From the

Institute of Physiology

Faculty of Veterinary Medicine, Leipzig University

RELATION BETWEEN METABOLIC STATE, MICROBIAL COMMUNITY STRUCTURE AND METHANE PRODUCTION IN DAIRY COWS

Inaugural-Dissertation

to obtain the degree of a

Doctor medicinae veterinariae (Dr. med. vet.)

from the Faculty of Veterinary Medicine

Leipzig University

submitted by

Anita Bielak

from Wiesbaden

Leipzig, 2019

Mit Genehmigung der Veterinärmedizinischen Fakultät der Universität Leipzig

Dekan: Prof. Dr. Walter Brehm

Betreuer: Prof. Dr. Rainer Cermak
PD Dr. Björn Kuhla

Gutachter: Prof. Dr. Rainer Cermak, Veterinär-Physiologisches Institut, Veterinärmedizinische Fakultät der Universität Leipzig, Leipzig
PD Dr. Björn Kuhla, Institut für Ernährungsphysiologie "Oskar Kellner", Leibniz Institut für Nutztierbiologie, Dummerstorf
Prof. Dr. Quendrin Zebeli, Institut für Tierernährung und funktionelle Pflanzenstoffe, Veterinärmedizinische Universität Wien, Wien

In Zusammenarbeit mit dem Leibniz Institut für Nutztierbiologie, Dummerstorf

Tag der Verteidigung: 21. Mai 2019

Den Kühen

CONTENTS

1	Introduction	1
2	Background	2
2.1	Greenhouse Gases	2
2.2	Dairy cows and their importance to food production	3
2.3	Rumen functions	5
2.3.1	Anatomy and Physiology	5
2.3.2	Rumen microbes	7
2.3.2.1	Bacteria	8
2.3.2.2	Archaea	11
2.3.3	Short-chain fatty acids	12
2.3.4	Methane formation	15
2.4	Interrelationship between methane and host animal physiology	15
2.4.1	Physiologic aspects affecting methane formation	15
2.5	Effects of feed composition and feed contents on methane production	16
2.5.1	Relationship of ruminal short-chain fatty acids and methane production	17
2.5.2	Milk fatty acids to estimate methane emission	19
2.6	Description of methods	20
2.6.1	Methane Measurement	20
2.6.2	Sampling of rumen contents	21
2.6.3	Methods to identify microbes	22
2.7	Objective and realization of the studies	23
3	Publications	26
3.1	First Publication	26
3.1.1	Supplement first Publication	40
3.2	Second Publication	42
3.2.1	Supplement second Publication	56
4	Discussion	60
4.1	Assessment of experimental design	60
4.1.1	Animals	60
4.1.2	Feed	61

4.1.3	Rumen fluid	61
4.1.4	Blood and milk metabolites	62
4.2	Assessment of results	62
4.2.1	Variance of methane emissions	62
4.2.2	Rumen short-chain fatty acids and methane	65
4.2.3	Acetate in the cows' metabolism and methane production	66
4.2.4	Fat mobilization in early lactation	67
4.2.5	NEFA in the context of metabolism	68
4.2.6	Rumen microbes	69
4.2.6.1	Microbial community change over time	70
4.2.6.2	Community differences between individuals	71
4.2.6.3	Relationship between microbes and methane production levels	72
4.2.7	Further considerations	74
5	Conclusions	75
6	Summary	77
7	Zusammenfassung	79
8	References	81

ABBREVIATIONS

ACTH	Adrenocorticotrophic hormone
BCS	Body condition score
CCK	Cholecystokinin
cDNA	Complementary DNA
CV	Coefficient of variation
DM	Dry matter
DMI	Dry matter intake
DNA	Deoxyribonucleic acid
ECM	energy corrected milk yield
FAO	Food and Agriculture Organization of the United Nations
FBN	Leibniz Institute for Farm Animal Biology
GFE	Gross feed efficiency
GHG	Greenhouse gases
HFC	Hydrofluorocarbon
IPCC	Intergovernmental panel on climate change
LCFA	Long-chain fatty acids
NADPH	Reduced form of nicotinamide adenine dinucleotide phosphate
NDF	Neutral detergent fiber
NEB	Negative energy balance
NEFA	Non-esterified fatty acids
OTU	Operational taxonomic units
PCR	Polymerase chain reaction
PFC	Perfluorocarbon
RFI	Residual feed intake
RNA	Ribonucleic acid
rRNA	Ribosomal ribonucleic acid
SCFA	Short-chain fatty acids
SE	Standard Error
TAG	Triacylglycerol
VLDL	Very low-density lipoproteins

1 INTRODUCTION

One of the biggest threats to the environment is global warming, a major aspect of climate change. Methane (CH_4) emission from ruminants is thought to contribute considerably to this phenomenon, drawing strategies for mitigation of CH_4 production from ruminants into research focus besides the well-known fact that CH_4 emissions imply an energy loss for the animals.

Ruminants possess a large forestomach compartment essential for their digestion. This compartment, called the reticulorumen, harbors a complex ecosystem of microbes, which are responsible for the breakdown of plant material into nutrients that are usable by the animal. A major product of these fermentation processes are short-chain fatty acids (SCFA), an important energy source for the host. The three most abundant SCFA are acetate, propionate and butyrate. Whereas the production of acetate is accompanied by the release of hydrogen, the formation of propionate is a hydrogen consuming process (MOSS et al. 2000). Most of methanogenic archaea, a prokaryotic subgroup of the rumen microbiota, utilize the released hydrogen for the reduction of CO_2 and for their own energy generation, thereby producing methane. Thus, the actual SCFA pattern, especially the (acetate + butyrate) / propionate ratio in the rumen, is directly related to ruminal CH_4 production (AGUINAGA CASAÑAS et al. 2015, MOHAMMED et al. 2011).

The metabolic status of the animal determines the relative utilization of different SCFA by various organs of the host. For example, during the negative energy balance in early lactation, dairy cows intensively catabolize their body fat resulting in increased circulating concentrations of non-esterified fatty acids (NEFA) (SCHÄFF et al. 2013). Apart from addressing the glucose shortage, the mobilized body fat is used as an alternative to SCFA by the mammary gland for milk fat synthesis. An altered utilization of SCFA, especially a diminished use of acetate or increased use of propionate, could therefore affect the fermentation pattern in the rumen and reduce CH_4 production.

Different CH_4 production levels of dairy cows can be directly attributed to the rumen microbial community. Depending on the amount, the feeding regime and the composition of feed, microbial species with distinct metabolic profiles can thrive, resulting in variable patterns of SCFA (BELANCHE et al. 2012) and, consequently yielding different CH_4 production (LETTAT et al. 2013). However, even for animals on the same

diet different levels of CH_4 emission and CH_4 yield, i.e. methane normalized to dry matter feed intake (CH_4/DMI), were shown (KITTELMANN et al. 2014, SHI et al. 2014). The reasons for different CH_4 yields originate directly from the activity and abundance of specific microbes in the rumen which in turn are influenced by host-individual factors. For example, the genetic background of the host was shown to influence the rumen microbiome and CH_4 yield in various ruminant species (GOOPY et al. 2014, KITTELMANN et al. 2014, ROEHE et al. 2016).

Furthermore, rumen physiology plays an important role in CH_4 emission. Increased retention time of feed in the reticulorumen allows a longer contact time for microbes with substrate, resulting in higher CH_4 production (ELLIS et al. 2008, MOSS et al. 2000, SHI et al. 2014). DIAS et al. (2011) have shown that rumination behavior and the number of chews per bolus can also influence passage kinetics.

In order to investigate the effects of longevity and metabolic status on the variation in CH_4 yield between cows it is necessary to measure CH_4 emissions repeatedly throughout the lactation cycle in order to distinguish between high and low emitters.

Therefore, this study aimed to investigate the relationship between CH_4 emission and the hosts' metabolism with special emphasis on mean retention time and rumen SCFA. In addition, differences in microbial communities in dairy cows with different CH_4 emission levels fed the same feed and kept under the same housing conditions were investigated. To this end, repeated measurements on the same cows were carried out.

2 BACKGROUND

2.1 Greenhouse Gases

Earth's atmosphere contains molecules that have the ability to absorb and emit radiation within the thermal infrared range which leads to decreased loss of heat in the surface-troposphere system, the so called "Greenhouse effect" (PLANTON 2013). This is foremost a natural phenomenon that keeps the earth's surface temperature at about +14°C in average and enables life on our planet. Increased atmospheric concentrations of greenhouse gases (GHG) trap heat in the atmosphere and lead to an increased infrared opacity; this increased greenhouse effect will have consequences for the global climate. GHG are both of natural and anthropogenic origin. The primary GHG are water

vapor (H_2O), carbon dioxide (CO_2), nitrous oxide (N_2O), methane (CH_4) and ozone (O_3). Halocarbons and other chlorine and bromine containing substances are anthropogenic and also function as greenhouse gases. The Kyoto Protocol names six GHG that need to be tackled to mitigate climate warming: CO_2, N_2O, CH_4, sulphur hexafluoride (SF_6), hydrofluorocarbons (HFCs) and perfluorocarbons (PFCs) (PLANTON 2013).

CH_4 is the simplest alkane, an odorless and colorless gas. It can be found in large quantities below the earth's surface as the main component of natural gas, in form of methane hydrate in deep sea, but also in the atmosphere in varying concentrations. In 2016 the global CH_4 concentration amounted to an average of 1842.99 parts per billion (ppb) (DLUGOKENCKY 2017).

About 58% of the globally emitted CH_4 is of anthropogenic origin, but also wetlands (30%), oceans, lakes and rivers (7%) as well as termites and other arthropods, wildlife, wildfires and permafrost (5%) contribute to methane emission (KNAPP et al. 2014). CH_4 emissions are especially dangerous for the climate, because it's global warming potential is 28 times greater than CO_2 for a horizon of 100 years, and 84 times greater for a horizon of 20 years as stated in the 5th assessment report of the intergovernmental panel on climate change (IPCC), and therefore even small amounts contribute considerably to global warming (MYHRE et al. 2013). The reaction of methane with hydroxyl radicals ($HO^{\bullet}$) in the troposphere functions as the main sink for CH_4 (MOSS et al. 2000). The livestock sector accounts for 37% of anthropogenic CH_4 emissions (STEINFELD et al. 2006), meaning that the ruminant livestock sector alone is responsible for 87 – 94 million tonnes (10^{12} g) of CH_4 per year (CIAIS et al. 2013). According to FAO (2010), the global contribution of the dairy sector to anthropogenic GHG emission amounts to 4%.

2.2 Dairy cows and their importance to food production

Ruminant livestock is kept in most continents of the world. Depending on the environmental conditions however, breeds, husbandry and purpose of the animals differ. The great advantage of ruminant livestock is their ability to digest and utilize plant materials and low-quality forages as well as waste products from the food industry, which are not suited for human nutrition.

The digestive characteristics enabling this possibility of nutrition are due to the unique anatomy and physiology of the gastrointestinal tract as discussed in section 2.3.1, leading to the naming of this group of animals. The digestive and fermentative processes that take place in the rumen are of essential importance for the energetic and metabolic requirements of the host organism.

The present work focuses on dairy cows, a subgroup of livestock ruminants. They are kept for milk production purposes, therefore regular calvings are required and longevity is desired. Evaluations of their methane emissions, especially with regard to emissions per unit of product, have to consider the different periods of productivity or non-productivity, respectively, as well as the diet changes these animals undergo throughout their live spans. Rearing of calves until their first lactation has to be considered separately and related to the productive periods of the animal. Also, the dry cow periods have to be considered differently than lactating cows. Dairy cows in late gestation and shortly after calving are usually in a state of negative energy balance (NEB), which is caused by the energy demand for the growing fetus and the onset of lactation on top of the normal maintenance requirements exceeding dietary energy intake (PARK et al. 2011). The NEB is intensified by the fact that dairy cows decrease their dry matter intake (DMI) in the last days of pregnancy until parturition and only slowly adapt to the increased need of energy and nutrients after calving. Within normal ranges of energy requirement and adjusted diets dairy cows, like many other mammals, can compensate the increased energy demand with the mobilization of body reserves (INGVARTSEN & ANDERSEN 2000).

Another aspect to consider is the fact that dairy cows are subjected to many dietary changes during their life span: during weaning from milk to solid feeds, to a diet for growing calves and heifers, to lactation diets adapted to the reproductive status as well as transition rations; sometimes cattle are also allowed to graze. Depending on the feeding regimen applied on a farm, the deployed concentrate levels also vary with production system, they relate to milk yield and they are adapted over the course of lactation. Those changes in diet composition greatly influence ruminal fermentation and the ruminal ecosystem (CERSOSIMO et al. 2016, WANG et al. 2012).

Ruminal fermentation leads to production and emission of CH_4, which cannot be utilized any further by the host animal, hence the energy of 0,036 MJ/l CH_4 is lost and GHG emission is increased. Therefore, if mitigation strategies are considered, one has to

walk the line between decreasing energy loss and GHG emissions on the one hand and ensuring productive ruminal fermentation on the other hand. Feed efficiency of dairy cows is a measure to calculate milk production efficiency, and many definitions exist for this parameter. The most common ones are gross feed efficiency (GFE), which relates the energy corrected milk yield to DMI, and residual feed intake (RFI), which is the difference between individual feed consumption relative to feed consumption of other animals on the same feed at the same production level (JEWELL et al. 2015). Intensifying livestock production was shown to decrease CH_4 emission per unit of product, because increasing the product per cow dilutes CH_4 formed during fermentation of feed for maintenance (VAN MIDDELAAR et al. 2014). This means higher milk yields, greater longevity and better feed efficiency have favorable effects on emission levels of livestock systems (WALL et al. 2010). The assessment of a dairy production system has to consider all animals including those during the rearing period and dry cows, as well as their manure and other waste, their feed and other resources. Nevertheless, digestive processes in the rumen still render the lion's share of CH_4 emission from ruminant livestock.

2.3 Rumen functions

2.3.1 Anatomy and Physiology

The rumen is one of three proventriculi of domesticated ruminants and the largest compartment of their digestive tract. SALOMON (2015) describes it as a very voluminous chamber, stretching from the 8th rib to the pelvic area of the left side of the animal. Thereby this organ takes up more than half of the abdominal cavity in adult animals and is composed of several muscular sacs lined with stratified squamous epithelium forming finger-like papillae to increase the absorptive surface.

It is separated with muscle pillars corresponding to grooves on the outside into dorsal and ventral sac and two caudal blind sacs. The reticulum, another chamber of the forestomachs in ruminants located just cranially of the rumen, is also separated from the rumen with a muscular fold. The reticulum needs to be considered as a functional unit together with the rumen, referred to as reticulorumen. In adult Holstein cattle the reticulorumen can have a feed uptake capacity of 125 to 170 kg (PARK et al. 2011).

The esophagus inlet is located just dorsally between reticulum and rumen, and 15-20 cm ventrally of the esophageal inlet the ostium ruminoreticulare is located, opening into the third compartment, the omasum.

Periodic contractions of the rumen wall mix the digestive content. The animal regurgitates portions of the digesta to thoroughly chew it before swallowing it again and adding a fair amount of saliva during this procedure. These processes ensure proper access of the microbes to their substrates in the feed, as well as a relatively constant pH in the rumen.

Innervation of the rumen is achieved via the Autonomic Nervous System, controlling periodic contractions and the regurgitating of ingesta to be chewed repetitively, a process called rumination. Reticuloruminal motility is predominantly regulated via vagal reflexes (TITCHEN 1976). The three most important factors that increase motility of the reticulorumen are feed intake, moderate distension of the reticulorumen and a low pH in the abomasum. Decreased motility is caused by increased concentration of short-chain fatty acids in the rumen, distention of the abomasum or low pH in the duodenum. Furthermore, fever and pain can decrease motility by stimulating the brain stem directly or via activation of the sympathoadrenal system, which releases neurotransmitters like adrenaline, hypothalamic corticoliberine and adrenocorticotrophic hormone (ACTH) having direct and indirect effects on the sympathic nervous system (KANIA et al. 1999).

Reticuloruminal contractions as well as the composition of diet and amount of physically effective fiber result in an extensive stratification of the rumen content, the formation of a gas cap in the dorsal sac, a liquid phase in the ventral part and a thick-packed mat of fibrous solids in between (ZEBELI et al. 2012).

This segregation takes place due to the different particle sizes and density of ingesta components, resulting in greater retention times for larger particles. Passage rates of feed through the reticulorumen should therefore be considered separately for solids and liquids. Usually liquid retention time is about 12 hours, whereas solids retention time varies around 18 – 72 hours depending on particle size distribution (BREVES et al. 2015). Passage rate and retention times are also influenced by rumen size (GOOPY et al. 2014), feed intake levels and diet composition (PARK et al. 2011).

Fermentative processes within the rumen are fueled by the substrates in feed ingested by the animal. Furthermore, the animal is able to use its metabolite urea in the rumen

for microbial protein synthesis besides of excreting it into urine like other mammals. Dietary N-supply and dietary composition, especially carbohydrate composition and fermentable energy supply, influence the amount of urea transferred into the gut (REYNOLDS & KRISTENSEN 2008). Large amounts of gas are produced during fermentation processes, and this gas accumulates in the dorsal part of the rumen. It is vital for ruminants to eliminate the rumen gas, which is achieved by a process called ructus or belching.

A prerequisite for unimpaired ruminal fermentation is balancing fermentation conditions by secretion of saliva for pH control, by chewing and ruminating, by the excretion of fermentation gases by the ructus, by regulating the passage of digesta due to specific rumen and intestinal motility patterns, as well as by the absorption of fermentation products. Due to these fermentation processes, rumen temperature can exceed the rectal body temperature by up to two degrees Celsius (BRADE 2013). Disturbances of factors controlling rumen homeostasis can lead to an imbalanced microbial community, resulting in unfavorable metabolic substrate composition, which in turn can lead to performance losses of the host or even to metabolic disorders.

2.3.2 Rumen microbes

Ruminal fermentation is the microbial digestion of plant material to produce short-chain fatty acids, serving as energy source for the ruminant, but also other substances such as lactate, succinate, ammonia (NH_3), hydrogen sulfide (H_2S), hydrogen (H_2) and CO_2. This process is performed by the highly complex interaction of many microorganisms within the rumen. Understanding the dynamics of ruminal digestion is essential to elucidate influential parameters on methane formation and its importance for the host.

The rumen is one of the most phylogenetically complex ecosystems, consisting of protozoa, fungi, bacteria, archaea and some non-cellular life such as phages (FIRKINS & YU 2015) along with the occasionally occurring parasitic helminths or transitional organism, the latter just passing through without taking part in metabolic breakdown. The microbial community forms a symbiosis with the host, because the ruminant can utilize many of the microbially produced products for its own metabolism as well as it

also can digest the microbial biomass itself in subsequent compartments of its digestive tract (BREVES et al. 2015).

Rumen content harbors about 10^{11} microbial cells per gram, but only a few microbial species have been cultured up to date (HENDERSON et al. 2013). Those microbes belong to the taxonomic domains eukaryotes (Protozoa and Fungi), as well as prokaryotes (Bacteria and Archaea) and noncellular life (viruses) (FIRKINS & YU 2015). High influx of substrate and high efflux rates of metabolites from the rumen combined with predominant anaerobic conditions lead to the formation of a specialized ecosystem. However, a stable microbial community has to develop from birth until adulthood, since ruminants are born with only a fraction of the microbial colonization they feature as adult animals after full development of the reticulorumen. Already at 14 days of age all major types of rumen bacteria can be detected in the developing reticulorumen of young ruminants (YÁÑEZ-RUIZ et al. 2015). Ruminal colonization takes place sequentially, for example the early detectable *Proteobacteria* are gradually replaced over time by *Bacteroidetes* as the main phyla (YÁÑEZ-RUIZ et al. 2015).

YÁÑEZ-RUIZ et al. (2015) summarized the works by KITTELMANN et al. (2014) and WEIMER et al. (2010) demonstrating that after oral or via rumen cannula supplementation of different bacteria, antibiotic treatments or after ingesta swapping, the fermentation profile and microbial community composition will return to its original state, which supports the idea of a specific microbiota composition in a host.

2.3.2.1 Bacteria

Depending on the components and chemical composition of feed the composition of the bacterial community in terms of species abundance, diversity and richness varies. Bacteria in the rumen account for > 95% of total ruminal microorganisms (SANDRI et al. 2014) amounting to 10^{10} – 10^{11} bacterial cells/ml in fully developed rumen (KOIKE & KOBAYASHI 2009, SIROHI et al. 2012). They show a high degree of biodiversity. Molecular tools and bioinformatics allow to distinguish high numbers of operational taxonomic units (OTU), only few of which have been characterized, cultured or even named so far. It is estimated that 300 – 400 phylotypes can be found in the rumen (MORGAVI et al. 2012).

HENDERSON et al. (2015) described recently that similar bacteria are abundant in the rumen of various animal species all over the world, on a variety of feeds and under different climate conditions, which supports the idea of a core rumen microbiome. *Prevotella*, *Butyrivibrio*, and *Ruminococcus*, as well as unclassified Lachnospiraceae, Ruminococcaceae, Bacteroidales, and Clostridiales are the dominant rumen bacteria, however, the abundance of these bacterial groups varies among individual animals, species, diets and geographical locations. The phyla Bacteroidetes, Firmicutes and Proteobacteria and members of their lower taxonomic levels were also described as the core microbiome by other researches (FIRKINS & YU 2015, JAMI & MIZRAHI 2012, KASPAROVSKA et al. 2016, PETRI et al. 2013).

Rumen bacteria can be separated into fractions of spatial distribution. Bacterial cells are suspended in the liquid phase or planktonic, some are attached to the epithelium of the rumen wall, while others are loosely (biofilm) or tightly (embedded, fixed) attached to digesta particles. All these fractions exhibit different community diversities (KONG et al. 2010, ZEBELI et al. 2012).

The metabolism of the different bacterial species is only partly explored yet. Because of the extremely low O_2 partial pressure in the rumen, most of the bacteria are obligate anaerobes. However, in young ruminants facultative anaerobe and even aerobe species can be detected (JAMI et al. 2013).

Rumen bacteria can be classified into various functional groups: fibrolytic or cellulolytic, amylolytic, proteolytic, lipolytic, acetogenic, ureolytic and tanninolytic (HENDERSON et al. 2015, SIROHI et al. 2012) and many more (see also Table 1).

Common fibrolytic species are *Fibrobacter succinogenes*, *Ruminococcus flavefaciens*, *Ruminococcus albus*, *Butyrivibrio fibrisolvens*, *Prevotella ruminicola*, *Eubacterium cellulosolvens* and *Eubacterium ruminantium*. Furthermore, *F. succinogenes*, *R. flavefaciens* and *R. albus* are specific cellulolytic species which possess a high ability to digest fiber (KOIKE & KOBAYASHI 2009). Although these bacteria all share the ability to break down fiber, they differ in the surface enzymes facilitating the attachment to particles, which is essential for initiation of digestion and fibrolysis.

Table 1: Fermentative properties of rumen bacteria (ALLISON 2004)

Species	Function*	Products**
Fibrobacter succinogenes	CL, AL	F, A, S
Ruminococcus albus	CL, XL	F, A, E, H, C
Ruminococcus flavefaciens	CL, XL	F, A, S, H
Butyrivibirio fibrisolvens	CL, XL, PRL	F, A, L, B, E, H, C
Streptococcus bovis	AL, SS, PRL	L, A, F
Ruminobacter amylophilus	AL, PL, PRL	F, A, S
Prevotella ruminicola	AL, XL, PL, PRL	F, A, P, S
Succinimonas amylolytica	AL, DL	A, S
Selenomonas ruminantium	AL, SS, GU, LU, PRL	A, L, P, H, C
Lachnospira multiparus	PL, PRL, AL	F, A, E, L, H, C
Succinovibirio dextrinosolvens	PL, DL	F, A, L, S
Methanobrevibacter spp. ruminantium and *smithii*	MG, HU	M
Treponema bryantii	PL, SS	F, A, L, S, E
Megasphaera elsdenii	SS, LU	A, P, B, V, CP, H, C
Lactobacillus spp.	SS	L
Anaerovibirio lipolytica	LL, GU	A, P, S
Eubacterium ruminantium	SS	F, A, B, C
Oxalobacter formigenes	OD	F, C
Wolinella succinogenes	HU	S, C

* CL: cellulolytic, AL: amylolytic, XL: xylanolytic, PRL: proteolytic, SS: major soluble sugar fermenter, PL: pectinolytic, DL: dextrionolytic, GU: glycerol utilizing, LU: lactate utilizing, MG: methanogenic, HU: hydrogen utilizer, LL: lipolytic, OD: oxalate degrading

** F: formate, A: acetate, S: succinate, E: ethanol, H: hydrogen, C: carbon dioxide, L: lactate, B: butyrate, P: propionate, M: methane, V: valerate, CP: caproate

In general, fiber fermenting bacteria grow slower than starch fermenting bacteria. They use ammonia as their source of nitrogen, whereas starch fermenting bacteria also use amino acids or peptides (RUSSEL et al. 1992).

Important amylolytic species include *Selenomonas ruminantium* and *Steptococcous bovis*, both of which produce lactate. *Anaerovibrio lipolytica* is an example of a lipolytic species (BELANCHE et al. 2012).

Many species of the Prevotella genus are proteolytic, however, its omnipresence in the rumen across a variety of diets suggests a substantial metabolic adaptability and diversity of this genus (PETRI et al. 2013).

Fermentation of feed to short-chain fatty acids (SCFA) requires a complex interaction of bacteria. In this context, MORGAVI et al. (2012) distinguished between primary and secondary fermenters. For example, fibrolytic species such as *Fibrobacter succinogenes* degrades cellulose to succinate (primary fermenters), which in turn serves as substrate for *Selenomonas ruminantium*, producing propionate (secondary fermenters) (KOIKE & KOBAYASHI 2009).

2.3.2.2 Archaea

The microorganisms present in the rumen that belong to the archaea domain are obligate anaerobes. Their numbers reach as much as 10^7 – 10^9 cells /ml rumen content, but only seven species have been successfully cultured so far (SIROHI et al. 2012). Like rumen bacteria, ruminal archaea can be found on the epithelium of the rumen wall, attached to particles, suspended in the liquid phase of ingesta or symbiotically associated with protozoa (JANSSEN & KIRS 2008, MORGAVI et al. 2012).

Most of the archaeal species can be assigned to the phyla Euryarchaeota and Crenarchaeota, further phyla are known but usually not found in the rumen. The phylum Euryarchaeota can be subdivided into seven orders, including 31 genera.

The unique feature of most ruminal archaea is the ability to produce methane, therefore, they are often referred to as methanogens. Until recently all currently known methanogens were ascribed to the phylum Euryachaeota (ST-PIERRE et al. 2015). However, recent publications suggest that methane metabolism also occurs in the phylum Bathyarchaeota, formerly attributed to the Miscellaneous Crenarchaeota Group (EVANS et al. 2015). In addition, gene sequences associated with methanogenesis were found in a newly proposed phylum Verstraetearchaeota (VANWONTERGHEM et al. 2016). These latter phyla are intrinsic to environmental samples and have not been described as rumen habitants yet.

Methanobacteriales and Methanomassiliicoccales, both belonging to the phylum Euryarchaeota, are the dominant archaeal orders in the rumen (SEEDORF et al. 2015).

Based on a meta-analysis JANSSEN & KIRS (2008) demonstrated that the archaeal community structure varied between host animal species, diets and geographical locations. In contrast, HENDERSON et al. (2015) described that the archaeal community is highly conserved.

Most of the ruminal archaea use CO_2 for methanogenesis, but some species are able to metabolize methylamines, methanol, formate or acetate (ST-PIERRE et al. 2015, see also section 2.3.4). The most abundant methanogens in the rumen belong to the hydrogenotrophic genus *Methanobrevibacter* and the methylotrophic genus *Methanosphaera* (SEEDORF et al. 2015). Further genera of the Methanomassilicoccales order are frequently identified in rumen samples and suggested to be methylotroph (ST-PIERRE et al. 2015). The only rarely detected *Methanobacterium* and *Methanosarcina* use carbon monoxide (CO) as substrate for methanogenesis, an energetically highly favorable reaction although CO levels in the rumen are low (ELLIS et al. 2008).

Despite their low numbers in the rumen compared to bacteria, the role of methanogens is very important for rumen function. Removal of [2H] from the rumen is necessary to maintain the ruminal redox potential. For the bacterially mediated oxidation of reduction equivalents ($NADH + H^+ \rightarrow NAD^+ + H_2$), which are necessary for glycolysis, it is crucial to eliminate the electrons. Archaea contribute substantially to the elimination of electrons by removing [2H] bound in CH_4 from the rumen. This may also protect the rumen from acidification leading to health impairment, since H^+ lowers the pH. Furthermore, removing the hydrogens inhibitory effect on rumen fermentation leads to increased metabolic rates and a nutritionally more favorable pattern of SCFA (see section 2.3.3) (JANSSEN & KIRS 2008, ST-PIERRE et al. 2015).

2.3.3 Short-chain fatty acids

The major source of energy in ruminal metabolism is derived from a variety of short-chain fatty acids. The three most important SCFA are acetate, propionate and butyrate, making up 80 % of the metabolic energy intake (ASCHENBACH et al. 2009).

Many ruminal microbes degrade plant fiber components like cellulose, hemicellulose, pectin and lignin but also non-structural carbohydrates such as sugars and starch. After hydrolysis of the complex fibers into glucose equivalents, glucose is converted

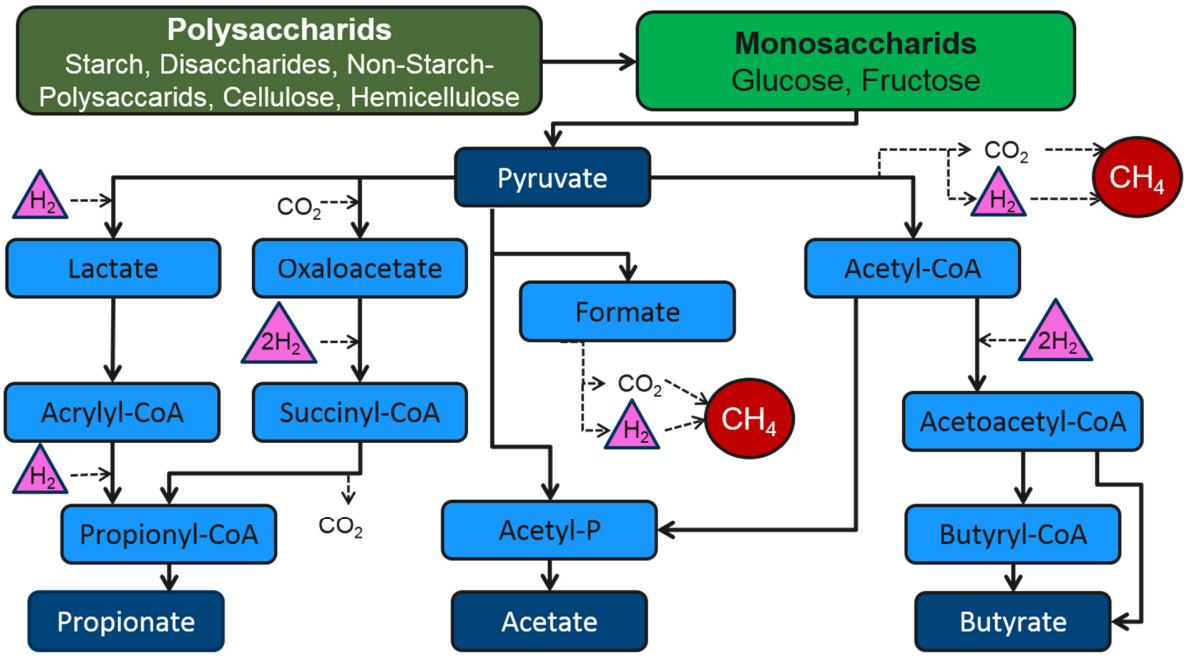


Figure 1: Simplified reaction scheme illustrating the formation of the three major short chain fatty acids during ruminal fermentation

by anaerobic glycolysis or the pentose-phosphate-cycle into pyruvate. Pyruvate as a central intermediate substrate for a large number of microbial organisms serves as a precursor for the production of the SCFA acetate, propionate and butyrate. Pyruvate can also be utilized to produce ethanol, valerate or lactate, but in much lesser quantities (compare Table 1).

The synthesis of the three major SCFA is illustrated in Figure 1. For the production of acetate or butyrate, pyruvate is first decarboxylated to acetyl-CoA, which can be achieved by different pathways. Pyruvate formate lyase catalyzes the reaction of pyruvate to acetyl-phosphate and formate. However, formate rarely accumulates but is quickly degraded to CO_2 and [2H] or directly reduced by rumen methanogens (HEGARTY & GERDES 1999). Pyruvate ferredoxin oxidoreductase catalyzes the cleavage of pyruvate forming acetyl-CoA, CO_2 and [2H]. The resulting acetyl-CoA can be converted into acetyl-phosphate, which in turn is dephosphorylated by acetate kinase yielding acetate.

The synthesis of butyrate from 2 molequivalents of acetyl-CoA is a reductive process requiring 2 molequivalents of [2H] (MOSS et al. 2000).

Furthermore, propionate is produced from pyruvate within two major microbial metabolic pathways, the succinate pathway and the acrylate pathway. In the succinate

pathway oxaloacetate, malate, fumarate and succinate are intermediate metabolites. Within the conversion pathway from pyruvate to propionyl-CoA 2 mol [2H] are used. The acrylate pathway first reduces pyruvate to lactate, which then again is transformed to propionyl-CoA. This pathway also uses 2 mol [2H]. Propionyl-CoA is the direct precursor for propionate.

The concentrations and ratios in which SCFA are produced are not constant, but depend greatly on the fiber types of the diet, especially the neutral detergent fiber (NDF) content, the amount of ingested feed and the site of starch digestion (e.g. rumen protected starch which is not fermented in the rumen but is digested in the small intestine) (NOZIÈRE et al. 2011). The groundwork to the relationship between feed and fermentation pattern was done by DEMEYER & VAN NEVEL (1975), and many more studies since then have shown that diets rich in starch increase the rate of ruminal fermentation. This increased starch fermentation increases propionate production and thus the propionate / acetate ratio (JIAO et al. 2014, JOHNSON & JOHNSON 1995, MCALLISTER et al. 1996). Particle size and chemical fiber content, especially NDF, are the two parameters used to determine the physically effective fiber in a diet. Physically effective fibers in the feed influence the fermentation patterns by altering chewing time and therefore saliva flux into the rumen, thereby affecting rumen pH and consequently microbial activity (ZEBELI et al. 2012).

The produced SCFA are almost completely absorbed across the ruminal wall (CERSOSIMO et al. 2016). Absorption of SCFA from the ruminal lumen follows a variety of different pathways, including passive diffusion and active and passive transport mechanisms with the aid of diverse apical transport proteins (ASCHENBACH et al. 2009). A portion of butyrate is metabolized to beta-hydroxybutyrate in epithelial cells of the rumen wall before entering circulation.

Absorbed propionate is the main precursor for hepatic gluconeogenesis (46 – 73%) (DIJKSTRA 1994), a significant metabolic pathway especially for ruminants, since the glucose absorption from the gut is usually very low in these animals. Acetate, butyrate and beta-hydroxybutyrate can deliver energy to the mammary gland, adipose or muscular tissues for example. Dairy cows use these substrates as precursors for milk fat production in the mammary gland (PALMQUIST 2006).

2.3.4 Methane formation

The methane production represents a loss of feed energy for the host animal amounting to 5.23 -6.99% (ARNDT et al. 2015) or of 2 – 12% (JOHNSON & JOHNSON 1995) of the gross energy (GE) intake of dairy cows.

Although the production of methane is rather unfavorable in efficient production systems, it is an important process to eliminate hydrogen from the rumen. Hydrogen accumulation in the rumen inhibits microbial growth and fiber fermentation (ROOKE et al. 2014). The largest sink for hydrogen in the rumen are methanogens, keeping the partial pressure of H_2 generally very low at 1-10 Pa (ELLIS et al. 2008) which is also necessary to allow for the oxidation of reducing equivalents by generation of H_2.

Most of the methanogens utilize CO_2 as final electron acceptor according to the following equation:

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

Other methanogens transfer the hydrogen to methyl- and dimethylamines (POULSEN et al. 2013) forming methane and ammonia as follows:

$$CH_3NH_2 + H_2 \rightarrow CH_4 + NH_3$$

A minor portion of methanogens reduce acetate or methanol (FERRY 1992), or oxidize ethanol (HOEDT et al. 2016) for methane production:

$$CH_3COOH \rightarrow CH_4 + CO_2$$

$$4\ CH_3OH \rightarrow 3\ CH_4 + CO_2 + 2\ H_2O$$

$$2\ CH_3OH + CH_3CH_2OH \rightarrow 2\ CH_4 + H_2O + CH_3COOH$$

Methanogenesis is essential for all ruminal archaea, as it is their sole pathway to generate energy form anaerobic respiration (ROUVIERE & WOLFE 1988).

2.4 Interrelationship between methane and host animal physiology

2.4.1 Physiologic aspects affecting methane formation

MOSS et al. (2000) stated that the residence time of feed in the rumen influences methane emission, since a longer contact time between microbes and substrates allows for a more intensive degradation and, therefore, methane production. On the other hand, a rapid digesta passage rate favors propionate production and, therefore, higher [2H] consumption, making less hydrogen available for CH_4 production. The

ruminal passage rate is mostly determined by feed intake, by the particle size of the feed (DIAS et al. 2011, HRISTOV et al. 2013, OKINE et al. 1998) and by rumen motility (OKINE et al. 1989). The volume of saliva produced increases as a function of feeding and rumination time (BAILEY & BALCH 1961). Saliva buffering capacity ensures favorable fermentation conditions in the rumen, and, therefore, the amount of produced saliva is positively correlated with total CH_4 emissions (PINARES-PATIÑO et al. 2007). An independent effect of rumination alone excluding saliva production on CH_4 production could not be detected by WATT et al. (2015), but this group suspected a change in DMI and associated modifications in grazing behavior as a reason for different CH_4/rumination ratios.

The rumen volume is positively correlated with feed intake (DIJKSTRA 1993). GOOPY et al. (2014) demonstrated a direct relationship between a lower methane yield in sheep and a lower rumen size that was coupled with a different layer constitution of rumen content.

Additionally, genetic traits of the host, which influence feed conversion efficiency, DMI or the microbiota, seem to exert some control over the ruminal fermentation process (ROEHE et al. 2016, XIANG et al. 2016).

2.5 Effects of feed composition and feed contents on methane production

CH_4 emission increases with DMI (JOHNSON & JOHNSON 1995). Many different models, linear and non-linear, have been described to explain this relationship, for example the linear equation by MILLS et al. (2003):

$$CH_4 \text{ (MJ/d)} = 5.93 \text{ (SE 1.60)} + 0.92 \text{ (SE 0.08)} \times \text{DMI (kg/d)}$$

$$r^2 = 0.60$$

Non-linear equations improve the prediction of CH_4 emissions in comparison to linear equations (MILLS et al. 2003).

MOSS et al. (2000) summarize the general characteristics of diets and their impact on methane production. Mature dried forages as well as coarsely chopped rather than finely ground or pelleted feedstuff increase CH_4 yield. JENTSCH et al. (2007) deduced a formula to estimate CH_4 energy from the digestible nutrient intake:

CH_4 (kJ) = 1.62 (±0.02) x digestible crude protein (g) – 0.38 (±0.52) x digestible crude fat (g) + 3.78 (±0.18) x digestible crude fiber (g) + 1.49 (±0.06) x digestible N-free extract + 1142 (±196)

$r^2 = 0.896$

Also, due to their chemical composition, different feedstuffs have different methane production potentials. In vitro tests can be used to compare feeds, plants and additives regarding their fermentation characteristics and CH_4 production. KIM et al. (2012) compared 35 feeds and proposed that the total organic carbon level in a feed could be used as a proxy to estimate CH_4 production.

Ruminal fermentation of starch and sugars decreases CH_4 production, whereas increased fiber fermentation is accompanied with a rise in CH_4 production (MOSS et al. 2000).

Most dietary, rumen degradable fat undergoes microbial lipolysis. The resulting unsaturated long-chain fatty acids (LCFA) are extensively hydrogenated. As a result the composition of fatty acids in the ruminal outflow greatly differs from the fatty acid composition of the diet (DOREAU & CHILLIARD 1997).

Biohydrogenation of unsaturated LCFA is an alternative hydrogen sink and therefore competes with methanogenesis. LCFA can be directly toxic to methanogens (ELLIS et al. 2008), decrease the activity of fibrolytic bacteria and interfere with microbial growth (DOREAU & CHILLIARD 1997) resulting in decreased fiber breakdown and therefore CH_4 production. Dietary fats and oils are able to coat the fiber surface, which limits the physical access of microbes to the fibers and other macronutrients. Consequently, ruminal NDF digestibility is decreased. Furthermore, incorporation of lipids into the diet decreases DMI, leading to decreased CH_4 production (HRISTOV et al. 2013). Finally feed additives such as monensin as well as nitrates or tannins affect methanogenic pathways, e.g. by altering proportions of microbial species, and thus have methane mitigation potential (HRISTOV et al. 2013).

2.5.1 Relationship of ruminal short-chain fatty acids and methane production

It has been shown that total SCFA concentration in the rumen follows the same diurnal pattern as CH_4 production (BRASK et al. 2015). However, the production ratio of the three most important SCFA, as shown in section 2.3.3, results in different amounts of

CH_4 produced. MOSS et al. (2000) outlined the reasons for this: CH_4 formation is a [2H] consuming process, like the production of propionate. By contrast, the pathways forming acetate or butyrate are [2H] releasing reactions. Therefore, the production of CH_4 in the rumen is stoichiometrically linked to the molar percentages of SCFA.
(DEMEYER & VAN NEVEL 1975) deduced a stoichiometric relationship between the three most important short-chain fatty acids of ruminal fermentation and methane production.

$$2A + P + 4B = 4M + 2P + 2B$$

(Where A, B, P and M represent the number of moles acetate, butyrate, propionate and CH_4, respectively.)

In line with this, MOHAMMED et al. (2011) were able to detect negative correlations of CH_4 with mol % of rumen propionate on the one hand and positive correlations of CH_4 with acetate, the acetate / propionate ratio, and the (acetate + butyrate) / propionate ratio on the other hand. Similar results were observed in other studies (e.g. BENCHAAR et al. 2013, HASSANAT et al. 2014).
In vitro tests revealed that the amount and proportion of acetate, propionate, butyrate and isobutyrate can be used to very accurately predict (r^2 = 0.95) the volume of fermentative CH_4 and CO_2 gases, and that this is true for a wide range of feeds (BLÜMMEL et al. 1999).
Different models have been proposed to estimate the concentrations of SCFA being formed in ruminal fermentation from the composition of the diet in order to predict ruminal CH_4 production (ALEMU et al. 2011, ANNISON & BRYDEN 1998, BRASK et al. 2015). ALEMU et al. (2011) showed that using stoichiometric prediction equations resulted in unsatisfactory performance for all tested models (mean squared prediction error 9.8 – 16.7 %). For the most part this is caused by the poor agreement of observed and predicted proportions of SCFA. The authors concluded that SCFA production rates would be better suited to predict CH_4 production, but those are very difficult to determine due to the dynamics of absorption and efflux from the rumen (ALEMU et al. 2011). For the accurate determination of the production rates isotope dilution procedures are required as used in the study by ANNISON & BRYDEN (1998).
BRASK et al. (2015) developed a model predicting CH_4 production from SCFA concentrations. Their model included a ruminal carbon balance, which can only be measured in animals equipped with rumen and intestinal fistulas.

In summary, the inaccuracy of predicting SCFA patterns from feed composition or the requirement for very invasive and labor-intensive methods are probably the reasons why mathematical equations based on SCFA concentrations are not widely used for the estimation of CH_4 production.

2.5.2 Milk fatty acids to estimate methane emission

The required fatty acids for milk triglyceride synthesis arise from two different sources. Acetate and butyrate as ruminal fermentation products serve as carbon sources for *de novo* fatty acid synthesis. Additionally, acetate is a primary source for the production of the reducing equivalent NADPH (PALMQUIST 2006), which in turn is necessary for the synthesis of triglycerides. Additionally, absorbed dietary long-chain fatty acids or long-chain fatty acids mobilized from body tissues can be incorporated in milk triglycerides. These fatty acids are transported in the bloodstream as triglycerides in chylomicrons or very low-density lipoproteins (VLDL) and conveyed across the blood-milk barrier into the mammary gland.

The *de novo* synthesis of milk fatty acids results predominantly in chain lengths of 4 to 16 carbon atoms, whereas the circulating fatty acids usually consist of 16 to 18 carbon atoms. Consequently, the fatty acid pattern in milk depends largely on the availability of fatty acid sources. Long-chain fatty acids are decreasingly mobilized towards peak lactation and hence the transfer of blood triglycerides into milk fat declines (PALMQUIST 2006).

As mentioned in the previous section, the increased production of acetate is paralleled by higher CH_4 emissions, showing a link between methane emission and milk fatty acid composition. CHILLIARD et al. (2009) fed cows different forms of linseed products and observed a strong positive correlation between CH_4 production and saturated fatty acids with 6 to 16 carbon atoms in milk. This and other studies were included in the meta-analysis by VAN LINGEN et al. (2014), that summarized significant relationships between the proportion of certain milk fatty acids and methane yield. Milk fatty acids of shorter chain length (C6 – C16) were positively related to CH_4/DMI, whereas longer saturated and unsaturated fatty acids (C18:0, C18:1, C18:2) were negatively related to CH_4/DMI. In an attempt to predict CH_4 yield from milk fatty acid concentrations, VAN

LINGEN et al. (2014) described a prediction equation with a correlation coefficient of $r^2 = 0.54$:

$$CH_4 \text{ (g/kg DMI)} = 23.39 + 9.74 \times \text{C16:0-iso} - 1.06 \times \text{trans-10+11 C18:1} - 1.75 \times \text{cis-9, 12 C18:2.}$$

DIJKSTRA et al. (2016) tested this equation in dairy cows on grass-silage based diets, and showed the correlation coefficient in that scenario only amounted to 0.35. MOHAMMED et al. (2011) described an equation with a correlation coefficient of 0.9 that included concentrations of certain milk fatty acids (C9 – C17:1, C16:0) and also protozoans:

$$CH_4 \text{ (g/d)} = -910.8 \times \text{C9-17:1} + 331.2 \times \text{C16:0 iso} + 0.0001 \times \text{sum of total Entodiniomorphs*} + 242.5$$

*Entodinimorphs make up 99% of the ruminal protozoa, numbers are determined using a counting chamber (BEAUCHEMIN et al. 2009)

2.6 Description of methods

2.6.1 Methane Measurement

The measurement of CH_4 production from ruminants using respiration chambers is currently the most accurate and reliable technique. Often, respiration chamber measurements are used as a reference for evaluating the accuracy of other CH_4 measurement techniques. The most commonly used systems are open circuit chambers (HAMMOND et al. 2016). BHATTA et al. (2007) described the importance of the technical features that a respiration chamber has to fulfil. As a principle, gas concentrations of the incoming air and of the exhaust air as well as the air flow through the chamber are measured (HAMMOND et al. 2016). Emissions from the animal are mixed with the incoming air and the concentration difference of gases between the incoming air and exhaust air are multiplied by the airflow to calculate the amount of emissions. To warrant this, chambers have to be sealable for the quantification of in- and outflowing gases like CH_4, CO_2 and O_2. The facilities used in the presented trials fulfil this requirement. Also, within the chamber used in this study the animal can be restrained for further experimental procedures or during manure removal. The unit is designed and provisioned to ensure normal behavior including milking, feeding,

drinking and resting. Also, feed intake of the animal in the chamber can be monitored in real time with load cells under the troughs (WAGHORN & PINARES 2014) and water intake is determined with a water meter. Physical activity of the animals such as standing up and lying down can also be measured. This is quite relevant, since rising from a lying position releases CH_4 from the rumen in a surge and increases CH_4 concentrations in the chamber (personal communication from MICHAEL DERNO, Dummerstorf, 09.07.2018).

Recently, HAMMOND et al. (2016) and HILL et al. (2016) reviewed the currently used techniques distinguishing between breath sampling methods with or without a tracer, laser detection instruments and meteorological techniques. The usage of respiration chambers is costly and requires extensive resources to install and maintain the facilities. Therefore, the demand for techniques that are suitable to measure CH_4 from a larger group of animals or in the field has brought forward a number of other devices and methods. However, CH_4 measurement techniques produce more reliable emission data than estimation of CH_4 production with equation models using for example SCFA concentrations in rumen fluid (ALEMU et al. 2011).

2.6.2 Sampling of rumen contents

For sampling rumen contents different techniques are available. The easiest method is probably the sampling of whole rumen contents in the abattoir after slaughter, allowing to withdraw very large sample sizes. However, the process of slaughter usually mixes the rumen contents, and their natural stratification can be lost. To sample the same individual animal repeatedly, other methods have to be used. Under research conditions rumen cannulation is a preferred method (RAMOS-MORALES et al. 2014), allowing the repeated sampling or continuous measurements in different areas within the reticulorumen, e.g. from the liquid, solid, gaseous or epimural phases. However, the procedure to equip an animal with a permanent rumen cannula is quite an invasive surgery and requires high expertise and husbandry effort. A less invasive procedure is ruminocentesis, by which the outer skin and the rumen wall are punctuated with a large-bore needle through which a small amount of rumen fluid or gas can be sampled, but this method is not recommended for repeated samples over a short collection period.

Esophageal tubes that are inserted into the rumen via the mouth or nose are a minimally invasive alternative technique. This method can be used repetitively and under field conditions, although such tubes cannot be used for continuous sampling. Sample sizes of up to a few liters of rumen fluid can be obtained but care must be taken to avoid contamination of the rumen fluid with saliva. If the experimenter is experienced and the collection is smooth and rapid without much need for correction of tube insertion (RAMOS-MORALES et al. 2014), the salivary contamination can be kept to a minimum. The insertion depth and sampling site within the rumen influences the sample pH as well as the concentration of SCFA, minerals and trace elements and also the microbial composition (SHEN et al. 2012). This is due to the fact that the dilution of rumen contents with saliva, water and freshly ingested feed is higher closer to the orifice of the esophagus than in the caudal or ventral compartments of the rumen. However, when the esophageal tubing reached the ventral rumen sack, HERNANDEZ-RAQUET et al. (2016) observed no differences in the microbial community when compared to samples obtained via the rumen cannula.

2.6.3 Methods to identify microbes

The traditional way to identify microbes, especially bacteria, is through culturing of individual strains. This method generates larger quantities of the examined strain to be able to perform biochemical tests and to determine the chemical composition, which aids in identification of microbes and their functions (KOBAYASHI et al. 2004). Although culturing is still indispensable for obtaining pure isolates of microbes, the use of molecular techniques for the identification of microbial species has several advantages. High throughput possibilities, comparatively low capital and labor costs, and the ability to detect unculturable species make modern molecular techniques feasible for multi sample identification and quantification of microbes (HENDERSON et al. 2013). The most common approach to identify rumen microbes is the sequence analysis of the amplified 16S/18S rRNA gene (SIROHI et al. 2012). For all molecular methods DNA or RNA extraction from the sample is required, and usually the DNA of interest is amplified with a polymerase chain reaction (PCR), resulting in amplicons.

A variety of extraction methods exists, and usually they all utilize a combination of mechanical forces and chemicals to extract DNA or RNA from within the cells. The PCR

method uses many cycles of first melting the DNA double helix into single strands, then cooling down and employing an enzyme (DNA polymerase) to assemble a new DNA strand from added nucleotides using the single-stranded DNA as a template. By using specifically targeted primers (short DNA fragments), selected regions of the DNA can be chosen for amplification. Specific primers can shorten the length of the fragments and therefore the time and expenses for analysis. Sequencing determines the order of the nucleotides in the DNA fragment, and is followed by DNA sequence analysis. The sequence analysis is the necessary bioinformatic step to assign meaning to the nucleotide sequences, for example to define genes on the fragment or to assign sequences to taxonomies.

The rapid development of molecular techniques has led to a large variety of methods. HENDERSON et al. (2013) compared different DNA extraction protocols and discovered that they differed significantly with regard to DNA yields, purity and different microbial community structures even when using the same sample. This group also concluded that chloroform-based methods result in purer DNA, and that methods that include a mechanical lysis step usually lead to more sheared DNA with lower molecular weight. DENG et al. (2008) reviewed methods of nucleic acid amplification, cloning and sequencing. These are the necessary steps to obtain information on the 16S/18S rRNA/rDNA sequence information, which then was processed with different software (e.g. QUIIME, MOTHUR) and clone libraries (e.g. GreenGenes, SILVA) to assess abundances and phylogenetic information on the examined microbial community. The software is used to filter the obtained sequences, to process the raw data and to perform sequence alignment or searches against biological databases to assign taxonomy. Currently the most important ongoing sequencing project for rumen microbes is the Global Rumen Census (http://www.rmgnetwork.org/global-rumen-census.html) and the Hungate 1000 (http://www.rmgnetwork.org/ hungate1000.html) (TAPIO et al. 2017).

2.7 Objective and realization of the studies

The tight interrelationship between the metabolism of the host, the processes of microbial fermentation in the rumen and the resulting CH_4 production is currently only partly understood and therefore many current research approaches are dedicated to

this topic. The factor with the largest influence on CH_4 emission from ruminants – feed – has been extensively researched throughout the years involving the impact of DMI, diet composition and feed additives. Only recently genetic and metabolic effects of the animal itself on fermentation within its rumen have come into focus. Modern technologies, primarily high-throughput DNA sequencing, have quickly advanced our understanding on the microbial composition and their fermentation performances in the rumen, and we increasingly gain insight into the complexity of the rumen microbial network. However, strategies to mitigate CH_4 emissions from the livestock sector can be only successful if the metabolic network culminating in CH_4 production is better understood than today.

In this context, the current study investigated the influence of the cow's metabolic status on markers of ruminal fermentation. In the first paper the hypothesis that cows with increased body fat mobilization have a lower CH_4 production than their less mobilizing age matched herd mates was tested. It was assumed that increased plasma NEFA concentrations due to an increased body fat mobilization would result in a lower utilization of circulating acetate. Consequently, a lower utilization of plasma acetate would result in a lower ruminal acetate production which, in turn, would diminish ruminal CH_4 production. This hypothesis was investigated in a herd of 20 Holstein Frisian dairy cows throughout their first lactation. Blood, milk and rumen fluid were sampled and analyzed photometrically or by gas chromatography to depict the metabolic status of the animals (for details see Publication 1). Also, feed and dry matter intake, milk yield, body weight, back fat thickness and a number of health indicators were recorded to further assess the metabolic status of the animals and to possibly eliminate sick animals from the study. Feed samples were analyzed for dry matter (DM) and chemical composition. For the measurement of whole-body energy metabolism and CH_4 emission, respiration chambers were used to obtain accurate values. The chambers allowed measurements concerning CH_4 production as well as calculations of CH_4 yield, fat and carbohydrate oxidation.

In the second paper the question was asked if the increase of CH_4 yield with progressing lactation was driven by a change in the microorganisms producing CH_4 or CH_4-precursor substances, specifically archaea and bacteria, independently of the diet. A further objective was to compare the microbiome structure of cows with high and low CH_4 yields in late lactation, i.e. in a time period when the influence of fat

mobilization can be neglected. Immediate processing of rumen fluid after sampling was performed at the laboratory at the Leibniz Institute for Farm Animal Biology (FBN) and consisted of filtering the obtained fluid, determination of pH and centrifugation. The resulting microbe containing pellet was snap-frozen at -80°C for later shipment, and the supernatant was frozen at -20°C for the measurement of SCFA via gas chromatography. Preparation and analysis of the microbial DNA in the rumen fluid pellets was conducted by project partners at the University College Dublin, Ireland. They used the PCSA DNA extraction method, which is short for Phenol-chloroform plus bead-beating method. The Phenol-chloroform is added prior to bead-beating step. This is a simple and quick protocol that provides high yields of DNA. Further PCR was conducted with primers targeting the V4 region of rRNA and amplicon sequencing on the Illumina Miseq platform (see Publication 2).

3 PUBLICATIONS

3.1 First Publication

Body fat mobilization in early lactation influences methane production in dairy cows

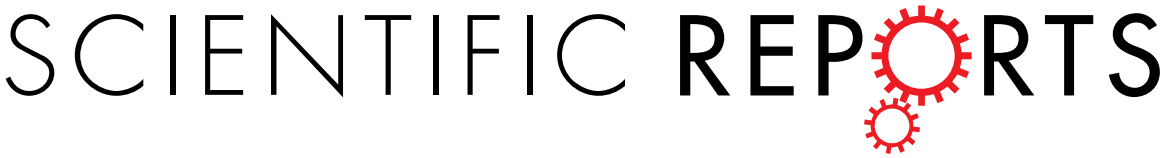
A. Bielak, M. Derno, A. Tuchscherer, H.M. Hammon, A. Susenbeth, B. Kuhla

Scientific Reports 2016; 6:28135

Own contribution:

- rumen fluid, milk, feed and blood sample collection in late lactation (trial started before I joined the research group at the FBN)
- record of animal data
- data assembly and digitalization
- selection of statistical models in cooperation with A. Tuchscherer and B. Kuhla
- statistical data evaluation with SAS (provision of statistical models by A. Tuchscherer)
- compilation of figures and tables
- selection of topic, data content and choice of presentation of the publication
- manuscript draft writing
- contribution to revision of manuscript after first review

www.nature.com/scientificreports



OPEN

Body fat mobilization in early lactation influences methane production of dairy cows

Received: 06 December 2015
Accepted: 01 June 2016
Published: 16 June 2016

A. Bielak[1], M. Derno[1], A. Tuchscherer[2], H. M. Hammon[1], A. Susenbeth[3] & B. Kuhla[1]

Long-chain fatty acids mobilized during early lactation of dairy cows are increasingly used as energy substrate at the expense of acetate. As the synthesis of acetate in the rumen is closely linked to methane (CH_4) production, we hypothesized that decreased acetate utilization would result in lower ruminal acetate levels and thus CH_4 production. Twenty heifers were sampled for blood, rumen fluid and milk, and CH_4 production was measured in respiration chambers in week −4, +5, +13 and +42 relative to first parturition. Based on plasma non-esterified fatty acid (NEFA) concentration determined in week +5, animals were grouped to the ten highest (HM; NEFA > 580 μmol) and ten lowest (LM; NEFA < 580 μmol) mobilizing cows. Dry matter intake (DMI), milk yield and ruminal short-chain fatty acids did not differ between groups, but CH_4/DMI was lower in HM cows in week +5. There was a negative regression between plasma NEFA and plasma acetate, between plasma NEFA and CH_4/DMI and between plasma cholecystokinin and CH_4/DMI in week +5. Our data show for the first time that fat mobilization of the host in early lactation is inversely related with ruminal CH_4 production and that this effect is not attributed to different DMI.

The fermentation of poly- and monosaccharides in the rumen results in the formation of pyruvate which in turn serves as intermediate substrate for the production of short-chain fatty acids, primarily acetate, propionate and butyrate. Propionate synthesis is a hydrogen consuming process, whereas the conversion of pyruvate to acetate or butyrate is accompanied by the production of hydrogen. Methanogenic microbiota utilize the released hydrogen to reduce CO_2 yielding in the formation of CH_4[1]. Accordingly, the ruminal acetate or the (acetate + butyrate) : propionate ratio is highly related with daily CH_4 emission (g/d) or CH_4 yield expressed as g per unit of dry matter intake (g/kg DMI)[2,3]. In the post-absorptive metabolism of dairy cows, propionate is primarily used for hepatic gluconeogenesis, whereas acetate is activated to form acetyl-CoA serving as the main energy-providing substrate for the host. In lactating cows, acetate and butyrate may also serve as precursors for de novo milk fat (C4 – C16) synthesis by the mammary gland. Thus, CH_4 and precursors for de novo milk fatty acids arise from the same biochemical pathway in the rumen. Therefore concentrations of milk fatty acids de novo synthesized from acetate, e.g. C6, C8, C10 and C16 or the sum of these saturated fatty acid concentrations are indicators for the level of acetate production in the rumen and were found to be positively related to CH_4 emission or CH_4 yield[3–5].

The (acetate + butyrate) : propionate ratio in the rumen decreases when the diet is supplemented with oilseeds rich in C18 fatty acids, resulting in reduced CH_4 production[6]. The CH_4 suppressing effect of dietary long-chain fatty acids may be due to decreased fiber digestibility or reduced DMI[7]. In lactating animals, C18 milk fatty acid concentrations increased at the expense of shorter chain milk fatty acids upon supplementing the diet with oilseeds[6,7]. Hence, concentrations of C18 milk fatty acids were found to be negatively related to CH_4 yield[3–5]. However, neither DMI, feed composition, ruminal short-chain fatty acids nor milk fatty acids alone are sufficient to accurately predict CH_4 emission[3], indicating that either the microbial composition or traits of the host may influence methanogenesis in the rumen. Only recently Ricci *et al.*[8] considered, besides dietary characteristics, the physiological stage of the animal to improve the prediction of CH_4 emissions.

During the transition from late pregnancy to early lactation, high-yielding dairy cows do not ingest enough feed to meet the nutrient and energy requirements for milk production and consequently enter into negative

[1]Leibniz Institute for Farm Animal Biology (FBN), Institute of Nutritional Physiology "Oskar Kellner", Wilhelm-Stahl-Allee 2, 18196 Dummerstorf, Germany. [2]Leibniz Institute for Farm Animal Biology (FBN), Institute of Genetics and Biometry, Wilhelm-Stahl-Allee 2, 18196 Dummerstorf, Germany. [3]Institute of Animal Nutrition and Physiology, Christian-Albrechts-Universität zu Kiel, Hermann-Rodewald-Straße 9, 24118 Kiel, Germany. Correspondence and requests for materials should be addressed to B.K. (email: b.kuhla@fbn-dummerstorf.de)

energy balance. During this time of lactation provision of energy by dietary derived acetate is lowest and as an adaptive response cows mobilize their body fat reserves leading to increased concentrations of circulating long-chain, non-esterified fatty acids (NEFA). These mobilized NEFA are mainly composed of C16 and C18 and are intensively oxidized to allocate energy but also used by the mammary gland for milk fat synthesis[9]. Plasma NEFA concentrations paralleled the concentrations of milk C18, while the concentration of milk fatty acids synthesized from acetate remained unaffected or were inversely associated with plasma NEFA levels until week 7 after parturition[9]. Thus, the increase in plasma NEFA concentrations reduces the level of acetate utilization, at least in the mammary gland, but whether plasma NEFA concentrations are also negatively related to CH_4 yield remains to be investigated. Herein, we hypothesized that increased body fat mobilization resulting in higher plasma NEFA concentrations would negatively affect metabolic acetate utilization, and as a kind of negative feed-back regulation, acetate production in the rumen and consequently CH_4 production.

Results

Animals and Diets. Per design, animals were grouped according to their plasma NEFA concentrations at the time of the respiration chamber measurements in week 5 ± 0.2 of early lactation in which plasma NEFA concentrations amounted to 811.2 ± 61.9 μmol/L for the ten highest (HM; NEFA > 580 μmol) and 379.1 ± 61.9 μmol/L for the ten lowest (LM; NEFA < 580 μmol) mobilizing cows ($P < 0.001$; Supplemental Fig. 1a). The HM cows had also greater total amounts of plasma NEFA in the time period two weeks before until six weeks after the respiration chamber measurement in week 5 ± 0.2 ($P = 0.007$, Supplemental Fig. 1b), indicating slower increase but longer-lasting fat mobilization compared to LM cows who showed earlier, shorter and less intensive peak concentration far (i.e. 4 weeks) before respiration chamber measurement (702.3 ± 123.7 μmol/L; Supplemental Fig. 1a). The body weight of HM and LM cows ranged from 610 kg to 665 kg 4 weeks before parturition and changed over time ($P < 0.001$), but was not different between groups (Fig. 1a; Supplemental Table S1). From late pregnancy until week 13 post partum (p.p.) body weight declined to 556 ± 112 kg after which it increased again. DMI increased after calving ($P < 0.001$), but without difference between groups at any time point (Fig. 1b; Supplemental Table S1). In week 4 ante partum (a.p.) animals ingested about 7.1 kg of DM per d, which increased to 13.2 kg, 14.5 kg and 15.2 kg in week 5, 13 and 42 p.p., respectively, without difference between groups at times indicated (Supplemental Table S1). Body condition score (BCS) and back fat thickness (BFT) were not different between groups, but changed over time ($P < 0.001$; Fig. 1c,d; Supplemental Table S1). However, the group × time interaction for BFT was significant ($P = 0.02$; Fig. 1d). Precisely, HM cows had a higher BFT at calving ($P = 0.007$) and a higher loss of back fat until week 5 p.p. ($P = 0.006$, Fig. 1d).

Energy corrected milk yield (ECM) increased from week 1 until week 4 p.p. and decreased for all cows over time ($P < 0.001$) from 28.8 ± 1.1 kg in week 5 p.p. to 27.6 ± 0.8 kg in week 13 p.p. to 24.8 ± 0.9 kg in week 42 p.p., but again there was no differences between HM and LM cows (Fig. 1e, Supplemental Table S1). Milk fat decreased from week 5 p.p. to week 13 p.p. in both cow groups and was significantly lower in HM compared to LM cows in week 13 p.p. (HM 3.72 and LM 4.34 ± 0.13%; $P = 0.03$; Supplemental Table S1).

Feed digestibility determined in week 6 p.p. was comparable between groups (77.4 ± 0.3% in HM and LM cows; $P > 0.1$). The mean retention time (MRT) in week 6 p.p. amounted to 27.5 ± 0.6 h in HM cows and to 27.0 ± 0.6 h in LM cows without difference ($P = 0.54$) between groups.

Plasma, Milk and Rumen fluid. Plasma NEFA concentrations were greater in HM than LM cows not only in week 5 ($P < 0.001$), but still tended to be higher in week 13 after parturition ($P = 0.1$, Fig. 2a). Plasma beta-hydroxybutyrate (BHBA) concentrations increased after parturition ($P < 0.01$), but there were no differences ($P = 0.18$) between groups (Fig. 2b). Plasma acetate concentrations continuously increased over time ($P < 0.001$) and were lower in HM compared to LM cows (0.67 and 0.85 ± 0.06 mmol/L, respectively) in week 5 p.p. ($P = 0.04$; Fig. 2c). Accordingly, plasma NEFA and plasma acetate concentrations showed a significant ($P < 0.001$) inverse relationship in week 5 p.p. (Fig. 2d). Plasma cholecystokinin (CCK) concentrations increased after parturition ($P < 0.01$) and tended to be lower in HM compared to LM cows in week 5 p.p. ($P = 0.1$; Fig. 2e). Ruminal acetate concentrations varied with time (each $P < 0.05$) and pair-wise comparison for each time point revealed that HM cows tended ($P = 0.087$) to have higher rumen acetate concentrations in week 4 a.p. only (Fig. 3a). Ruminal propionate and butyrate, the (acetate + butyrate) : propionate ratio and ruminal pH were not influenced by time or group (Fig. 3b–e). In addition, linear regression between ruminal and plasma acetate was not significant (Fig. 3f; $P = 0.57$).

Methane, Fat and Carbohydrate Oxidation. Total CH_4 production increased over time ($P < 0.001$), but was not different between groups (Fig. 4a) and CH_4 yield did not change over time (Fig. 4b). However, in week 5 p.p. HM cows tended to have lower CH_4 emission when related to DMI (29.7 and 32.8 ± 1.3 L/kg, respectively; $P = 0.1$; Fig. 4b) or to neutral detergent fiber (NDF) (86.0 and 95.3 ± 3.5 L/kg, respectively; $P = 0.08$; Fig. 4c) than LM cows. But when CH_4 emission was related to ME intake, groups did not differ at any time point (Fig. 4d). Methane emission per kilogram ECM decreased over time ($P < 0.01$), but was not different between groups (Fig. 4e).

Net carbohydrate oxidation (COX) continuously increased until week 42 of lactation and tended to be different ($P = 0.09$) between cow groups in week 13, but not in week 5 p.p. (Fig. 5a). After parturition net fat oxidation (FOX) decreased over time in both groups ($P < 0.001$; Fig. 5b). The pair-wise comparison in week 5 p.p. showed that HM cows tended to have a higher fat oxidation than LM cows (1783 and 1352 ± 140 g/d, respectively; $P = 0.058$; Fig. 5a), but there was no significant regression between net FOX and CH_4 ($P = 0.37$) or CH_4/DMI ($P = 0.78$), respectively (Fig. 5c,d).

Linear regression between CH_4 production and plasma NEFA concentration in week 5 p.p. was not significant (Fig. 6a; $P = 0.17$), whereas we found an inverse relationship between CH_4 yield expressed as CH_4/DMI ($P = 0.002$) or CH_4/NDF ($P = 0.005$) with plasma NEFA concentrations of individual cows in week 5 p.p.

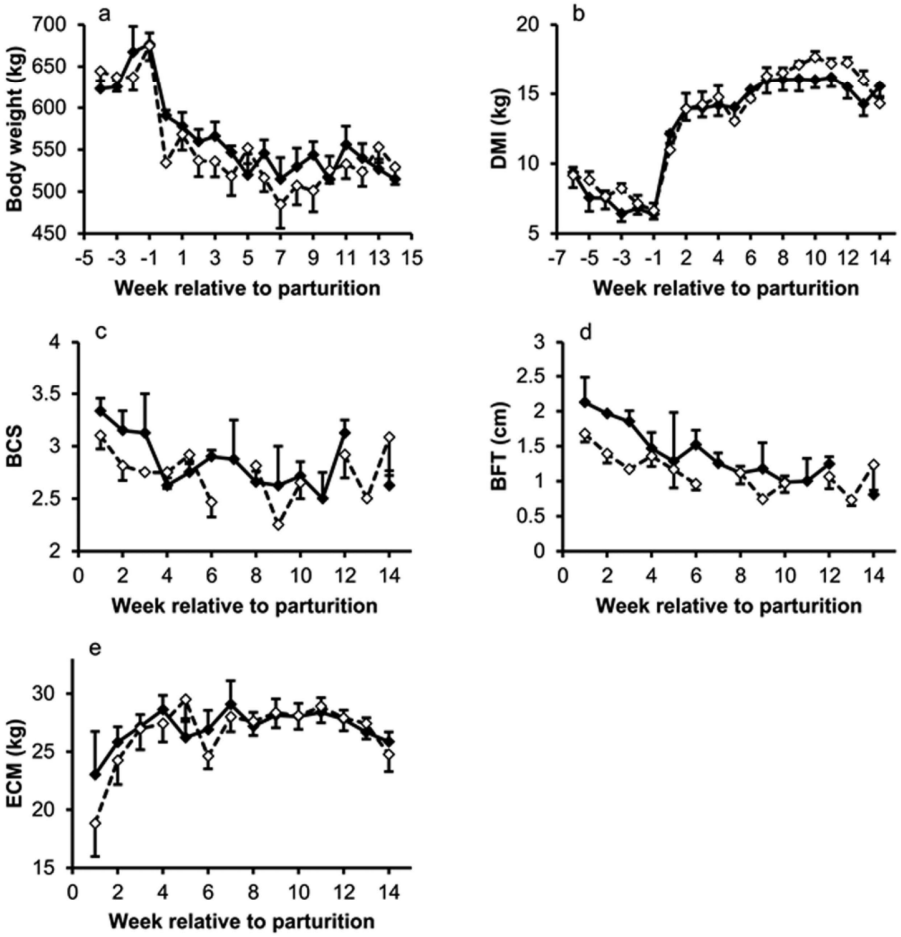


Figure 1. Body weight, DMI, BCS, BFT and ECM of high (◆, solid line; n = 10) and low mobilizing (◊, dashed line; n = 10) cows during the period of first lactation. ANOVA calculated time × group interactions were: (**a**) $P = 0.18$, (**b**) $P = 0.04$, (**c**) $P = 0.60$, (**d**) $P = 0.02$, (**e**) $P = 0.15$. Data are shown as LSM ± SE.

(Fig. 6c,e). These regressions were not evident in week −4, +13 and +42 relative to parturition (data not shown). Furthermore, we were not able to detect any significant relationship between CH_4 production and ruminal acetate concentrations (Supplemental Fig. 2).

The best fitted curve for a relationship between CCK and CH_4, CH_4/DMI or CH_4/NDF was a two parametrical exponential function, but the coefficients of determination only reached $R^2 = 0.06$, $R^2 = 0.23$ and $R^2 = 0.17$, respectively (Fig. 6b,d,f).

Discussion

We hypothesized that increased body fat mobilization in early lactation associated with reduced acetate utilization of the host would negatively affect ruminal acetate concentration and CH_4 production. To examine this hypothesis we retrospectively grouped the cows according to their plasma NEFA concentrations in early lactation (i.e. week 5) to high and low mobilizing cows and determined CH_4 production at different physiological stages throughout the lactation cycle. Both HM and LM cows had a continuous increase in daily CH_4 production from one month before parturition to lactation week 42. This course does not parallel the biphasic course of CH_4 production predicted by mid-infrared spectra validated by the SF_6 technique[10]. A decrease in CH_4 production in late lactation however, may be initiated by a diet change often applied during that time, while our cows were kept on the same diet throughout lactation. Alterations in CH_4 emission during the lactation cycle can be due to the increasing rumen capacity during early lactation, accompanied by a passage rate that is decoupled from DMI, as well as changes in the digestibility of feed[11]. In agreement with their greater plasma NEFA concentrations, HM cows had the greater loss of back fat thickness during the first five weeks of lactation. Recent studies showed that elevated plasma NEFA concentrations around parturition are utilized for about 40% of milk fat synthesis during

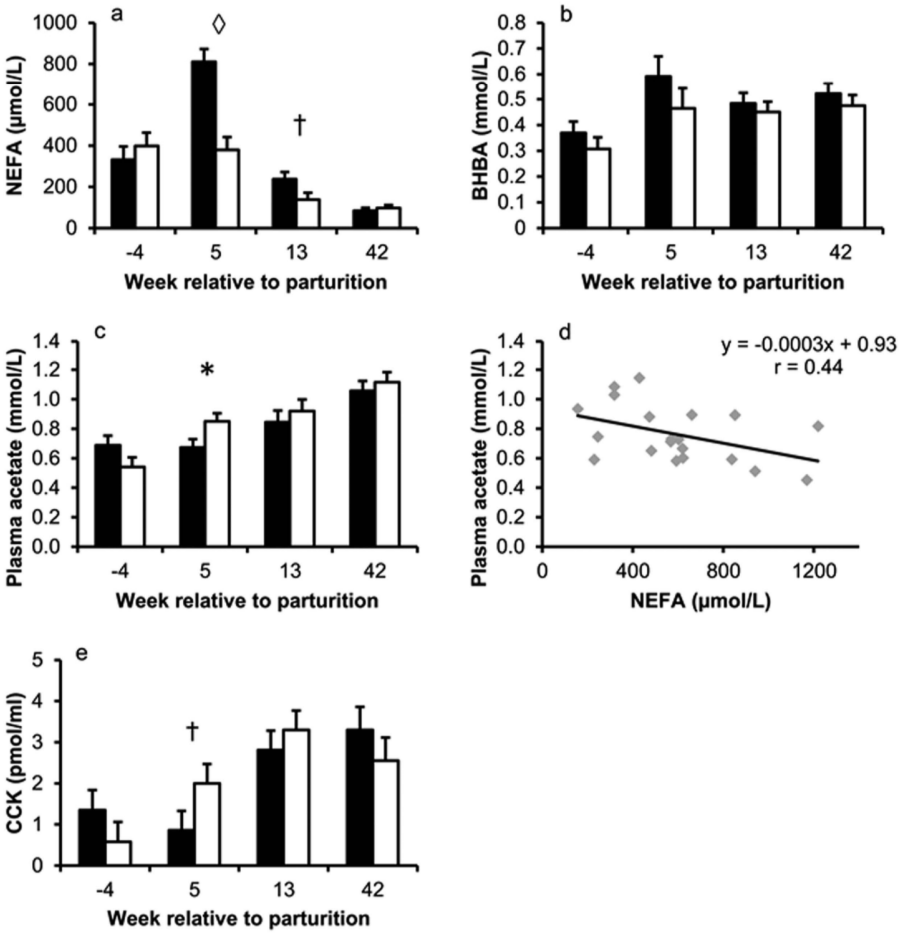


Figure 2. Plasma NEFA, BHBA and acetate concentrations at different time points, and regression between plasma acetate and plasma NEFA concentrations in week 5 p.p. Cows grouped as high mobilizing are marked ■ (n = 10), cows grouped as low mobilizing are marked □ (n = 10). (**a**) ◊ Indicates the time point of grouping the animals. (**b**) Time $P = 0.01$, group $P = 0.18$, time × group $P = 0.87$, ANOVA. (**c**) Time $P < 0.001$, group $P = 0.32$, time × group $P = 0.13$, ANOVA; * indicates $P = 0.04$, Tukey-Test. (**d**) Slope $P = 0.053$, n = 20. (**e**) Time $P = 0.003$, group $P = 0.95$, time × group $P = 0.12$, ANOVA; † indicates $P = 0.1$. Data in bar charts are shown as LSM ± SE.

early lactation[12,13]. Other authors discovered that cows with greater fat mobilization produce higher milk fat contents and thus a higher ECM in early lactation[14]. In our study, ECM and milk fat content did not differ between groups in week 5 p.p. Reasons for this may be the difference in the dynamic of fat mobilization with LM cows showing an early but short and less intensive peak NEFA concentration while HM cows had slowly increasing but longer-lasting fat mobilization (Supplemental Fig. 1). Another reason may be because the extent of fat mobilization reflected by plasma NEFA concentrations is much lower in first lactating cows as compared to cows with more parities. It is well established that increased utilization of NEFA for milk fat synthesis accounts not only for an increased total milk fat content but also for a higher proportion of long-chain fatty acids on the expense of short- and medium-chain fatty acids *de novo* synthesized from acetate or butyrate[13]. Hence, the mammary gland utilizes less acetate and as a consequence one might expect rising plasma acetate concentrations. Instead we observed lower plasma acetate concentrations in HM cows in week 5 p.p. which argues against our hypothesis proposed above - higher plasma NEFA concentrations would negatively affect metabolic acetate utilization - and led us to waive milk fatty acid profile analysis.

The lower plasma acetate concentrations in HM cows in week 5 p.p., but not at the other time points investigated, suggest that HM and LM cows differ in their acetate oxidation, acetate absorption or ruminal acetate production rates, and that these differences are specific for the early lactation period only. Acetate originates from carbohydrate fermentation and thus COX can be seen as an estimate for acetate oxidation. We found no

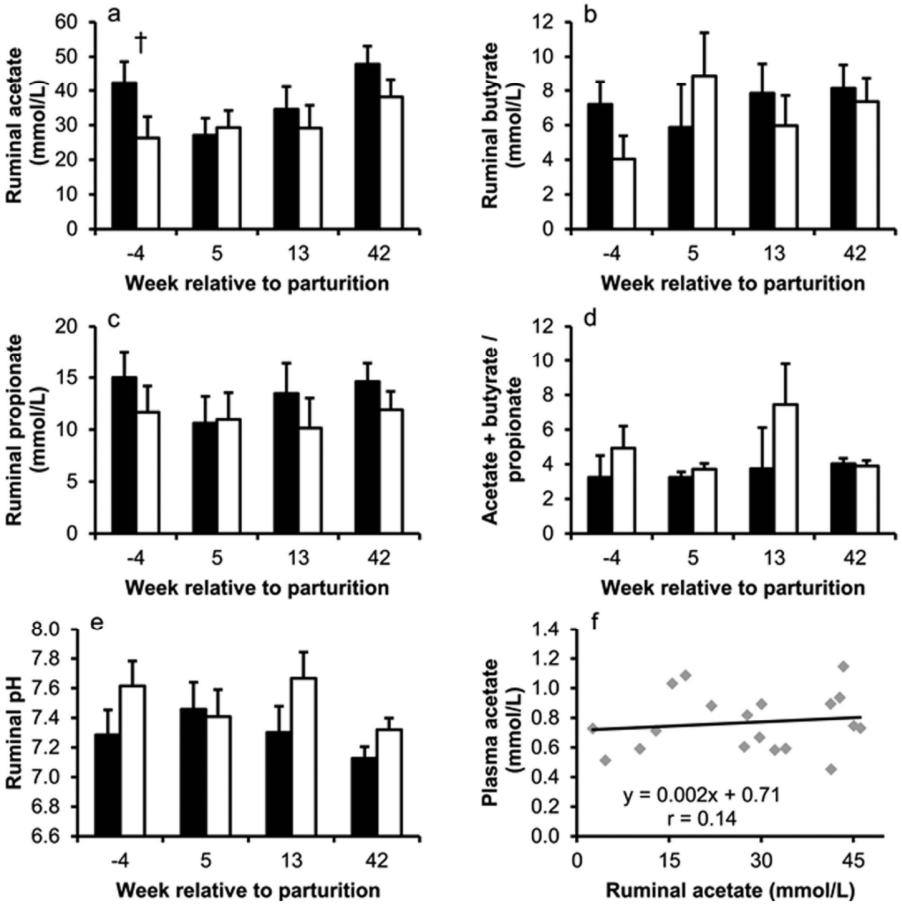


Figure 3. Ruminal variables as group means at the times of respiration chamber measurements in cows with high (■; n = 10) and low (□; n = 10) plasma NEFA concentrations in week 5 p.p. and regression between plasma and ruminal acetate concentrations measured in week 5 p.p. (**a**) Time $P = 0.045$, group $P = 0.045$, time × group $P = 0.44$, ANOVA; † indicates $P = 0.09$; Tukey-Test. (**b**) Time $P = 0.71$, group $P = 0.55$, time × group $P = 0.61$, ANOVA. (**c**) Time $P = 0.61$, group $P = 0.25$, time × group $P = 0.81$, ANOVA. (**d**) Time $P = 0.17$, group $P = 0.11$, time × group $P = 0.48$, ANOVA. (**e**) Time $P = 0.16$, group $P = 0.12$, time × group $P = 0.57$, ANOVA. (**f**) Slope $P = 0.57$. Data in bar charts are shown as LSM ± SE.

differences in COX between groups in week 5 p.p., suggesting that metabolic acetate oxidation should be similar between HM and LM cows at this time. In addition, comparable COX between groups in week 5 p.p. also reflects the same level of DMI of HM and LM cows.

The absorption of acetate through the ruminal epithelium into the blood stream follows partly mass action diffusion and partly active protein mediated transporters[15,16]. The former depends largely on ruminal pH, capillary blood flow to the epithelium and the size of the epithelial surface area. Equally sized and aged cows fed the same diet are also expected to have a comparable rumen size and epithelial surface area. Also, the comparable rumen pH of HM and LM cows argues against a different acetate diffusion rate. Therefore, different active acetate transport rates in the two cow groups influenced by increased circulating NEFA concentrations are a credible feasibility, but this assumption remains to be investigated. The fact that we did not observe a significant relationship between acetate concentrations in plasma and in ruminal fluid enforces the idea that diminished active transport of acetate across the rumen wall contributes to reduced plasma acetate concentration in HM cows during early lactation. This assumption is supported by Dijkstra *et al.*[17], who reported that ruminal concentrations of short chain fatty acids do not reflect their production rates. Other factors such as absorption rates, ruminal pH and the microbial populations account for this discrepancy[17]. Ultimately, we can only speculate whether the ruminal acetate production rate or the microbial composition differed temporally between HM and LM cows in week 5 p.p.

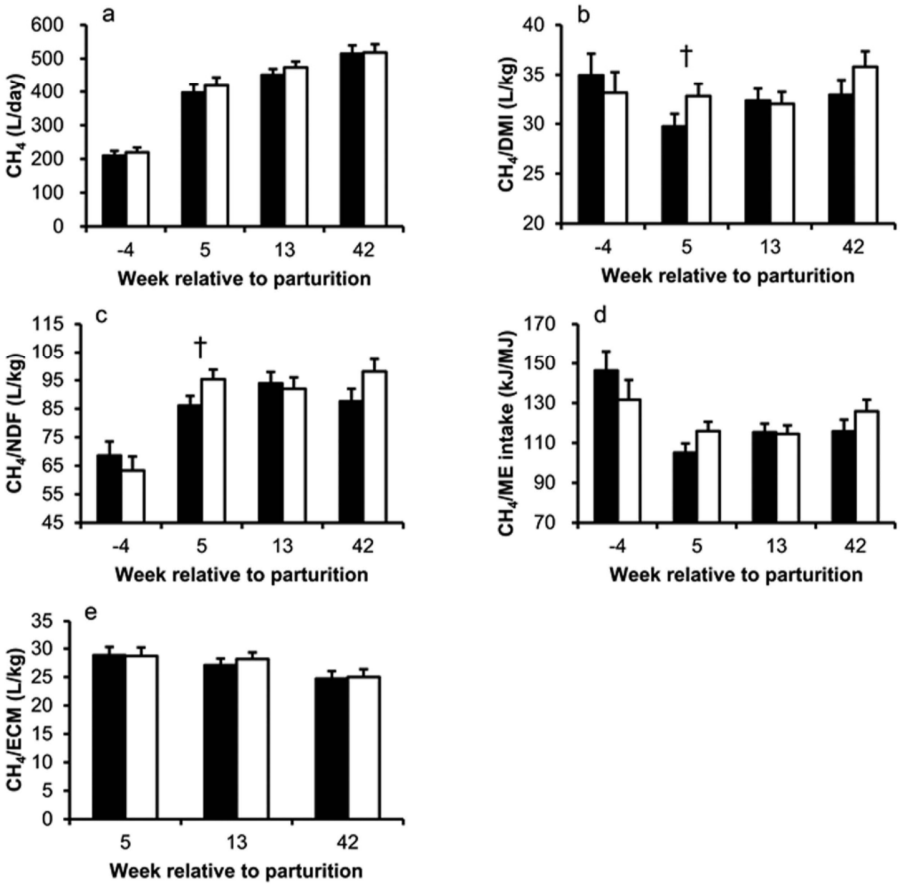


Figure 4. Daily CH_4 production, CH_4 per dry matter intake (CH_4/DMI), CH_4 per neutral detergent fiber (CH_4/NDF), CH_4 per metabolizable energy (ME) intake (CH_4/ME intake), and CH_4 per energy corrected milk yield (CH_4/ECM) in cows with high (■; n = 10) and low (□; n = 10) plasma NEFA concentrations in week 5 p.p. (**a**) Time $P < 0.001$, group $P = 0.51$, time × group $P = 0.91$, ANOVA. (**b**) Time $P = 0.17$, group $P = 0.54$, time × group $P = 0.14$, ANOVA; † indicates $P = 0.1$, Tukey-Test. (**c**) Time $P < 0.001$, group $P = 0.66$, time × group $P = 0.91$, ANOVA; † indicates $P = 0.08$, Tukey-Test. (**d**) Time $P < 0.001$, group $P = 0.44$, time × group $P = 0.09$, ANOVA. (**e**) Time $P < 0.023$, group $P = 0.82$, time × group $P = 0.14$, ANOVA. Data in bar charts are shown as LSM ± SE.

Interestingly we did not find a relationship between the ruminal acetate concentration and CH_4 emission in the present study. This is in accordance with findings in sheep[18], although other authors described a positive relationship between CH_4 yield and ruminal acetate concentration or CH_4 yield and acetate : propionate ratio[19–21]. However, the latter studies involved different diets that were fed to the animals, whereas all cows in the present study received the same diet at each time point investigated. Thus, a diet effect accounting for the rumen acetate to CH_4 yield relationship described[19–21] cannot be excluded.

As our initial hypothesis - plasma NEFA concentrations would negatively affect metabolic acetate utilization - could not be confirmed, we examined whether the extent of fat mobilisation is associative with different passage rates accounting for the different CH_4 yield between in HM and LM cows. Increase in DMI usually increases the passage rate of the digesta through the gastro-intestinal tract, or vice versa decreases MRT, which in turn reduces feed digestibility and CH_4 emission per unit feed ingested[22–24]. Conversely, CH_4 within the gut has been shown to slow digesta transit and influence gut motility in dogs, guinea pigs and humans[25]. We found comparable MRT in the rumen-intestinal tract and feed digestibility in LM and HM cows, which is in line with the comparable DMI of both groups. The small differences in CH_4 yield between cow groups in early lactation can therefore not be explained by MRT measured over the whole rumen-intestinal tract. Different studies have shown that the passage rate through the entire gastro-intestinal tract is not proportional to the reticulorumen passage of indigestible NDF in Holstein cows fed a corn silage based diet[26,27]. The precise measurement of the reticulorumen passage rate, however, requires the use of cannulated animals, which could not be realized in the present study. Goopy *et al.*[28] described that sheep with a lower MRT of particulate and liquid matter in the rumen produced less CH_4 despite

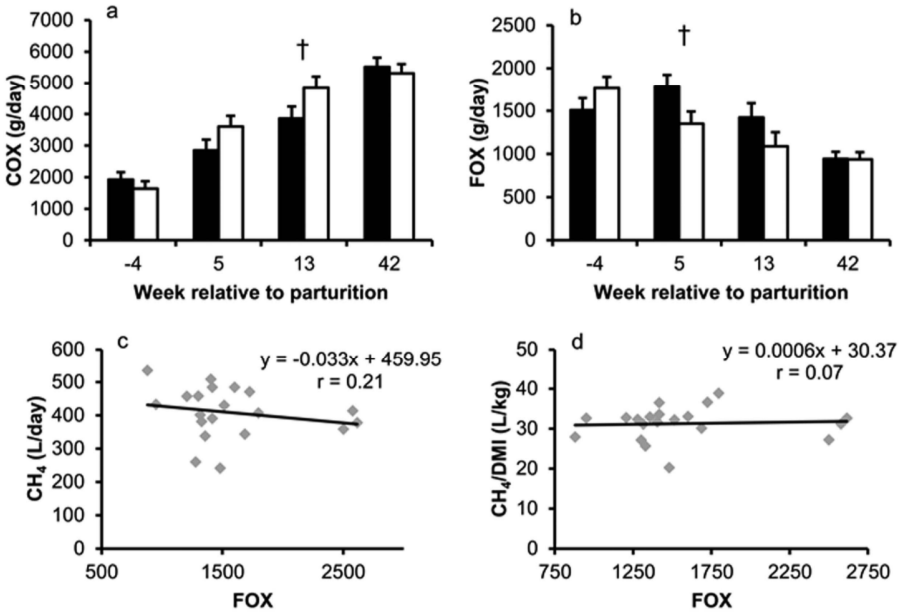


Figure 5. Carbohydrate oxidation (COX) and fat oxidation (FOX) at different time points in cows with high (■; n = 10) and low (□; n = 10) plasma NEFA concentrations in week 5 p.p.; and linear regression of FOX and CH_4 yield (n = 20). (**a**) Time $P < 0.001$, group $P = 0.24$, time × group $P = 0.08$, ANOVA; † indicates $P = 0.09$; Tukey-Test. (**b**) Time $P < 0.001$, group $P = 0.14$, time × group $P = 0.22$, ANOVA; † indicates $P = 0.06$; Tukey-Test. (**c**) Slope $P = 0.37$. (**d**) Slope $P = 0.78$. Data in bar charts are shown as LSM ± SE.

the fact that their DMI was comparable with sheep possessing a higher ruminal MRT and CH_4 yield. Sheep with lower CH_4 yield had smaller rumen volumes[28], however, general differences in the rumen size of HM and LM cows are unlikely because differences in CH_4 yield between the two groups occurred only in week 5 p.p. and did not persist throughout whole lactation.

It might be that differences in CH_4 yield between HM and LM cows in week 5 p.p. are owed to temporary differences in the MRT of the reticulorumen only, and not of the gastrointestinal tract as a whole, although we were not able to measure the reticulorumen-specific MRT in the present study. Dias *et al.*[29] and Oshita *et al.*[30] have shown that the fractional outflow rate of particulate matter from the reticulorumen was positively correlated with total chews, and that the decrease in particle size caused by chewing facilitates particle flow through the digestive tract. We did not evaluate chewing behavior of the animals, but because CH_4 yield tended to differ only temporarily between HM and LM cows and both groups received the same diet, it seems unlikely that differences in chewing account for different MRT of the reticulorumen and therefore the statistical trend observed for CH_4 yield in week 5 pp.

Possible causes for the approximately 10% different CH_4 yield in HM and LM cows during early lactation may be temporary differences at the systemic side of the rumen-intestinal tract, e.g. differences in fat metabolism of the cows which affect the MRT of the reticulorumen and thus digestive processes. Several previous studies have shown that hormones related to fat metabolism and fat accretion can directly or indirectly influence gut motility. For example ghrelin and motilin stimulate gastric motility, accelerate gastric emptying and small intestinal transit time[31,32]. In an earlier study we have shown that HM cows have greater preprandial ghrelin concentrations than LM cows, and that this difference was particularly prominent during early lactation and less in late pregnancy[14]. This data indicates that HM cows have a higher motility at the proximal rumen-intestinal tract, conclusively reduced feed retention time in the rumen and therefore tend to have less CH_4 yield in week 5 p.p. Moreover, 3rd to 5th lactating HM cows investigated in the earlier study[14] had significantly less CH_4 yield as compared to their LM counterparts in week 2 p.p. but not in week 6 a.p. (data not published yet) supporting the findings of the present study. However, whether administration of ghrelin directly affects CH_4 emission remains to be examined in future studies. In contrast to ghrelin, intravenous infusion of cholecystokinin has been shown to depress frequency of reticular contractions and rumen motility[33,34]. Thus, lower CCK plasma concentrations in HM compared to LM cows in week 5 p.p. further underscores greater rumen motility which should promote an acceleration of ruminal passage rate in cows with greater fat mobilisation in early lactation.

Leptin, a hormone secreted by adipose tissue, interacts with the vagus nerve and the release of CCK and has a complex effect on motility of the gastrointestinal tract, e.g. by delaying gastric emptying and transit time of the ingesta through the small intestine[31]. It has been described that LM cows have lower plasma leptin concentrations than HM cows, but only before and not after parturition[35]. Thus, different CH_4 yields observed as a trend between groups in week 5 p.p. can therefore not be explained by different plasma leptin concentrations.

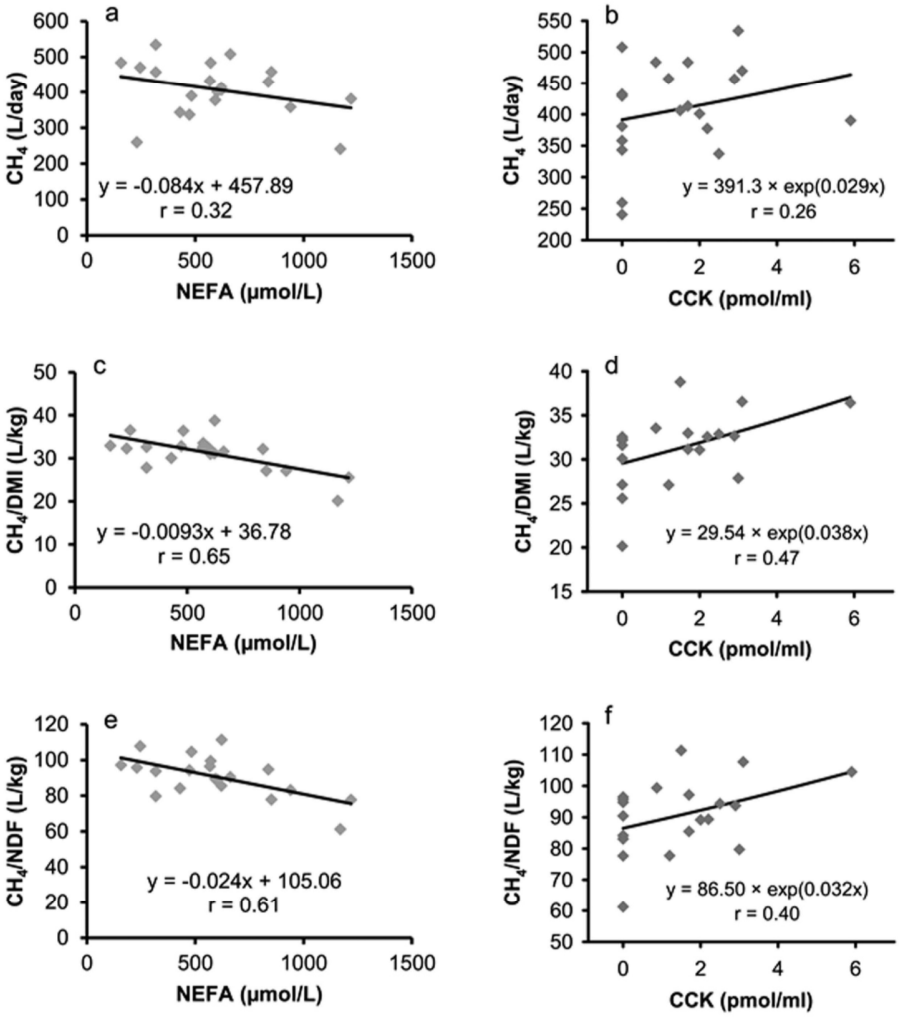


Figure 6. Linear regression between plasma NEFA concentration and daily CH_4 production either expressed as L/d, L/kg DMI, or L/kg NDF, respectively and 2 parametrical exponential regression ($y = a \times e^{(bx)}$)between plasma CCK concentrations and CH_4 production (n = 20). (**a**) Slope $P = 0.17$. (**b**) $P < 0.0001$ for a; $P = 0.28$ for b. (**c**) Slope $P = 0.002$. (**d**) $P < 0.0001$ for a; $P = 0.03$ for b. (**e**) Slope $P = 0.005$. (**f**) $P < 0.0001$ for a; $P = 0.07$ for b.

Intravenous infusion of acetate did not modify gastric motility[36] and conclusively it seems unlikely that differences in plasma acetate concentrations of HM and LM cows p.p. would affect motility of the gastrointestinal tract. The effect of circulating long-chain fatty acids on rumen or gut motility is not known, however, enterocytes are able to oxidize long-chain fatty acids to activate enteric neurons signaling to reduce feed intake[37]. Greater plasma NEFA concentrations and FOX rates in HM cows during early lactation argue against this mechanism because DMI is comparable to LM cows. This opens the question whether circulating NEFA may influence the motility of the gastrointestinal tract and consequently CH_4 yield.

A further question to be answered is concerning the fate of the hydrogen in HM cows producing less CH_4 in week 5 p.p. Possibly, hydrogen is increasingly transferred towards propionate although ruminal propionate concentrations were not different between groups. But keeping in mind that the total amount of propionate produced in the rumen exceeds the total amount of CH_4 by far, conclusions on the hydrogen transfer towards propionate are difficult to draw. It is also conceivable that in HM cows less hydrogen is transferred to the butyrate producing pathway as indicated by the numerically lower ruminal butyrate concentrations in HM cows in week 5 p.p. and that this hydrogen is released via eructation.

Component (g/kg DM)	Far-off	Close-up	Lactation		
Grass silage	793	160	181		
Corn silage		432	325		
Grass hay	66	102	32		
Barley straw	133	63	38		
Corn kernels		45	70		
Grain mix			39		
Extracted soy meal		54	21		
Extracted canola seed meal		68	38		
Feed lime			4		
MF 2000 (concentrate)[1]		65	220		
Mineral 9522[2]			9		
Prenatal TR40[3]	11	11			
Chemical analysis		4 weeks a.p.	5 weeks p.p.	13 weeks p.p.	42 weeks p.p.
Crude ash	(g/kg DM)	86	69	68	73
Crude protein	(g/kg DM)	177	162	157	156
Crude fiber	(g/kg DM)	231	167	171	166
Crude fat	(g/kg DM)	21	31	30	32
Sugar	(g/kg DM)	10	26	29	21
Starch	(g/kg DM)	100	255	252	254
NDF	(g/kg DM)	501	345	347	371
ADF	(g/kg DM)	296	208	211	210
N free extracts	(g/kg DM)	407	494	494	494
ME	(MJ/kg DM)	9.6	11.3	11.2	11.3
NE_L	(MJ/kg DM)	5.7	7.0	6.9	7.1

Table 1. Diet components and analysis of the total mixed ration (TMR) ingested during stays in respiration chamber. [1]MF 2000 (Vollkraft Mischfutterwerke GmbH, Güstrow, Germany): 7.1 MJ NE_L/kg, 24% crude protein, 3.3% crude fat, 6.2% crude fiber, 8.4% crude ash, 0.7% calcium, 0.5% phosphorus, 0.65% sodium, vitamins A, D_3, E, calcium iodate, cobalt carbonate, manganese oxide, zinc oxide, sodium selenite. [2]Rinderstolz 9522 (Salvana GmbH, Sparrieshoop, Germany): 92% crude ash, 20% calcium, 5% phosphorus, 8% sodium, 6% magnesium, vitamins A, D_3, E, copper sulfate, zinc oxide, manganese oxide, calcium iodate, sodium selenite, cobalt carbonate. [3]Prenatal TR 40 (Salvana GmbH, Sparrieshoop, Germany): 75% crude ash, 4% calcium, 6% phosphorus, 15% magnesium, 8% sodium, vitamins A, D_3, E, zinc oxide, manganese oxide, copper sulfate, calcium iodate, sodium selenite, cobalt carbonate.

Materials and Methods

Animals, feeding and milking. Twenty pregnant German Holstein heifers were kept in a free stall at the Leibniz-Institute for Farm Animal Biology (FBN), Dummerstorf, Germany and monitored until 291 DIM (SE ± 1.5) of their first lactation. 6 weeks prior to their expected calving date heifers were transferred to the straw bedded calving box and returned to the free stall on the first day after calving. All animals were treated in accordance with the guidelines for the use of animals as experimental subjects of the State Government in Mecklenburg-Western Pomerania. All experimental protocols were approved by the local animal ethics committee (Landesamt für Landwirtschaft, Lebensmittelsicherheit und Fischerei Mecklenburg-Vorpommern; approval No. 7221.1-1.-053/13).

Heifers had free access to water and the diet was offered as a standard ruminant total mixed ration (TMR) for ad libitum intake. 21 days before expected calving date animals were switched from the far-off to the close-up diet, and after parturition they received the lactation diet (Table 1). Individual daily feed intake was recorded as disappearance of feed from troughs connected to an electronic scale to which access was controlled by individual transponder (Roughage Intake Control, Insentec, Marknesse, The Netherlands). Based on the analysis of the individual TMR ration components, diet compositions for the far-off, close-up and lactation period were formulated and calculated according to the feeding standards of the German Society of Nutrition Physiology (GfE)[38]. An additional TMR sample was taken at the time of respiration chamber measurements (see below) for the determination of dry matter (DM) and diet composition. Analyses were conducted by the Landwirtschaftliche Untersuchungs- und Forschungsanstalt (LUFA) in Rostock, Germany. Chemical composition of TMR samples are shown in Table 1.

Feed energy was calculated according to Boguhn *et al.*[39] and GfE[38]:

$$\mathrm{GE\ (MJ/kg\ DM)} = 0.0239\ \mathrm{XP} + 0.0398\ \mathrm{XL} + 0.0201\ \mathrm{XF} + 0.0175\ \mathrm{XX} \quad (1)$$

$$\mathrm{ME\ (MJ/kg\ DM)} = 6.0756 + 0.19123\ \mathrm{XL} + 0.02459\ \mathrm{XP} - 0.000038\ \mathrm{XF}^2 - 0.002139\ \mathrm{XL}^2 - 0.000060\ \mathrm{XP}^2 \quad (2)$$

$$NE_L\,(MJ/kg\ DM) = 0.6\ [1 + 0.004\ (q - 57)]\ ME, \quad (3)$$

where GE is gross energy, ME is metabolizable energy, NE_L is net energy lactation, XP is crude protein, XL is crude fat, XF is crude fibre, XX is N free extracts and q = ME/GE x 100.

Body weight of the animals was measured automatically after milking and recorded as weekly means, and immediately before each indirect calorimetric measurement.

BFT measurements were conducted ultrasonographically (Titan Ultrasound System, SonoSite Inc., USA) from 1 until 14 weeks after calving at 14-day intervals. Additionally, BCS was determined at the same day on a 5 point scale according to Schroder and Staufenbiel[40]. Animals were milked twice daily at 04:30 h and 16:30 h and milk yield was recorded automatically. Milk samples from evening and morning milking were pooled and analyzed weekly by the Landeskontrollverband für Leistungs- und Qualitätsprüfung Mecklenburg-Vorpommern e.V. for milk composition. To calculate the ECM the following formula according to Reist *et al.*[41] was used:

$$\begin{aligned} ECM\ (kg) = {} & [0.038 \times \text{crude fat (g)} + 0.024 \times \text{crude protein (g)} \\ & + 0.017 \times \text{lactose (g)}] \times \text{milk (kg)}/3.14. \end{aligned} \quad (4)$$

Indirect Calorimetry. For CH_4 measurement animals were transferred into open-circuit respiration chambers[42] in week 4 a.p. and in week 5, 13 and 42 p.p. (SE ± 0.2 weeks). Animals were halter-trained and well adapted before measurements in the chamber, meaning habituation at least three times until the animal appeared relaxed and displayed regular behavior such as eating, ruminating and lying down. Within the chambers animals were kept in tie-stall at 15 °C, a dark–light cycle from 06:00 h to 19:00 h, milked twice daily if lactating and had access to fresh water. After an overnight stay allowing gas exchange equilibration, measurement of gas concentrations started at 07:00 h and lasted for 24 h. The CH_4 and CO_2 concentrations in the chamber were analyzed by infrared absorption and the O_2 concentration was measured paramagnetically (SIDOR, SICK MAIHAK GmbH, Reute, Germany) as described recently by Derno *et al.*[42]. Air flow through the chambers was recorded with a differential-pressure type V-cone flow meter (McCrometer, Hemet, CA, USA). Fermentative CO_2 ($C_{CO_2\ ferm.}$) production was estimated from CH_4 production according to Chwalibog *et al.*[43]:

$$V_{CO_2\ ferm.}(L) = 1.7 \times V_{CH4}\ (L) \quad (5)$$

in which the factor 1.7 is constant for a variety of diet compositions[44].

Metabolic CO_2 ($CO_{2\ metab.}$) was calculated as difference between total and fermentative CO_2 production:

$$V_{CO_2 metab.} = V_{CO_2} - V_{CO_2\ ferm.} \quad (6)$$

COX and FOX were calculated as described by Derno *et al.*[42]:

$$COX\ (g) = (4.75\ V_{CO_2\ metab.}\ (L) - 3.23\ V_{O2}\ (L) - 2.60\ N_U\ (g)), \quad (7)$$

$$FOX\ (g) = (1.69\ V_{O2}\ (L) - 1.69\ V_{CO_2\ metab.}\ (L) - 2.03\ N_U\ (g)), \quad (8)$$

where N_U is urine N excretion. N_U was not measured and set to zero accepting an error of about 10%[43] for both COX and FOX.

Feed was given twice at 07:30 h and 15:00 h. Feed intake in the chamber was determined by feed disappearance measured by using a scale connected to an electronic registration device. Data was collected every 6 min for 24 consecutive hours. Animals were milked in the chamber at 07:00 h and 16:30 h and the milk yield was recorded.

Blood sampling. Animals were blood sampled at 07:00 h in the morning immediately before transferring into the respiration chambers by puncture of the Vena jugularis externa using BD Vacutainers containing potassium ethylenediaminetetraacetate (Greiner bio-one, Frickenhausen, Germany). Additional blood samples were taken weekly from week −3 to +12 relative to parturition (Supplemental Figure S1). Immediately after collection the vials were processed in a centrifuge at 4 °C for 20 minutes at 1,300 × g. Plasma was harvested and stored at −20 °C until analysis. Plasma concentrations of NEFA and BHBA were analyzed photometrically (Abx Pentra 400, Horiba ABX SAS, Montpellier, France) using kit no. 436-91995 for NEFA (Wako Chemicals GmbH, Neuss, Germany)and kit RB 1008 (Labor und Technik, Berlin, Germany) for BHBA. Plasma acetate concentrations were determined as chloroethyl ester derivative on a gas chromatography-flame ionization detector instrument (GC-FID, Series 2010, Shimadzu Corp., Kyoto, Japan) on a 25 m RTX-1701 column according to Kristensen *et al.*[45]. CCK was measured using a double antibody radioimmuno assay (Wizard 1470 Automatic Gamma Counter, Perkin Elmer, Waltham, USA) according to Relling and Reynolds[46].

Ruminal fluid. Ruminal fluid was collected immediately before animals were transferred into respiration chambers using an esophageal tube system attached to a vacuum pump. Rumen fluid was sieved (mash size 0.7–1.0 mm) and pH was determined using a glass electrode (Roth, Karlsruhe, Germany). The filtrate was centrifuged at 4 °C for 10 min at 4,000 g. Rumen fluid short-chain fatty acid concentrations were measured in the supernatant using a gas chromatograph (GC-FID, Series 17A, Shimadzu Corp., Kyoto, Japan), equipped with a 25 m FFAP column according to Ryan[47].

Gastrointestinal passage rate and digestibility. Analysis of gastrointestinal passage rate was performed in week 6 p.p. Cows were fed 15 g titanium dioxide (TiO_2; pelleted with corn meal in a ratio of 1:2) twice daily for five days. Starting four days after begin of TiO_2 application, feces samples (approx. 600 g) were taken twice daily at 08:30 h and 16:00 h for five days. Daily samples were pooled and stored at −20 °C until drying and analysis. Feces DM was determined after drying the samples at 65 °C for 72 h. TiO_2 was analyzed according to the method described by Brandt and Allam[48]. For the calculation of the MRT of digesta we used the formula described by Voigt *et al.*[49]:

$$MRT = \frac{\sum_{i=1}^{n}(t_i x m_i)}{\sum_{i=1}^{n} m_i} \quad (9)$$

where m_i is the amount of TiO_2 excreted at the *i*th sample and t_i is the time elapsed between dosing and the mid-point of the *i*th collection interval.

The Kjeldahl procedure was used to determine fecal N content with small modifications[50]. Fecal CP was calculated on an organic matter (OM) basis:

$$\text{CP (g/kg OM)} = \text{N (g/kg OM)} \times 6.25. \quad (10)$$

The OM digestibility of the ration was estimated using the formula of Lukas *et al.*[51]:

$$\text{OM digestibility, \%} = 79.76\text{–}107.7^{(-0.01515 * \text{fecal CP (g/kg OM)})}. \quad (11)$$

Data handling and Statistics. Animals were grouped according to their plasma NEFA concentrations at the time of the respiration chamber measurements in early lactation (week 5 ± 0.2 p.p.) to the ten highest mobilizing (HM; plasma NEFA > 580 μmol/L) and the ten lowest mobilizing (LM; plasma NEFA < 580 μmol/L) cows. Weekly means of BFT and BCS were calculated from two consecutive measurements performed at 14 day intervals.

The data analysis was generated using SAS software, Version 9.3 for Windows, SAS Institute Inc., Cary, NC, USA. Variables BCS, BFT, DMI, CH_4, milk, rumen fluid and plasma parameters were analyzed by repeated measurement ANOVA with the MIXED procedure of SAS/STAT software. The ANOVA models contained the fixed factors group (levels: HM, LM), time and the interaction group × time. The levels of the repeated variable time for milk data are weeks 5 p.p., 13 p.p., 42 p.p. and, for all other variables weeks 4 a.p., 5 p.p., 13 p.p., 42 p.p. Additionally, the levels of the repeated variable time for the variable DMI are weekly from 6 weeks a.p. to 14 weeks p.p, for the variable BW weekly from 4 weeks a.p. to 14 weeks p.p., and for the variables BCS, BFT, ECM are weekly from 1 week to 14 weeks p.p. Repeated measures on the same animal were taken into account by the REPEATED statement of the MIXED procedure and the type for the block diagonal residual covariance matrix was unstructured for the calculations with three or four time points and compound symmetry for the weekly calculations. Least-squares means (LSM) and their standard errors (SE) were computed for each fixed effect in the models, and all pairwise differences of LS-means were tested by the Tukey-Kramer procedure. The SLICE statement of the MIXED procedure was used for performing partitioned analyses of the LS-means for the interaction group × time. The MIXED procedure was also used to test the variable area under the curve (AUC) for the fixed factor group.

Linear relationships between variables NEFA, ruminal acetate and plasma acetate, CH_4, CH_4/DMI and CH_4/NDF in week 5 p.p. were estimated and tested with the REG procedure of SAS/STAT software. Exponential relationships between variables CCK and CH_4, CH_4/DMI, CH_4/NDF in week 5 p.p. were estimated and tested with the NLIN procedure of SAS/STAT software using the model formula $y = a + b^{(cx)}$. Effects and differences were declared significant if $P < 0.05$ and trends as $P < 0.1$.

Conclusions

We investigated the individual CH_4 production of first lactating dairy cows to characterize the impact of body fat mobilization during the exceptional metabolic state in early lactation on ruminal fermentation characteristics. While ruminal acetate concentrations proved to be unrelated to levels of CH_4 production, cows with high body fat mobilization and low plasma acetate concentrations tended to have lower CH_4/DMI and CH_4/NDF production rates in early lactation than less mobilizing cows. Lower plasma CCK concentrations in early lactation accounts for increased rumen motility and a faster digesta passage through the rumen (without affecting whole rumen-intestinal MRT) and therefore the lower CH_4 yield of high mobilizing cows. The direct relationship between plasma CCK concentrations and CH_4 yield as well as the inverse relationship between plasma NEFA concentrations and CH_4 yield offers a new perspective on the interaction between host metabolism and rumen fermentation.

References

1. Demeyer, D. I. & Van Nevel, C. J. Methanogenesis and integrated part of carbohydrate fermentation and its control. in: McDonald, I. W. & Warner, A. C. I. (eds.) *Digestion and Metabolism in the Ruminant.* The University of New England Publishing Unit, Armidale, N.S.W., pp. 366–382, ISBN 0-85834-086-0 (1975).
2. Casanas, M. A. A. *et al.* Methyl-coenzyme M reductase A as an indicator to estimate methane production from dairy cows. *J. Dairy Sci.* **98,** 4074–4083, doi: 10.3168/jds.2015-9310 (2015).
3. Mohammed, R., McGinn, S. M. & Beauchemin, K. A. Prediction of enteric methane output from milk fatty acid concentrations and rumen fermentation parameters in dairy cows fed sunflower, flax, or canola seeds. *J. Dairy Sci.* **94,** 6057–6068, doi: 10.3168/jds.2011-4369 (2011).

4. Chilliard, Y., Martin, C., Rouel, J. & Doreau, M. Milk fatty acids in dairy cows fed whole crude linseed, extruded linseed, or linseed oil, and their relationship with methane output. *J. Dairy Sci.* **92,** 5199–5211, doi: 10.3168/jds.2009-2375 (2009).
5. van Lingen, H. J., Crompton, L. A., Hendriks, W. H., Reynolds, C. K. & Dijkstra, J. Meta-analysis of relationships between enteric methane yield and milk fatty acid profile in dairy cattle. *J. Dairy Sci.* **97,** 7115–7132, doi: 10.3168/jds.2014-8268 (2014).
6. Johnson, K. A. *et al.* The effect of oilseeds in diets of lactating cows on milk production and methane emissions. *J. Dairy Sci.* **85,** 1509–1515 (2002).
7. Martin, C., Rouel, J., Jouany, J. P., Doreau, M. & Chilliard, Y. Methane output and diet digestibility in response to feeding dairy cows crude linseed, extruded linseed, or linseed oil. *J. Anim. Sci.* **86,** 2642–2650, doi: 10.2527/jas.2007-0774 (2008).
8. Ricci, P., Rooke, J. A., Nevison, I. & Waterhouse, A. Methane emissions from beef and dairy cattle: Quantifying the effect of physiological stage and diet characteristics. *J. Anim. Sci.* **91,** 5379–5389, doi: 10.2527/jas.2013-6544 (2013).
9. Lerch, S. *et al.* Rapeseed or linseed in dairy cow diets over 2 consecutive lactations: Effects on adipose fatty acid profile and carry-over effects on milk fat composition in subsequent early lactation. *J. Dairy Sci.* **98,** 1005–1018, doi: 10.3168/jds.2014-8578 (2015).
10. Vanlierde, A. *et al.* Hot topic: Innovative lactation-stage-dependent prediction of methane emissions from milk mid-infrared spectra. *J Dairy Sci* **98,** doi: 10.3168/jds.2014-8436 (2015).
11. Park, A. F. *et al.* Characterization of ruminal dynamics in Holstein dairy cows during the periparturient period. *J. Anim. Physiol. Anim. Nutr.* **95,** 571–582, doi: 10.1111/j.1439-0396.2010.01085.x (2011).
12. Adewuyi, A. A., Gruys, E. & van Eerdenburg, F. Non esterified fatty acids (NEFA) in dairy cattle. A review. *Vet. Q.* **27,** 117–126 (2005).
13. Palmquist, D. L. In *Advanced Dairy Chemistry Volume 2 Lipids* (eds Fox, P. F. & McSweeney, P. L. H.) Ch. 2, 43–92, ISBN 978-0-387-28813-0 (Springer, US, 2006).
14. Borner, S. *et al.* Plasma ghrelin is positively associated with body fat, liver fat and milk fat content but not with feed intake of dairy cows after parturition. *J. Endocrinol.* **216,** 217–229, doi: 10.1530/joe-12-0384 (2013).
15. Kendall, P. E. & McLeay, L. M. Excitatory effects of volatile fatty acids on the *in vitro* motility of the rumen of sheep. *Res. Vet. Sci.* **61,** 1–6, doi: 10.1016/s0034-5288(96)90101-0 (1996).
16. Storm, A. C., Kristensen, N. B. & Hanigan, M. D. A model of ruminal volatile fatty acid absorption kinetics and rumen epithelial blood flow in lactating Holstein cows. *J. Dairy Sci.* **95,** 2919–2934, doi: 10.3168/jds.2011-4239 (2012).
17. Dijkstra, J. Production and absorption of volatile fatty-acids in the rumen. *Livestock Production Science* **39,** 61–69, doi: 10.1016/0301-6226(94)90154-6 (1994).
18. Robinson, D. L., Goopy, J. & Hegarty, R. S. Can rumen methane production be predicted from volatile fatty acid concentrations? *Animal Production Science* **50,** 630–636, doi: 10.1071/an09214 (2010).
19. Hassanat, F. *et al.* Replacing alfalfa silage with corn silage in dairy cow diets: Effects on enteric methane production, ruminal fermentation, digestion, N balance, and milk production. *J. Dairy Sci.* **96,** 4553–4567, doi: 10.3168/jds.2012-6480 (2013).
20. Benchaar, C. *et al.* Effects of increasing amounts of corn dried distillers grains with solubles in dairy cow diets on methane production, ruminal fermentation, digestion, N balance, and milk production. *J. Dairy Sci.* **96,** 2413–2427, doi: 10.3168/jds.2012-6037 (2013).
21. Lettat, A., Hassanat, F. & Benchaar, C. Corn silage in dairy cow diets to reduce ruminal methanogenesis: Effects on the rumen metabolically active microbial communities. *J. Dairy Sci.* **96,** 5237–5248, doi: 10.3168/jds.2012-6481 (2013).
22. Benchaar, C., Rivest, J., Pomar, C. & Chiquette, J. Prediction of methane production from dairy cows using existing mechanistic models and regression equations. *J. Anim. Sci.* **76,** 617–627, doi: /1998.762617x (1998).
23. Okine, E. K., Mathison, G. W., Kaske, M., Kennelly, J. J. & Christopherson, R. J. Current understanding of the role of the reticulum and reticulo-omasal orifice in the central of digesta passage from the ruminoreticulum of sheep and cattle. *Canadian Journal of Animal Science* **78,** 15–21, doi: 10.4141/A97-021 (1998).
24. Sauvant, D. & Noziere, P. The quantification of the main digestive processes in ruminants: the equations involved in the renewed energy and protein feed evaluation systems. *Inra Productions Animales* **26,** 327–346, doi: 10.1017/S1751731115002670 (2013).
25. Pimentel, M. *et al.* Methane, a gas produced by enteric bacteria, slows intestinal transit and augments small intestinal contractile activity. *Am. J. Physiol.-Gastroint. Liver Physiol.* **290,** G1089–G1095, doi: 10.1152/ajpgi.00574.2004 (2006).
26. Kramer, M., Lund, P. & Weisbjerg, M. R. Rumen passage kinetics of forage- and concentrate-derived fiber in dairy cows. *J. Dairy Sci.* **96,** 3163–3176, doi: 10.3168/jds.2012-6146 (2013).
27. Warner, D., Dijkstra, J., Hendriks, W. H. & Pellikaan, W. F. Passage kinetics of C-13-labeled corn silage components through the gastrointestinal tract of dairy cows. *J. Dairy Sci.* **96,** 5844–5858, doi: 10.3168/jds.2013-6694 (2013).
28. Goopy, J. P. *et al.* Low-methane yield sheep have smaller rumens and shorter rumen retention time. *Br. J. Nutr.* **111,** 578–585, doi: 10.1017/s0007114513002936 (2014).
29. Dias, R. S. *et al.* Relationships between chewing behavior, digestibility, and digesta passage kinetics in steers fed oat hay at restricted and ad libitum intakes. *J. Anim. Sci.* **89,** 1873–1880, doi: 10.2527/jas.2010-3156 (2011).
30. Oshita, T., Sudo, K., Nonaka, K., Kume, S. & Ochiai, K. The effect of feed regimen on chewing time, digesta passage rate and particle size distribution in Holstein non-lactating cows fed pasture ad libitum. *Livest. Sci.* **113,** 243–250, doi: 10.1016/j.livsci.2007.04.001 (2008).
31. Yarandi, S. S., Hebbar, G., Sauer, C. G., Cole, C. R. & Ziegler, T. R. Diverse roles of leptin in the gastrointestinal tract: Modulation of motility, absorption, growth, and inflammation. *Nutrition* **27,** 269–275, doi: 10.1016/j.nut.2010.07.004 (2011).
32. Muller, T. D. & Tschop, M. H. Ghrelin - a key pleiotropic hormone-regulating systemic energy metabolism. *Endocr Dev* **25,** 91–100, doi: 10.1159/000346590 (2013).
33. Kermani, R. Z. & Rezaiee, A. The effects of intravenous cholecystokinin, secretin and pentagastrin on electromyographic activity of the rumen in sheep. *Regul. Pept.* **45,** 371–377, doi: 10.1016/0167-0115(93)90363-d (1993).
34. Grovum, W. L. Factors affecting the voluntary intake of food by sheep 3. The effect of intravenous infusions of gastrin, cholecystokinin and secretin on motility of the reticulo-rumen and intake *Br. J. Nutr.* **45,** 183–201, doi: 10.1079/bjn19810091 (1981).
35. Pires, J. A. A., Delavaud, C., Faulconnier, Y., Pomies, D. & Chilliard, Y. Effects of body condition score at calving on indicators of fat and protein mobilization of periparturient Holstein-Friesian cows. *J. Dairy Sci.* **96,** 6423–6439, doi: 10.3168/jds.2013-6801 (2013).
36. Cuche, G., Cuber, J. C. & Malbert, C. H. Ileal short-chain fatty acids inhibit gastric motility by a humoral pathway. *Am. J. Physiol.-Gastroint. Liver Physiol.* **279,** G925–G930, WOS:000090062700011 (2000).
37. Langhans, W., Leitner, C. & Arnold, M. Dietary fat sensing via fatty acid oxidation in enterocytes: possible role in the control of eating. *Am. J. Physiol.-Regul. Integr. Comp. Physiol.* **300,** R554–R565, doi: 10.1152/ajpregu.00610.2010 (2011).
38. GfE (German Society of Nutrition Physiology) Ausschuss für Bedarfsnormen der Gesellschaft für Ernährungsphysiologie. *Empfehlungen zur Energie- und Nährstoffversorgung der Milchkühe und Aufzuchtrinder (Recommended energy and nutrient supply for dairy cows and growing cattle*). Vol. 13, ISBN-13: 978-3769005912 (DLG-Verlag, 2004).
39. Boguhn, J., Kluth, H., Steinhöfel, O., Peterhänsel, M. & Rodehutscord, M. Nutrient digestibility and prediction of metabolizable energy in total mixed rations for ruminants. *Archives of Animal Nutrition* **57,** 253–266, doi: 10.1080/00039420310001594405 (2003).
40. Schroder, U. J. & Staufenbiel, R. Methods to determine body fat reserves in the dairy cow with special regard to ultrasonographic measurement of backfat thickness. *J. Dairy Sci.* **89,** 1–14, doi: 10.3168/jds.S0022-0302(06)72064-1 (2006).
41. Reist, M. *et al.* Concentrate feeding strategy in lactating dairy cows: Metabolic and endocrine changes with emphasis on leptin. *J. Dairy Sci.* **86,** 1690–1706, doi: 10.3168/jds.S0022-0302(03)73755-2 (2003).

42. Derno, M. *et al.* Short-term feed intake is regulated by macronutrient oxidation in lactating Holstein cows. *J. Dairy Sci.* **96,** 971–980, doi: 10.3168/jds.2012-5727 (2013).
43. Chwalibog, A., Jensen, K. & Thorbek, G. Oxidation of nutrients in bull calves treated with beta-adrenergic agonists. *Archives of Animal Nutrition-Archiv Fur Tierernahrung* **49,** 255–261, ISSN: 0003-942X (1996).
44. Blummel, M., Aiple, K. P., Steingass, H. & Becker, K. A note on the stoichiometrical relationship of short chain fatty acid production and gas formation *in vitro* in feedstuffs of widely differing quality. *Journal of Animal Physiology and Animal Nutrition-Zeitschrift Fur Tierphysiologie Tierernahrung Und Futtermittelkunde* **81,** 157–167, doi: 10.1046/j.1439-0396.1999.813205.x (1999).
45. Kristensen, N. B., Pierzynowski, S. G. & Danfaer, A. Net portal appearance of volatile fatty acids in sheep intraruminally infused with mixtures of acetate, propionate, isobutyrate, butyrate, and valerate. *J. Anim. Sci.* **78,** 1372–1379, doi: /2000.7851372x (2000).
46. Relling, A. E. & Reynolds, C. K. Feeding rumen-inert fats differing in their degree of saturation decreases intake and increases plasma concentrations of gut peptides in lactating dairy cows. *J. Dairy Sci.* **90,** 1506–1515, doi: 10.3168/jds.S0022-0302(07)71636-3 (2007).
47. Ryan, J. P. Determination of volatile fatty-acids and some related compounds in ovine rumen fluid, urine, and blood-plasma, by gas-liquid-chromatography. *Analytical Biochemistry* **108,** 374–384, doi: 10.1016/0003-2697(80)90602-8 (1980).
48. Poppe, S. & Allam. Workshop zu Problemen der Verdauungsphysiologie beim Wiederkäuer (Rostock, 16.-17. 1. 1986). *Arch. Anim. Nutr* **37,** 451–466, doi: 10.1080/17450398709425369 (1987).
49. Voigt, J., Jentsch, W., Kuhla, S., Matthes, H. D. & Derno, M. Rumen fermentation and retention time of the digests in growing cattle of the breeds Black-White Dairy Cattle, Galloway, and Highland. *Arch. Tierz.-Arch. Anim. Breed.* **43,** 609–620, WOS:000165330200007 (2000).
50. Glindemann, T., Tas, B. M., Wang, C., Alvers, S. & Susenbeth, A. Evaluation of titanium dioxide as an inert marker for estimating faecal excretion in grazing sheep. *Anim. Feed Sci. Technol.* **152,** 186–197, doi: 10.1016/j.anifeedsci.2009.04.010 (2009).
51. Lukas, M., Sudekum, K. H., Rave, G., Friedel, K. & Susenbeth, A. Relationship between fecal crude protein concentration and diet organic matter digestibility in cattle. *J. Anim. Sci.* **83,** 1332–1344, doi: /2005.8361332x (2005).

Acknowledgements

We thank C. Arlt, H. Pröhl, U. Wiedemuth, Dr. T. Viergutz, R. Grahl and the staff at the 'Tiertechnikum' (FBN Dummerstorf) for biochemical sample analysis, sample collection and assistance with animal care. This study was conducted in frame of the JPI FACCE program and financially supported by the German Federal Ministry of Food and Agriculture (BMBL) through the Federal Office for Agriculture and Food (BLE), grant number 2814ERA04A. Furthermore, part of this study was supported by the Deutsche Forschungsgemeinschaft (DFG; KU 1956/4-1). The publication of this article was funded by the Open Access fund of the Leibniz Association.

Author Contributions

A.B. and B.K. wrote the main manuscript text. Figures and tables were created by A.B., H.M.H. and B.K. designed the study and performed plasma and rumen fluid analyses. M.D. performed the methane measurements, A.S. analyzed the passage rate and digestibility, and A.T. performed the statistical calculations.

Additional Information

Supplementary information accompanies this paper at http://www.nature.com/srep

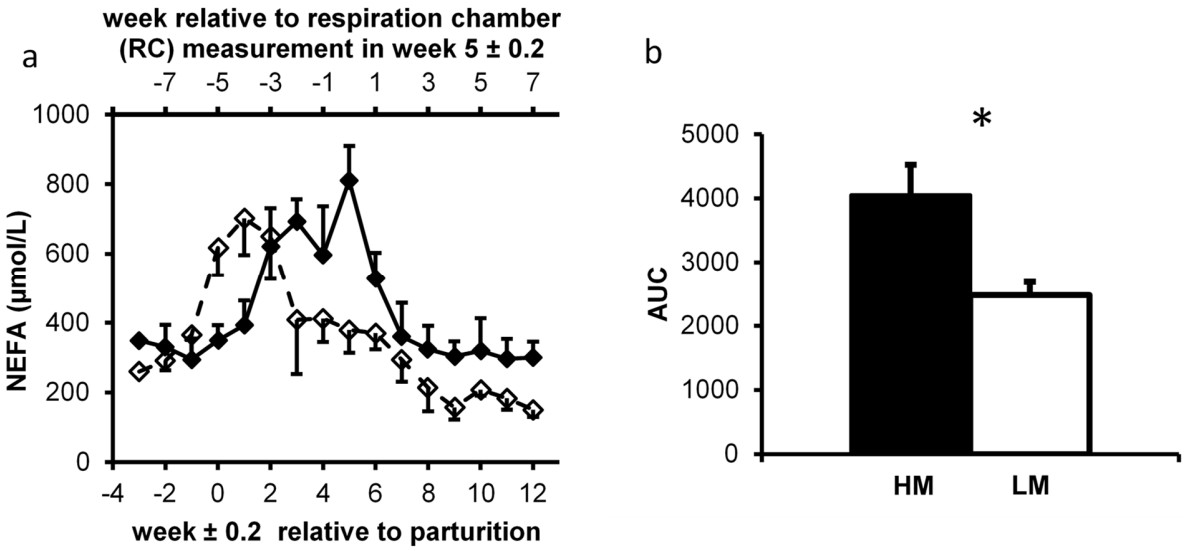
Competing financial interests: The authors declare no competing financial interests.

How to cite this article: Bielak, A. *et al.* Body fat mobilization in early lactation influences methane production of dairy cows. *Sci. Rep.* **6**, 28135; doi: 10.1038/srep28135 (2016).

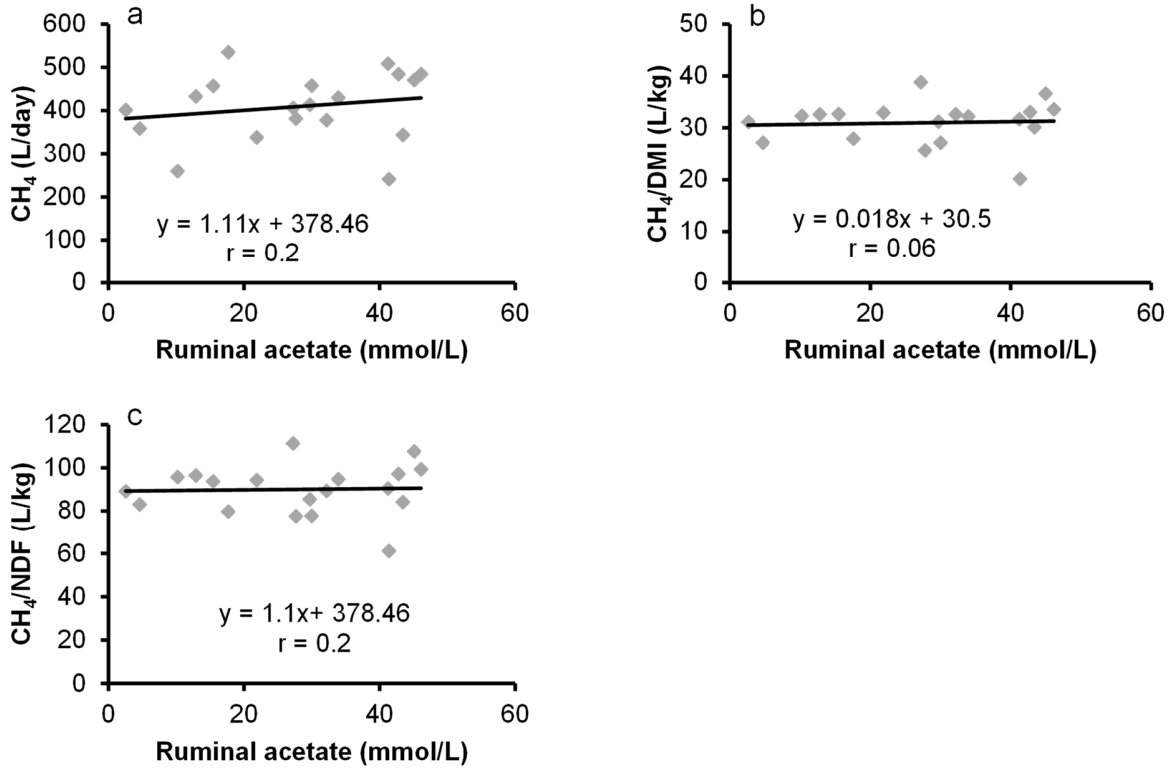
3.1.1 Supplement first Publication

Body fat mobilization in early lactation influences methane production of dairy cows

A. Bielak, M. Derno, A. Tuchscherer, H.M. Hammon, A. Susenbeth, B. Kuhla



Supplemental Figure S1.
a: Plasma NEFA of high (♦, solid line; n = 10) and low mobilizing (◊, dashed line; n = 10) cows in relation to the respiration chamber measurement in early lactation, and in relation to weeks relative to parturition. ANOVA calculated time×group interaction $P < 0.001$.
b: Area under the curve (AUC) of the graph in Figure 1a calculated for high (■; n = 10) and low mobilizing (□; n = 10) cows for the time period 2 weeks before until 6 weeks after the respiration chamber measurement in week 5 ± 0.2 post partum. $P = 0.007$
Data in bar chart is shown as LSM ± SE.



Supplemental Figure S2. Linear regression between ruminal acetate concentration and daily CH_4 production either expressed as L/d, L/kg DMI, or L/kg NDF, respectively (n=19).
a: slope *P* = 0.42
b: slope *P* = 0.80
c: slope *P* = 0.42

Supplemental Table S1. Animal and performance data of high mobilizing (HM) and low mobilizing (LM) cows during stays in respiration chamber

	4 weeks a.p.			5 weeks p.p.			13 weeks p.p.			42 weeks p.p.			Statistics ANOVA, *P* values		
Group	HM	LM	STE	HM	LM	STE	HM	LM	STE	HM	LM	STE	Time	Group	Time×Group
Body weight (kg)	626.4	631.9	±11.7	568.7	552.9	±16.1	557.7	553.8	±15.8	627.7	636.3	±20.0	< 0.001	0.88	0.65
DMI (kg)	6.5	7.6	±0.7	13.5	12.9	±0.8	14.2	14.8	±0.6	15.8	14.6	±0.7	< 0.001	0.97	0.09
BCS	-	-		2.8	2.7	±0.2	2.7	2.7	±0.2	-	-		0.37	0.71	0.44
BFT (cm)	-	-		1.5†	1.2†	±0.1	1.0	0.9	±0.1	-	-		< 0.001	0.21	0.03
Milk yield (kg/day)	-	-		27.03	26.22	±1.37	28.16	26.81	±1.16	23.49	22.43	±1.42	< 0.001	0.47	0.94
Milk fat (%)	-	-		4.69	4.95	±0.37	3.72*	4.34*	±0.13	4.18	4.71	±0.23	0.003	0.06	0.74
Milk protein (%)	-	-		3.07	3.09	±0.08	3.15	3.36	±0.05	3.78	3.85	±0.08	< 0.001	0.17	0.09
Milk lactose (%)	-	-		4.91	4.94	±0.06	5.00	4.99	±0.05	4.94	4.81	±0.05	0.002	0.66	0.06
ECM (kg/day)	-	-		28.81	28.70	±1.48	27.05	28.14	±1.18	24.68	25.18	±1.36	0.002	0.79	0.80

† P = 0.07 Tukey-Kramer
* P = 0.03 Tukey-Kramer

3.2 Second Publication

Variations in methane yield and microbial community profiles in the rumen of dairy cows as they pass through stages of first lactation

Tamsin Lyons* & Anita Bielak*, Evelyn Doyle, Björn Kuhla

*These authors contributed equally to this work

Journal of Dairy Science 2018; 101:5102-5114

Own contribution:

- oral rumen fluid collection in late lactation (trial was already initiated before I joined the research group at the FBN)
- record of animal performance and CH_4 data
- first surveying statistical analysis of data (t-Test) as a basis for animal grouping
- statistical evaluation of diet composition, feed intake, SCFA concentrations, selected genus abundances
- coordination and execution of rumen fluid sample preparation and shipment to UCD, Ireland for microbiological Analysis
- selection of topic, data content and choice of presentation of the publication
- compilation of tables and supplementary tables
- manuscript writing in cooperation with T. Lyons, B. Kuhla and E. Doyle
- contribution to revision of manuscript after first review

J. Dairy Sci. 101:5102–5114
https://doi.org/10.3168/jds.2017-14200

Variations in methane yield and microbial community profiles in the rumen of dairy cows as they pass through stages of first lactation

Tamsin Lyons,*[1] Anita Bielak,†[1] Evelyn Doyle,*[2] and Björn Kuhla*[2]
*School of Biology and Environmental Science & Earth Institute, University College Dublin, Dublin, D04 N2E5, Ireland
†Institute of Nutritional Physiology "Oskar Kellner," Leibniz Institute for Farm Animal Biology (FBN), Dummerstorf, 18196, Germany

ABSTRACT

Considerable interest exists both from an environmental and economic perspective in reducing methane emissions from agriculture. In ruminants, CH_4 is produced by a complex community of microorganisms that is established in early life but can be influenced by external factors such as feed. Although CH_4 emissions were thought to be constant once an animal reached maturity, recent studies have shown that CH_4 yield significantly increases from early to late lactation in dairy cows. The aim of this study was to test the hypothesis that increases in CH_4 yield over the lactation cycle are related to changes in rumen microbial community structure. Nine cows were monitored throughout their first lactation cycle. Methane and dry matter intake were measured to calculate CH_4 per dry matter intake (CH_4 yield) and ruminal fluid was collected during early, mid, and late lactation. A significant difference in bacterial and archaeal community structure during early and late lactation was observed. Furthermore, when ruminal short-chain fatty acid concentrations were measured, the ratio of acetate and butyrate to propionate was significantly higher in late lactation compared with early lactation. Propionate concentrations were higher in cows with low CH_4 yield during late lactation, but no differences were observed in bacterial or archaeal community structures. *Prevotella* dominated the rumen of cows followed by *Succinclasticum*; *Treponema*, *Fibrobacter*, *Ruminococcus*, and *Bifidobacterium* were also in high abundance relative to other bacterial genera. In general, positive correlations were stronger between the most relatively abundant bacterial genera and acetate and butyrate concentrations in the cows with high CH_4 and weaker between these genera and propionate concentration. This study indicates that increased CH_4 yield in late lactation is reflected in significant changes in microbial community structure.
Key words: dairy cow, methane, rumen microbiome, methanogens, short-chain fatty acids

INTRODUCTION

The microbial ecosystem in the rumen of cattle is highly complex, consisting of many microbial species acting together to convert plant materials into nutrients, primarily short-chain fatty acids (**SCFA**). These fermentation products, predominantly acetate, propionate, and butyrate, are essential for host maintenance and growth (Hobson and Stewart, 1997; Van Houtert, 1993). However, bacterial fermentation also creates by-products, namely hydrogen and carbon dioxide, that cannot be used by the host and are converted to CH_4 by methanogenic archaea (Hobson and Stewart, 1997). The extent of CH_4 produced by archaea is therefore dependent on the level of metabolic by-products formed by other microbial species.

Methane is of no energetic use to the host and is released into the environment through eructation (Dougherty, 1968; Anderson et al., 1987). This is a major environmental problem, as CH_4 is a potent greenhouse gas (**GHG**) that contributes to global warming. Global CH_4 emissions originating from enteric fermentation account for 17% of global CH_4 emissions, whereas dairy cows are estimated to contribute 3.3% to overall anthropogenic GHG emissions (Knapp et al., 2014). In addition, CH_4 represents a 5 to 7% loss of feed energy for dairy cows on commonly fed diets (Arndt et al., 2015), which negatively affects animal productivity. Feed energy loss due to CH_4 is predominantly influenced by the level of feed intake and dietary composition and, to a lesser extent, by feed additives or antimethanogen vaccines (Atakora et al., 2011; Knapp et al., 2014; Sun et al., 2015; Roehe et al., 2016). Implementing strategies to reduce CH_4 emissions from dairy cows would thus be beneficial from both an environmental and economic perspective.

Received November 17, 2017.
Accepted February 13, 2018.
[1]These authors contributed equally to this work.
[2]Corresponding authors: b.kuhla@fbn-dummerstorf.de and evelyn.doyle@ucd.ie

In the livestock industry, GHG mitigation strategies have largely focused on diet and dietary supplements as a means of reducing CH_4 emissions (de Menezes et al., 2011; Ellison et al., 2014; Cobellis et al., 2016). Although diet has a strong effect on rumen microbial community structure, this effect is often inconsistent and short-lived, making it difficult to create a universal dietary strategy (Yáñez-Ruiz et al., 2015). Furthermore, it is not well understood what type of microbial community profile leads to low CH_4 production. Host genetics have also been shown to influence rumen microbial community structure and CH_4 emissions (Weimer et al., 2010; Goopy et al., 2014). Ranking of sires based on CH_4 yield did not change when they were fed a high concentrate-based diet compared with a medium concentrate-based diet (Roehe et al., 2016), indicating that the influence of host genetics on CH_4 yield did not change with diet.

As microbes in the rumen are responsible for producing the CH_4, information on the factors shaping microbial community composition is needed to inform strategies designed to manipulate these communities to achieve long-term, consistent reduction of enteric CH_4 production. There has been considerable focus on the rumen microbiome of cows in the first year of life, as the rumen microbiome is thought to be relatively stable once mature (Rey et al., 2014; Li et al., 2012; Jami et al., 2013). It has been suggested that early life may represent a window during which the establishing rumen microbiome can be manipulated with long-term effects (Yáñez-Ruiz et al., 2015; Abecia et al., 2014). However, CH_4 emissions vary during different periods of an animal's lifetime; for example, CH_4 levels have been reported to increase by up to 35% from early to late lactation (Bielak et al., 2016; Garnsworthy et al., 2012), but this increase is primarily due to an increase in DMI, the main driver of the CH_4 production. Targeting specific periods of increased CH_4 production may facilitate the development of short-term interventions that can contribute to an overall strategy for the mitigation of CH_4 levels from agriculture. Jewell et al. (2015) reported that bacterial community structure in cows was dynamic over 2 lactation cycles, with specific operational taxonomic units (**OTU**) associated with high and low milk production efficiency; however, those authors did not monitor CH_4 emissions during their study. The aim of the current study was to monitor CH_4 emission per unit of DMI (CH_4/DMI = CH_4 yield), SCFA, and microbial community dynamics of cows during lactation to determine if any observed changes in CH_4 yields across lactation stages were driven by changes in rumen microbiome structure. It was hypothesized that spikes in CH_4 yields would be accompanied by a marked difference in bacterial and archaeal community composition compared with periods when CH_4 yields were lower. Nine cows from the same dairy herd were tracked during their first lactation cycle, with CH_4 production measured in respiration chambers and ruminal fluid samples collected for microbial and SCFA analysis during early, mid, and late lactation (5, 13, and 42 wk postpartum, respectively). In addition, rumen microbial profiles of cows from the dairy herd that were identified as either producing high or low CH_4 yield during the late lactation stage were compared to determine if significant differences in their rumen microbiome could be observed.

MATERIALS AND METHODS

Animals and Feed

A group of 9 German Holstein dairy cows of the same age in first lactation were used for the present study. All animals were treated in accordance with the State Government guidelines for the use of animals as experimental subjects in Mecklenburg-Western Pomerania. All experimental protocols were approved by the local animal ethics committee (Landesamt für Landwirtschaft, Lebensmittelsicherheit und Fischerei Mecklenburg-Vorpommern; approval No. 7221.1–1.-053/13).

Animals were kept in a freestall of the Leibniz Institute for Farm Animal Biology (FBN) in Dummerstorf, Germany, with ad libitum access to fresh water and feed offered as TMR. To investigate whether the microbial community profile changes over the course of lactation and not in response to an altered ration composition, the diet was formulated to ensure a constant nutrient and energy composition over the course of lactation. Individual daily feed intake was recorded via automated weighing troughs and the TMR was sampled at each respiration chamber measurement for the determination of DM and nutrient composition. Feed analysis was performed by the Landwirtschaftliche Untersuchungs- und Forschungsanstalt (LUFA) in Rostock, Germany. Dietary ingredient and nutrient compositions are listed in Supplemental Table S1 (https://doi.org/10.3168/jds.2017-14200).

Methane Measurements in Respiration Chambers

Cows calved between October 2014 and April 2015. In wk 5, 13, and 42 (±0.2 wk; SE) of lactation animals were transferred from the freestall into open-circuit respiration chambers as described previously (Bielak et al., 2016). Before the actual gas exchange measure-

ments, the animals were adapted several times to the rubber mat-floored chambers to ensure normal feeding and lying behavior. Prior to entering the chamber, cows were weighed on an electronic scale. Animals were transferred into the chambers around midday to allow overnight gas equilibration. On the next day, gas exchange measurements began at 0700 h and lasted for the next 48 h. The daily mean of the measuring period lasting from 0700 to 0659 h of the next day was calculated. Two adjacent chambers separated by a common glass window ensured visual contact with the neighboring animal. In the chambers, cows were tied and feed intake was measured by feed disappearance from a feeding trough connected to an electronic scale. Cows had ad libitum access to water. Animals were fed twice daily at 0730 and 1500 h and milked at 0700 and 1700 h. Milk samples from the preceding evening and the morning milking were pooled and milk composition was analyzed by the Landeskontrollverband für Leistungs- und Qualitätsprüfung Mecklenburg-Vorpommern e.V. (Güstrow, Germany). Energy-corrected milk yield was calculated as

$$\text{ECM (kg)} = [0.038 \times \text{crude fat (g)} + 0.024 \times \text{CP (g)} + 0.017 \times \text{lactose (g)}] \times \text{milk (kg)}/3.14.$$

The temperature within the chamber was kept at 15°C with a dark–light cycle from 0600 to 1900 h. Data collection was automatically conducted every 6 min. The CH_4 concentration in the air flow out of the chambers was measured by infrared absorption (Sidor, Sick Maihak GmbH, Reute, Germany) as described recently (Derno et al., 2013). The recovery test for CH_4 revealed 99.8%.

Rumen Fluid Sampling and Analyses

Rumen fluid sampling was conducted as described by Bielak et al. (2016). About 2 h before the transfer into respiration chambers, animals were sampled for rumen fluid via an esophageal tube system connected to a vacuum pump. The first ~100 mL was discarded to eliminate potential contamination with saliva. The subsequent 200 mL of fluid was collected and immediately stored on ice until processing. After sieving (mash size = 0.7–1.0 mm) and pH measurement using a glass electrode (Roth, Karlsruhe, Germany), the filtrate was centrifuged at 4,000 × g and 4°C for 10 min and the resulting pellet was immediately frozen at −80°C. The ruminal fluid sample was freeze-dried before DNA extraction. In the supernatant, SCFA concentrations were analyzed using a GC (GC-FID, Series 17A, Shimadzu Corp., Kyoto, Japan) equipped with a 25-m free fatty acid phase column according to (Ryan, 1980).

DNA Extraction and Amplicon Sequencing

The DNA of the rumen samples was extracted from all 9 animals at sampling time points at wk 5, 13, and 42 postpartum (27 samples in total) using the PCSA method described by Lueders et al. (2004), with phenol chloroform added before the bead beating step. The DNA concentration and purity were assessed using a Nanodrop spectrophotometer (Thermo Scientific, Waltham, MA), and DNA was stored at −20°C until needed. Bacterial and archaeal community structures were determined using amplicon sequencing on an Illumina Miseq platform (Illumina, San Diego, CA), using the method described by Kozich et al. (2013). Briefly, once DNA was extracted and purified, 1 μL of target DNA was added to a well on a 96-well PCR plate already containing 17 μL of Accuprime Pfx Supermix (Invitrogen, Thermo Fisher Scientific, Dublin, Ireland) and 2 μL of a primer set targeting the V4 region of the 16S rRNA gene (Kozich et al., 2013). The PCR conditions consisted of a hot start at 95°C for 2 min, followed by denaturation at 95°C for 20 s, annealing at 55°C for 15 s, and extension at 72°C for 5 min (30 cycles) with a final extension at 72°C for 10 min. The PCR products were visualized on a 1.2% (wt/vol) agarose gel (Roche Diagnostic, Basel, Switzerland); PCR products were then purified and concentrations normalized using the SequalPrep Normalization Plate Kit (Invitrogen) according to manufacturer's instructions (www.thermofisher.com/order/catalog/product/A1051001) and an amplicon pool was created using 5 μL of PCR product from each sample. The concentration of the pool was then determined using a Qubit fluorometer and dsDNA kit (Thermo Fisher Scientific) to ensure that at least 100 ng of DNA was present. The amplicon pool was then sent to the Centre for Genomic Research, University of Liverpool (Liverpool, UK), for sequencing on an Illumina Miseq platform. Sequence files associated with each sample have been submitted to the NCBI Sequence Read Archive (Accession no. PRJNA393984).

MiSeq sequencing data were initially processed using the mothur program v.1.32.1 developed by Schloss et al. (2009). Illumina adapter sequences were trimmed by cutadapt ver.2.1.1 using option -O 3 (Martin, 2011). Sickle ver.1.2 (Joshi and Fass, 2011) was used to further trim the data with a quality score of ≥20. Reads <10 bp after trimming were removed. Each read was then trimmed to a maximum of 275 bp and ambiguous

Table 1. Methane emission and its relation to input and output traits of 9 dairy cows measured in wk 5, 13, and 42 postpartum

Item	wk 5	wk 13	wk 42	*P*-value
CH_4 (L/d)	434.3 ± 50.3[a]	450.9 ± 56.0[a]	540.5 ± 48.4[b]	0.001
CH_4/DMI (L/kg)	32.2 ± 2.3[a]	33.8 ± 3.7[a]	36.7 ± 3.6[b]	0.044
DMI (kg/d)	13.6 ± 0.6[a]	13.41 ± 0.53[a]	14.80 ± 0.40[a]	0.082
ECM (kg/d)	29.47 ± 1.34[a]	27.07 ± 1.11[a]	25.12 ± 1.05[a]	0.019
ECM/DMI (kg/kg)	2.23 ± 0.18	2.05 ± 0.12	1.71 ± 0.10	0.001
CH_4/ECM (L/kg)	15.10 ± 1.01[a]	16.89 ± 0.92[a]	21.98 ± 1.22[b]	<0.001
CH_4/NDF (L/kg)	92.6 ± 8.3[a]	97.2 ± 12.0[a]	75.1 ± 8.5[b]	<0.001
BW (kg)	575.75 ± 12.65[a]	571.28 ± 14.34[a]	647.78 ± 15.99[b]	<0.001

[a,b]Different superscript letters within row indicate significance ($P < 0.05$, Tukey honestly significant difference).

bases were removed. Sequences which contained homopolymer runs >8 bases were discarded. After trimming, identical sequences were grouped into unique sequences. Chimeric sequences were identified using the UCHIME algorithm (Edgar et al., 2011) within mothur and were then removed. Sequences were assigned to OTU using the cluster command and the average neighbor algorithm. All subsequent OTU-based analyses were performed using a cutoff of 0.03. Taxonomy was assigned to all remaining aligned sequences by comparing processed data to the silva128 databases for bacteria and archaea independently (arb-silva.de/silva-license-information; Quast et al., 2013). A rarefaction curve was constructed to ensure sufficient sequencing depth had been achieved (Supplemental Figure S1; https://doi.org/10.3168/jds.2017-14200).

Statistical Analysis

Means for each of the 3 lactation stages were calculated for CH_4 yield, SCFA concentrations, ECM, and CH_4/ECM. An ANOVA and post hoc Tukey HSD tests were carried out within the R software environment (www.r-project.org) using the lsmeans package to analyze differences between means. Multivariate analysis was carried out using Primer-E v.7 software with the Permanova add-on (Quest Research Limited, Auckland, New Zealand). Similarity matrices were constructed for samples using Bray-Curtis similarities on standardized, fourth root-transformed abundance data. Distance-based permutational multivariate ANOVA (Anderson et al., 2001) was then performed to test the null hypothesis that no differences existed in microbial community structure across time or CH_4 yield group at a significance level of $\alpha = 0.05$ based on 9,999 possible permutations. The nonmetric multidimensional scaling plots were constructed to visualize the data. Similarity percentages were calculated using Bray-Curtis similarities to evaluate the level to which each genus contributed to the difference in community structures in the rumen across time.

RESULTS

CH_4 Yield and Performance Parameters

Individual DMI and CH_4 production were measured for each cow (n = 9) during early (5 wk postpartum), mid (13 wk postpartum), and late lactation (42 wk postpartum) to calculate CH_4 yield. Although DMI did not change over time (Table 1), methane yield differed significantly between stages ($P = 0.001$), with highest levels (40.96 L/kg of DMI) recorded during late lactation. Changes in CH_4 yields were observed between early and late stage lactation ($P = 0.002$, Tukey HSD) and between mid and late stage lactation ($P = 0.009$, Tukey-HSD). We found no significant difference between early and mid lactation; therefore, we focused on the mid and late lactation stages to determine possible explanations for the shift in CH_4 yield between these 2 time points (Table 1). The pattern of higher CH_4 yields in mid and late lactation was still apparent when CH_4 was corrected for ECM yield (expressed as CH_4/ECM, L/kg; Table 1). Energy-corrected milk yield and gross feed efficiency (expressed as ECM/DMI) decreased, whereas BW and methane emission intensity (defined as CH_4/ECM) increased from early to late lactation (each $P < 0.05$; Table 1).

Rumen Microbial Community Structures

The DNA was extracted from rumen fluid collected from all cows during early, mid, and late stage lactation and amplicon libraries constructed based on the V4 region of the 16S rRNA gene and sequenced using the Illumina MiSeq platform. Following initial processing, an average of 315,617 good-quality sequences were obtained, with sequence numbers per sample ranging from 45,771 to 1,607,531 (median = 262,413). A total of 346 unique OTU that could be taxonomically classified to genus level were identified across all animals. Twenty-three distinct bacterial phyla were identified in the rumen of cows during all stages of lactation. *Bacteroidetes* (42–48%) and *Firmicutes* (30–31%) dominated

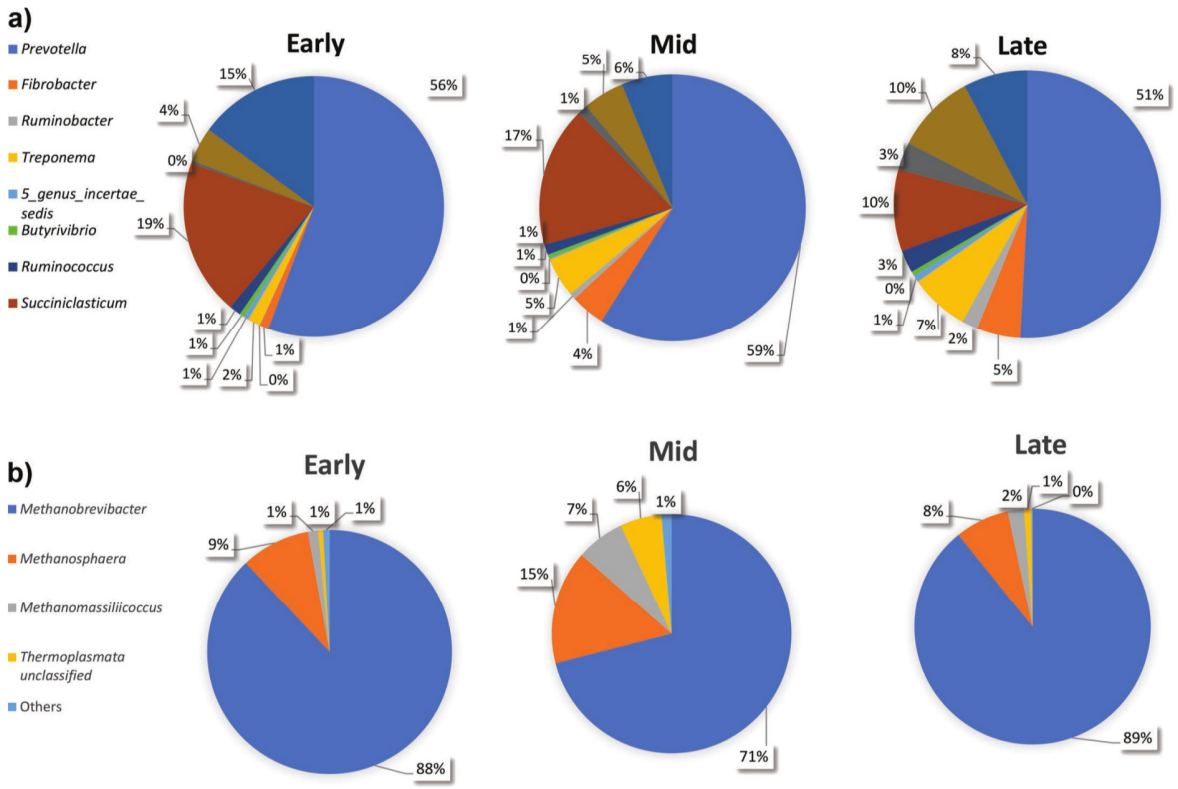


Figure 1. Relative abundance of (a) bacterial and (b) archaeal genera in the rumen of cows in early-, mid-, and late-phase lactation. Similarity percentages analysis of these data (Table 2 and Supplemental Table S4; https://doi.org/10.3168/jds.2017-14200) identified which genera contributed to dissimilarity between bacterial community structures of cows in mid- and late-stage lactation. Color version available online.

the rumen microbiome of cows at all lactation stages. *Proteobacteria* were also present in relatively high abundance (5–11%) across all stages (Figure 1a). Of the bacterial genera detected, *Prevotella* was by far the relatively most abundant genus, accounting for 51 to 59% of total bacterial sequences, with *Succiniclasticum* accounting for a further 10 to 19%. The relative abundances of *Treponema*, *Fibrobacter*, *Ruminococcus*, and SR1 genus *incertae sedis* were also high compared with other bacterial genera. It should be noted that 1.9% of sequences detected in the rumen of cows could not be taxonomically assigned at any level and differences observed were not due to any bias associated with DNA recovery levels.

Only 8 genera of archaea were identified in the rumen of cows across all lactation stages. *Methanobrevibacter* was by far the most relatively abundant (71–89%), followed by *Methanosphaera* (8–15%). The relative abundance of *Methanomassiliicoccus* and an unclassified genus of *Thermoplasmata* appeared to increase as lactation stage advanced (Figure 1b). The remaining sequences were assigned to unclassified genera of *Methanobacteriaceae*, *Methanosarcinaceae*, *Euryarchaeota*, and *Nitrososphaera*.

Changes in Rumen Microbiome Profile and SCFA Concentrations over Lactation Cycle

Although the rumen microbiome was dominated by similar bacterial and archaeal genera throughout the lactation cycle, the overall microbial community structure did change over time. Differences in bacterial community structure were identified at all taxonomic levels but became most pronounced at genus level. Shannon diversity indices differed significantly between mid and late lactation ($P = 0.001$; Supplemental Table S2; https://doi.org/10.3168/jds.2017-14200) and permutational multivariate ANOVA analysis revealed significant ($P = 0.0014$) differences in bacterial community structure in the rumen of cows as they passed through

Table 2. Similarity percentage analysis of bacterial genera accounting for 19.34% of dissimilarity calculated between the ruminal community structures of cows in mid- and late-stage lactation

Genus	Contribution to community dissimilarity (%)	Relative abundance (%)	
		Mid lactation	Late lactation
Acetobacter	1.10	0.54	0.12
Bifidobacterium	1.08	0.74	0.79
Lactobacillus	1.03	0.68	0.24
Arthrobacter	0.95	0.52	0.16
Clostridium IV	0.94	0.52	0.82
Chloroflexi unclassified	0.90	0.26	0.29
Streptococcus	0.88	0.75	0.43
SR1 genus *incertae sedis*	0.88	1.01	1.31
Weissella	0.87	0.47	0.11
Sharpea	0.85	0.37	0.41
Rheinheimera	0.84	0.42	0.15
Treponema	0.81	1.24	1.34
Ruminobacter	0.80	0.99	0.88
Fusobacterium	0.79	0.42	0.16
Pediococcus	0.78	0.34	0.00
Acidaminococcus	0.77	0.34	0.43
Lactococcus	0.76	0.38	0.05
Leuconostoc	0.74	0.33	0.03
Flavobacterium	0.73	0.37	0.09
Escherichia/Shigella	0.72	0.25	0.18
Brevundimonas	0.72	0.32	0.03
Devosia	0.70	0.31	0.04
5 Genera *incertae sedis*	0.69	0.85	1.01

stages of lactation. Bacterial community structures in the rumen of mid-lactation cows were significantly ($P = 0.0007$) different from those in late lactation, as were those present in the rumen during early lactation and late lactation ($P = 0.02$; Supplemental Table S3; https://doi.org/10.3168/jds.2017-14200). A similar difference was seen with archaeal community structure, which differed significantly ($P = 0.042$) in the rumen of cows in early lactation compared with those in late lactation. Similarity percentage analysis indicated that the top 5 contributors to the dissimilarity between bacterial community profiles in cows at different stages of lactation were *Acetobacter*, *Bifidobacterium*, *Lactobacillus*, *Arthrobacter*, *and Clostridium* IV spp. (Table 2).

In early, mid, and late lactation, rumen acetate (32.2 ± 3.4, 34.8 ± 7.5, and 45.9 ± 6.7 mmol/L), propionate (11.8 ± 1.6, 11.3 ± 2.2, and 12.7 ± 2.3 mmol/L), and butyrate (9.7 ± 3.3, 7.2 ± 1.8, and 8.4 ± 1.7 mmol/L) concentrations, respectively, did not differ by stage of lactation; however, a significant ($P = 0.012$) difference was observed in the ratio of acetate and butyrate to propionate between cows in mid and late stage lactation (Figure 2).

Pearson correlation coefficient (r) was used to determine if any significant correlations could be observed between SCFA profiles in the rumen and the relative abundance of any of the 9 most relatively abundant bacterial genera (Table 3). In general, the relative abundance of all bacterial genera was strongly negatively correlated with acetate, butyrate, and propionate in early lactation, and this correlation weakened as cows progressed through the lactation cycle. *Ruminococcus* was an exception, as the relative abundance of this genus displayed a weak positive correlation to acetate, butyrate, and propionate in early lactation, but this changed to a stronger positive correlation in late lactation, particularly with butyrate.

Rumen Microbiome Structure and SCFA Concentrations in Low Versus High CH_4-Yielding Cows

Although CH_4 yield increased as the lactation cycle progressed (Table 1), no individual cow could be identified as a persistently extremely low or high CH_4-emitter across all lactation stages. However, clear differences could be observed in CH_4 yields from different cows during late lactation (42 wk postpartum), when CH_4 yields were highest. Therefore, 3 cows that produced low (mean 29 L/kg of DMI) and 3 that produced high (mean 38 L/kg of DMI) CH_4 yields during this stage of lactation were selected for SCFA and rumen microbial community structure analyses to investigate whether any differences could be observed between the 2 cohorts of animals from the same herd. A significant ($P = 0.032$) difference in propionate concentration was observed between the high and low CH_4-yielding cows, with a higher concentration of this SCFA detected in

the rumen of cows emitting less CH_4 yield (Figure 3a). A decrease in the acetate and butyrate-to-propionate ratio was noted in cows emitting lower levels of CH_4, although this difference was not significant ($P = 0.08$; Figure 3b). No differences in acetate or butyrate concentrations were detected between high and low CH_4 yielders. Despite the differences observed in CH_4 yields between the low and high CH_4-emitting cows, no differences were observed in bacterial ($P = 0.3$) or archaeal ($P = 0.8$) community structures. *Prevotella* (55.96%) dominated the rumen of cows, with *Succinclasticum* (15.35%) *Treponema*, *Fibrobacter*, *Ruminococcus*, and *Bifidobacterium* also in high abundance relative to other bacterial genera. Strong positive correlations were observed between the relative abundance of *Fibrobacter*, *Treponema*, and SR1 genus *incertae sedis* and all 3 SCFA in the rumen of cows emitting lower levels of CH_4 (Table 4). Furthermore, in this group, strong negative correlations were detected between the relative abundance of *Succiniclasticum* and all 3 SCFA. In the rumen of cows emitting high levels of CH_4, strong negative correlations existed between *Bifidobacterium* and acetate and butyrate concentrations, whereas *Fibrobacter* and *Succiniclasticum* were positively correlated with all 3 SCFA and *Prevotella* had a strong positive correlation with propionate concentration. Although some exceptions were noted, in general positive correlations were stronger between the most relatively abundant bacterial genera and concentrations of acetate and butyrate in higher emitters, whereas correlations were weaker between bacterial genera and concentrations of propionate.

DISCUSSION

Are Changes in CH_4 Yield Driven by Shifts in Rumen Microbial Community Structure?

For beef cattle, it has been shown that repeatability of CH_4 measurements decreases with increasing time between 2 measurements (Donoghue et al., 2016). Therefore, in the current study, each cow was measured at the same 3 time points [i.e., early (5 wk postpartum), mid (13 wk postpartum), and late (42 weeks postpartum) lactation] by using the most accurate method, respiration chambers. Although respiration chambers can be prohibitively expensive, they are the most reliable tool available for measuring enteric CH_4 emissions and are widely used (Yan et al., 2010; Hellwing et al., 2012). Similar to previous studies (Garnsworthy et al., 2012; Bielak et al., 2016), we showed that CH_4 yields from dairy cows are not constant during the lactation

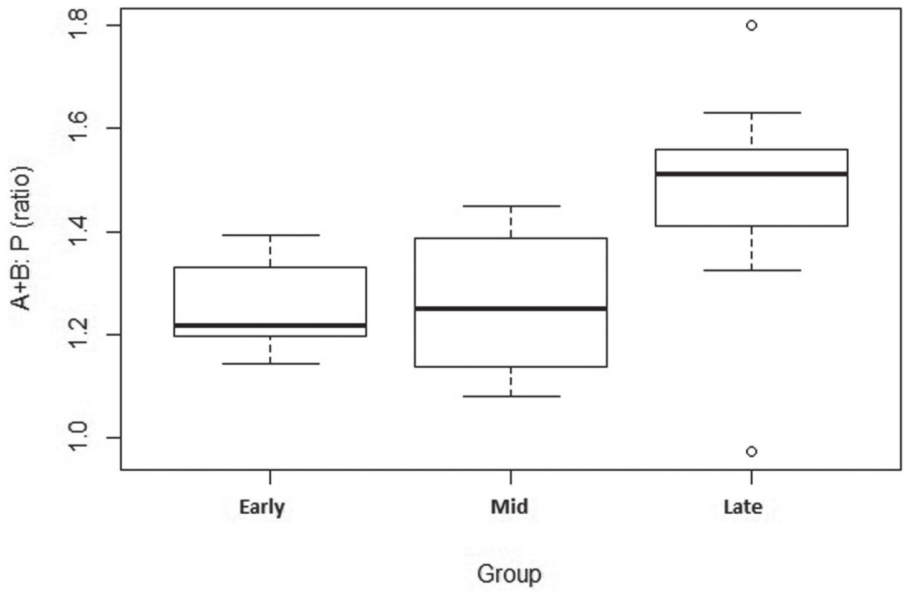


Figure 2. Acetate and butyrate-to-propionate ratio (A+B:P) measured in rumen fluid of cows during early, mid and late lactation. Each box depicts the interquartile range of the data; the horizontal line in each box shows the median, which separates the upper quartile (25%) from the lower quartile (25%). The whiskers (vertical capped lines) represent a further upper and lower 25%, and the circles represent outliers in the data set.

Table 3. Pearson correlation coefficients[1] between the relative abundance of sequences assigned to each bacterial genus and short-chain fatty acid concentrations (acetate, butyrate, and propionate) in the rumen of cows during early, mid, and late lactation

	Early			Mid			Late		
Genus	Acetate	Butyrate	Propionate	Acetate	Butyrate	Propionate	Acetate	Butyrate	Propionate
Prevotella	−0.529	−0.418	−0.610	−0.398	−0.340	−0.498	−0.302	−0.174	−0.179
Fibrobacter	−0.341	−0.307	−0.397	−0.040	0.055	−0.165	−0.017	0.181	0.110
Ruminobacter	0.305	0.097	0.203	0.516	0.606	0.418	0.472	0.661	0.420
Treponema	−0.518	−0.492	−0.625	−0.212	−0.151	−0.266	−0.486	−0.293	−0.341
5 Genera *incertae sedis*	−0.032	−0.358	−0.161	−0.198	−0.292	−0.236	−0.226	−0.219	−0.367
Butyrivibrio	−0.425	−0.397	−0.478	−0.378	−0.431	−0.437	−0.419	−0.251	−0.354
Ruminococcus	−0.499	−0.286	−0.457	−0.450	−0.439	−0.519	−0.153	−0.081	−0.015
Succiniclasticum	−0.537	−0.577	−0.655	−0.298	−0.189	−0.389	−0.245	−0.126	−0.177
SR1 genus *incertae sedis*	−0.269	−0.309	−0.327	−0.040	0.014	0.097	−0.282	−0.183	−0.300
Unclassified phylum	−0.681	−0.519	−0.717	−0.361	−0.302	−0.421	−0.485	−0.279	−0.288

[1]Calculated at a 95% confidence interval.

period, but increase from early to late lactation. This is in contrast to the relatively constant CH_4 yields measured in nonlactating adult sheep (Shi et al., 2014).

Although energy nutrient composition of the diet was constant over the course of lactation, a significant difference in bacterial community structure was detected ($P = 0.001$), and this shift was most pronounced between mid and late lactation. This is in contrast to the widely held opinion that, once established, the rumen microbiome is relatively stable in adult cows except in cases of major disruptions caused by antibiotics or major feed alterations (Rey et al., 2014). Michelland et al. (2011) reported that ruminal bacterial communities never appeared to be in a steady state in nonlactating cows, whereas Jewell et al. (2015) similarly reported that the rumen bacterial community composition in

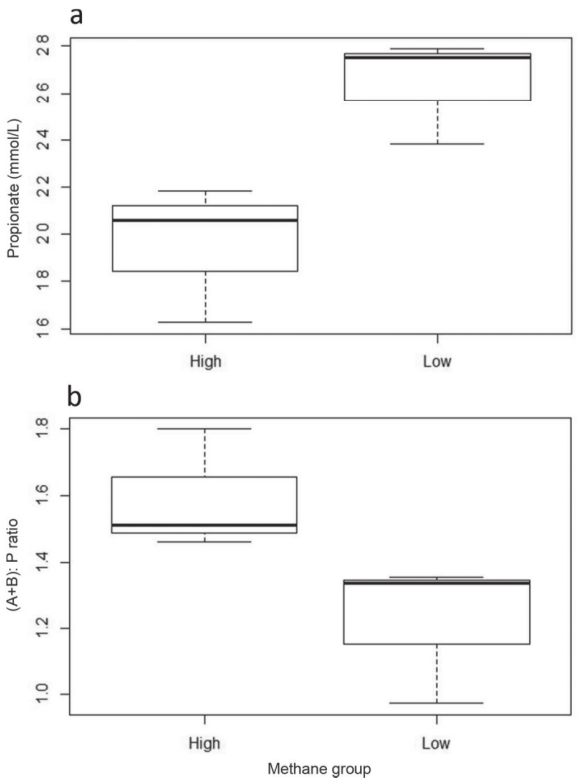


Figure 3. Concentration of VFA (a) propionate and (b) acetate and butyrate-to-propionate ratio (A+B:P) measured in the rumen of cows producing high (mean 38 L/kg of DMI) levels of CH_4 (high emitters) and cows producing low (mean 29 L/kg of DMI) levels of CH_4 (low emitters). Each box depicts the interquartile range of the data; the horizontal line in each box shows the median, which separates the upper quartile (25%) from the lower quartile (25%). The whiskers (vertical capped lines) represent a further upper and lower 25%.

Table 4. Pearson correlation coefficients[1] between the relative abundance of sequences assigned to each bacterial genus and short-chain fatty acid concentrations (acetate, butyrate, and propionate) in the rumen of cows producing high (mean 38 L/kg of DMI) levels of CH_4 (high emitters) and cows producing low (mean 29 L/kg of DMI) levels of CH_4 (low emitters)

	High emitters			Low emitters		
Item	Acetate	Butyrate	Propionate	Acetate	Butyrate	Propionate
Bifidobacterium	−0.667	−0.704	−0.099	0.517	0.215	0.457
Prevotella	0.528	0.484	0.927	0.467	0.157	0.405
Fibrobacter	0.959	0.943	0.940	0.795	0.948	0.835
Treponema	0.125	0.075	0.687	0.882	0.986	0.912
5 Genera *incertae sedis*	0.680	0.716	0.115	0.411	0.095	0.347
Butyrivibrio	0.706	0.740	0.151	0.364	0.045	0.299
Succiniclasticum	0.834	0.805	0.99	−0.993	−0.976	−0.999
SR1 genus *incertae sedis*	0.670	0.707	0.103	0.993	0.902	0.982
Unclassified phylum	0.801	0.831	0.294	0.660	0.383	0.607

[1]Calculated at a 95% confidence interval.

dairy cows was dynamic over the course of lactation and suggested that certain profiles favor high production efficiency in cows; however, CH_4 yield as a component determining feed energy utilization efficiency by the host was not measured. Gross feed efficiency of cows in the present study declined from early to late lactation, but whether correlation exists between the bacterial community structure and the numerously known feed efficiency indicators such as ECM/DMI, NE_L/ECM, or (NE_L − ECM)/metabolic BW needs to be determined in future studies. However, the level of ECM production in early lactation is in part decoupled from DMI, as cows produce milk to some extent from mobilized body reserves during this time (Bielak et al., 2016).

Although the concentrations of acetate, butyrate, and propionate in the rumen did not appear to change significantly between mid and late lactation, the ratio of acetate and butyrate to propionate was significantly higher in late lactation. This correlates with the sharp increase in CH_4 emissions measured in late lactation. Mohammed et al. (2011) also reported a strong correlation between enteric CH_4 emissions and the ratio of acetate and butyrate to propionate in the rumen of dairy cows and concluded that the ratio could be used as a predictor of CH_4 production. Microbial metabolism resulting in acetate and butyrate production creates hydrogen as a by-product, whereas synthesis of propionate consumes hydrogen (Van Soest, 1994). Increased levels of acetate and butyrate compared with propionate could result in overall increased levels of hydrogen in the rumen and, therefore, increased substrate availability for CH_4 production by methanogens.

Even though the same 6 bacterial genera (*Prevotella*, *Succiniclasticum*, *Treponema*, *Fibrobacter*, SR1 genus *incertae sedis*, *and Ruminococcus*) were the most relatively abundant in the rumen of cows in both mid and late lactation, their distribution changed in late lactation when CH_4 yields were higher. These bacterial genera are commonly found in the rumen, with *Prevotella*, *Fibrobacter*, *Succiniclasticum*, and *Ruminococcus* considered part of the core bovine rumen microbiome (Henderson et al., 2015; Lima et al., 2015). *Ruminococcus albus* and *Ruminococcus flavefaciens*, both cellulolytic bacteria, are the most abundant species of *Ruminococcus* present in the rumen. In pure culture, *R. albus* is known to produce ethanol, acetate, formate, and hydrogen during cellulose metabolism, whereas *R. flavefaciens* forms similar metabolites but produces succinate instead of ethanol (Russell et al., 2008). *Acetobacter*, a bacterial genus which also produces acetate by oxidizing sugars (Balch et al., 1977), was one of the top contributors to dissimilarity and showed largest relative abundances in the rumen of cows in mid lactation compared with late lactation. However, acetate concentrations were not significantly different in mid and late lactation, suggesting that these genera and their metabolites are not solely responsible for the increased CH_4 yield seen in late lactation. Members of the candidate phylum SR1 have only recently been discovered in the rumen (Lima et al., 2015). Animals fed a dietary supplement (linseed oil) designed to decrease CH_4 production were shown to have lower abundances of SR1 genus *incertae sedis* in their rumen (Veneman et al., 2015). *Bifidobacterium* was also a significant contributor to differences in bacterial community structures, with a higher relative abundance appearing in late lactation. This genus is known to confer numerous health benefits, such as vitamin production, modulation of immune system response, pathogen inhibition, and regulation of intestinal homeostasis (Mayo and Van Sinderen, 2010). No clear association between *Bifidobacterium* and CH_4 levels has been reported, and the relative increase may merely reflect a return to host homeostasis at the end of lactation. However, this bacterial genus is important for dietary carbohydrate degradation and produces lactic acid (Pokusaeva et al., 2011), which is further

metabolized to either propionate or acetate by microbial species in the rumen (Ladd and Walker, 1965). An increased relative abundance of *Bifidobacterium* may therefore result in increased levels of these SCFA. *Lactobacillus* and *Arthrobacter* were both relatively less abundant in late lactation, whereas *Clostridium* IV was relatively more abundant. Similar to *Bifidobacterium*, lactobacilli produce lactic acid that is subsequently converted to propionate or acetate (Ladd and Walker, 1965; Han et al., 2014), whereas *Clostridium* IV are butyrate producers (Lopetuso et al., 2013). Hydrogen availability is the limiting factor for CH_4 production by methanogens (Baker, 1999), and acetate and butyrate synthesis leads to the production of hydrogen whereas propionate synthesis consumes hydrogen. (Van Soest, 1994). Therefore, increases in the relative abundance of microbial species producing propionate and decreases in the relative abundance of microbial species producing metabolites leading to acetate or butyrate would have a knock-on effect on overall CH_4 yield.

None of the dominant bacterial genera appeared to be positively correlated with propionate. In fact, all genera, with the exception of *Ruminococcus*, were negatively correlated with acetate, butyrate, and propionate throughout lactation, although this correlation did weaken in late lactation. *Ruminococcus* are cellulolytic bacteria capable of producing both acetate and succinate, a propionate precursor (Ladd and Walker, 1965). The predominately negative correlations observed may indicate that these bacterial genera are using the SCFA or competing for nutrients with acetate, butyrate, and propionate producers. Although some studies have proposed roles for specific bacteria based on observed correlations (Sandri et al., 2014), it is difficult to draw conclusions based on correlations given the complex nature of the rumen microbial community and the lack of information concerning metabolic pathways employed by particular microbes. Furthermore, rumen fungi and ciliate protozoa also produce acetate, butyrate, and propionate during anaerobic digestion of feed (Martin et al., 2010). Although most research on this topic has focused on bacterial community composition, as they are typically the most abundant and metabolically diverse members of the rumen microbiome, ciliate protozoa can account for up to half of rumen microbial biomass and therefore strongly influence overall VFA production (Newbold et al., 2015); these organisms no doubt have contributed to the concentrations of acetate, butyrate, and propionate produced in the current study.

Archaeal community structure, at genus level, also changed significantly ($P = 0.04$) in the rumen of dairy cows between mid and late lactation. Although archaea with diverse metabolisms exist and are found in a range of habitats, those detected in the rumen thus far are all strictly anaerobic methanogens (Janssen and Kirs, 2008). In a pattern similar to that observed with bacterial communities, the most abundant archaeal genera remained the same, but the distribution shifted. *Methanobrevibacter* were found to be in a higher relative abundance in the rumen of cows in late lactation, whereas *Methanosphaera*, *Methanomassilicoccus*, and an unclassified genus of *Thermoplasmata* were all in a lower relative abundance. *Methanobrevibacter* have previously been associated with high CH_4 production. Zhou et al. (2011) reported high abundances of certain species of *Methanobrevibacter* in animals producing high CH_4 yields; however, this may reflect species-level differences and the current study only has information at genus level. As methanogens rely on metabolites produced by other microbial species in the rumen, changes observed in archaeal community structure may be a consequence of the changes occurring in bacterial community composition and changes in protozoal or fungal abundance or species richness.

Does the Rumen Microbial Profile Differ Between Low and High CH_4-Yielding Cows in Late Lactation?

Similar to previous studies (Garnsworthy et al., 2012; Bielak et al., 2016), overall CH_4 emissions from the dairy herd used in the current study increased from early to late lactation. Increased rumen volume and particulate passage rate have been associated with higher CH_4 yields (Goopy et al., 2014). Although rumen volume and particulate retention time were not measured in our study, rumen DM volume and rumen capacity have been shown to increase in cows up to 30 d postpartum compared with late lactation (72 d preparturition) volumes (Park et al., 2001; Reynolds et al., 2004). Increasing CH_4 yields throughout lactation could therefore, in part, be explained by increased rumen volume. Although overall CH_4 emissions were lower in early lactation and higher in late lactation, individual cows emitted varying CH_4 yields and no group of cows produced consistently lower amounts of CH_4 yields at all time points throughout the lactation cycle when compared with the herd as a whole. Comparison of the rumen microbiome of cows producing low versus high CH_4 yields during late lactation revealed no significant differences in bacterial or archaeal community structures. Differences were observed in the relative abundance of some bacterial genera. *Succiniclasticum*, which have been reported to convert succinate to propionate in the rumen (van Gylswyk, 1995), appeared to be relatively more abundant in cows producing lower levels of CH_4. In line with our result, Wallace et al. (2015) found that *Succinivibrionaceae* was 4-fold less abundant in high CH_4-emitting animals. Increased lev-

els of propionate have been linked to lower levels of CH_4 production, and propionate concentrations were found to be significantly ($P = 0.03$) higher in the rumen of cows producing low levels of CH_4 in the current study. In addition, the ratio of acetate and butyrate to propionate concentrations appeared to be reduced in the rumen of low emitters, although this reduction was not significant ($P = 0.08$). Despite its reported link with propionate production, no clear relationship between *Succiniclasticum* with propionate could be seen; this genus was negatively correlated with all 3 SCFA in cows that produced low levels of CH_4 and positively correlated with cows that produced high levels of CH_4. This finding again highlights the complexity of metabolism in the rumen and the difficulty in identifying the role(s) specific bacteria may be playing based on relative abundance alone and the possible role of protozoa and fungi. The rumen microbiome contains a high level of microbial diversity and therefore harbors a high level of functional redundancy, with many species carrying out overlapping activities (Weimer, 2015). Similar phylogenetic profiles in the rumen may be misleading, as the functional profiles may be decidedly different. Shi et al. (2014) performed metagenomic and metatranscriptomic sequencing of rumen samples from 4 high versus 4 low CH_4-yielding sheep and found that methanogen abundance was similar in both groups, but transcription of methanogenesis pathway genes was significantly increased in sheep with high CH_4 yield. This may explain why microbial communities appear largely unchanged in the rumen of dairy cows even when they produce higher levels of CH_4 yield.

It is known that host genetics influence rumen microbiome structure and that animal-to-animal variation exists (Li et al., 2009; Henderson et al., 2015; Roehe et al., 2016). Therefore, when examining potential drivers of low or high CH_4 production, it is important to examine overall trends in the herd rather than focusing on individual animals. Because CH_4 yield varies considerably between individuals and increased significantly in late lactation, we focused on the average change over time. Short-term dietary interventions in early life aimed at manipulating the microbiome to create stable, desirable community compositions that persist throughout life are already being investigated and are yielding encouraging results (Abecia et al., 2014; Yáñez-Ruiz et al., 2015; Lyons et al., 2017). The identification of significantly higher CH_4 yield in late lactation in the current study could mark this period as an additional target for interventions designed to reduce overall CH_4 emissions from the livestock sector. Dietary interventions are expensive, and it would be largely impractical to implement them throughout the animal production cycle; therefore, identifying periods where short-term dietary intervention results in meaningful CH_4 reductions could be a better approach for mitigation strategies in this sector.

In conclusion, CH_4 yield increased significantly in late lactation and was accompanied by a shift in bacterial and archaeal community structure and propionate concentration in the rumen. This suggests that changes in the microbial community structure, which cause changes in the SCFA profile contribute to increases in CH_4 yield. However, due to the complex nature of the rumen microbiome, individual microorganisms cannot be singled out as solely accountable for changes in CH_4 yield. Rather, shifts in the microbial communities as a whole appear to be responsible. Furthermore, our study highlights late lactation as a critical period where short-term intervention could be useful to reduce overall CH_4 emissions from the dairy sector.

ACKNOWLEDGMENTS

We thank M. Derno, H. Pröhl, and the staff at the "Tiertechnikum" and the bovine experimental unit at the FBN Dummerstorf for assistance in respiration chamber measurements, animal care and handling, sample collection, and sample preparation. This study was conducted in frame of the Joint Programming Initiative on Agriculture, Food Security and Climate Change (FACCE-JPI) program (RumenStability project) and financially supported by the German Federal Ministry of Food and Agriculture (BMBL) through the Federal Office for Agriculture and Food (BLE), grant number 2814ERA04A and the Irish Department of Agriculture Fisheries and Marine's Agricultural Greenhouse Gas Research Initiative for Ireland (Dublin, Ireland; Project no. 10/RD/GHG-IRELAND/716).

REFERENCES

Abecia, L., E. Ramos-Morales, G. Martinez-Fernandez, A. Arco, A. I. Martin-Garcia, C. J. Newbold, and D. R. Yáñez-Ruiz. 2014. Feeding management in early life influences microbial colonisation and fermentation in the rumen of newborn goat kids. Anim. Prod. Sci. 54:1449–1454.

Anderson, K. L., T. G. Nagaraja, J. L. Morrill, T. B. Avery, S. J. Galitzer, and J. E. Boyer. 1987. Ruminal microbial development in conventionally or early-weaned calves. J. Anim. Sci. 64:1215–1226.

Anderson, L. P., J. A. Paterson, R. P. Ansotegui, M. Cecava, and W. Schmutz. 2001. The effects of degradable and undegradable intake protein on the performance of lactating first-calf heifers. J. Anim. Sci. 79:2224–2232.

Arndt, C., J. M. Powell, M. J. Aguerre, P. M. Crump, and M. A. Wattiaux. 2015. Feed conversion efficiency in dairy cows: Repeatability, variation in digestion and metabolism of energy and nitrogen, and ruminal methanogens. J. Dairy Sci. 98:3938–3950.

Atakora, J. K. A., S. Moehn, and R. O. Ball. 2011. Enteric methane produced by finisher pigs is affected by dietary crude protein content of barley grain based, but not by corn based, diets. Anim. Feed Sci. Technol. 166–67:412–421.

Baker, S. K. 1999. Rumen methanogens and inhibition of methanogenesis. Aust. J. Agric. Res. 50:1293–1298.

Balch, W. E., S. Schoberth, R. S. Tanner, and R. S. Wolfe. 1977. *Acetobacterium,* a new genus of hydrogen-oxidizing, carbon dioxide-reducing, anaerobic bacteria. Int. J. Syst. Evol. Microbiol. 27:355–361.

Bielak, A., M. Derno, A. Tuchscherer, H. M. Hammon, A. Susenbeth, and B. Kuhla. 2016. Body fat mobilization in early lactation influences methane production of dairy cows. Sci. Rep. 6:28135.

Cobellis, G., Z. Yu, C. Forte, G. Acuti, and M. Trabalza-Marinucci. 2016. Dietary supplementation of *Rosmarinus officinalis* L. leaves in sheep affects the abundance of rumen methanogens and other microbial populations. J. Anim. Sci. Biotechnol. 7:27.

de Menezes, A. B., E. Lewis, M. O'Donovan, B. F. O'Neill, N. Clipson, and E. M. Doyle. 2011. Microbiome analysis of dairy cows fed pasture or total mixed ration diets. FEMS Microbiol. Ecol. 78:256–265.

Derno, M., G. Nürnberg, P. Schön, A. Schwarm, M. Röntgen, H. M. Hammon, C. C. Metges, R. M. Bruckmaier, and B. Kuhla. 2013. Short-term feed intake is regulated by macronutrient oxidation in lactating Holstein cows. J. Dairy Sci. 96:971–980.

Donoghue, K. A., T. Bird-Gardiner, P. F. Arthur, R. M. Herd, and R. S. Hegarty. 2016. Repeatability of methane emission measurements in Australian beef cattle. Anim. Prod. Sci. 56:213.

Dougherty, R. W. 1968. Eructaion in ruminants. Ann. N. Y. Acad. Sci. 150:22–26. https://doi.org/10.1111/j.1749-6632.1968.tb19026.x.

Edgar, R. C., B. J. Haas, J. C. Clemente, C. Quince, and R. Knight. 2011. UCHIME improves sensitivity and speed of chimera detection. Bioinformatics 27:2194–2200.

Ellison, M. J., G. C. Conant, R. R. Cockrum, K. J. Austin, H. Truong, M. Becchi, W. R. Lamberson, and K. M. Cammack. 2014. Diet alters both the structure and taxonomy of the ovine gut microbial ecosystem. DNA Res. 21:115–125.

Garnsworthy, P. C., J. Craigon, J. H. Hernandez-Medrano, and N. Saunders. 2012. Variation among individual dairy cows in methane measurements made on farm during milking. J. Dairy Sci. 95:3181–3189.

Goopy, J. P., A. Donaldson, R. Hegarty, P. E. Vercoe, F. Haynes, M. Barnett, and V. H. Oddy. 2014. Low-methane yield sheep have smaller rumens and shorter rumen retention time. Br. J. Nutr. 111:578–585.

Han, H., Y. Ogata, Y. Yamamoto, S. Nagao, and N. Nishino. 2014. Identification of lactic acid bacteria in the rumen and feces of dairy cows fed total mixed ration silage to assess the survival of silage bacteria in the gut. J. Dairy Sci. 97:5754–5762.

Hellwing, A. L. F., P. Lund, M. R. Weisbjerg, M. Brask, and T. Hvelplund. 2012. Technical note: Test of a low-cost and animal-friendly system for measuring methane emissions from dairy cows. J. Dairy Sci. 95:6077–6085.

Henderson, G., F. Cox, S. Ganesh, A. Jonker, W. Young, and P. H. Janssen. 2015. Rumen microbial community composition varies with diet and host, but a core microbiome is found across a wide geographical range. Sci. Rep. 5:14567.

Hobson, P. N., and C .S. Stewart. 1997. The Rumen Microbial Ecosystem. Blackie Academic & Professional, London, UK. https://doi.org/10.1007/978-94-009-1453-7.

Jami, E., A. Israel, A. Kotser, and I. Mizrahi. 2013. Exploring the bovine rumen bacterial community from birth to adulthood. ISME J. 7:1069–1079.

Janssen, P. H., and M. Kirs. 2008. Structure of the archaeal community of the rumen. Appl. Environ. Microbiol. 74:3619–3625.

Jewell, K. A., C. A. McCormick, C. L. Odt, P. J. Weimer, and G. Suen. 2015. Ruminal bacterial community composition in dairy cows is dynamic over the course of two lactations and correlates with feed efficiency. Appl. Environ. Microbiol. 81:4697–4710.

Joshi, N. A., and J. N. Fass. 2011. Sickle: A sliding-window, adaptive, quality-based trimming tool for FastQ files. Version 1.33. Accessed Oct. 7, 2017. https://github.com/najoshi/sickle.

Knapp, J. R., G. L. Laur, P. A. Vadas, W. P. Weiss, and J. M. Tricarico. 2014. Enteric methane in dairy cattle production: quantifying the opportunities and impact of reducing emissions. J. Dairy Sci. 97:3231–3261.

Kozich, J. J., S. L. Westcott, N. T. Baxter, S. K. Highlander, and P. D. Schloss. 2013. Development of a dual-index sequencing strategy and curation pipeline for analyzing amplicon sequence data on the MiSeq Illumina sequencing platform. Appl. Environ. Microbiol. 79:5112–5120.

Ladd, J. N., and D. J. Walker. 1965. Fermentation of lactic acid by the rumen microorganism, *Peptostreptococcus elsdenni.* Ann. N. Y. Acad. Sci. 119:1038–1047.

Li, M., G. B. Penner, E. Hernandez-Sanabria, M. Oba, and L. L. Guan. 2009. Effects of sampling location and time, and host animal on assessment of bacterial diversity and fermentation parameters in the bovine rumen. J. Appl. Microbiol. 107:1924–1934.

Li, R. W., E. E. Connor, C. Li, R. L. Baldwin VI, and M. E. Sparks. 2012. Characterization of the rumen microbiota of pre-ruminant calves using metagenomic tools. Environ. Microbiol. 14:129–139.

Lima, F. S., G. Oikonomou, S. F. Lima, M. L. S. Bicalho, E. K. Ganda, J. C. de Oliveira Filho, G. Lorenzo, P. Trojacanec, and R. C. Bicalhoa. 2015. Prepartum and postpartum rumen fluid microbiomes: Characterization and correlation with production traits in dairy cows. Appl. Environ. Microbiol. 81:1327–1337.

Lopetuso, L. R., F. Scaldaferri, V. Petito, and A. Gasbarrini. 2013. Commensal Clostridia: Leading players in the maintenance of gut homeostasis. Gut Pathog. 5:23.

Lueders, T., M. Manefield, and M. W. Friedrich. 2004. Enhanced sensitivity of DNA- and rRNA-based stable isotope probing by fractionation and quantitative analysis of isopycnic centrifugation gradients. Environ. Microbiol. 6:73–78.

Lyons, T., T. Boland, S. Storey, and E. Doyle. 2017. Linseed oil supplementation of lambs' diet in early life leads to persistent changes in rumen microbiome structure. Front. Microbiol. 8:1656.

Martin, C., D. P. Morgavi, and M. Doreau. 2010. Methane mitigation in ruminants: From microbe to the farm scale. Animal 4:351–365.

Martin, M. 2011. Cutadapt removes adapter sequences from high-throughput sequencing reads. EMBnet J. 17:10–12.

Mayo, B., and D. Van Sinderen, ed. 2010. *Bifidobacteria* Genomics and Molecular Aspects. Caister Academic Press, Poole, UK.

Michelland, R. J., V. Monteils, S. Combes, L. Cauquil, T. Gidenne, and L. Fortun-Lamothe. 2011. Changes over time in the bacterial communities associated with fluid and food particles and the ruminal parameters in the bovine rumen before and after a dietary change. Can. J. Microbiol. 57:629–637.

Mohammed, R., S. M. McGinn, and K. A. Beauchemin. 2011. Prediction of enteric methane output from milk fatty acid concentrations and rumen fermentation parameters in dairy cows fed sunflower, flax, or canola seeds. J. Dairy Sci. 94:6057–6068.

Newbold, C. J., G. de la Fuente, A. Belanche, E. Ramos-Morales, and N. R. McEwan. 2015. The role of ciliate protozoa in the rumen. Front. Microbiol. 6:1313.

Park, A. F., J. M. DeFrain, E. C. Titgemeyer, and E. E. Ferdinand. 2001. Changes in rumen capacity of dairy cows during the periparturient period. Kansas Agricultural Experiment Station Research Reports. https://doi.org/10.4148/2378-5977.3231.

Pokusaeva, K., G. F. Fitzgerald, and D. van Sinderen. 2011. Carbohydrate metabolism in *Bifidobacteria.* Genes Nutr. 6:285–306.

Quast, C., E. Pruesse, P. Yilmaz, J. Gerken, T. Schweer, P. Yarza, J. Peplies, and F. O. Glöckner. 2013. The SILVA ribosomal RNA gene database project: improved data processing and web-based tools. Nucleic Acids Res. 41:D590–596.

Rey, M., F. Enjalbert, S. Combes, L. Cauquil, O. Bouchez, and V. Monteils. 2014. Establishment of ruminal bacterial community in dairy calves from birth to weaning is sequential. J. Appl. Microbiol. 116:245–257.

Reynolds, C. K., B. Dürst, B. Lupoli, D. J. Humphries, and D. E. Beever. 2004. Visceral tissue mass and rumen volume in dairy cows during the transition from late gestation to early lactation. J. Dairy Sci. 87:961–971.

Roehe, R., R. J. Dewhurst, C. A. Duthie, J. A. Rooke, N. McKain, D. W. Ross, J. J. Hyslop, A. Waterhouse, T. C. Freeman, M. Watson,

and R. J. Wallace. 2016. Bovine host genetic variation influences rumen microbial methane production with best selection criterion for low methane emitting and efficiently feed converting hosts based on metagenomic gene abundance. PLoS Genet. 12:e1005846.

Russell, J. B., R. E. Muck, and P. J. Weimer. 2008. Quantitative analysis of cellulose degradation and growth ofcellulolytic bacteria in the rumen. FEMS Microbiol. Ecol. 67:183–197.

Ryan, J. P. 1980. Determination of volatile fatty-acids and some related compounds in ovine rumen fluid, urine, and blood-plasma, by gas-liquid-chromatography. Anal. Biochem. 108:374–384.

Sandri, M., C. Manfrin, A. Pallavicini, and B. Stefanon. 2014. Microbial biodiversity of the liquid fraction of rumen content from lactating cows. Animal 8:572–579.

Schloss, P. D., S. L. Westcott, T. Ryabin, J. R. Hall, M. Hartmann, E. B. Hollister. R. A. Lesniewski, B. B. Oakley, D. H. Parks, C. J. Robinson, J. W. Sahl, B. Stres, G. G. Thallinger, D. J. Van Horn, and C. F. Weber. 2009. Introducing mothur: Open-source, platform-independent, community-supported software for describing and comparing microbial communities. Appl. Environ. Microbiol. 75:7537–7541.

Shi, W., C. D. Moon, S. C. Leahy, D. Kang, J. Froula, S. Kittelmann, C. Fan, S. Deutsch, D. Gagic, W. J. Kelly, R. Atua, C. Sang, P. Soni, D. Li, C. S. Pinares-Patino, J. C. McEwan, P. H. Janssen, F. Chen, A. Visel, Z. Wang, G. T. Attwood, and E. M. Rubin. 2014. Methane yield phenotypes linked to differential gene expression in the sheep rumen microbiome. Genome Res. 24:1517–1525.

Sun, X., G. Henderson, F. Cox, G. Molano, S. J. Harrison, D. Luo, P. H. Janssen, and D. Pacheco.. 2015. Lambs fed fresh winter forage rape (*Brassica napus* L.) emit less methane than those fed perennial ryegrass (*Lolium perenne* L.), and possible mechanisms behind the difference. PLoS One 10:e0119697.

van Gylswyk, N. O. 1995. *Succiniclasticum ruminis* gen. nov., sp. nov., a ruminal bacterium converting succinate to propionate as the sole energy-yielding mechanism. Int. J. Syst. Bacteriol. 45:297–300.

Van Houtert, M. 1993. The production and metabolism of volatile fatty acids by ruminants fed roughages: A review. Anim. Feed Sci. Technol. 43:189–225.

Van Soest, P. J. 1994. Nutritional Ecology of the Ruminant. Cornell University Press, New York, NY.

Veneman, J. B., S. Muetzel, K. J. Hart, C. L. Faulkner, J. M. Moorby, H. B. Perdok, and C. J. Newbold. 2015. Does dietary mitigation of enteric methane production affect rumen function and animal productivity in dairy cows? PLoS One 10:e0140282.

Wallace, R. J., J. A. Rooke, N. McKain, C.-A. Duthie, J. J. Hyslop, D. W. Ross, A. Waterhouse, M. Watson, and R. Roehe. 2015. The rumen microbial metagenome associated with high methane production in cattle. BMC Genomics 16:839.

Weimer, P. J. 2015. Redundancy, resilience, and host specificity of the ruminal microbiota: implications for engineering improved ruminal fermentations. Front. Microbiol. 6:296.

Weimer, P. J., D. M. Stevenson, H. C. Mantovani, and S. L. Man. 2010. Host specificity of the ruminal bacterial community in the dairy cow following near-total exchange of ruminal contents. J. Dairy Sci. 93:5902–5912.

Yan, T., C. S. Mayne, F. G. Gordon, M. G. Porter, R. E. Agnew, D. C. Patterson, C. P. Ferris, and D. J. Kilpatrick. 2010. Mitigation of enteric methane emissions through improving efficiency of energy utilization and productivity in lactating dairy cows. J. Dairy Sci. 93:2630–2638.

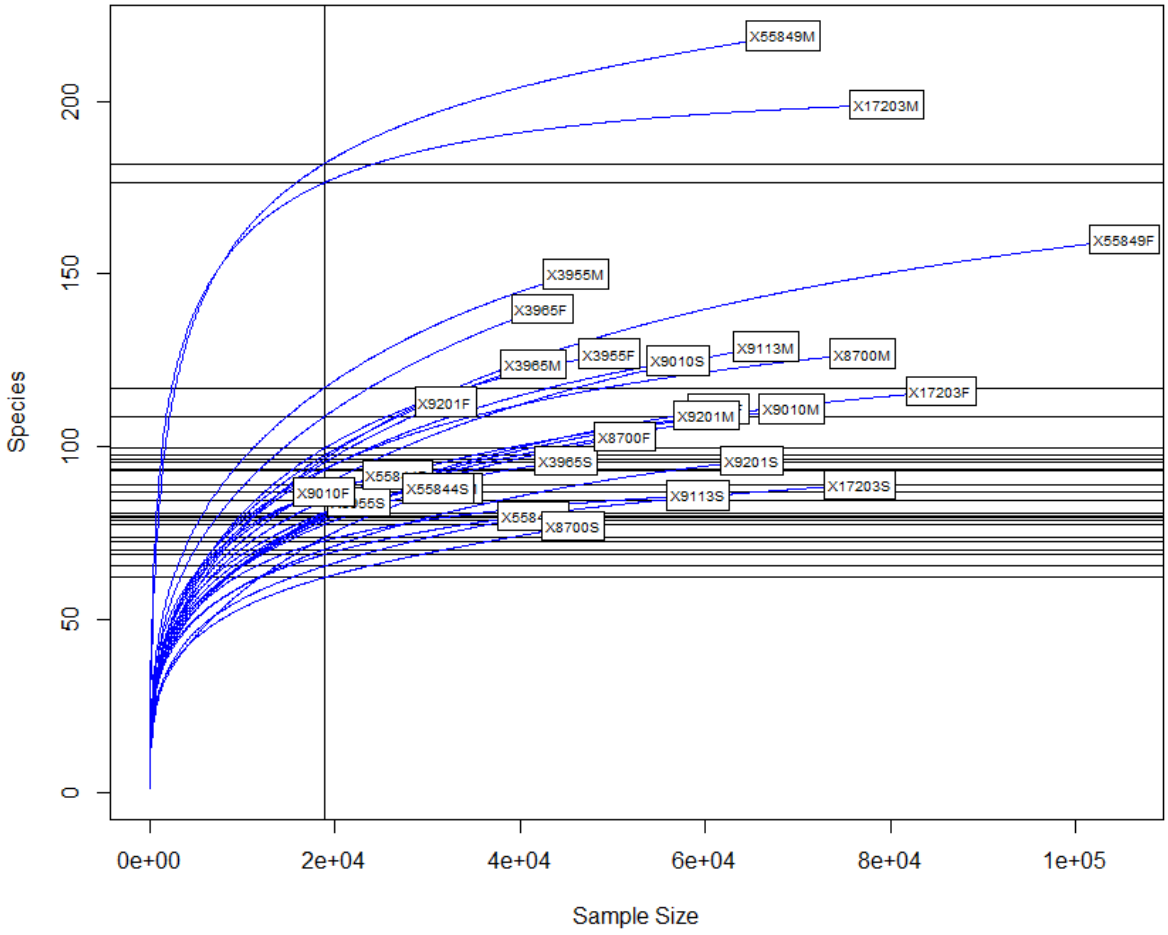
Yáñez-Ruiz, D. R., L. Abecia, and C. J. Newbold. 2015. Manipulating rumen microbiome and fermentation through interventions during early life: a review. Front. Microbiol. 6:1133.

Zhou, M., Y. H. Chung, K. A. Beauchemin, L. Holtshausen, M. Oba, T. A. McAllister, and L. L. Guan. 2011. Relationship between rumen methanogens and methane production in dairy cows fed diets supplemented with a feed enzyme additive. J. Appl. Microbiol. 111:1148–1158.

3.2.1 Supplement second Publication

Variations in methane yield and microbial community profiles in the rumen of dairy cows as they pass through stages of first lactation

T. Lyons, A. Bielak, E. Doyle and B. Kuhla



Supplemental Figure 1. Rarefaction curves for all samples. Labels are cow tags and letters for time of lactation (F = week 5, M = week 13, S = week 42). Vertical line indicates the size of the smallest sample (18855 sequences).

Supplementary Table S1. Ingredients and nutrient composition (g/kg DM), and energy content (MJ/ kg DM) of the diet fed to 9 first lactating cows over the course of first lactation.

Item	
Grass silage	184.4
Corn silage	335.0
Grass hay	29.4
Barley straw	28.5
Corn kernels	75.5
Wheat grains	19.0
Sugar beet pulp	37.7
Extracted soy meal	18.1
Extracted canolaseed meal	29.2
Feed lime	3.8
MF 2000 (concentrate)	230.9
Mineral 9522	8.5
Crude ash	70.7
Crude protein	157.8
Crude fiber	168.7
Crude fat	31.0
Sugar	25.0
Starch	249.7
NDF	360.1
ADF	212.4
NfE	492.7
GE	17.0
ME	11.2
NEL	7.0

Supplementary Table S2. Average species richness, species evenness and Shannon diversity indices calculated for **a)** bacterial and **b)** archaeal communities in the rumen of dairy cows in early, mid and late lactation and **c)** Tukey test results (based on ANOVA test $P = 0.002$) showing bacterial Shannon diversity indices differed significantly between mid-lactation and late lactation.

a)

Lactation group	Species richness	Species evenness	Shannon diversity
Early	28.23±4.91	0.95±0.003	4.48±0.17
Mid	32.40±7.12	0.96±0.009	4.66±0.31
Late	22.77±2.90	0.95±0.003	4.23±0.13

b)

Lactation group	Species richness	Species evenness	Shannon diversity
Early	2.87±0.80	0.90±0.01	1.78±0.26
Mid	2.58±0.60	0.90±0.01	1.69±0.23
Late	2.63±0.28	0.89±0.02	1.70±0.11

c)

	Shannon diversity	Species richness	Species evenness
Early-Mid	0.26	0.05	0.3
Mid-Late	0.001	0.3	0.03
Early-Late	0.06	0.05	0.4

Supplementary Table S3. Permutational analysis of variance (PERMANOVA) results showing significant differences in bacterial community structure between lactation stages.

PERMANOVA main

Source	df	SS	MS	Pseudo-F	*P* (perm)
Lactation stage	2	2227.8	1113.9	2.1149	0.0014*
Res	24	12640	526.68		
Total	26	14868			

PERMANOVA pairwise

Groups	t	P (perm)	Unique perms	*P* (MC)
Early & Mid	1.2324	0.1341	8116	0.1532
Early & Late	1.3105	0.0212*	8171	0.0768
Mid & Late	1.7556	0.0007*	8145	0.0147

Supplementary Table S4. Similarity percentage (SIMPER) analysis of archaeal genera accounting for 76.15% of dissimilarity calculated between the ruminal community structures of cows in mid and late stage lactation.

Genus	Contribution to community dissimilarity (%)	Relative abundance (%)	
		Mid lactation	Late lactation
Thermoplasmata unclassified	18.31	1.33	1.50
Methanomassiliicoccus	13.39	1.48	1.50
Methanosarcinaceae unclassified	13.00	0.44	0.56
Methanobacteriaceae unclassified	11.71	0.49	0.56
Methanosphaera	10.34	1.88	1.60
Nitrososphaera unclassified	9.41	0.28	0.01

4 DISCUSSION

The aim of the present study was to enhance the understanding of the responsible processes for naturally occurring variations in CH_4 production of ruminants. The level of CH_4 emission was linked to the metabolic status of the cow, especially with regard to the status of negative energy balance in early lactation that is characterized by an increased fat mobilization. In addition, changes in the microbiome structure were examined as a potential cause for the variability of CH_4 emissions throughout lactation and between cows. This study showed that dairy cows kept under identical husbandry conditions and fed as much as possible the same diet throughout lactation varied in CH_4 yield and that their rumen microbial community structure changed during the course of lactation. Despite differences in the microbial communities, the differences in CH_4 yield were not related to microbial abundance but rather to the metabolic status of the animals.

4.1 Assessment of experimental design

4.1.1 Animals

In the presented trial 18 and 9 cows were used for CH_4 measurements and simultaneous CH_4 and microbial data collection, respectively. In terms of statistics the number of sampled animals was rather low. Statistical power of a test depends on sample size (number of individuals in this case), on the significance criterion and on the magnitude of the effect that is considered relevant. Husbandry of large farm animals is labor intensive, requires high staff expenses and the animals' life expectancy and production cycles are much longer than those in commonly used laboratory animals. These factors limit the sample size when using cows in such experiments. Thus, studies involving CH_4 measurements in cattle often present data from as little as four or even less animals (BELANCHE et al. 2012, HAMMOND et al. 2014, PARK et al. 2011, WEIMER et al. 2010). Especially the availability of respiration chambers for CH_4 measurements limits the number of animals that can be studied in a certain period of time. In a similar trial set-up to ours (DANIELSSON et al. 2017), 73 cows were sampled for the CH_4 data presented, and in a subgroup of 21 cows rumen microbial community

and SCFA were analyzed. However, this large number of animals could only be realized by using an infrared CH_4 analyzer installed in the concentrate trough of the milking robot, which provided only short time concentration measurements of exhaled CH_4.

4.1.2 Feed

Great effort was put into the calculations of rations. It was intended to feed all animals a uniform diet throughout the productive period, and a second uniform diet during the dry period. Nevertheless, accessibility to forages and silages varied because silo size was limited. Thus, roughage from different silos had to be used throughout the trial. Consequently, the amount of concentrate and mineral components in the ration had to be adjusted in order to meet nutrient requirements of the animals. Also, calving dates of our cows and, accordingly, diet change from gestation to lactation ration had to be spread over a period of nine weeks. Therefore, provision of an identical feed for all animals at the respective measuring times could not be ensured and thus feed composition could have been a confounding factor in this study.

4.1.3 Rumen fluid

The analysis of microbes in rumen fluid performed in the present study involved only bacteria and archaea. Rumen fungi and protozoa were not analyzed. While prokaryotic bacteria contribute to the breakdown of plant polymers in the rumen, archaea are producers of CH_4. Eukaryotes also contribute substantially to fermentation processes in the rumen, producing propionate, acetate and butyrate. They are part of the complex ecosystem and metabolic network in the rumen. However, for the analysis of fungi and protozoa different primer sets from the ones used for bacteria and archaea are required, and amplicon mixing to simultaneously pyrosequence the whole community had been advisable (KITTELMANN et al. 2013). Due to the additional expenditure of time and resources for these processes it was decided to concentrate on bacteria and archaea.

Ruminal microbes can be found dispersed in the rumen fluid, attached to feed particles or to rumen epithelium. They form a sub-community in each of these locations. The sampling method used only provided the fluid phase and very small dispersed particles

and did not take the other locations into account. TERRÉ et al. (2013) stated that regardless of the sampling technique (stomach tubing or rumen cannula), comparisons of bacterial communities are acceptable as long as the method applied is the same for all samples. In addition, the exact sampling site within the ruminal lumen might vary when using the stomach tubing, depending on animal size and insertion depth (SHEN et al. 2012). Ruminal fermentation parameters, such as metabolite concentrations, differ depending on the location within the rumen sac, as does pH. Due to potential contamination with saliva when using stomach tubes, the first 150ml of gathered fluid were discarded in this study as recommended by SHEN et al. (2012).

4.1.4 Blood and milk metabolites

Blood metabolite concentrations were used to designate cows to metabolic groups and also quantify some metabolic situations like the NEB. Especially the plasma NEFA concentration is a valid parameter for these assumptions, although the body condition score (BCS) at calving and parity influence the extent of the NEFA peak (PIRES et al. 2013). The cows in the present study were all first parity, but groups differed in the loss of back fat after parturition. As expected this difference in fat mobilization was reflected in their plasma NEFA concentrations.

Milk constitution and metabolites were useful for determining energy turnover and nutritive balance. Also, these parameters were necessary to relate the CH_4 production to productivity. Data from both blood and milk metabolites aided further in the early identification of ill – and thus for the trial ineligible - cows.

4.2 Assessment of results

4.2.1 Variance of methane emissions

To minimize confounding effects on methane emission, a great effort was put into choosing a group of animals with most similar biological preconditions including age, breed, performance parameters and reproductive status. The similarity between animals fed the same diet in the presented study resulted in only moderate but still

present differences in CH_4 production between individuals. That provided the basis to investigate metabolic and microbiological causes for differences in CH_4 yield.

CH_4 emission and CH_4 yield are not constant for individuals but vary in repeated measurements. For dairy cows, changes in CH_4 emission over time are related to changes in their productivity cycle during the transition from lactation to the dry-off period and vice versa.

During the dry period CH_4 production of dairy cows decreases relative to the preceding lactation period. Then, it exponentially increases after parturition until about week 10 – 12 of lactation and slowly decreases afterwards until late lactation (GARNSWORTHY et al. 2012). In accordance with GARNSWORTHY et al. (2012) and BELL et al. (2014), animals investigated in the present study increased CH_4 production from dry period to late lactation (Publication 1, Figure 4a). CH_4 yield increased from early lactation to late lactation and was markedly higher in the dry period than during early lactation (Publication 2, Table 1; Publication 1, Figure 4b). BELL et al. (2014) described an increase in CH_4 production in early lactation followed by relatively stable values in mid to late lactation. DMI of cows follows the same dynamic over the course of lactation. When CH_4 production was corrected for DMI, CH_4 yields were higher in late lactation than in early lactation (ROBERTSON & WAGHORN 2002). Similar to the results in this study, PINARES-PATIÑO et al. (2007) showed that lactating cows have much lower CH_4 yields than dry cows. However, total CH_4 production is higher when the animals are lactating. The difference in CH_4 production and CH_4 yield between dry and lactating animals is usually explained with different diets. As outlined in section 2.4.2 the ration components greatly influence CH_4 production. Since diets for dry cows are usually formulated with a lower concentrate and higher fiber content than rations for lactating cows, CH_4 production per kg DM is higher in the former. Additionally, cows ingest lower amounts of DM during the dry period versus the lactating period, and the DMI of dry cows further decreases until parturition. Dry cow rations contain higher grass silage and lower corn silage proportions in comparison to lactation rations. This comes along with a lower starch content, lower digestibility and, therefore, a decreased palatability (BAUMONT 1996). In addition, hormonal changes in late gestation and the decrease of available abdominal space due to the growing conceptus impacts DMI negatively (INGVARTSEN & ANDERSEN 2000). After parturition rumen capacity increases and, concurrently with the increase in DMI, CH_4 production rises during early lactation.

However, PINARES-PATIÑO et al. (2007) showed that the differences in CH_4 production and CH_4 yield for dry and lactating cows also occurs without a diet change.

Although the main differences in CH_4 emission are attributable to DMI and ration composition, different levels of CH_4 production were described for individual animals on the same diet in studies by BELL et al. (2014), GARNSWORTHY et al. (2012) and GRAINGER et al. (2007). The observed coefficients of variation (CV; unpublished data) for CH_4 production between animals ranged from 9% in late lactation to 12% in early and peak lactation (Publication 2), and from 12% in peak lactation to 18% in early lactation and the dry period (Publication 1). For CH_4 yield CV were similar and ranged from 7% in early lactation to 11% in peak lactation (Publication 2) and from 11% in peak lactation to 15% in the dry period (Publication 1). These data are comparable to CV values reported in earlier studies. BLAXTER & CLAPPERTON (1965) observed a CV for CH_4 production between animals on the same diet of 7 – 8 % for sheep and cows (measured in respiration chambers). With the use of SF_6 technique and the sampling of exhaled air during milking a CV of about 19% for *ad libitum* fed dairy cows was reported (GARNSWORTHY et al. 2012, GRAINGER et al. 2007).

A variation of CH_4 production can also be observed within animals using repeated measurements. GRAINGER et al. (2007) reported a CV of 4.3 – 6.1% for CH_4 emission within individuals, depending on measurement technique. GARNSWORTHY et al. (2012) reported a CV of 11.5% within cows and attributed both the variability within a cow and between cows to body weight, milk yield, parity and week of lactation. The CV of CH_4 production for the animals investigated in the present studies ranged between 6 – 44%, the CV for CH_4 yield ranged between 3.5 – 16.5%. However, these numbers include both the dry and lactation period and, thus, also a diet change.

Ranking of cows according to their CH_4 production or CH_4 yield was performed in quest of high and low emitting animals. The repeatability of the ranking in repetitive measurements is only moderate to low, though, according to MÜNGER & KREUZER (2008), PICKERING et al. (2015), and PINARES-PATIÑO et al. (2007). It further decreases with increased time between measurements (DONOGHUE et al. 2016, PICKERING et al. 2015). While some studies were able to demonstrate a consistent ranking of high or low CH_4 emitting cows on the same diet in repetitive measurements (BELL et al. 2014, GARNSWORTHY et al. 2012, RISCHEWSKI et al. 2017), others did not observe any consistencies (MÜNGER & KREUZER 2008). Although ROEHE et al. (2016) assumed that

ranking of sires would not change according to diet, CARBERRY et al. (2014) and GOOPY & HEGARTY (2004) reported that the ranking of high and low emitting sheep was altered when animals were transitioned to a different ration.

The reliability of ranking depends on the differences in CH_4 production between animals (VLAMING et al. 2008), the number of animals investigated (GARNSWORTHY et al. 2012) and the used measuring technique (HAMMOND et al. 2014). Repeatability can further be improved using longer measuring periods as arise for example when using methane hood measurement techniques versus the GreenFeed system (TROY et al. 2016). Although the respiration chamber delivers CH_4 data from a comparably very long period of 24 hours, the cows in the present study also lacked consistency in CH_4 production in repeated measurements. No single animal was at all time points either extremely high or extremely low in CH_4 production or CH_4 yield during its lactation cycle.

4.2.2 Rumen short-chain fatty acids and methane

As proposed in the mid 70s by DEMEYER & VAN NEVEL (1975) and outlined in section 2.3.3, the production of different SCFAs is accompanied by different amounts of consumed and released [2H] and CO_2, the substrates used by the majority of archaea to produce CH_4. A variety of studies showed a positive relationship between the ruminal acetate concentration or the acetate to propionate ratio, respectively, and CH_4 emission from animals fed different diets (BENCHAAR et al. 2013, LETTAT et al. 2013, MOHAMMED et al. 2011, SUN et al. 2015, VENEMAN et al. 2015). For the animals of the present study, fermentation patterns were expected to be comparable. Nevertheless, ruminal SCFA concentrations changed throughout lactation and the ratio of (acetate + butyrate) : propionate was higher in late than in early lactation, despite the fact that the cows received a comparable ration composition (Publication 2).

In late lactation, higher rumen propionate concentrations were observed in the low CH_4 yield group than in the high CH_4 yield group (Publication 2, Figure 3a). Acetate and butyrate concentrations were not different between high or low CH_4 emitting cows, but the ratio of (acetate + butyrate) : propionate tended to be higher in the group of cows with high CH_4 yield. According to these findings, acetate concentrations in rumen fluid are of limited value for estimating CH_4 emission in early and in late lactation.

Moreover, ruminal acetate concentrations were higher in high vs. low mobilizing cows before parturition, and comparable after calving, arguing against the hypothesized negative feed-back mechanism.
However, the concentration of SCFA does not parallel their production rates (DIJKSTRA 1994). That means that drawing conclusions from SCFA concentrations on SCFA production rates and their underlying fermentative processes could be inaccurate. Furthermore, BRASK et al. (2015) showed that concentrations of SCFA and their proportion vary throughout the day in relation to feeding times and the resulting intensity of feed intake. Therefore, the measured SCFA concentration from a single rumen fluid sample, as taken in the present study, is only of limited use for drawing conclusions on the level of methane production. This is even more relevant since the cows had *ad libitum* access to feed and the time interval between latest feed ingestion and rumen fluid sampling could not be determined. This assumption is further supported by the meta-analysis performed by ALEMU et al. (2011), concluding that enteric CH_4 production cannot be accurately predicted from SCFA concentrations. In order to quantify SCFA production rates a tracer study would be needed to relate the amount of SCFA and CH_4 produced per day (see section 2.4.3). However, snap shot measured SCFA concentrations directly reflect the metabolic conditions of the microbiota analyzed in corresponding rumen fluid samples.

4.2.3 Acetate in the cows' metabolism and methane production

The experiments described in Publication 1 were based on the hypothesis that increased body fat mobilization would result in decreased acetate utilization by the mammary gland; that in turn should act as a negative feedback for acetate production in the rumen, which would be accompanied by a decrease in CH_4 production.
Indeed, the results of this study showed an inverse relationship between fat mobilization and CH_4 production. However, plasma acetate concentrations were lower in cows with high body fat mobilization in early lactation, although one would have expected elevated plasma acetate concentrations if less acetate was metabolized. Possible reasons for this finding include different ruminal absorption rates for acetate, alternative metabolic pathways for this SCFA in other organs and different ruminal passage rates. The latter seems to be the most reasonable explanation, although

partial passage rates for distinct gastro-intestinal compartments were not determined in this study.

Hormones related to fat metabolism like leptin, ghrelin and cholecystokinin (CCK) were described to influence gastric motility, namely ghrelin increasing gastric motility with leptin and CCK having contradictory effects (MÜLLER & TSCHÖP 2013, YARANDI et al. 2011). In high mobilizing cows lower concentrations of CCK and higher concentrations of ghrelin were measured (Publication 1, BORNER et al. 2013), supporting the hypothesis of increased rumen passage rates in high mobilizing cows. However, to enlighten the effects of body fat mobilization on acetate metabolism and CH_4 production a further experimental approach involving 13C-labled acetate administration needs to be performed.

4.2.4 Fat mobilization in early lactation

High yielding dairy cows are particularly affected by the metabolic challenges of the periparturient period. As outlined in section 2.2, shortly before until about 12 weeks after calving, cows experience a period of NEB, whose duration depends on the amount of energy corrected milk yield (ECM) produced, the energy density of the diet, on DMI and on maintenance requirements. To meet the increased energy requirements, animals mobilize stored energy reserves. These include glycogen stores in liver and muscle, which are quickly depleted and only short-term energy suppliers though. In contrast, body fat can serve as a long-term energy store. Mobilization of body fat leads to increased plasma concentrations of NEFA (GRUMMER 1993). Plasma NEFA concentrations were used to distinguish between high and low mobilizing cows (Publication 1) and this grouping was also reflected in the consumption of body fat and the resulting drop of BCS in early lactation. The mobilization of body fat is a highly dynamic process. In some cows NEFA concentrations were slowly rising and remained moderately elevated over a longer period of time (e.g. 2 weeks). Other animals showed a sharp and intensive rise is NEFA concentrations. Some individuals developed more than one NEFA peak in the first weeks of lactation. Therefore, not all cows had their highest NEFA peak in the first week after parturition.

MOYES (2012) reported that NEFA can be included in an index to predict physiological imbalance and therefore the risk for disease. Metabolic disorders in the periparturient

period of dairy cows belong to the most common medical problems in modern milk production. This includes primary metabolic disorders, e.g. ketosis, fatty liver syndrome and hypocalcemia. Also, pathological conditions that are secondary to metabolic disorders have to be taken into account. These latter include for example displaced abomasum, increased incidence of mastitis and endometritis or lameness. The origins of these diseases indirectly linked to metabolic disorders can originate from a weakened immune system due to NEB or from toxin absorption and loss of hematologic homeostasis due to ruminal acidosis among other coherences. The cows used in the presented study showed high incidence of *retentio secundinarium* (10 animals), two cows presented with displaced abomasum very shortly after the first calving (2 days), and all of them had dermatitis digitalis in varying extents throughout lactation. However, the frequency of the disorders was not different between the two groups. Many of the periparturient disorders are ultimately linked to the NEB (RUKKWAMSUK et al. 1999), of whose degree plasma NEFA concentrations are a good indicator.

Simultaneously to the NEB, an inverse relationship between plasma NEFA concentrations and CH_4 yield was observed (Publication 1), which was not evident later in lactation or during the dry period. This indicates that the host animals' metabolic status might affect rumen fermentation and the involved microorganisms.

4.2.5 NEFA in the context of metabolism

Usually, high NEFA concentrations are associated with higher milk fat content, and therefore higher ECM (PALMQUIST 2006), both of which was not the case in the present study.

The differences of milk fatty acid profiles in cows with high or low methane emission have been extensively described in literature (e.g. DIJKSTRA et al. 2016, VAN GASTELEN et al. 2015, VAN LINGEN et al. 2014, MOHAMMED et al. 2011, VANLIERDE et al. 2015). Based on these studies it can be assumed that high methane production is accompanied by higher acetate utilization for milk fatty acid synthesis in the mammary gland. As discussed in the first paper, a better availability of NEFA in high mobilizing cows should diminish the relative use of acetate for milk fat synthesis and therefore result in rising plasma acetate concentrations. PALMQUIST (2006) stated that a net

uptake of NEFA into the mammary gland occurs with high NEFA concentrations. Rising NEFA concentrations could, therefore, replace acetate as a source for milk fat synthesis. However, in contrast to this idea, high mobilizing and low CH_4 yielding animals had lower plasma acetate concentrations compared to low mobilizing cows in the present study. Furthermore, no relationship between plasma and rumen fluid acetate concentration was detected. Therefore, the concentration of acetate in blood plasma may not be directly linked to the production of acetate by the ruminal microorganisms.

4.2.6 Rumen microbes

The rumen harbors a rich community of anaerobic microbes, whose spectrum of species and composition differs from that in the hindgut. The variety and population count of microbial species can be discriminated between different sections of the digestive system and among host species.

Despite of existing differences in microbial abundance and community spectrum, the need to degrade fiber and conversion of complex carbohydrates to SCFA results in the colonization by several predominant microbial organisms including *Bacteroides* and *Ruminococcus* both in rumen and hindgut (ALLISON 2004).

The bacterial community structure of the cows investigated herein was dominated by the genera *Prevotella*, *Succiniclasticum*, *Treponema*, *Fibrobacter*, *Ruminococcus*, *Ruminobacter*, and *Butyrivibrio*, many of which belong to the phyla Firmicutes and Bacteroidetes. Most of them were identified as the minimal "core microbiome" of Holstein (JEWELL et al. 2015, LIMA et al. 2015) and Jersey (HERNANDEZ-RAQUET et al. 2016) dairy cows, Angus heifers (PETRI et al. 2013) or Aberdeen-Angus and Limousin beef cattle (WALLACE et al. 2015). The overall community structure and individual microbe abundances may differ between these breeds, though.

The archaeal genera *Methanobrevibacter*, *Methanomassiliicoccales* and *Methanosphaera* dominated nearly all samples in the study by HENDERSON et al. (2015). In accordance with those results, the vast majority of archaeal organisms identified in the present study belong to the genus *Methanobrevibacter* (Publication 2, Fig. 1b), which is in line with previous reports (e.g. CARBERRY et al. 2014, DANIELSSON et al. 2012, ST-PIERRE et al. 2015, WALLACE et al. 2015).

Protozoa communities were not analyzed in the present study, however (HENDERSON et al. 2015) further reports that white protozoal communities were usually variable among animal species and geographical locations, Archaea were the most conserved group of organisms, which seems to be true for the presented results as well.

4.2.6.1 Microbial community change over time

From birth until adulthood the rumen interior changes dramatically from an almost sterile environment to an extremely diverse microbial ecosystem (JAMI et al. 2013, YÁÑEZ-RUIZ et al. 2015). Even after establishment of the adult rumen function, the bacterial diversity continues to increase when the animal passes through metabolic, environmental and dietary changes (JEWELL et al. 2015, PITTA et al. 2014). However, unless those major interventions like anti-methanogen vaccination, diet changes or feed additive supplementation occur, the mature rumen microbiome is believed to be relatively stable. Still, recent research shows that part of the microbial community appears to be dependent on the metabolic state or on the genetics of the host (PETRI et al. 2013, ROEHE et al. 2016).

In the present study the bacterial and the archaeal community structure changed from early until late lactation despite the fact that cows received a very similar diet over the entire sampling period (Publication 2). Comparable results were obtained by MICHELLAND et al. (2011), who found the bacterial community structure changed in non-lactating Holsteins cows over a period of 56 days. Yet, against expectations, bacterial species richness and the Shannon diversity index decreased from mid to late lactation (Publication 2, Table S2). In the present study, the greatest contributors to the dissimilarity in the microbial community composition were bacteria that were not among the most prevalent genera, namely *Acetobacter*, *Bifidobacterium*, *Lactobacillus*, *Arthrobacter* and *Clostridium_IV spp* (Publication 2, Table 2).

These findings suggest that under otherwise unaltered environmental conditions the abundance of single bacterial genera may be sensitive towards metabolic changes occurring during the course of lactation.

4.2.6.2 Community differences between individuals

The individual cows of the present study had different abundances of bacterial and archaeal genera, as well as varying SCFA concentrations in the rumen fluid while being fed the same ration. This observation confirms an element of individuality in the microbial community structure and metabolism. A possible explanation for this individual community structure might be the ability of some bacteria to compensate for the metabolism of others. Some strains within a genus possess different metabolic activities than others, resulting in fluctuations in the occupation of different niches and varying product amounts. For example, the HD4 strain of *Selenomonas ruminantium* prefers to metabolize glucose but is also able to utilize lactate. The organism can either be a lactate producer or utilizer (RUSSELL & BALDWIN 1978). Furthermore, *S. bovis* usually produces acetate, formate and ethanol but is also able to change its metabolism to provide lactate if the host is fed a high concentrate diet (CASTILLO-GONZÁLEZ et al. 2014). The metabolic versatility of microbes could therefore enable different sets of organisms to form a stable community, depending on the microbial environment.

It is well established that diet has a crucial impact on the microbial community structure in the rumen (BELANCHE et al. 2012, CARBERRY et al. 2014, LIMA et al. 2015). For example, a diet with a high starch content increases the abundance of amylolytic bacteria (LETTAT et al. 2013). The adaption of the microbiome to the ingested feed is not permanent, however. The effect of diet on the microbial community can be short lived and is often inconsistent (YÁÑEZ-RUIZ et al. 2015). LETTAT et al. (2013) concluded that the "effect of diets on the growth of cellulolytic bacteria is variable and multifactorial", and WEIMER et al. (1999) demonstrated that the cellulolytic bacterial populations in the rumen were rather dependent on the individual cow than on the diet type. The microbial community is even able to change back to its original composition after the rumen content of a cow was nearly completely exchanged with the content of a donor animal (WEIMER et al. 2010). Thus, there seems to exist a strong individual microbiota specificity. That individual specifity might be used as an indicator for selecting high and low CH_4 emitting animals from a herd.

Another explanation for microbial community differences between animals on the same diets are influences of the host. Molecules circulating in blood are known to influence gut microbiota, for example lipopolysaccharides in dairy cattle (JING et al. 2014) or

unsaturated fatty acid triacylglycerols (TAGs) in rats (HODIN et al. 2012). Studies by CARBERRY et al. (2014), HERNANDEZ-RAQUET et al. (2016), and ROEHE et al. (2016) showed genetic influences on microbial community compositions regardless of diet. Furthermore, at least for humans and laboratory animals a direct effect of host hormonal status (like estrous cycle or pregnancy) on gut microbiota has been established (MULAK et al. 2014).

4.2.6.3 Relationship between microbes and methane production levels

This study focused on the abundance and diversity of archaea and bacteria present in rumen fluid of dairy cows and did not include ruminal eukaryotes. Archaea are the sole group of microorganisms able to produce CH_4, but they depend on substrates provided by other microorganisms. While *Methanobrevibacter* reduce CO_2 with H_2 to produce CH_4, *Methanosphaera spp.* are unable to use these substrates but rely on the reduction of alcohols (HOEDT et al. 2016, ST-PIERRE et al. 2015).

As outlined in section 2.3.2 and section 5.6.2, bacterial species generate diverse metabolites and variable amounts of H_2 and CO_2 and, consequently, archaea produce different amounts of CH_4. This may lead to a range of CH_4 emission levels in a herd of cows on the same diet.

KITTELMANN et al. (2014) investigated the microbiome in sheep with varying CH_4 productions and concluded that specific bacterial community types can be attributed to high or low CH_4 emitting animals. Another study on sheep (SHI et al. 2014) identified comparable archaeal community structures in extremely high and low CH_4 emitting rams, but the low CH_4 yielding animals had elevated *Methanosphaera spp.* and lower relative abundances of *Methanobrevibacter gottschalkii*. The microbial communities of dairy cows with different CH_4 yields on the same diet have not been compared before, although very recently a similar approach was published (DANIELSSON et al. 2017). The group by DANIELSSON et al. (2017) conducted a trial using a larger herd of 73 dairy cows from which they retrospectively selected 6 high, 8 medium and 7 low CH_4 emitters. They detected that both methanogenic and bacterial community structures correlate with CH_4 production in mid and late lactation although CH_4 yields were not different between groups. A similar correlation between archaeal abundances and CH_4 emissions was also described by others (DANIELSSON et al. 2012, SHI et al. 2014,

WALLACE et al. 2014, ZHOU et al. 2011), but, likely due to only small inter-individual differences, those findings could not be confirmed in the present study.

Some studies also addressed the bacterial abundance and its relationship with CH_4 emission. A lower proportion of Proteobacteria and a higher abundance of Actinobacteria were found in high CH_4 emitting cows (DANIELSSON et al. 2017, WALLACE et al. 2015). The rationale of these authors was that a higher abundance of H_2 producing bacteria should result in a higher CH_4 production. However, metabolic capabilities of bacterial families are diverse, and not all species present in the rumen are metabolically fully characterized yet (SESHADRI et al. 2018). Furthermore, the contribution of eukaryotes and the presence of yet undetected bacterial species can lead to varying concentrations of CH_4 precursors, making it difficult to draw implications from the abundance of selected bacterial genera to CH_4 production. On the other hand, the comparatively few metabolic pathways leading to CH_4 formation and the exclusive role of methanogens suspect a direct relationship between archaeal abundance and CH_4 production.

The present study followed a similar attempt as DANIELSSON et al. (2017) and WALLACE et al. (2015) and tried to identify differences in microbial communities between cows with either high or with low CH_4 yields as described in the second paper. A difference in community structure was not found between the two extremes, although some bacterial genera actually differed in their relative abundance. For example, the propionate producer *Succiniclastium* (VAN GYLSWYK 1995) was higher abundant in cows with a lower CH_4 yield. Further, increased levels of propionate were linked with lower CH_4 yields. However, neither for high nor low CH_4 yielding cows in late lactation a consistent correlation was detected between *Succiniclasticum* abundances and propionate concentrations in the rumen fluid (Publication 2).

Therefore, the consideration of bacterial or archaeal abundances alone to assign individual cows to the high or low CH_4 yielding group is therefore not possible. SIROHI et al. (2012) suggested that metatranscriptomic and metabolomic approaches as performed in previous studies (ROEHE et al. 2016, SHI et al. 2014, WALLACE et al. 2014, ZHAO et al. 2014) could improve the understanding of the metabolic activity as well as the interactions within the microbial community in the ruminal community. This could help to enlighten the interrelationship between the microbial community and CH_4 production.

4.2.7 Further considerations

The presented work focused on measuring methane emission from rumen and on postruminal fermentation processes of dairy cows by utilizing whole body respiration chambers.

According to BRASK et al. (2015) about 4% of methane production in dairy cows takes place postruminally, which is lower than the 13% reported in sheep (MURRAY et al. 1976). Besides species differences, this large discrepancy might be due to the high fermenting intensity in the rumen of dairy cows fed an energy-dense diet based on conserved roughages compared to cows on pasture.

RICCI et al. (2014) described the three routes CH_4 can be eliminated from the animal: First emission of CH_4 from the hindgut via flatus (about 2% of the total CH_4 emission); second, release of CH_4 from the forestomach via eructation, and, third, absorption of CH_4 from the rumen and lower gut into the blood stream followed by elimination via the lungs during expiration. The latter two are often summarized as exhaled CH_4. HAMMOND et al. (2016) reviewed the proportions of CH_4 on the different production sites and elimination routes; ruminant species and timespan between feeding and measurement seem to influence the proportions. Nevertheless, with a proportion of 98% the major part of the produced CH_4 is eliminated via exhalation. By using whole body CH_4 measurements and breath sampling techniques at the same time (e.g. the SF_6 method on an animal in a respiration chamber) the amount of CH_4 produced in the rumen and exhaled can be estimated.

Although intestinal CH_4 production contributes just a small proportion of emission in ruminants - compared to monogastric animals - the intestinal digestibility can still be a relevant source for variation of CH_4 production in dairy cows (BRASK et al. 2015). Thus, caution has to be taken when drawing conclusions between ruminal digestive processes with the involved microbial community structures and the measured methane yields.

Passage rate or mean retention time of particles in the digestive system seems to be a main factor explaining the individual differences of methane emission in our cows. Slower partial passage rates through the reticulorumen and a longer fermentation time are perceived as the most probable explanation for the higher CH_4 yield in cows with low fat mobilization. This assumption originates in the observation that hormones

related to fat metabolism influence gut motility. Indeed lower concentrations of CCK were found in high mobilizing cows. GROVUM (1981) described a dose-related depressing effect of CCK on the motility of the reticulum. RELLING et al. (2011) could not confirm an increase of passage rate by intravenous CCK infusion though. Contrary to humans, CCK plasma levels in dairy cows are not elevated after feeding (FURUSE et al. 1991). Thus, an impact of time between the latest feed intake and blood sampling in this study can be neglected. However, ruminal motility is controlled by many factors including feed intake, diet composition, health status and a variety of hormones and metabolites, also ruminal CH_4 formation influences motility of the reticulorumen (DITTMANN et al. 2016). The marker study using titanium dioxide (TiO_2) showed no differences in whole-tract passage rates between the animals (Publication 1). However, it would have been desirable to determine partial passage rates for liquid and solid phases in different gastro-intestinal compartments. If future studies pursue this approach, multi-fistulated cows will be needed for accurate measurements of fractional passage rates, and repeated sampling periods throughout the lactation cycle. The investigation of fractional passage rates in combination with microbial community structures could also provide further clarification of the divergent SCFA concentrations between animals.

Also, for a precise description of metabolic processes and interaction, metabolite concentrations in the digestive tract should not be evaluated isolated, but in correlation with microbial measures, ideally including metabolomic approaches. More research is needed to extend our understanding of the interaction between archaea, bacteria and eukaryotes. Detailed knowledge of microbes and their metabolism network will improve the comprehension of these factors influencing on ruminal fermentation and, therefore, CH_4 production.

5 CONCLUSIONS

The repeated measurements and samplings of the same cows at different stages of lactation gave valuable insight in the variability of CH_4 yields and CH_4 production and their associations to the metabolic state of the host, also on rumen microbial diversity and abundance and on alterations of microbial communities.

It was possible to link CH_4 yields in early lactation to blood plasma NEFA concentrations as a measure of fat mobilization, and to show that ruminal acetate concentrations during this critical time of NEB were unrelated to CH_4 production.

The observed changes in the microbial community over the course of the first lactation indicated a variability of the mature microbiome that is unrelated to diet composition or environmental conditions. No correlation between the bacterial and archaeal community structure to CH_4 yield was found. However, lower concentrations of propionate were found in the rumen fluid of high CH_4 yielding cows compared to low yielding cows in late lactation.

6 SUMMARY

Author: Anita Bielak

Title: Relation between metabolic state, microbial community structure and methane production in dairy cows

Institution: Institute of Veterinary Physiology, Faculty of Veterinary Medicine, University of Leipzig and Institute of Nutritional Physiology, Leibniz Institute for Farm Animal Biology Dummerstorf

Submitted: January 2019

Bibliographic indications: 80 pages, 2 publications, 1 table, 1 diagram, 153 references

Keywords: methane, dairy cow, NEFA, rumen microbiome

Introduction: Methane (CH_4) production in dairy cows is influenced by a variety of environmental and host-specific factors, among which dry matter intake (DMI) and ration composition have the greatest impact. The major part of CH_4 is produced in the rumen by Archaea. The short-chain fatty acid (SCFA) acetate is also produced in the rumen by microbial fermentation and can be used by the host to synthesize milk fat in the mammary gland. The production of acetate is correlated with ruminal CH_4 production. Milk fat can also be synthesized from non-esterified fatty acids (NEFA) and triacylglycerol that originate from endogenous fat stores of dairy cows, especially during times of fat mobilization.

Aims of the study: This study checked the hypothesis that a higher fat mobilization during early lactation decreases ruminal acetate production by replacing acetate for milk fat synthesis and, thus, decreases synthesis of CH_4. Another aim of this study was to test the hypothesis that increases in CH_4 yield (CH_4/DMI) over the course of lactation are associated with changes in rumen microbial community profile, and that high and low CH_4 emitting cows differ in their bacterial and archaeal community structure.

Animals, Materials and Methods: A herd of 20 Holstein cows was studied during the course of their first lactation; feed intake and diet composition was monitored. Blood and rumen fluid were repeatedly sampled throughout the trial. Plasma NEFA concentrations were analyzed by photometrical analysis, and rumen SCFA concentrations by gas chromatography. Individual CH_4 production was measured in respiration chambers at four times during the observation period. In a subgroup of 9 cows, rumen fluid samples from 3 timepoints during lactation were subjected to DNA

extraction and bacterial and archaeal amplicons of the 16S-rRNA-gene were sequenced. The bacterial and archaeal community structures in the rumen fluid were described, and the rumen microbiome composition linked to CH_4 yield. Statistical analysis was conducted using repeated measurement ANOVA and Tukey tests, as well as Pearsons' correlation for selected parameters. Microbial data was further treated with multivariate analyses (PERMANOVA) and Bray-Curtis dissimilarities were determined.

Results: Total CH_4 production increased significantly over time from an average 208 L/day during the dry period to 516 L/day in late lactation. The level of fat mobilization, expressed as blood plasma NEFA concentrations, and CH_4 yield showed an inverse relationship in early lactation ($p = 0.002$). High mobilizing cows (NEFA > 580 µmol/L) tended to show higher ruminal acetate concentrations than low mobilizing cows (NEFA < 580 µmol/L) only before parturition and not during lactation. Despite a diet composition that was kept as constant as possible throughout the lactation, the microbial community changed significantly over time as indicated by a decrease in species richness and species evenness. However, in late lactation when CH_4 yield was highest, no difference in bacterial or archaeal community structure could be detected between the three highest CH_4 yielding cows and the three lowest CH_4 yielding cows. The ratio of (acetate + butyrate) / propionate in rumen fluid changed significantly with progressing lactation from 3.5 to 4.4, accompanied by an increase in CH_4 production from 434.3 L/d to 540.5 L/d. However, no correlation between the concentration of ruminal SCFA and CH_4 yield was found.

Conclusions: The metabolic state of the animal, especially the degree of fat mobilization during times of negative energy balance, had an impact on CH_4 yield. Also, the microbial community composition in the rumen and its metabolic network is adaptable and changes over time. However, in this study individual microorganisms could not be identified to serve as predictor for CH_4 emission from dairy cows at the moment. Rather, shifts in the microbial communities as a whole appear to be responsible for the changes in CH_4 yield.

7 ZUSAMMENFASSUNG

Verfasser: Anita Bielak

Titel: Zusammenhang zwischen Stoffwechselstatus, mikrobieller Gemeinschaftsstruktur und Methanproduktion bei Milchkühen

Institution/Klinik: Veterinär-Physiologisches Institut, Veterinärmedizinische Fakultät, Universität Leipzig und Institut für Ernährungsphysiologie, Leibniz-Institut für Nutztierbiologie Dummerstorf

Eingereicht: Januar 2019

Bibliographische Angaben: 80 Seiten, 2 Publikationen, 1 Abbildung, 1 Tabelle, 153 Literaturangaben

Schlüsselwörter: Methan, Milchkuh, NEFA, Pansenmikrobiom

Einleitung: Die Produktion von Methan (CH_4) bei Milchkühen wird durch eine Vielzahl von umwelt- und wirtsspezifischen Faktoren beeinflusst, wobei Trockensubstanzaufnahme (DMI) und Rationszusammensetzung die größte Auswirkung haben. Der größte Teil des CH_4 wird von Archaeen im Pansen produziert. Auch die kurzkettige Fettsäure (SCFA) Acetat wird im Pansen durch mikrobielle Fermentation gebildet und kann vom Wirtstier zur Milchfettsynthese im Euter verwendet werden. Die Acetatbildung im Pansen korreliert mit der CH_4-Produktion. Allerdings kann Milchfett auch aus freien Fettsäuren (NEFA) und Triacylglycerolen endogenen Ursprungs synthetisiert werden, insbesondere aus mobilisiertem Körperfett.

Ziele der Untersuchungen: In dieser Studie wurde die Hypothese überprüft, dass eine Verdrängung des zur Milchfettbildung genutzten Acetats durch eine höhere Körperfettmobilisation in der Frühlaktation die ruminale Acetatproduktion senkt und damit die Bildung von CH_4 verringert. Ein weiteres Ziel war zu untersuchen, ob der Anstieg der CH_4-Ausbeute (CH_4/DMI) im Laktationsverlauf mit einer Veränderung des Mikrobioms assoziiert ist, und ob sich Kühe mit hoher oder niedriger CH_4-Emission in ihrer Bakterien- und Archaeen-Zusammensetzung unterscheiden.

Tiere, Material und Methoden: 20 Holstein Kühe wurden in ihrer ersten Laktation untersucht; ihre Futteraufnahme und Rationszusammensetzung wurde analysiert. Im Verlauf des Versuchs wurden mehrfach Blut- und Pansensaftproben gewonnen. Die Plasma-NEFA-Konzentrationen wurden photometrisch, die Pansen-SCFA-Konzentrationen mittels Gaschromatographie analysiert. Während des

Beobachtungszeitraums wurde an 4 Zeitpunkten die individuelle CH_4-Produktion in Respirationskammern erfasst. In einer Untergruppe von 9 Kühen wurden Pansensaftproben von 3 Zeitpunkten während der Laktation einer DNA-Extraktion unterzogen und bakterielle und archaeale Amplifikate des 16S-rRNA-Gens wurden sequenziert. Die Bakterien- und Archaeenpopulation im Pansensaft wurde beschrieben und das Pansenmikrobiom der CH_4-Ausbeute gegenübergestellt. Statistische Auswertungen wurden mit repeated measurements ANOVA und Tukey-Tests, sowie mit der Pearsons` Korrelation für ausgewählte Parameter durchgeführt. Mikrobielle Daten wurden mit multivariaten Analysen (PERMANOVA) weiterverarbeitet und Bray-Curtis-Unähnlichkeiten ermittelt.

Ergebnisse: Die gesamte CH_4-Produktion stieg signifikant von durchschnittlich 208 l/Tag in der Trockenperiode auf 516 l/Tag in der Spätlaktation an. Der Grad der Körperfettmobilisation, ausgedrückt als Plasma-NEFA-Konzentration, und die CH_4-Ausbeute waren in der Frühlaktation negativ korreliert ($p = 0{,}002$). Kühe mit hoher Fettmobilisation (NEFA > 580 µmol/l) neigten nur vor der Kalbung, aber nicht während der Laktation zu höheren Pansenacetat-Konzentrationen als Tiere mit niedriger Mobilisation (NEFA < 580 µmol/l). Trotz einer weitgehend gleichbleibenden Rationszusammensetzung während der Laktation änderte sich das Mikrobiom mit der Zeit signifikant, was sich in einer Abnahme des Artenreichtums und der Biodiversität zeigte. In der Spätlaktation, als die CH_4-Ausbeute am höchsten war, gab es keinen Unterschied in der bakteriellen oder archaealen Populationsstruktur zwischen den drei Kühen mit der schwächsten und den dreien mit der stärksten CH_4-Ausbeute.

Parallel zum Anstieg der CH_4-Produktion von 434,3 l/Tag auf 540,5 l/Tag veränderte sich das Verhältnis von (Acetat + Butyrat) / Propionat im Pansensaft mit dem Fortschreiten der Laktation von 3,5 auf 4,4. Dennoch war kein Zusammenhang zwischen der Konzentration der ruminalen SCFA und der CH_4-Ausbeute festzustellen.

Schlussfolgerungen: Der Stoffwechselzustand des Tieres, insbesondere der Grad der Körperfettmobilisierung bei negativer Energiebilanz, nahm Einfluss auf die CH_4-Ausbeute. Die Zusammensetzung des Mikrobioms im Pansen und dessen Stoffwechselnetzwerk veränderte sich mit der Zeit. Es war jedoch in dieser Studie nicht möglich, einzelne Mikroorganismen als Prädiktor für die CH_4-Emission von Milchkühen zu identifizieren. Vielmehr scheinen Verschiebungen der mikrobiellen Gemeinschaften insgesamt für die Veränderung der CH_4-Ausbeute verantwortlich zu sein.

8 REFERENCES

Aguinaga Casañas MA, Rangkasenee N, Krattenmacher N, Thaller G, Metges CC, Kuhla B. Methyl-coenzyme M reductase A as an indicator to estimate methane production from dairy cows. J Dairy Sci. 2015; 98:4074–83.

Alemu AW, Dijkstra J, Bannink A, France J, Kebreab E. Rumen stoichiometric models and their contribution and challenges in predicting enteric methane production. Anim Feed Sci Technol. 2011; 166–167:761–78.

Allison MJ. Microbiology of Fermentative Digestion in the Rumen and Large Intestine. In: Reece WO, editor. Dukes' Physiology of Domestic Animals. 12th ed. Ithaca: Comstock Publishing Associates a division of Cornell University Press; 2004. p. 475–87.

Annison EF, Bryden WL. Perspectives on ruminant nutrition and metabolism. Nutr Res Rev. 1998; 11:173–98.

Arndt C, Powell JM, Aguerre MJ, Crump PM, Wattiaux MA. Feed conversion efficiency in dairy cows: Repeatability, variation in digestion and metabolism of energy and nitrogen, and ruminal methanogens. J Dairy Sci. 2015; 98:3938–50.

Aschenbach JR, Bilk S, Tadesse G, Stumpff F, Gäbel G. Bicarbonate-dependent and bicarbonate-independent mechanisms contribute to nondiffusive uptake of acetate in the ruminal epithelium of sheep. Am J Physiol Gastrointest Liver Physiol. 2009; 296:G1098–107.

Bailey CB, Balch CC. Saliva secretion and its relation to feeding in cattle. Br J Nutr. 1961; 15:383–402.

Baumont R. Palatability and feeding behaviour in ruminants. A review. Ann Zootech. 1996; 45:385–400.

Beauchemin KA, McGinn SM, Benchaar C, Holtshausen L. Crushed sunflower, flax, or canola seeds in lactating dairy cow diets: Effects on methane production, rumen fermentation, and milk production. J Dairy Sci. 2009; 92:2118–27.

Belanche A, de la Fuente G, Pinloche E, Newbold CJ, Balcells J. Effect of diet and absence of protozoa on the rumen microbial community and on the representativeness of bacterial fractions used in the determination of microbial protein synthesis. J Anim Sci. 2012; 90:3924–36.

Belanche A, Moorby J, Pinloche EJR, Newbold CJ, Newbold CJ. Shifts in the Rumen Microbiota Due to the Type of Carbohydrate and Level of Protein Ingested by Dairy Cattle Are Associated with Changes in Rumen Fermentation. J Nutr. 2012; 142:1684–92.

Bell MJ, Potterton SL, Craigon J, Saunders N, Wilcox RH, Hunter M, et al. Variation in enteric methane emissions among cows on commercial dairy farms. Animal. 2014; 8:1540–6.

Benchaar C, Hassanat F, Gervais R, Chouinard PY, Julien C, Petit H V, et al. Effects of increasing amounts of corn dried distillers grains with solubles in dairy cow diets on methane production, ruminal fermentation, digestion, N balance, and milk production. J Dairy Sci. 2013; 96:2413–27.

Bhatta R, Enishi O, Kurihara M. Measurement of methane production from ruminants. Asian-Aust J Anim Sci. 2007; 20:1305–18.

Blaxter KL, Clapperton JL. Prediction of the amount of methane produced by ruminants. Br J Nutr. 1965; 19:511–22.

Blümmel M, Aiple K-P, Steingabeta H, Becker K. A note on the stoichiometrical relationship of short chain fatty acid production and gas formation in vitro in feedstuffs of widely differing quality. J Anim Physiol Anim Nutr (Berl). 1999; 81:157–67.

Börner S, Derno M, Hacke S, Kautzsch U, Schaff C, ThanThan S, et al. Plasma ghrelin is positively associated with body fat, liver fat and milk fat content but not with feed intake of dairy cows after parturition. J Endocrinol. 2013; 216:217–29.

Brade W. Milcherzeugung unter den Bedingungen des Klimawandels - Möglichkeiten zur Vermeidung oder Minderung des Hitzestresses. Berichte über Landwirtschaft - Zeitschrift für Agrar und Landwirtschaft. 2013 Jan 1; 91.

Brask M, Weisbjerg MR, Hellwing ALF, Bannink A, Lund P. Methane production and diurnal variation measured in dairy cows and predicted from fermentation pattern and nutrient or carbon flow. Animal. 2015; 9:1795–806.

Breves G, Pfannkuche H, Diener M, Aschenbach JR, Leonhard-Marek S, Martens H, et al. Magen-Darm-Trakt. In: von Engelhardt W, Breves G, Diener M, Gäbel G, editors. Physiologie der Haustiere. 5th ed. Stuttgart: Enke im Hippokrates Verlag GmbH; 2015. p. 345–450.

Carberry CA, Waters SM, Kenny DA, Creevey CJ. Rumen methanogenic genotypes differ in abundance according to host residual feed intake phenotype and diet type. Appl Environ Microbiol. 2014; 80:586–94.

Castillo-González AR, Burrola-Barraza ME, Domínguez-Viveros J, Chávez-Martínez A. Rumen microorganisms and fermentation. Arch Med Vet. 2014; 46:349–61.

Cersosimo LM, Bainbridge ML, Kraft J, Wright A-DG. Influence of periparturient and postpartum diets on rumen methanogen communities in three breeds of primiparous dairy cows. BMC Microbiol. 2016; 16:78.

Chilliard Y, Martin C, Rouel J, Doreau M. Milk fatty acids in dairy cows fed whole crude linseed, extruded linseed, or linseed oil, and their relationship with methane output. J Dairy Sci. 2009; 92:5199–211.

Ciais P, Sabine C, Bala G, Bopp L, Brovkin V, Canadell J, et al. In: Climate Change 2013: The Physical Science Basis. Contribution of Working Group I to the Fifth Assessment Report of the Intergovernmental Panel on Climate Change; 465–570.

Danielsson R, Schnürer A, Arthurson V, Bertilsson J. Methanogenic population and CH4 production in swedish dairy cows fed different levels of forage. Appl Environ Microbiol. 2012; 78:6172–9.

Danielsson R, Dicksved J, Sun L, Gonda H, Müller B, Schnürer A, et al. Methane Production in Dairy Cows Correlates with Rumen Methanogenic and Bacterial Community Structure. Front Microbiol. 2017; 8:1–15.

Demeyer DI, Van Nevel CJ. Methanogenesis, an Integrated Part of Carbohydrate Fermentation, and its Control. In: McDonald IW, Warner ACI, editors. Proceedings of the IV International Symposium on Ruminant Physiology, Sydney, Australia, August 1974. Sydney, Australia: The University of New England Publishing Unit; 1975. p. 366–82.

Deng W, Xi D, Mao H, Wanapat M. The use of molecular techniques based on ribosomal RNA and DNA for rumen microbial ecosystem studies: A review. Mol Biol Rep. 2008; 35:265–74.

Dias RS, Patino HO, López S, Prates E, Swanson KC, France J. Relationships between chewing behavior, digestibility, and digesta passage kinetics in steers fed oat hay at restricted and ad libitum intakes. J Anim Sci. 2011; 89:1873–80.

Dijkstra J, Van Gastelen S, Antunes-Fernandes EC, Warner D, Hatew B, Klop G, et al. Relationships between milk fatty acid profiles and enteric methane production in dairy cattle fed grass- or grass silage-based diets. Anim Prod Sci. 2016; 56:541–8.

Dijkstra J. Production and absorption of volatile fatty acids in the rumen. Livest Prod Sci. 1994; 39:61–9.

Dijkstra J. Mathematical Modelling and Integration of Rumen Fermentation Processes. Wageningen Agricultural University; 1993.

Dittmann MT, Hammond KJ, Kirton P, Humphries DJ, Crompton LA, Ortmann S, et al. Influence of ruminal methane on digesta retention and digestive physiology in non-lactating dairy cattle. Br J Nutr. 2016; 116:763–73.

Dlugokencky E. NOAA/ESRL. Trends in Atmospheric Methane. 2017 [cited 2017 Jul 7]. www.esrl.noaa.gov/gmd/ccgg/trends_ch4/

Donoghue KA, Bird-Gardiner T, Arthur PF, Herd RM, Hegarty RS. Repeatability of methane emission measurements in Australian beef cattle. Anim Prod Sci. 2016 Feb 9; 56:213.

Doreau M, Chilliard Y. Digestion and metabolism of dietary fat in farm animals. Br J Nutr. 1997; 78 Suppl 1:S15–35.

Ellis JL, Dijkstra J, Kebreab E, Bannink A, Odongo NE, McBride BW, et al. Aspects of rumen microbiology central to mechanistic modelling of methane production in cattle. J Agric Sci. 2008; 146:213–33.

Evans PN, Parks DH, Chadwick GL, Robbins SJ, Orphan VJ, Golding SD, et al. Methane metabolism in the archaeal phylum Bathyarchaeota revealed by genome-centric metagenomics. Science. 2015; 350:434–8.

Ferry JG. Biochemistry of Methanogenesis. Crit Rev Biochem Mol Biol. 1992; 27:473–503.

Firkins JL, Yu Z. Ruminant nutrition symposium: How to use data on the rumen microbiome to improve our understanding of ruminant nutrition. J Anim Sci. 2015; 93:1450–70.

Food and Agriculture Organization of the United Nations (FAO). Greenhouse Gas Emissions from the Dairy Sector, A Life Cycle Assessment. 2010.

Furuse M, Yang SI, Choi YH, Kawamura N, Takahashi A, Okumura J. A note on plasma cholecystokinin concentration in dairy cows. Anim Prod. 1991; 53:123–5.

Garnsworthy PC, Craigon J, Hernandez-Medrano JH, Saunders N. Variation among individual dairy cows in methane measurements made on farm during milking. J Dairy Sci. 2012; 95:3181–9.

van Gastelen S, Antunes-Fernandes EC, Hettinga K a, Klop G, Alferink SJJ, Hendriks WH, et al. Enteric methane production, rumen volatile fatty acid concentrations, and milk fatty acid composition in lactating Holstein-Friesian cows fed grass silage- or corn silage-based diets. J Dairy Sci. 2015; 98:1915–27.

Goopy JP, Hegarty RS. Repeatability of methane production in cattle fed concentrate and forage diets. J Anim Feed Sci. 2004; 13:75–8.

Goopy JP, Donaldson A, Hegarty R, Vercoe PE, Haynes F, Barnett M, et al. Low-methane yield sheep have smaller rumens and shorter rumen retention time. Br J Nutr. 2014;111(4):578–85.

Grainger C, Clarke T, McGinn SM, Auldist MJ, Beauchemin KA, Hannah MC, et al. Methane emissions from dairy cows measured using the sulfur hexafluoride (SF_6) tracer and chamber techniques. J Dairy Sci. 2007; 90:2755–66.

Grovum WL. Factors affecting the voluntary intake of food by sheep 3. The effect of intavenous infusions of gastrin, cholecystokinin and secretin on motility of the reticulo-rumen and intake. Br J Nutr. 1981; 45:183–201.

Grummer RR. Etiology of Lipid-Related Metabolic Disorders in Periparturient Dairy Cows. J Dairy Sci. 1993; 76:3882–96.

van Gylswyk NO. Succiniclasticum ruminis gen. nov., sp. nov., a ruminal bacterium converting succinate to propionate as the sole energy-yielding mechanism. Int J Syst Bacteriol. 1995; 45:297–300.

Hammond KJ, Crompton LA, Bannink A, Dijkstra J, Yáñez-Ruiz DR, O'Kiely P, et al. Review of current in vivo measurement techniques for quantifying enteric methane emission from ruminants. Anim Feed Sci Technol. 2016; 219:13–30.

Hammond KJ, Humphries DJ, Crompton LA, Green C, Reynolds CK. Methane emissions from cattle: Estimates from short-term measurements using a GreenFeed system compared with measurements obtained using respiration chambers or sulphur hexafluoride tracer. Anim Feed Sci Technol. 2014; 203:41–52.

Hassanat F, Gervais R, Massé DI, Petit H V, Benchaar C. Methane production, nutrient digestion, ruminal fermentation, N balance, and milk production of cows fed timothy silage- or alfalfa silage-based diets. J Dairy Sci. 2014; 97:6463–74.

Hegarty RS, Gerdes R. Hydrogen production and transfer in the rumen. Recent Adv Anim Nutr Aust. 1999; 12:37–44.

Henderson G, Cox F, Ganesh S, Jonker A, Young W, Abecia L, et al. Rumen microbial community composition varies with diet and host, but a core microbiome is found across a wide geographical range. Sci Rep. 2015; 5:14567.

Henderson G, Cox F, Kittelmann S, Miri VH, Zethof M, Noel SJ, et al. Effect of DNA extraction methods and sampling techniques on the apparent structure of cow and sheep rumen microbial communities. PLoS One. 2013; 8:e74787.

Hernandez-Raquet G, Piao H, Fernando SC, Ha P, Cl A, Mj M, et al. Rumen Bacterial Community Composition in Holstein and Jersey Cows Is Different under Same Dietary Condition and Is Not Affected by Sampling Method. Front Microbiol. 2016; 7:1206.

Hill J, McSweeney C, Wright ADG, Bishop-Hurley G, Kalantar-zadeh K. Measuring Methane Production from Ruminants. Trends in Biotechnology. 2016; 34:26–35.

Hodin CM, Visschers RGJ, Rensen SS, Boonen B, Damink SWMO, Lenaerts K, et al. Total Parenteral Nutrition Induces a Shift in the Firmicutes to Bacteroidetes Ratio in Association with Paneth Cell Activation in Rats. J Nutr. 2012; 142:2141–7.

Hoedt EC, Cuiv PO, Evans PN, Smith WJM, McSweeney CS, Denman SE, et al. Differences down-under: alcohol-fueled methanogenesis by archaea present in Australian macropodids. ISME J. International Society for Microbial Ecology; 2016; 10:2376–88.

Hristov AN, Oh J, Firkins JL, Dijkstra J, Kebreab E, Waghorn G, et al. SPECIAL TOPICS-Mitigation of methane and nitrous oxide emissions from animal operations: I. A review of enteric methane mitigation options. J Anim Sci. 2013; 91:5045–69.

Ingvartsen KL, Andersen JB. Integration of metabolism and intake regulation: a review focusing on periparturient animals. J Dairy Sci. 2000; 83:1573–97.

Jami E, Israel A, Kotser A, Mizrahi I. Exploring the bovine rumen bacterial community from birth to adulthood. ISME J. 2013; 7:1069–79.

Jami E, Mizrahi I. Composition and similarity of bovine rumen microbiota across individual animals. PLoS One. 2012; 7:1–8.

Janssen PH, Kirs M. Structure of the archaeal community of the rumen. Appl Environ Microbiol. 2008; 74:3619–25.

Jentsch W, Schweigel M, Weissbach F, Scholze H, Pitroff W, Derno M. Methane production in cattle calculated by the nutrient composition of the diet. Arch Anim Nutr. 2007; 61:10–9.

Jewell KA, McCormick CA, Odt CL, Weimer PJ, Suen G. Ruminal Bacterial Community Composition in Dairy Cows Is Dynamic over the Course of Two Lactations and Correlates with Feed Efficiency. Appl Environ Microbiol. 2015; 81:4697–710.

Jiao HP, Dale AJ, Carson AF, Murray S, Gordon AW, Ferris CP. Effect of concentrate feed level on methane emissions from grazing dairy cows. J Dairy Sci. 2014; 97:7043–53.

Jing L, Zhang R, Liu Y, Zhu W, Mao S. Intravenous lipopolysaccharide challenge alters ruminal bacterial microbiota and disrupts ruminal metabolism in dairy cattle. Br J Nutr. 2014; 112:170–82.

Johnson KA, Johnson DE. Methane emissions from cattle. J Anim Sci. 1995; 73:2483–92.

Kania BR, Zaremba-rutkowska M, Romanowicz K. Experimental intestinal stress induced by duodenal distention in sheep. J Anim Feed Sci. 1999; 8:233–45.

Kasparovska J, Pecinkova M, Dadakova K, Krizova L, Hadrova S, Lexa M, et al. Effects of isoflavone-enriched feed on the rumen microbiota in dairy cows. PLoS One. 2016; 11:e0154642.

Kim MJ, Lee JS, Kumar S, Rahman MM, Shin JS, Ra CS. Indirect Estimation of CH4 from Livestock Feeds through TOCs Evaluation Apparatus for in vitro fermentation. Asian-Aust J Anim Sci. 2012; 25:496–501.

Kittelmann S, Pinares-Patiño CS, Seedorf H, Kirk MR, Ganesh S, McEwan JC, et al. Two different bacterial community types are linked with the low-methane emission trait in sheep. PLoS One. 2014; 9:e103171.

Kittelmann S, Seedorf H, Walters WA, Clemente JC, Knight R, Gordon JI, et al. Simultaneous Amplicon Sequencing to Explore Co-Occurrence Patterns of Bacterial, Archaeal and Eukaryotic Microorganisms in Rumen Microbial Communities. PLoS One. 2013; 8:e47879.

Knapp JR, Laur GL, Vadas P a, Weiss WP, Tricarico JM. Invited review: Enteric methane in dairy cattle production: quantifying the opportunities and impact of reducing emissions. J Dairy Sci. 2014; 97:3231–61.

Kobayashi Y, Koike S, Taguchi H, Itabashi H, Kam DK, Ha JK. Recent Advances in Gut Microbiology and Their Possible Contribution to Animal Health and Production - A Review. Asian-Aust J Anim Sci. 2004; 17:877–84.

Koike S, Kobayashi Y. Fibrolytic rumen bacteria: Their ecology and functions. Asian-Aust J Anim Sci. 2009; 22:131–8.

Kong Y, Teather R, Forster R. Composition, spatial distribution, and diversity of the bacterial communities in the rumen of cows fed different forages. FEMS Microbiol Ecol. 2010; 74:612–22.

Lettat A, Hassanat F, Benchaar C. Corn silage in dairy cow diets to reduce ruminal methanogenesis: effects on the rumen metabolically active microbial communities. J Dairy Sci. 2013; 96:5237–48.

Lima FS, Oikonomou G, Lima SF, Bicalho MLS, Ganda EK, de Oliveira Filho JC, et al. Prepartum and postpartum rumen fluid microbiomes: Characterization and correlation with production traits in dairy cows. Appl Environ Microbiol. 2015; 81:1327–37.

van Lingen HJ, Crompton L a, Hendriks WH, Reynolds CK, Dijkstra J. Meta-analysis of relationships between enteric methane yield and milk fatty acid profile in dairy cattle. J Dairy Sci. 2014; 97:7115–32.

McAllister T, Cheng K-J, Okine EK, Mathison GW. Dietary, environmental and microbiological aspects of methane production in ruminants. Can J Anim Sci. 1996; 76:231–43.

Michelland RJ, Monteils V, Combes S, Cauquil L, Gidenne T, Fortun-Lamothe L. Changes over time in the bacterial communities associated with fluid and food particles and the ruminal parameters in the bovine rumen before and after a dietary change. Can J Microbiol. 2011; 57:629–37.

van Middelaar CE, Berentsen PBM, Dijkstra J, van Arendonk JAM, de Boer IJM. Methods to determine the relative value of genetic traits in dairy cows to reduce greenhouse gas emissions along the chain. J Dairy Sci. 2014; 97:5191–205.

Mills JA., Kebreab E, Yates CM, Crompton LA, Cammell SB, Dhanoa MS, et al. Alternative approaches to predicting methane emissions from dairy cows. J Anim Sci. 2003; 81:3141–50.

Mohammed R, McGinn SM, Beauchemin K. Prediction of enteric methane output from milk fatty acid concentrations and rumen fermentation parameters in dairy cows fed sunflower, flax, or canola seeds. J Dairy Sci. 2011; 94:6057–68.

Morgavi DP, Forano E, Martin C, Newbold CJ. Microbial ecosystem and methanogenesis in ruminants. Animal. 2012; 6:871.

Moss AR, Jouany J-P, Newbold J. Methane production by ruminants : its contribution to global warming (Review article). Ann Zootech. 2000; 49:231–53.

Moyes KM. Metabolic and production profiles of dairy cows in response to decreased nutrient density to increase physiological imbalance at different stages of lactation. J Dairy Sci. 2012; 95:2362–80.

Mulak A, Taché Y, Larauche M. Sex hormones in the modulation of irritable bowel syndrome. World J Gastroenterol. 2014; 20:2433–48.

Müller TD, Tschöp MH. Ghrelin-A key pleiotropic hormone-regulating systemic energy metabolism. Ghrelin Syst. 2013; 25:91–100.

Münger A, Kreuzer M. Absence of persistent methane emission differences in three breeds of dairy cows. In: Australian Journal of Experimental Agriculture. CSIRO Publishing; 2008. p. 77–82.

Murray BYRM, Bryant AM, Leng RA. Rates of production of methane in the rumen and large intestine of sheep. Br J Nutr. 1976; 36.

Myhre G, Shindell D, Bréon F-M, Collins W, Fuglestvedt J, Huang J, et al. Anthropogenic and Natural Radiative Forcing. In: Climate Change 2013: The Physical Science Basis. Contribution of Working Group I to the Fifth Assessment Report of the Intergovernmental Panel on Climate Change.; 659–740.

Nozière P, Glasser F, Sauvant D. In vivo production and molar percentages of volatile fatty acids in the rumen: a quantitative review by an empirical approach. Animal. 2011; 5:403–14.

Okine EK, Mathison GW, Kaske M, Kennelly JJ, Christopherson RJ. Current understanding of the role of the reticulum and reticulo-omasal orifice in the control of digesta passage from the ruminoreticulum of sheep and cattle. Can J Anim Sci. 1998; 78:15–21.

Okine EK, Mathison GW, Hardin RT. Effects of Changes in Frequency of Reticular Contractions on Fluid and Particulate Passage Rates in Cattle. J Anim Sci. 1989; 67:3388–96.

Palmquist DL. Milk Fat: Origin of Fatty Acids and Influence of Nutritional Factors Thereon. In: Advanced Dairy Chemistry, Volume 2 Lipids. 3rd Edition. New York: Springer; 2006. p. 43–92.

Park AF, Shirley JE, Titgemeyer EC, DeFrain JM, Cochran RC, Wickersham EE, et al. Characterization of ruminal dynamics in Holstein dairy cows during the periparturient period. J Anim Physiol Anim Nutr. 2011; 95:571–82.

Petri RM, Schwaiger T, Penner GB, Beauchemin KA, Forster RJ, McKinnon JJ, et al. Characterization of the core rumen microbiome in cattle during transition from forage to concentrate as well as during and after an acidotic challenge. PLoS One. 2013; 8:e83424.

Pickering NK, Oddy VH, Basarab J, Cammack K, Hayes B, Hegarty RS, et al. Animal board invited review: genetic possibilities to reduce enteric methane emissions from ruminants. Animal. 2015; 9:1431–40.

Pinares-Patiño CS, Waghorn GC, Machmüller A, Vlaming B, Molano G, Cavanagh A, et al. Methane emissions and digestive physiology of non-lactating dairy cows fed pasture forage. Can J Anim Sci. 2007; 87:601–13.

Pires JAA, Delavaud C, Faulconnier Y, Pomiès D, Chilliard Y. Effects of body condition score at calving on indicators of fat and protein mobilization of periparturient Holstein-Friesian cows. J Dairy Sci. 2013; 96:6423–39.

Pitta DW, Kumar S, Vecchiarelli B, Baker LD, Ferguson JD, Thomsen N, et al. Temporal dynamics in the ruminal microbiome of dairy cows during the transition period. J Anim Sci. 2014; 92:4014–22.

Planton S. Annex III: Glossary, In: Climate Change 2013: The Physical Science Basis. Contribution of Working Group I to the Fifth Assessment Report of the Intergovernmental Panel on Climate Change; 1447–66.

Poulsen M, Schwab C, Jensen BB, Engberg RM, Spang A, Canibe N, et al. Methylotrophic methanogenic Thermoplasmata implicated in reduced methane emissions from bovine rumen. Nat Commun. 2013; 4:1428.

Ramos-Morales E, Arco-Pérez A, Martín-García AI, Yáñez-Ruiz DR, Frutos P, Hervás G. Use of stomach tubing as an alternative to rumen cannulation to study ruminal fermentation and microbiota in sheep and goats. Anim Feed Sci Technol. 2014; 198:57–66.

Relling AE, Reynolds CK, Loerch SC. Effect of feeding fat or intrajugular infusion of glucagon-like peptide-1 and cholecystokinin on dry matter intake, digestibility, and digesta rate of passage in growing wethers. J Anim Sci. 2011; 89:168–78.

Reynolds CK, Kristensen NB. Nitrogen recycling through the gut and the nitrogen economy of ruminants: an asynchronous symbiosis. J Anim Sci. 2008; 86:293–305.

Ricci P, Chagunda MGG, Rooke J, Houdijk JGM, Duthie CA, Hyslop J, et al. Evaluation of the laser methane detector to estimate methane emissions from ewes and steers. J Anim Sci. 2014; 92:5239–50.

Rischewski J, Bielak A, Nürnberg G, Derno M, Kuhla B. Rapid communication: Ranking dairy cows for methane emissions measured using respiration chamber or greenFeed techniques during early, peak, and late lactation. J Anim Sci. 2017; 95:3154–9.

Robertson LJ, Waghorn GC. Dairy industry perspectives on methane emissions and production from cattle fed pasture or total mixed rations in New Zealand. Proc New Zeal Soc Anim Prod. 2002; 62:213–8.

Roehe R, Dewhurst RJ, Duthie CA, Rooke JA, McKain N, Ross DW, et al. Bovine Host Genetic Variation Influences Rumen Microbial Methane Production with Best Selection Criterion for Low Methane Emitting and Efficiently Feed Converting Hosts Based on Metagenomic Gene Abundance. PLoS Genet. 2016; 12:1–20.

Rooke JA, Wallace RJ, Duthie C-A, McKain N, de Souza SM, Hyslop JJ, et al. Hydrogen and methane emissions from beef cattle and their rumen microbial community vary with diet, time after feeding and genotype. Br J Nutr. 2014; 112:398–407.

Rouviere PE, Wolfe RS. Novel biochemistry of methanogenesis. J Biol Chem. 1988; 263:7913–6.

Rukkwamsuk T, Kruip TAM, Wensing T. Relationship between overfeeding and overconditioning in the dry period and the problems of high producing dairy cows during the postparturient period. Vet Q. 1999; 21:71–7.

Russel JB, O'Connor JD, Fox DG, Van Soest PJ, Sniffen CJ. A net carbohydrate and protein system for evaluating cattle diets: IV. Predicting amino acid adequacy. J Anim Sci. 1992; 70:3551–61.

Russell JB, Baldwin RL. Substrate preferences in rumen bacteria: evidence of catabolite regulatory mechanisms. Appl Environ Microbiol. 1978; 36:319–29.

Salomon F-V. Verdauungsapparat, Apparatus digestorius. In: Salomon F-V, Geyer H, Gille U, editors. Anatomie für die Tiermedizin. 3rd ed. Stuttgart: Enke im Hippokrates Verlag GmbH; 2015. p. 248–340.

Sandri M, Manfrin C, Pallavicini A, Stefanon B. Microbial biodiversity of the liquid fraction of rumen content from lactating cows. Animal. 2014; 8:572–9.

Schäff C, Börner S, Hacke S, Kautzsch U, Sauerwein H, Spachmann SK, et al. Increased muscle fatty acid oxidation in dairy cows with intensive body fat mobilization during early lactation. J Dairy Sci. 2013; 96:6449–60.

Seedorf H, Kittelmann S, Janssen PH. Few highly abundant operational taxonomic units dominate within rumen methanogenic archaeal species in New Zealand sheep and cattle. Appl Environ Microbiol. 2015; 81:986–95.

Seshadri R, Leahy SC, Attwood GT, Teh KH, Lambie SC, Cookson AL, et al. Cultivation and sequencing of rumen microbiome members from the Hungate1000 Collection. Nat Biotechnol. 2018; 36:359–67.

Shen J, Chai Z, Song L, Liu J, Wu Y. Insertion depth of oral stomach tubes may affect the fermentation parameters of ruminal fluid collected in dairy cows. J Dairy Sci. 2012; 95:5978–84.

Shi W, Moon CD, Leahy SC, Kang D, Froula J, Kittelmann S, et al. Methane yield phenotypes linked to differential gene expression in the sheep rumen microbiome. Genome Res. 2014; 24:1517–25.

Sirohi SK, Singh N, Dagar SS, Puniya AK. Molecular tools for deciphering the microbial community structure and diversity in rumen ecosystem. Appl Microbiol Biotechnol. 2012; 95:1135–54.

St-Pierre B, Cersosimo LM, Ishaq SL, Wright ADG. Toward the identification of methanogenic archaeal groups as targets of methane mitigation in livestock animals. Front Microbiol. 2015; 6:1–10.

Steinfeld H, Gerber P, Wassenaar TD, Castel V, de Haan C. Livestock's long shadow: environmental issues and options. Food & Agriculture Org.; 2006.

Sun X, Henderson G, Cox F, Molano G, Harrison SJ, Luo D, et al. Lambs fed fresh winter forage rape (Brassica napus L.) emit less methane than those fed perennial ryegrass (Lolium perenne L.), and possible mechanisms behind the difference. PLoS One. 2015; 10:1–16.

Tapio I, Snelling TJ, Strozzi F, Wallace RJ. The ruminal microbiome associated with methane emissions from ruminant livestock. J Anim Sci Biotechnol. 2017; 8:2–11.

Terré M, Castells L, Fàbregas F, Bach A. Short communication: Comparison of pH, volatile fatty acids, and microbiome of rumen samples from preweaned calves obtained via cannula or stomach tube. J Dairy Sci. 2013 Aug 1; 96:5290–4.

Titchen DA. The Nature and Control of Gastric Motility in Ruminants. Ann Rech Vétérinaires, INRA Ed. 1976; 7:165–8.

Troy SM, Duthie CA, Ross DW, Hyslop JJ, Roehe R, Waterhouse A, et al. A comparison of methane emissions from beef cattle measured using methane hoods with those measured using respiration chambers. Anim Feed Sci Technol. 2016; 211:227–40.

Vanlierde A, Vanrobays M-L, Dehareng F, Froidmont E, Soyeurt H, McParland S, et al. Hot topic: Innovative lactation-stage-dependent prediction of methane emissions from milk mid-infrared spectra. J Dairy Sci. 2015; 98:5740–7.

Vanwonterghem I, Evans PN, Parks DH, Jensen PD, Woodcroft BJ, Hugenholtz P, et al. Methylotrophic methanogenesis discovered in the archaeal phylum Verstraetearchaeota. Nat Microbiol. 2016; 1:16170.

Veneman JB, Muetzel S, Hart KJ, Faulkner CL, Moorby JM, Perdok HB, et al. Does dietary mitigation of enteric methane production affect rumen function and animal productivity in dairy cows? PLoS One. 2015; 10:e01400282.

Vlaming JB, Lopez-Villalobos N, Brookes IM, Hoskin SO, Clark H. Within- and between-animal variance in methane emissions in non-lactating dairy cows. Aust J Exp Agric. 2008; 48:124–7.

Waghorn G, Pinares C. Technical Manual on Respiration Chamber Designs. 2014.

Wall E, Simm G, Moran D. Developing breeding schemes to assist mitigation of greenhouse gas emissions. Animal. 2010; 4:366.

Wallace RJ, Rooke JA, McKain N, Duthie C-A, Hyslop JJ, Ross DW, et al. The rumen microbial metagenome associated with high methane production in cattle. BMC Genomics. 2015; 16:839.

Wallace RJ, Rooke JA, Duthie CA, Hyslop JJ, Ross DW, McKain N, et al. Archaeal abundance in post-mortem ruminal digesta may help predict methane emissions from beef cattle. Sci Rep. 2014; 4:5892.

Wang X, Li X, Zhao C, Hu P, Chen H, Liu Z, et al. Correlation between composition of the bacterial community and concentration of volatile fatty acids in the rumen during the transition period and ketosis in dairy cows. Appl Environ Microbiol. 2012; 78:2386–92.

Watt LJ, Clark CEF, Krebs GL, Petzel CE, Nielsen S, Utsumi SA. Differential rumination, intake, and enteric methane production of dairy cows in a pasture-based automatic milking system. J Dairy Sci. 2015 Jan 24; 98:7248–63.

Weimer PJ, Waghorn GC, Odt CL, Mertens DR. Effect of Diet on Populations of Three Species of Ruminal Cellulolytic Bacteria in Lactating Dairy Cows. J Dairy Sci. 1999; 82:122–34.

Weimer PJ, Stevenson DM, Mantovani HC, Man SLC. Host specificity of the ruminal bacterial community in the dairy cow following near-total exchange of ruminal contents. J Dairy Sci. 2010; 93:5902–12.

Xiang R, McNally J, Rowe S, Jonker A, Pinares-Patino CS, Oddy VH, et al. Gene network analysis identifies rumen epithelial cell proliferation, differentiation and metabolic pathways perturbed by diet and correlated with methane production. Sci Rep. 2016; 6:39022.

Yáñez-Ruiz DR, Abecia L, Newbold CJ. Manipulating rumen microbiome and fermentation through interventions during early life: A review. Front Microbiol. 2015; 6:1–12.

Yarandi SS, Hebbar G, Sauer CG, Cole CR, Ziegler TR. Diverse roles of leptin in the gastrointestinal tract: Modulation of motility, absorption, growth, and inflammation. Nutrition. 2011; 27:269–75.

Zebeli Q, Aschenbach JR, Tafaj M, Boguhn J, Ametaj BN, Drochner W. Invited review: Role of physically effective fiber and estimation of dietary fiber adequacy in high-producing dairy cattle. J Dairy Sci. 2012; 95:1041–56.

Zhao S, Zhao J, Bu D, Sun P, Wang J, Dong Z. Metabolomics analysis reveals large effect of roughage types on rumen microbial metabolic profile in dairy cows. Lett Appl Microbiol. 2014; 59:79–85.

Zhou M, Chung Y-H, Beauchemin KA, Holtshausen L, Oba M, McAllister TA, et al. Relationship between rumen methanogens and methane production in dairy cows fed diets supplemented with a feed enzyme additive. J Appl Microbiol. 2011; 111:1148–58.