}$	6	189.4	169.2	184.9	180.5	4.34	0.009	0.498	0.041	0.61	3.842
	$C_{10:0}$	10	189.4	187.5	175.4	173.0	4.16	<.0001	<.0001	0.835	0.908	1.462
	$C_{12:0}$	24	281.7	276.4	267.2	226.0	12.63	0.0014	0.0003	0.0307	0.745	5.245
75	$C_{10:0}$	24	218.5	210.9	193.2	183.2	8.08	0.006	0.001	0.852	0.628	6.182
	$C_{12:0}$	24	218.5	193.7	199.2	193.2	5.95	0.012	0.011	0.072	0.584	5.154
Digestibility (g/g DM)												
50	$C_{8:0}$	24	79.0	74.0	72.0	73.0	1.55	0.001	0.001	0.012	0.744	2.471
	$C_{10:0}$	6	62.0	56.0	52.0	55.0	2.10	0.003	0.002	0.01	0.681	5.088
		10	66.5	62.5	58.5	54.5	2.58	0	<.0001	0.855	0.789	4.421
	$C_{12:0}$	6	62.0	55.0	55.5	55.5	1.67	0.002	0.002	0.008	0.707	3.743
		10	66.5	55.0	59.0	57.0	2.51	<.0001	0.001	0.001	0.828	3.782

Table 2.4. (continued) Effects of variation MCFA concentration on selected variables

Grass silage	MCFA	IT (h)	MCFA concentration (%)				SEM	F Anova	F Linear	F Quadratic	R^2	CV
			0	1	3	5						
		24	79.0	70.5	68.0	68.5	2.56	<.0001	<.0001	<.0001	0.946	1.722
75	$C_{10:0}$	10	58.0	52.5	51.5	51.5	1.56	0.006	0.002	0.041	0.635	4.618
		24	75.5	71.5	69.0	67.0	1.83	<.0001	<.0001	0.144	0.886	1.811
	$C_{12:0}$	10	58.0	50.5	49.5	53.5	1.91	0.0004	0.008	<.0001	0.738	4.054
		24	75.5	67.5	66.5	66.0	2.23	0.0002	<.0001	0.0058	0.797	3.14

MCFA conc.= medium-chain fatty acid concentration, IT=incubation time, SEM=standard error of the means, CV=coefficient variation, P<0.05, n=4

Table 2.5. Regression equations –MCFA concentration (x) effect on CH_4 concentration (y; mmol g^{-1} digested DM)

Diet (grass silage %)	MCFA	IT (h)	Regression equation	Intercept SE	Intercept P	x SE / x^2 SE	x P / x^2 P
25	$C_{10:0}$	24	$Y = 2.534 + 0.261\ x$	2.534	<0.0001	0.263	0.339
			$Y = 5.534 + 0.261\ x - 0.178\ x^2$			0.084	0.055
	$C_{12:0}$	24	$Y = 2.572 - 0.267\ x$	0.242	<0.0001	0.389	0.505
50	$C_{10:0}$	10	$Y = 1.317 - 0.034\ x$	0.125	<0.0001	0.201	0.867
		24	$Y = 3.087 - 0.963\ x$	0.185	<0.0001	0.307	0.010
	$C_{12:0}$	10	$Y = 1.237 - 0.616\ x$	0.125	<0.0001	0.201	0.867
			$Y = 1.237 - 0.616\ x + 0.149\ x^2$			0.064	0.309
		24	$Y = 3.073 - 0.650\ x$	0.209	<0.0001	0.363	0.103

MCFA= medium-chain fatty acid, IT=incubation time, $P<0.05$, n=4

CHAPTER 3. In vitro investigation of the methane abatement effect of medium-chain fatty acids, included individually or in dual-component mixtures into diets varying in forage to concentrate ratio

T.W. Priambodo[1], J. Hummel[2], and K.-H. Südekum[1]

1) Institute of Animal Science, University of Bonn

2) Department of Animal Sciences, University of Göttingen

To be submitted

Abstract

Medium-chain fatty acids (MCFA), namely $C_{8:0}$, $C_{10:0}$, $C_{12:0}$, $C_{14:}$ were included individually or in dual component mixtures in diets consisting of 25, 50 and 75% forage (grass silage) at 5% (dry matter basis) MCFA concentration. The effects of MCFA on methane formation, short-chain fatty acid production, acetate:propionate ratio and protozoan numbers were measured at 6, 10 and 24 h of *in vitro* incubation using the Hohenheim gast test system. The diet with 50% forage responded most to dietary MCFA inclusion as regards suppressing lowering methane production and $C_{12:0}$ was the most effective agent, irrespective of the form of application (i.e., individual or two component mixture). The MCFA inclusion into different diets loweredshort-chain fatty acid production, and was accompanied by a lower acetate to propionate ratio reduction and less protozoa cells ($P< 0.05$).

Key words: in vitro, methane, medium-chain fatty acids, forage, diet type

Introduction

Annually, approximately 81 Tg of methane (CH_4) are eructated by ruminants to the atmosphere as a result of enteric fermentation and additionally 7 Tg CH_4 are emitted from manure of ruminants (Johnson 2002), a number which represents 25% of anthropogenic methane emission (Khalil 2000).

A large number of studies concerning methane emission mitigation strategies have been implemented, amongst others the dietary administration of fatty acids (FA). Fatty acids have been intensively investigated and proven as one of the most potent agents in suppressing ruminal methane formation (Czerkawski et al. 1966; van Nevel et al. 1967; McAllister et al. 1996). Among FA, medium-chain (MCFA) have turn out to be the most effective inhibitors relative to abating ruminant methane production, without major adverse effects on digestion and utilisation of energy and protein (Machmüller and Kreuzer 1999; Machmüller et al. 1998; Dohme et al. 2001; Fievez et al. 2010).

Several experiments, both *in vitro* and in vivo, have been conducted to assess the MCFA effectiveness, including studies to determine the adequate dose and form of MCFA application (Soliva et al. 2004; Goel et al. 2009; Fievez et al. 2010; van Zijderveld et al. 2011). Furthermore, Soliva et al. (2004), using a rumen simulation technique with a mixed diet, investigated the effect of lauric ($C_{12:0}$) and myristic acids ($C_{14:0}$) given individually or in mixture and provided information that a simultaneous supply of $C_{14:0}$ supported the $C_{12:0}$ effect in decreasing methane formation and methanogenics counts.

In addition to the use of dietary fats or FA, van Nevel and Demeyer (1966) reported that the substitution of structural by non-structural carbohydrates also serve as a factor which lower methane production in ruminants. Furthermore, Machmüller et al. (2001) reported that the efficacy of coconut oil in suppressing methane production was lower with an extensive-type diet compared with an intensive-type diet.

The current study was designed and conducted to provide comprehensive and detailed data regarding the action of MCFA when used individually or in two component mixtures, in mitigating methane production, and how MCFA effect rumen protozoa counts and short-chain FA (SCFA) production, dependent on diet and incubation time. Medium-chain fatty acids were included at 5%in of dietary dry matter (DM) in three diets consisting of 75, 50 or 25% forage (75F; 50F, 25F), with the remainder of the rations being concentrates. Data were generated with an *in vitro* system, namely

Hohenheim gas test (HGT), at 3 different times of incubation: 6, 10 and 24 h. The variables measured were: methane production, SCFA production, acetate:propionate ratio and protozoa counts.

Materials and methods

Diet preparation

Grass silage, maize grain and solvent-extracted soybean meal were finely ground (1-mm sieve size) and used to formulate three mixed diets with ratios (dry matter (DM) basis) of silage to concentrate of 75:25 (75F), 50:50 (50F) and 25:75 (25F). Medium-chain fatty acids, $C_{8:0}$ (aprylic acid), $C_{10:0}$ (capric acid), $C_{12:0}$ (lauric acid) and $C_{14:0}$ (myristic acid) (SAFC® Supply Solution; Sigma-Aldrich, St. Louis, MO, USA) were included in each diet at a concentration of 5% (DM basis). Two MCFA mixtures were obtained by blending 2.5% DM basisof each MCFA. The MCFA mixturesin the experiment were as follows: $C_{8:0}$:$C_{10:0}$; $C_{8:0}$:$C_{12:0}$; $C_{8:0}$:$C_{14:0}$; $C_{10:0}$:$C_{12:0}$; $C_{10:0}$:$C_{14:0}$; $C_{12:0}$:$C_{14:0}$.

Modified Hohenheim gas test

In vitro gas production was determined according to Menke and Steingass (1988). Rumen fluids were collected from two ruminally fistulated German Blackheaded Mutton sheep, fed a mixed diet of 600 g grass hay and 600 g mixed concentrate per day. Feed were offered in two equal meals at 07:00 and 19:00 h. Rumen fluid was collected prior to the morning feeding and strained through two layers of cheesecloth into pre-warmed and insulated flask. All laboratory handling of rumen fluid was carried out under a continuous flow of CO_2. Samples (200 ± 2 mg) were accurately weighed into a 100 ml glass syringe. The syringe pistons were lubricated with vaseline and inserted into the syringe. Four replicates of each treatment were incubated in two HGT runs which were carried out on different dates. Three blanks containing 30 ml of medium only were included in each run together with triplicates of a standard hay and standard concentrate obtained from the Institute of Animal Nutrition, University of Hohenheim, Germany. The syringes were placed in a rotor inside the incubator (39°C) with about one rotation per minute. The cumulative gas production as well as CH_4 was read at the following times: 6, 10 or 24h.At reading, each syringe was taken out from the incubator and the incubator's door closed to avoid temperature decrease.

Methane measurement

Methane concentration relative to total total gas volume (as %, v/v; range 0 to 40%) was measured using an infrared analyzer (Advanced Gasmitter® Pronova Analysentechnik GmbH & Co. KG, Berlin, Germany) at 6, 10 and 24 hours incubation time. Goel et al. (2008) and Jayanegara et al. (2009) applied the corresponding method for methane analysis in their experiments.

Methane measurement can be started as follows once the calibration processes has been completed. The syringe is taken out from HFT incubator; gas volume of the particular syringe is read and noted; the syringe is cooled down and stored on ice for approximately 20 minutes. A cooled syringe is withdrawn from the ice, connected with the inlet of the analyzer and the gas volume is noted again. The gas from the syringe is gently injected into the device; injecting moisture or liquid must be avoided. Syringe is gently disconnected from the analyzer inlet; CH_4 value (in %) is noted. The volume of the syringe after injection is also noted. The remaining gas in side the analyzer is sucked out using a syringe which is plugged to the analyzer outlet port. The CH_4-free condition inside the analyzer is indicated by 0% value; the analyzer is ready for the subsequent CH_4 measurement.

To increase the volume of measurement an additional dilution step is required for syringes which contain less than 40 ml gas by adjusting the piston position back to 40 ml syringe volume to let normal air join and mix with the gas inside the syringe.

Chemical analysis

The DM of feedstuffs and mixed diets were determined by freeze-drying (silage only) and subsequent oven-drying at 105 °C overnight. Dried feedstuffs were successively ground in mills with 3- and 1-mm screens. All feedstuffs and mixed diets were analysed for ash, crude protein and detergent fibre fractions. Starch content was determined by enzymatic hydrolysis of starch to glucose (Brandt et al. 1987), employing the heat-stable α-amylase Termamyl 120 L (Novo Industrials, Bagsværd, Denmark). The N was determined using Dumas procedure and crude protein calculated as N × 6.25. The neutral detergent fibre (NDF) analysis was conducted according to van Soest et al. (1991). Detergent fibre analyses were performed without the use of decalin. Sodium sulphite was omitted and triethylene glycol was used instead of 2-ethoxyethanol in the NDF procedure. The NDF values are expressed without residual ash and therefore designated NDFom. Ammonium was determined using the Kjeldahl method (without digestion step).

Protozoa enumeration

A Fuchs-Rosenthal counting chamber was used to count protozoan cells. Samples were preserved with 4% (v/v) formaldehyde. A 100 µl sample was inoculated into the Fuchs-Rosenthal chamber under the cover glass through side rift of the chamber. The protozoa were counted in 80 small rectangular prisms at 100x magnification. Total count was conducted in respectively, 80 small rectangular squares of Fuchs-Rosenthal chamber. The subsample on the other side/rafter of the chamber was counted in the same way. The cell calculation use the formula as follow:

Number of cells/ml = $(n1 + n2) \times (2 \times 10^3 \times d)^{-1}$

With:

n1 = number of cells counted in upper rafter

n2 = number of cells counted in lower rafter

d = dilution factor

Source: Couteau (1996).

Organic acid analysis

Organic acids encompassing acetic, propionic, iso-butyric, n-butyric, iso-valeric, n-valeric acids, and lactic and internal standard below were conducted using gas chromatography (GC; Auto System with Auto sampler, PE Nelson 600) method using column Optima FFAP 0,25µm, 25m x 0,32mm ID, Macherey & Nagel.

The frozen samples inside 2-ml Eppendorf tubes were thawed and shaken, followed by 15 min centrifugation at 18000 g. The filtrate was separated from the suspension, pipetted into a new Eppendorf tube, and mixed with 100 µl formic acid. After another centrifugation at 18000 g, filtrate was transferred into GC glass vial.

Statistical analysis

The general linear models (GLM) procdure (SAS software version 9.2, 2002-2008, SAS Institute Inc., Cary, NC, USA) was used to analyse according to model as follow:

$$Y_{ijklm} = \mu + M_i + F_j + C_k + T_l + \varepsilon_{ijklm.}$$

where Y is value of observation, µ is population mean, M is treatment MCFA ($_i$= 1, 2, 3, 4; 1 = $C_{8:0}$, 2 = $C_{10:0}$, 3 = $C_{12:0}$, 4 = $C_{14:0}$), F is forage:concentrate ratio ($_j$ = 1, 2, 3; 1 = 75:25, 2 = 50:50, 3 = 25:75), C is

MCFA forms ($_k$ = 1 and 2; 1 = single, 2 = mixture), T is incubation time ($_m$ = 1, 2, 3; 1 = 6 h, 2 = 10 h, 3 = 24 h), and ε_{ijklm} is the residual error.

Orthogonal contrasts test was used as follows: MCFA against control (C0N) and individual versus two mixture (CX) application of MCFA (CX). The overall least squares means were declared significant at $P < 0.05$ unless otherwise stated.

Results

Based on previous studies by Dohme et al. (2001) and Machmüller and Kreuzer (2005), MCFA in our experiment were supplemented at 5% of diet DM, to examine and compare the effectiveness of MCFA supplied individually single or in two component mixtures. The methane production relative to fermented feed DM, total SCFA production, the ration of acetate to propionate and protozoan counts were observed and recorded.

Methane production

The supplementation of MCFA in the 75F diet did not affect methane formation. Methane production was suppressed for two component MCFA mixtures with diet 50F at all incubation times, and diet 25F at 10 and 24 h incubation times.

Methane production was suppressed by MCFA in diet 50F at all incubation times, except $C_{14:0}$ at 6 and 10 h (Table 2). The most evident MCFA inhibition in diet 50F was observed at 24 h, with $C_{12:0}$ used alone as the most potent methane inhibitor. Two of the MCFA, $C_{8:0}$ at 6h, and $C_{12:0}$, at 24h incubation time, either included individually or in mixtures with other MCFA into the diets, lowered methane production.. Capric acid ($C_{8:0}$) in single form had greater inhibitory activity than when mixed with other MCFA, while $C_{12:0}$ combined with $C_{10:0}$ suppressed methane production more than when supplied individually or or in combination with other MCFA ($C_{8:0}$ or $C_{14:0}$).

The inclusion of MCFA into diet 25F decreased methane formation by up to 26% (mixture of $C_{12:0}$/$C_{14:0}$). Methane suppression was observed at all incubation times, except at 6 h. When $C_{10:0}$ and $C_{12:0}$ were supplied individually, they were more effective in reducing methane production than when combined with other MCFA.

Short-chain fatty acids

The inclusion of $C_{12:0}$, single or combination with other MCFA, into different diets reduced total ruminal SCFA production (Table 4). Exceptions occurred at the supplementation of $C_{12:0}$ in diet 75F at 10 h incubation time (no effects) and in diet 25F at 24 h incubation time (increased SCFA production). Capric acid ($C_{8:0}$) and $C_{14:0}$ had the same SCFA suppression pattern. Both MCFA reduced SFCA production at 6 h incubation time with diets 25F and 75F, at 10 h with diets 25F and 50F and at 24 h with diet 75F. Furthermore, the supplementation of $C_{10:0}$, reduced total SCFA production, when combined with diet 50F at 10 h incubation time and 75F (24 h). Both MCFA application forms (single and mixture) generated the same effect on SCFA production ($P<0.05$).

At 6 h incubation time, MCFA supplementation in diet 75F resulted in lower acetate to propionate ratio (Table 5). At the subsequent incubation times (10 h), a pronounced reduction of the acetate to propionate ratio was observed in diet 50F. At 24 h incubation $C_{10:0}$ and $C_{12:0}$ in single and two component mixture, reduced acetate to propionate ratio when combined with diet 25F, whilst $C_{8:0}$ and $C_{14:0}$ in both forms reduced the proportion in combination with diet 50F.

Protozoa population

The addition of the different MCFA irrespective of mode of application, i.e. individual or two component mixture, suppressed protozoan population in all diets (Table 3). The only two exceptions were $C_{8:0}$ with diet 75F at 10 h and with diet 25F at 24 h incubation, where the protozoan population suppression was negligible.

Discussion

Methane production

In this study, the methane suppressing effect of MCFA in both application forms, single and mixture, was most obviously observed in diet 50F. This is in line with results from previous studies. The application of MCFA in an intensive basal diet (with low structural carbohydrate) has been reported to have greater impact on methane reduction, as compared to an extensive type containing high structural carbohydrate (Dohme et al. 2001; Goel et al. 2009). Shifting diet type from forage-dominated to concentrate-dominated diet will lead to decreased CH_4 production per unit fermented substrate (Hungate 1966). Apart from the MCFA inclusion, the forage and concentrate ratio

adjustment is an effective means to manipulate ruminal fermentation pathways and reducing enteric methane production (Lovett et al. 2003).

Among the MCFA included in this study, $C_{12:0}$ and $C_{14:0}$ are the most prominent agents inhibiting CH_4 production. Our data is in agreement with previous results (Soliva et al. 2004), which showed a decrease of H_2 during application of both MCFAs. Hydrogen could have been utilized for the reduction of sulfate to sulfides, as well as hydrogen-consuming processes within the rumen and propionate formation. However, in the present study comparison of $C_{12:0}$ used individually and mixed with $C_{14:0}$ showed an almost analogue CH_4 inhibiting effect. In contrast, Soliva et al. (2004) found that the mixture of $C_{12:0}$ and $C_{14:0}$ magnified the CH_4 suppression rather than $C_{12:0}$ alone. In the rumen, the formation of acetic and butyric acids is accompanied by the production of H_2, whereas formation of propionic acid involves a net uptake of H_2. The different rate of H_2 availability in the rumen influences the formation CH_4 by methanogens (Whitelaw et al. 1984).

The present study is in agreement with previous findings which the individual application of $C_{12:0}$ had a stronger methane inhibitory effect than a mixture of $C_{12:0}$ and $C_{14:0}$, except when $C_{14:0}$ and $C_{12:0}$ were used in a 2:1 ratio (Soliva et al. 2004).

Short-chain fatty acids

A decrease in total SCFA production with different diets supplemented with MCFA, at different incubation times, supports results generated from previous experiments (Dohme et al. 2004; Goel et al. 2009; Castro-Montoya et al. 2012). Complementing findings from Dohme et al. (2007), who described the $C_{12:0}$ and $C_{14:0}$ inhibitory effect on total SCFA production, our investigation revealed pronounced effects of $C_{8:0}$ and $C_{10:0}$ in single and combined forms. The reduction of SFCA production might be due the to the decline of protozoan population (see Table 5) after MCFA supplementation, as rumen ciliates are responsible for the production of about 30 to 46% of total SCFA production (Cieślak et al. 2013). However, Jouany (1994) described that rumen fermentation shifted towards more propionate, as protozoan population declines.

Protozoa Population

The results of this study are agree with findings of Dohme et al. (2001), who foud that MCFA supplementation suppressed protozoan population. However, McGinn et al. (2004) stated that the addition of fat to ruminant diets decrease CH_4 losses by decreasing ruminally fermentable substrate,

providing an alternative of H_2 sink in the rumen and protozoan cell inhibition. Furthermore the reduction of protozoan count after the application of coconut oil at 5% was comparable, irrespective the dietary firage to concentrate ratio (Cieślak et al. 2006).

Conclusion

Supplementation of MCFA in the diet 50F containing equal proprotions of forage and concentrate generated an obvious suppression of methane production, irrespective of MCFA form of dietary inclusion (single or combined). When combined with diet 50F, $C_{12:0}$ reduced methane production most, compared to other MCFA. Short-chain fatty acid production was pronouncely affected by the application of $C_{8:0}$ and $C_{12:0}$. The ratio of acetate to propionate was affected by MCFA supplementation such that it varied over time and between the diets. Protozoan population was suppressed by supplementation of both forms of MCFA in combination with all diet types.

References

Brandt, M., Schuldt, A., Mannerkorpi, P., Vearasilp, T. 1987. Zur enzymatischen Stärkebestimmung im Darminhalt und Kot von Kühen mit hitzestabiler Amylase. Archives of Animal Nutrition 37: 455.

Castro-Montoya, J., de Campeneere, S., van Ranst, G., Fievez, V. 2012. Interactions between methane mitigation additives and basal substrates on *in vitro* methane and VFA production. Animal Feed Science and Technology 176: 47-60.

Couteau, P. 1996. Micro-Algae. Manual on the production and use of live food for aquaculture. FAO Fisheries Technical Paper No. 361. Rome, FAO.

Czerkawski, J. W., Blaxter, K.L., Wainman, F.W. 1966. The metabolism of oleic, linoleic and linolenic acids by sheep with reference to their effects on methane production. British Journal of Nutrition 20: 349-362.

Dohme, F., Machmüller, A., Wasserfallen, A., Kreuzer, M. 2001. Ruminal methanogenesis as influenced by individual fatty acids supplemented to complete ruminant diets. Letters in Applied Microbiology 32: 47-51.

Dohme , F., Machmüller, A., Sutter ,F., Kreuzer, M. 2004. Digestive and metabolic utilization of lauric, myristic and stearic acid in cows, and associated effects on milk fat quality. Archives of Animal Nutrition 58: 99-116.

Fievez, V., Goel, G., Boeckaert, C., Vlaeminck, B. 2010. Potential of fatty acids in mitigating ruminal methanogenesis. Dynamics in Animal Nutrition. J. Doppenberg, van der Aar, P. (Eds.). Wageningen, Wageningen Academic Publishers: 161-184.

Goel, G., Arvidsson, K., Vlaeminck, B., Bruggeman, G., Deschepper, K., Fievez, V. 2009. Effects of capric acid on rumen methanogenesis and biohydrogenation of linoleic and a-linolenic acid. Animal 3: 810-816.

Goel, G., Makkar, H.P.S., Becker, K. 2008. Changes in microbial community structure, methanogenesis and rumen fermentation in response to saponins-rich fraction from different plant materials. Journal of Applied Microbiology 105: 770-777.

Hungate, 1966. The rumen and its microbes. Academic Press, London: 533.

Jayanegara, A., Togtokhbayar, N., Makkar, H.P.S., Becker, K. 2009. Tannins determined by various methods as predictors of methane production reduction potential of plants by an *in vitro* rumen fermentation system. Animal Feed Science and Technology 150: 230-237.

Johnson, K. A., Kincaid, R.L., Westberg, H.H. 2002. The effect of oilseeds in diets of lactating cows on milk production and methane emissions. Journal of Dairy Science 85: 1509-1515.

Khalil, M. A. K. 2000. Atmospheric methane: an introduction. Atmospheric methane. Its role in the global environment. M. A. K. Khalil. Berlin, Heidelberg, New York, Springer: 351.

Lovett, D., S. Lovell, Stack, L., Callan, J., Finlay, M., Conolly, J., O'Mara, F. P. 2003. Effect of forage/concentrate ratio and dietary coconut oil level on methane output and performance of finishing beef heifers. Livestock Production Science 84: 135-146.

Machmüller, A., and Kreuzer, M. 1999. Methane suppression by coconut oil and associated effects on nutrient and energy balance in sheep. Canadian Journal of Animal Science 79: 65-72.

Machmüller, A. and Kreuzer, M. 2004. Impact of the methane-suppressing feed additive myristic acid on energy and calcium balance of sheep. Journal of Animal and Feed Science 13 Suppl.: 151-154.

Machmüller, A., Ossowski, D.A., Wannerm M., Kreuzer, M. 1998. Potential of various fatty feeds to reduce methane release from rumen fermentation *in vitro* (Rusitec). Animal Feed Science and Technology 71: 117-130.

McAllister, T. A., Okine, E.K., Mathison, G.W., Cheng, K.-J. 1996. Dietary, environmental and microbiological aspects of methane production in ruminants. Canadian Journal of Animal Science 76: 231-243.

McGinn, S.M., Beauchemin, K.A., Coates, T., Colombatto, D. 2004. Methane emissions from beef cattle: Effects of monensis, sunflower oil, enzymes, yeast and fumiric acid. Journal of Animal Science 82: 3346-3356.

Menke, K.H., Steingass, H. 1988. Estimation of the energetic feed value obtained from Chemical analysis and gas production using rumen fluid. Animal Research and Development 28:7-55.

Soliva, C. R., Meile, L., Cieślak, A., Kreuzer, M., Machmüller, A. 2004. Rumen simulation technique study on the interactions of dietary lauric and myristic acid supplementation in suppressing ruminal methanogenesis. British Journal of Nutrition 92: 689-700.

van Nevel, C. J., Demeyer, D.I. 1996. Control of rumen methanogenesis. Environmental Monitoring and Assessment 42: 73-97.

van Nevel, C. J., Demeyer, D.I., Henderickx, H.K. 1967. Effect of fatty acid derivates on rumen methane and propionate *in vitro*. Applied Microbiology 21: 365-366.

Van Soest, P. J., Robertson, J.B., Lewis, B.A. 1991. Methods for dietary fiber, neutral detergent fiber, and nonstarch polysaccharides in relation to animal nutrition. Journal of Dairy Science 74: 3583-3597.

van Zijderveld, S. M., Fonken, B., Dijkstra, J., Gerrits, W.J.J., Perdok, H.B., Fokkink, W., Newbold, J.R. 2011. Effects of a combination of feed additives on methane production, diet digestibility, and animal performance in lactating dairy cows. Journal of Dairy Science 94: 1445-1454.

Whitelaw, F.G., Margaret Eadie, J., Bruce, L.A., Shand, W.J. 1984. Methane formation in faunated and ciliate-free cattle and its relationship with rumen volatile fatty acid proportions. British Journal of Nutrition 52: 261-275.

Table 3.1**.** Chemical analysis of mixed diets and diet ingredients

Analysis	Diet			Feed		
(g kg^{-1} DM)	25F	50F	75F	Grass silage	Soybean meal	Maize grain
Dry matter (g kg^{-1})	897	897	892	899	904	914
Ash	66.1	79.2	83.3	106	80.9	21.6
Crude protein	183	194	165	106	515	144
Crude lipid	39.1	34.5	30.5	25.1	19.9	85.4
NDFom	289	349	465	603	-	-
ADFom	170	208	267	372	75.8	3.3
ADL	23.9	32	16	20.13	3.3	7.4
Starch (enzymatic)	31.85	24.63	6.92	1.17	1.98	30.66

Table 3.2. Methane mitigation effect of MCFA (mmol ml^{-1})

Incubation time (h)	6			10			24		
Diet	25F	50F	75F	25F	50F	75F	25F	50F	75F
CON	0.38	0.44	0.21	1.28	1.28	0.40	2.46	3.09	1.41
MCFA									
$C_{8:0}$	0.40	0.29	0.20	1.07	0.76	0.46	2.09	2.25	1.57
$C_{8:0}/C_{10:0}$	0.45	0.35	0.22	1.01	0.93	0.55	2.13	2.21	1.51
$C_{8:0}/C_{12:0}$	0.46	0.32	0.20	0.98	0.84	0.34	1.92	2.18	1.38
$C_{8:0}/C_{14:0}$	0.52	0.39	0.19	1.01	1.12	0.36	2.21	2.08	1.34
SEM	0.025	0.026	0.001	0.054	0.095	0.038	0.088	0.184	0.043
P-value CON vs. MCFA	0.031	<0.0001	0.755	0.005	0.037	0.641	0.014	0.006	0.769
P-value Single vs. mixture	0.376	0.004	0.712	0.376	0.298	0.443	0.953	0.746	0.335
$C_{10:0}$	0.48	0.38	0.23	1.06	0.74	0.36	1.79	1.89	1.32
$C_{8:0}/C_{10:0}$	0.45	0.35	0.22	1.01	0.93	0.55	2.13	2.21	1.51
$C_{10:0}/C_{12:0}$	0.54	0.34	0.25	1.01	0.63	0.41	2.46	2.24	1.64
$C_{10:0}/C_{14:0}$	0.45	0.42	0.22	1.00	1.01	0.46	2.36	2.63	1.50
SEM	0.026	0.019	0.007	0.053	0.113	0.033	0.128	0.206	0.054
P-value CON vs. MCFA	0.030	0.020	0.356	0.010	0.004	0.433	0.112	0.015	0.485
P-value Single vs. mixture	0.984	0.929	0.971	0.560	0.427	0.051	0.008	0.198	0.081

Table 3.2. (continued). Methane mitigation effect of MCFA (mmol.ml^{-1})

Incubation time (h)	6			10			24		
Diet	25F	50F	75F	25F	50F	75F	25F	50F	75F
$C_{12:0}$	0.47	0.38	0.21	1.07	0.69	0.45	1.71	1.66	1.44
$C_{8:0}/C_{12:0}$	0.46	0.32	0.20	0.98	0.84	0.36	1.92	2.18	1.41
$C_{10:0}/C_{12:0}$	0.54	0.34	0.25	1.01	0.76	0.41	2.46	2.24	1.65
$C_{12:0}/C_{14:0}$	0.56	0.39	0.22	0.95	0.88	0.48	2.45	2.37	1.42
SEM	0.032	0.021	0.009	0.509	0.103	0.021	0.161	0.23	0.046
P-value CON vs. MCFA	0.007	0.004	0.143	0.008	0.002	0.688	0.132	<.0001	0.504
P-value Single vs. mixture	0.370	0.166	0.334	0.344	0.336	0.400	0.030	0.033	0.613
$C_{14:0}$	0.47	0.44	0.24	1.11	0.83	0.53	2.15	2.48	1.65
$C_{8:0}/C_{14:0}$	0.52	0.39	0.19	1.01	1.12	0.36	2.21	2.08	1.34
$C_{10:0}/C_{14:0}$	0.45	0.42	0.22	1.00	1.03	0.46	2.36	2.63	1.47
$C_{12:0}/C_{14:0}$	0.56	0.39	0.22	0.95	0.88	0.48	2.45	2.37	1.42
SEM	0.031	0.011	0.008	0.059	0.082	0.03	0.063	0.65	0.052
P-value CON vs. MCFA	0.015	0.231	0.256	0.022	0.059	0.256	0.294	0.007	0.6205
P-value Single vs. mixture	0.351	0.161	0.002	0.275	0.277	0.002	0.259	0.586	0.0992

CON = control; SEM= standard error of the mean; n=4; P<0.05

Table 3.3. Rumen protozoa suppression effect of MCFA ($x10^5.ml^{-1}$)

Incubation time	6h			10h			24h		
Diet	25F	50F	75F	25F	50F	75F	25F	50F	75F
CON	1.30	0.48	0.65	0.93	0.70	0.23	0.63	0.45	0.35
MCFA									
$C_{8:0}$	0.68	0.28	0.38	0.55	0.45	0.18	0.35	0.30	0.30
$C_{8:0}/C_{10:0}$	0.68	0.30	0.40	0.45	0.33	0.13	0.50	0.30	0.20
$C_{8:0}/C_{12:0}$	0.58	0.30	0.28	0.35	0.38	0.10	0.55	0.23	0.20
$C_{8:0}/C_{14:0}$	0.78	0.28	0.28	0.40	0.50	0.15	0.60	0.15	0.20
SEM	0.137	0.038	0.068	0.104	0.064	0.022	0.049	0.049	0.032
P-value CON vs. MCFA	<.0001	0.002	<0.0001	<0.0001	0.002	0.053	0.052	0.007	<0.0001
P-value Single vs. mixture	1.000	0.754	0.108	0.023	0.525	0.278	0.005	0.287	<0.0001
$C_{10:0}$	0.55	0.28	0.23	0.23	0.28	0.10	0.25	0.28	0.10
$C_{8:0}/C_{10:0}$	0.68	0.30	0.40	0.45	0.33	0.13	0.50	0.30	0.20
$C_{10:0}/C_{12:0}$	0.78	0.18	0.23	0.38	0.30	0.10	0.20	0.40	0.18
$C_{10:0}/C_{14:0}$	0.95	0.45	0.23	0.48	0.23	0.10	0.35	0.23	0.10
SEM	0.13	0.056	0.082	0.117	0.085	0.025	0.08	0.04	0.046
P-value CON vs. MCFA	<.0001	0.015	<.0001	<0.0001	<0.0001	0.007	0.0002	0.0289	<0.0001
P-value Single vs. mixture	0.0175	0.618	0.167	0.0005	0.903	0.786	0.142	0.611	0.010

Table 3.3. (continued) Rumen protozoa suppression effect of MCFA ($x10^5.ml^{-1}$)

Incubation time	6h			10h			24h		
Diet	25F	50F	75F	25F	50F	75F	25F	50F	75F
$C_{12:0}$	0.38	0.20	0.10	0.13	0.28	0.13	0.10	0.40	0.15
$C_{8:0}/C_{12:0}$	0.58	0.30	0.28	0.35	0.38	0.10	0.55	0.23	0.20
$C_{10:0}/C_{12:0}$	0.38	0.18	0.23	0.38	0.30	0.10	0.20	0.40	0.18
$C_{12:0}/C_{14:0}$	0.75	0.27	0.30	0.70	0.23	0.10	0.28	0.23	0.18
SEM	0.153	0.053	0.091	0.141	0.085	0.025	0.102	0.047	0.035
P-value CON vs. MCFA	<.0001	0.0002	<.0001	<0.0001	<0.0001	0.003	<0.0001	0.024	0.0001
P-value Single vs. mixture	<.0001	0.360	0.001	<0.0001	0.903	0.367	0.010	0.058	0.356
$C_{14:0}$	1.03	0.20	0.23	0.50	0.35	0.15	0.45	0.58	0.20
$C_{8:0}/C_{14:0}$	0.78	0.28	0.28	0.40	0.50	0.15	0.60	0.15	0.20
$C_{10:0}/C_{14:0}$	0.95	0.45	0.23	0.48	0.23	0.10	0.35	0.23	0.10
$C_{12:0}/C_{14:0}$	0.75	0.27	0.30	0.70	0.25	0.10	0.28	0.23	0.18
SEM	0.099	0.055	0.079	0.96	0.088	0.024	0.068	0.08	0.04
P-value CON vs. MCFA	<0.0001	0.034	<.0001	<0001	<0.0001	0.023	0.006	0.101	<0.0001
P-value Single vs. mixture	<0.0001	0.118	0.316	0.723	0.730	0.426	0.536	0.001	0.1695

CON = control; SEM= standard error of the mean; n=4; P<0.05

Table 3.4. Effect of MCFA supplementation on ruminal SCFA production (mmol.l^{-1})

Incubation time	6h			10h			24h		
Diet	25F	50F	75F	25F	50F	75F	25F	50F	75F
CON	50.00	43.61	53.10	53.22	55.00	59.00	57.80	75.82	53.29
MCFA									
$C_{8:0}$	44.10	36.11	41.59	49.21	41.91	57.72	62.63	62.22	48.33
$C_{8:0/C10:0}$	45.23	35.82	48.14	49.84	42.56	54.65	60.43	63.91	49.78
$C_{8:0/C12:0}$	45.06	32.34	42.28	49.11	42.20	52.46	61.02	63.82	49.48
$C_{8:0/C14:0}$	45.69	41.11	45.18	49.45	47.52	55.52	61.69	69.64	48.77
SEM	1.029	2.016	2.107	0.774	2.512	1.150	0.815	2.458	0.878
P-value CON vs. MCFA	0.003	0.081	0.005	0.034	0.005	0.455	0.120	0.052	0.011
P-value Single vs. mixture	0.397	0.939	0.097	0.880	0.556	0.987	0.473	0.701	0.399
$C_{10:0}$	50.33	42.27	47.57	50.02	44.84	56.57	59.83	62.22	48.6
$C_{8:0/C10:0}$	45.23	35.82	48.14	49.84	42.56	54.65	60.59	63.91	49.78
$C_{10:0/C12:0}$	44.83	37.89	45.41	48.93	43.15	53.26	59.41	60.10	50.01
$C_{10:0/C14:0}$	47.74	37.37	47.25	45.58	44.72	54.48	59.79	34.56	48.10
SEM	1.151	1.503	1.284	0.821	2.280	1.003	0.462	6.765	0.906
P-value CON vs. MCFA	0.255	0.148	0.121	0.054	0.005	0.075	0.429	0.052	0.044
P-value Single vs. mixture	0.091	0.163	0.841	0.664	0.703	0.744	0.970	0.909	0.736

Table 3.4. (continued). Effect of MCFA supplementation on ruminal SCFA production (mmol.l^{-1})

Incubation time	6h			10h			24h		
Diet	25F	50F	75F	25F	50F	75F	25F	50F	75F
$C_{12:0}$	43.91	38.95	49.47	48.49	41.48	52.95	59.38	58.99	49.05
$C_{8:0/C12:0}$	45.06	32.34	42.28	49.11	42.20	52.46	60.24	63.82	49.48
$C_{10:0/C12:0}$	44.83	37.89	44.67	48.93	43.15	53.26	59.41	60.12	50.01
$C_{12:0/C14:0}$	44.76	38.10	43.97	49.68	43.28	55.10	58.85	60.21	48.23
SEM	1.090	1.793	1.854	0.855	2.492	1.198	0.401	3.115	0.870
P-value CON vs. MCFA	0.001	0.033	0.025	0.028	0.001	0.430	0.496	0.033	0.017
P-value Single vs. mixture	0.418	0.358	0.570	0.674	0.752	0.928	0.961	0.670	0.878
$C_{14:0}$	43.33	43.28	44.95	46.62	43.26	55.68	58.42	60.56	47.37
$C_{8:0/C14:0}$	45.69	47.53	45.18	49.45	47.52	55.52	62.73	64.94	48.77
$C_{10:0/C14:0}$	47.47	44.73	47.25	48.58	44.72	54.48	59.79	64.56	48.01
$C_{12:0/C14:0}$	44.76	43.30	43.97	49.68	43.28	55.10	58.85	60.21	48.23
SEM	1.169	0.805	1.640	1.703	2.202	0.789	0.866	2.826	1.063
P-value CON vs. MCFA	0.006	0.250	0.035	0.039	0.009	0.603	0.293	0.060	0.006
P-value Single vs. mixture	0.089	0.087	0.858	0.320	0.596	0.931	0.333	0.626	0.439

CON = control; SEM= standard error of the mean; n=4; P<0.05

Table 3.5. Effect of MCFA supplementation on acetate:propionate ratio

Incubation time	6h			10h			24h		
Diet	25F	50F	75F	25F	50F	75F	25F	50F	75F
CON	3.75	3.81	5.39	3.50	4.03	4.10	3.03	4.77	3.50
MCFA									
$C_{8:0}$	3.28	3.63	4.77	3.38	2.88	3.88	2.87	3.68	3.52
$C_{8:0/C10:0}$	3.27	3.60	4.00	3.35	2.93	3.79	2.90	3.76	3.41
$C_{8:0/C12:0}$	3.31	3.55	4.75	3.34	2.95	4.08	2.90	3.98	3.37
$C_{8:0/C14:0}$	3.31	3.76	4.90	3.41	2.91	4.14	2.87	3.84	3.54
SEM	0.092	0.049	0.223	0.029	0.223	0.069	0.03	0.197	0.033
P-value CON vs. MCFA	0.406	0.968	0.006	0.573	0.006	0.477	0.285	0.040	0.722
P-value Single vs. mixture	0.540	0.110	0.270	0.860	0.881	0.515	0.407	0.632	0.543
$C_{10:0}$	3.26	4.29	4.60	3.46	3.60	4.09	2.97	4.29	3.55
$C_{8:0/C10:0}$	3.27	3.63	4.00	3.35	2.93	3.79	2.90	3.76	3.41
$C_{10:0/C12:0}$	3.41	3.50	4.34	3.22	3.02	3.96	2.67	4.02	3.26
$C_{10:0/C14:0}$	3.11	3.59	4.29	3.26	2.65	3.98	2.89	3.49	3.30
SEM	0.108	0.144	0.237	0.054	0.25	0.056	0.061	0.221	0.056
P-value CON vs. MCFA	0.329	0.703	0.007	0.199	0.023	0.522	0.045	0.070	0.333
P-value Single vs. mixture	0.110	0.047	0.158	0.068	0.088	0.484	0.073	0.233	0.117

Table 3.5. (continued). Effect of MCFA supplementation on acetate:propionate ratio

Incubation time	6h			10h			24h		
Diet	25F	50F	75F	25F	50F	75F	25F	50F	75F
$C_{12:0}$	3.27	3.33	4.27	3.03	2.80	4.04	2.70	3.92	3.13
$C_{8:0/C12:0}$	3.31	3.55	4.75	3.43	2.95	4.08	2.90	3.98	3.37
$C_{10:0/C12:0}$	3.41	3.50	4.34	3.22	3.02	3.96	2.67	4.02	3.26
$C_{12:0/C14:0}$	3.27	3.58	4.35	3.28	2.84	3.99	2.95	3.80	3.37
SEM	0.091	0.077	0.210	0.083	0.229	0.026	0.071	0.172	0.062
P-value CON vs. MCFA	0.330	0.056	0.007	0.057	0.005	0.650	0.021	0.077	0.132
P-value Single vs. mixture	0.553	0.051	0.387	0.054	0.505	0.882	0.138	0.981	0.162
$C_{14:0}$	3.27	3.57	4.27	3.31	2.79	3.86	2.88	3.69	3.36
$C_{8:0/C14:0}$	3.21	3.76	4.90	3.45	3.21	4.14	2.99	3.84	3.54
$C_{10:0/C14:0}$	3.11	3.59	4.29	3.26	2.65	3.98	2.89	3.49	3.30
$C_{12:0/C14:0}$	3.27	3.58	4.35	3.28	2.84	3.99	2.95	3.80	3.37
SEM	0.111	0.051	0.221	0.048	0.249	0.049	0.029	0.221	0.045
P-value CON vs. MCFA	0.268	0.709	0.002	0.013	0.005	0.546	0.182	0.027	0.339
P-value Single vs. mixture	0.327	0.170	0.354	0.827	0.769	0.345	0.411	0.964	0.663

CON = control; SEM= standard error of the mean; n=4; P<0.05

CHAPTER 4. GENERAL CONCLUSIONS

The current study focused on identifying the appropriate combination between the type, concentration and application format of MCFA and diet (silage:concentrate proportions) in suppressing methane production, while avoiding negative effects on the application of MCFA on fermentation in the rumen. The outcomes from current study can be concluded as follows:

1. In order to gain significant effect of MCFA application on reduction of methane formation, selection of optimum silage: concentrate ratio is very crucial. Effect of the addition of MCFA on methane production inhibition in silage- dominated diet was very minor or even negligible. However, the use of concentrate feed in excessive amounts may be un favorable, technically and economically, when it is associated with an effort to suppress the production of methane.
2. Methane production decreases with the increasing concentration of MCFA applications. Nevertheless the impact of excessive use of MCFA also needs to be considered, especially in relation to rumen fermentation process. MCFA, particularly C_{12} and C_{14}, have specific inhibitory effect on methanogens, cellulolytic and amylolytic bacteria. Adding >5 to 6% of MCFA in the ration could depress fiber degradation in the rumen (Dong et al. 1997, Soliva et al. 2003). Furthermore, repellent odour as well as taste of such fatty acids could limit feed intake of animal (Bauer et al. 2006).
3. The configuration of MCFA application did not affect the effectiveness of MCFA on methane mitigation, as long as the MCFA was applied at an appropriate dose and in combination with an appropriate diet.
4. Further *in vivo* investigation to more specifically define the optimum combination between the type and concentration (within range of current study) of MCFA with specific feed needs to be conducted, followed by testing *in vivo*.

References

Bauer, E., Williams, B.A., Smidt, H., Mosenthin, R., Verstegen, M.W.A. (2006). Influence of dietary components on development of the microbiota in single-stomached species. Nutrition Research Reviews 19: 63-78.

Dong, Y., Bae, H.D., McAllister, T.A., Mathison, G.W., Cheng, K. J. 1997. Lipid-induced depression of methane production and digestibility in the artificial rumen system (RUSITEC). Canadian Journal of Animal Science 77: 269-278.

Soliva, C. R., Hindrichsen, I.K., Meile, L., Kreuzer, M., Machmüller, A. 2003.Effects of mixtures of lauric and myristic acid on rumen methanogens and methanogenesis *in vitro*. Letters in Applied Microbiology 37: 35-39.

Acknowledgment

Praise be to Allah, the Cherisher and Sustainer of the worlds.

I would like to express my sincere gratitude to my supervisor Prof. Dr. Karl-Heinz Südekum for his continuous guidance, constructive criticism, encouragement, and support throughout this work.

Very special thanks and appreciations go to Prof. Dr. Jürgen Hummel, for supporting me with his inestimable advices and opinions through my thesis. Also for his patience for introducing and guiding me in the lab especially at the early stage of my PhD study.

I would like also to express my deep gratitude to PD Dr. Joachim Clemens for his continuous remarks and valuable suggestions for improving the results of this work and also for criticizing this thesis as a second referee.

I gratefully acknowledge the assistance of Helga Koknat, Peter Müller, Stephan Knauf, Walter Diefenthal, Viktor Braun, Petra Jacquemin and Nadja Wahl.

To all my fellow (former) graduate students of animal nutrition group, especially Dr. Ahmed Abdel-Wareth, Dr. Muhammad Qaiser Riaz, Dr. Nina Gresner and Christian Böttger, I want to thank for your help, assistance and sincere friendship.

My thank goes also to all Indonesian-Malaysian students and family in Bonn, especially member of Indo-Malay Muslim Bonn (IMB) community, for their great supports and continuous prayers.

I wish to extend my final thanks to my wife Andri, my parents and parents in law. Their pray, patience, encouragement, together with their love have made this entire endeavour worthwhile. To my Fathiya and Latifah thank you for always being daddy's inspiration.