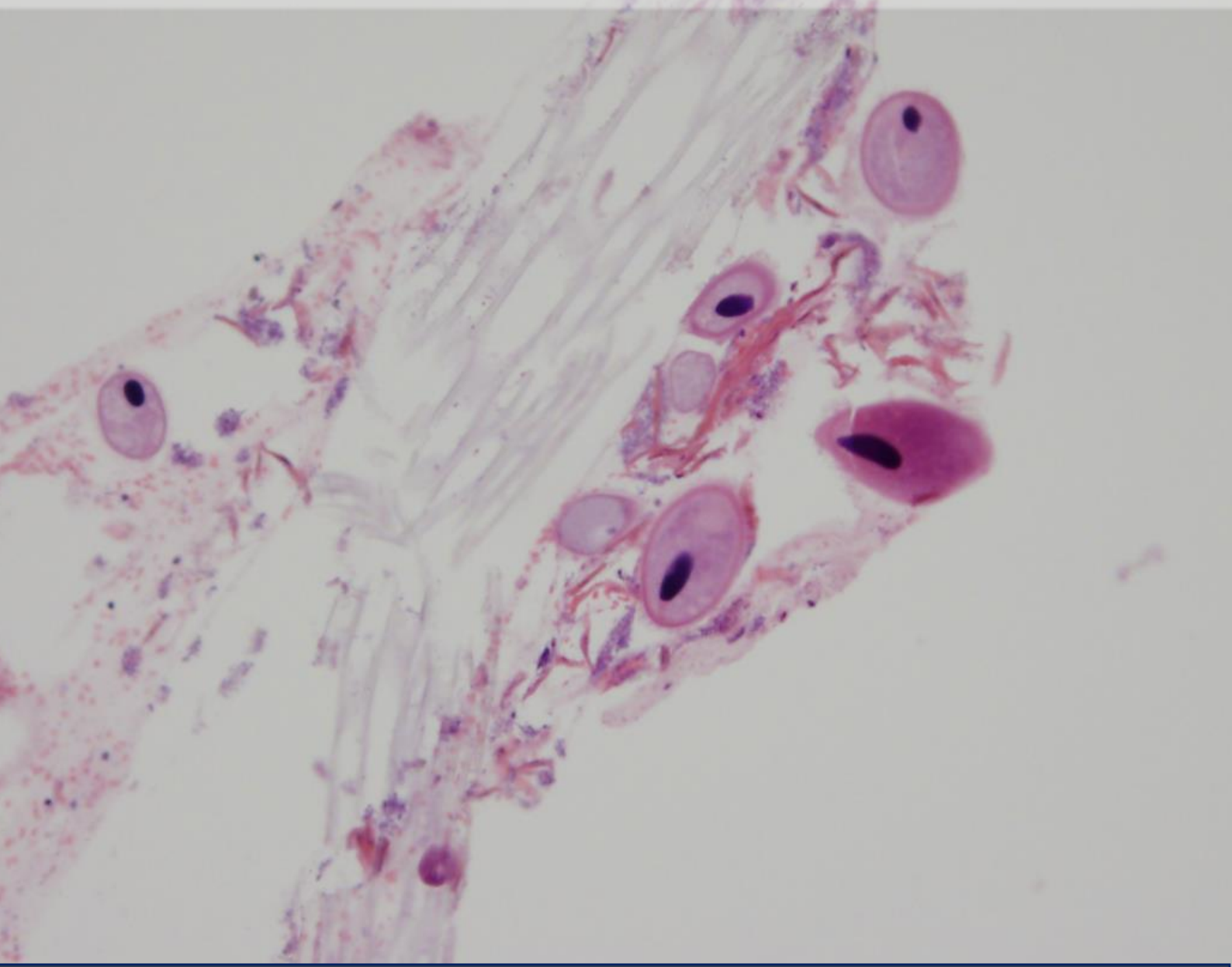
Georg-August-University Göttingen

Faculty of Agricultural Sciences – Animal Sciences

Ruminant Nutrition Group

# *Interrelations between feed, host and rumen microbiota in dairy cows*



Melanie Schären

Göttingen 2017

# *Interrelations between feed, host and rumen microbiota in dairy cows*

**Dissertation**

to attain the doctoral degree Dr. sc. agr.

in the Ph. D. Program for Agricultural Sciences in Göttingen (PAG)

at the Faculty of Agricultural Sciences

Georg-August-University Göttingen

submitted by

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born in Basel, Switzerland

Göttingen, September 2017

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## To my family

*Nimmer kann ich ruhig treiben,*
*Was die Seele stark erfaßt,*
*Nimmer still behaglich bleiben,*
*Und ich stürme ohne Rast.*

*Mich umwogt ein ewig Drängen,*
*Ew'ges Brausen, ew'ge Glut,*
*Kann sich nicht ins Leben zwängen,*
*Will nicht ziehn in glatter Flut.*

*Darum laßt uns alles wagen,*
*Nimmer rasten, nimmer ruhn.*
*Nur nicht dumpf so gar nichts sagen*
*Und so gar nichts woll'n und tun.*

*Nur nicht brütend hingegangen,*
*Ängstlich in dem niedern Joch,*
*Denn das Sehen und Verlangen*
*Und die Tat die bleibt uns doch!*

Excerpts from "Empfindungen", Karl Marx (1818 - 1883)

## Table of Contents

## List of Abbreviations

*(accounts for chapters 1. Background, 2. Aims of Study, and 5. Discussion)*

| | |
|---|---|
| a.p. | antepartum |
| AA | amino acids |
| CLA | conjugated linoleic acid |
| CRC | controlled-release capsule |
| DGGE | denaturing gradient gel electrophoresis |
| dsDNA | double stranded DNA |
| EAAB | epithelium-associated archaea and bacteria |
| FA | fatty acid |
| LAAB | liquid-associated archaea and bacteria |
| LPS | lipopolysaccharides |
| NGS | next generation sequencing |
| $NH_3$ | ammonia |
| OTU | operational taxonomic unit |
| p.p. | postpartum |
| PAAB | particle-associated archaea and bacteria |
| PMR | partially mixed ration |
| PUFA | polyunsaturated fatty acid |
| RFI | residual feed intake |
| RT-qPCR | quantitative real-time PCR |
| SARA | subacute ruminal acidosis |
| SSCP | single-strand conformation polymorphism |
| ssDNA | single-stranded DNA |
| TGGE | temperature gradient gel electrophoresis |
| TMR | total mixed ration |
| T-RFLP | terminal restriction fragment length polymorphism |
| VFA | volatile fatty acid |

## Summary

Melanie Schären, *"Interrelations between feed, host and rumen microbiota in dairy cows"*

The adaptability of the rumen microbiome to new nutritional situations is a key feature in ruminant survival strategy. Different studies and reviews describe the high redundancy and resilience of the rumen microbiome allowing the fermentation and nutrient extraction from a wide range of feedstuffs. They further highlight the strong host effect and that many questions concerning the temporal, spatial and microbial dynamics involved are still unanswered. The aim of this thesis was therefore to investigate different factors influencing the rumen microbiome and their interrelations. Three different studies were performed, each examining a different aspect in the rumen host-microbiome interplay: the adaptation to a new diet, the influence of anti-ketogenic feed additives, and the interrelations with phenotypic characteristics of the host. The database for the three studies was formed by rumen microbiota samples which were analyzed by a DNA-fingerpriting technique (single-strand conformation polymorphism, SSCP) and next generation sequencing (16S rRNA gene amplicon sequencing using the Illumina MiSeq platform).

For the first study samples were collected from three different sites in the rumen (liquid, fiber mat and epithelium) at three points in time, in a trial involving the transition from a silage- and concentrate-based ration to pasture in spring. To investigate the influence of anti-ketogenic feed additives on the rumen microbiome, rumen liquid samples were collected during a trial performed to investigate the influence of monensin and essential oils on health, production and rumen fermentation of transition dairy cows. For the third study, a large dataset of 36 healthy dairy cows in the first weeks of their lactation was analyzed for interrelations concerning the rumen microbiome, production, behavior, rumen fermentation, metabolic, and immunological variables.

The first study confirmed that the concept of a "core and variable microbiome" accounts for all three locations in the rumen and that the ration fed has the largest influence on the rumen microbiome compilation. The first trial further illustrated that a ration change from a concentrate- and silage-based ration to pasture influences the microbiome at all three locations, opposite the generally acknowledged hypothesis that the epithelium-associated prokaryotes remain more consistent throughout dietary changes. The data also suggests that the alterations observed in the rumen microbiome across a ration change cannot solely be accounted to the time needed for the different microbial species to adapt to the new substrate, but also to temporal aspects in behavioral and physiological alterations of and in the host. In the second study, we show that the feed additive monensin alters the "core microbiome" and confirm that the reason for the ineffectiveness of essential oils can most likely be attributed to

the adaptability of the rumen microbiome. Different aspects of the mode of action and the prokaryotes affected are discussed. In the first study, we were able to statistically proof the concept of the “variable or individual microbiome” for different prokaryotes. In the final study, it was hypothesized that the feed intake behavior of the host could be responsible for this “individual microbiome” through induction of alterations in the rumen fermentation profile. This hypothesis was however not confirmed. Nevertheless, several previously described interrelations between the abundance of certain rumen prokaryotes and production traits were confirmed.

Throughout the three studies different methodological aspects are discussed in detail, possible bottlenecks and key-influencing factors are identified, and it is illustrated that caution needs to be taken when interpreting and comparing microbiome sequencing data. A major finding of the presented studies is that prokaryotes which are phylogenetically close do not necessarily exhibit functional communality. This aspect has been largely ignored in previous studies and stresses the importance of functional characterization aside taxonomic classification.

It is concluded that future studies should not only involve more sophisticated methods to characterize the rumen microbiome as well as phenotypic attributes of its host, but also focus on an array of previously insufficiently investigated aspects, such as the interrelations between the microbiota and its hosts metabolism, the role of the low abundant microbial species and the rumen wall associated microbiota, the interrelations between the different rumen microorganisms and the role of the lower-gut microbiota.

## Zusammenfassung

Melanie Schären, *"Aspekte zur Wechselbeziehung zwischen Futter, Wirt und Pansenmikrobiom der Milchkuh"*

Die schnelle Anpassung des Pansenmikrobioms an eine neue Ration gehört zu den Schlüsselmerkmalen der Überlebensstrategie der Wiederkäuer. Verschiedene Studien haben die Redundanz und Elastizität des Pansenmikrobioms beschrieben wodurch die Fermentation und Nährstoffextraktion aus einer breiten Palette von Futtermitteln ermöglicht wird. Des Weiteren wurde der starke Einfluss des Wirtsindividuums auf das Pansenmikrobiom beschrieben und festgestellt, dass viele Fragestellungen bezüglich der zeitlichen, räumlichen und mikrobiellen Dynamik weitestgehend ungeklärt sind. Ziel der vorliegenden Arbeit war es deshalb die verschiedenen Faktoren zu untersuchen welche das Pansenmikrobiom beeinflussen und die Zusammenhänge näher zu beleuchten. Dazu wurden drei Studien durchgeführt, die jeweils einen anderen Aspekt im Wirt-Mikrobiom Zusammenspiel betrachten: die Anpassung an eine neue Ration, der Einfluss von anti-ketogenen Futterzusatzstoffen und den Zusammenhang von phänotypischen Merkmalen des Wirtes mit dem Pansenmikrobiom. Die Pansenmikrobiomproben aus allen drei Studien wurden mittels einer DNA-fingerprinting (single-strand conformation polymorphism, SSCP) und einer „Next-Generation Sequencing" Methode (16S rRNA Gen Amplikon Sequenzierung mittels der Illumina MiSeq Plattform) untersucht.

Für die erste Untersuchung wurde das Pansenmikrobiom an drei verschiedenen Stellen beprobt (Flüssigkeit, Futterpartikel und Epithel). Dies wurde im Rahmen eines Versuches durchgeführt, in dem die Umstellung von einer Kraftfutter- und Silage-basierten Fütterung (Stallhaltung) auf Weide und deren Einfluss auf den Metabolismus der Milchkuh im Fokus stand. Um den Einfluss von anti-ketogenen Futterzusatzstoffen auf das Pansenmikrobiom zu untersuchen wurden Proben in einem Versuch gesammelt, in dem der Einfluss von Monensin und ätherischen Ölen auf die Leistung, Tiergesundheit und Pansenfermentation der Milchkuh im Transitzeitraum betrachtet wurde. Für die dritte Studie wurde ein umfangreicher Datensatz bestehend aus Daten zu Leistung, Fressverhalten, Pansenmikrobiom und -fermentation, Metabolismus und Immunsystem von 36 gesunden Milchkühen im frühen Zeitraum ihrer Laktation ausgewertet.

Die erste Studie bestätigte das Konzept des „Kern- und variablen Mikrobioms" („core and variable microbiome") und dass dieses für alle drei beprobten Lokalisationen gilt. Des Weiteren zeigten die beiden ersten Studien, dass die größte veränderliche Wirkung von der Futterzusammensetzung ausgeht. Der erste Versuch zeigte auch, dass der Übergang von einer Kraftfutter- und Silage-basierten Fütterung hin zur Weide das Mikrobiom an allen drei

Lokalisationen im Pansen in ähnlichem Umfang verändert. Dies stellt ein besonders interessantes Resultat dar, da bisher angenommen wurde, dass das wandständige Mikrobiom Futtereinflüssen nur wenig unterworfen ist. Die Daten lassen auch vermuten, dass der benötigte Zeitraum zur Anpassung des Pansenmikrobioms an eine neue Ration nicht nur von der Anpassung der einzelnen Mikrobenspezies an das neue Substrat abhängig ist, sondern auch von Veränderungen im Verhalten und Metabolismus des Wirtes.

In der zweiten Studie konnte gezeigt werden, dass der Futterzusatzstoff Monensin das „Kernmikrobiom“ des Pansens verändert und dass die fehlende Wirkung von ätherischen Ölen höchstwahrscheinlich auf eine Gewöhnung und Anpassung des Pansenmikrobioms zurückzuführen ist. Des Weiteren werden verschiedene Aspekte zur Wirkweise von Monensin und den betroffenen Prokaryoten diskutiert. Das Konzept des „variablen oder individuellen Mikrobioms“ wurde in der ersten Studie statistisch untermauert. In der finalen Studie wurde dann der Hypothese nachgegangen ob dieses „individuelle Mikrobiom“ auf Unterschiede im Fressverhalten der Tiere zurückzuführen ist. Dies konnte nicht bestätigt werden. Jedoch konnten viele zuvor beschriebene Zusammenhänge zwischen der Abundanz von bestimmten Prokaryoten und Leistungsmerkmalen bestätigt werden.

In allen drei Studien werden verschiedene methodische Aspekte im Detail diskutiert, Probleme und Schlüsselfaktoren identifiziert und illustriert, dass bei der Interpretation und dem Vergleich von Mikrobiom Sequenzierdaten verschiedene Punkte zu berücksichtigen sind. Eine wichtige Beobachtung, welche in den verschiedenen hier dargelegten Studien gemacht wurde ist, dass phylogenetisch nah verwandte Prokaryotenspezies nicht zwingend ähnliche funktionale Merkmale aufweisen. Dieser Aspekt wurde bisher nur wenig erforscht und diskutiert und zeigt die Notwendigkeit einer funktionellen Charakterisierung neben der taxonomischen Klassifizierung auf.

Zusammenfassend wird festgestellt, dass zukünftige Studien sich die in den letzten 1-2 Jahren auf dem Markt angekommenen modernen Sequenziermethoden zu Nutze machen sollten um das Pansenmikrobiom besser und genauer zu charakterisieren. Dies sollte im Zusammenhang mit einer genauen Erfassung von phänotypischen Merkmalen des Wirtes erfolgen. Weiterhin sollten bisher ungenügend erforschte Aspekte näher beleuchtet werden, wie z.B. der Zusammenhang zwischen dem Pansenmikrobiom und dem Stoffwechsel des Wirtes, die Rolle der wenig abundanten Spezies und des Pansenwand-assoziierten Mikrobiom, die Wechselwirkungen zwischen den verschiedenen Pansenmikroorganismen und die Rolle des Darmmikrobioms.

# 1. Background

## 1.1. Introduction

Ruminants have been among the first animals domesticated by mankind and their inherent ability to transform plant forages into high-quality foods for humans has made them the most important livestock (Van Soest, 1994, Oltenacu Branford, 2004, Morgavi et al., 2013). This is only possible due to a symbiotic relationship between the ruminant and its microbes which perform a pregastric fermentation of the ingested plant material (Mizrahi, 2013). In the midth of the 20$^{th}$ century Robert Hungate, his graduate students and colleagues studied this unique and thus far relatively unexplored ecosystem (Chung and Bryant, 1997, Morgavi et al., 2013). Their findings and knowledge were summarized and published in 1966 in the book "The rumen and its microbes" (Hungate, 1966). For many years this book formed the reference work in the field of microbial ecology since research was limited to culture-based techniques (McCann et al., 2014a). In the last two decades, our understanding of the rumen microbial ecosystem has evolved and also changed considerably with the upcoming of molecular techniques such as PCR and DNA-fingerprinting methods (Dohrmann et al., 2004, Kim et al., 2011b). Recently DNA sequencing methods (next generation sequencing, **NGS**) have become affordable and are being widely used to characterize microbial communities. It is thought that these methods will revolutionize our insight in microbial dynamics and function (McCann et al., 2014a).

## 1.2. Rumen physiology

During ingestion of feed the cow performs the first step of digestion by crushing the feed particles, thereby enhancing the surface and breaking cell walls, and mingling it with saliva (Bailey and Balch, 1961, Mizrahi, 2013). As soon as the feed particles arrive in the first forestomach, the reticulorumen (Figure 1), they are colonized by different microorganisms within minutes (Martin et al., 1993, Edwards et al., 2007). Huws et al. (2016) have shown that the colonization of fresh perennial ryegrass is biphasic, with a first event 1-2 h and a second 4-8 h after ingestion, with different bacteria species involved. The feed is then hydrolyzed and fermented by the different rumen microbes, which results in the production of volatile fatty acids (**VFA**), mainly acetate, propionate and butyrate, the gases carbon dioxide and methane (Bergman, 1990). The three VFA are present at a ratio of 65:20:15, a concentration between 60 and 150 mM, and cover 80 % of the animal´s daily energy requirements (Bergman, 1990). Plant protein is hydrolysed by the rumen bacteria into smaller peptides, amino acids (**AA**) and deaminated into ammonia (**$NH_3$**). The peptides and AA are used by the rumen microorganisms for growth and provide the animal with valuable microbial protein, which is resorbed in the lower intestines, whereas $NH_3$ diffuses freely across the rumen wall (Pfeffer and Hristov, 2005). The reticulorumen can therefore be seen as a large fermentation vessel, containing

approximately 60-100 kg of feed (Hartnell and Satter, 1979, Schären et al., 2016). Studies have shown that the turnover rate of the rumen ingesta occurs at a rate of approx. 4 % and is dependent on the diet fed (Hartnell and Satter, 1979, Evans, 1981).

To support the fermentation process the cow regurgitates the partially digested feed (cud) and chews it over (Mizrahi, 2013). This process, called rumination, does not only facilitate the decrease of particle size and degradation, but also assists in keeping the ruminal pH stable due to the further mingling with saliva (Bergman, 1990, Aschenbach et al., 2011, Mizrahi, 2013). To remove the carbon dioxide and methane from the rumen, the cow regularly eructates these gases (Mizrahi, 2013), whereas the VFA are for a large part (~88 %) absorbed by the rumen epithelium (Bergman, 1990). The rumen wall has enhanced its surface through evolution by the formation of papillae (Dirksen et al., 1984, Graham and Simmons, 2005) and the surface area of these papillae can adapt to alterations in VFA production within days by an increase or decrease in cell division and elongation (Liebich et al., 1987, Gäbel et al., 2002, Bannink et al., 2012, Martens et al., 2012, Dieho et al., 2016a, Schären et al., 2016). But not only VFA are absorbed across the rumen epithelium through different active and passive processes, also an influx and/or absorption of water, bicarbonate and other electrolytes occurs, depending on the osmotic state of the rumen content (Aschenbach et al., 2011). Further, also urea can be actively transported from the blood to the lumen (urea recycling, depending of the dietary N content), supplying the rumen microorganisms with N, thereby increasing the microbial protein synthesis and allowing to augment diets low in N (Pfeffer and Hristov, 2005). These different regulatory mechanisms contribute to a stable anaerobic environment within a range of a pH of 5.5-7.0, temperature of 38-40 °C and a low reduction potential of 0.15-0.4 V (Russell, 2002, Mizrahi, 2013).

To guarantee a continuous mingling of the total content there are complex cyclic contractions in the reticulorumen, that average about 1/min throughout the day (Sellers and Stevens, 1966). Even though the total content is continuously shifted, gradient formation occurs due to gravity and the differences in density between feed particles and rumen fluid. Generally, dry matter content and particle size are higher, and pH lower, at the dorsal site, and decrease and increases towards the ventral site of the rumen, respectively (Tafaj et al., 2004, Storm and Kristensen, 2010). Small particle sizes and fluid leave the rumen through the rumino-omasal orifice and reach the omasum, a smaller oblate sphere-formed forestomach, mainly responsible for reabsorption of fluid, bicarbonate, VFA and transfer of ingesta to the abomasum (Gray et al., 1954, Stevens et al., 1960, Sellers and Stevens, 1966). Thereafter the ingesta are digested in the abomasum and small intestines, similarly to monogastric animals. Several authors describe the production of lysozyme (an enzyme that degrades bacterial cell walls) by abomasal cells, ascribing it an adaptive function to the foregut fermentation system (Mizrahi,

2013, Morgavi et al., 2013). The digestions of the rumen microorganism and the absorption of the microbial protein, has been estimated to contribute between one-half to three-quarters of the absorbed amino-acids in the ruminant (Clark et al., 1992, Mizrahi, 2013).

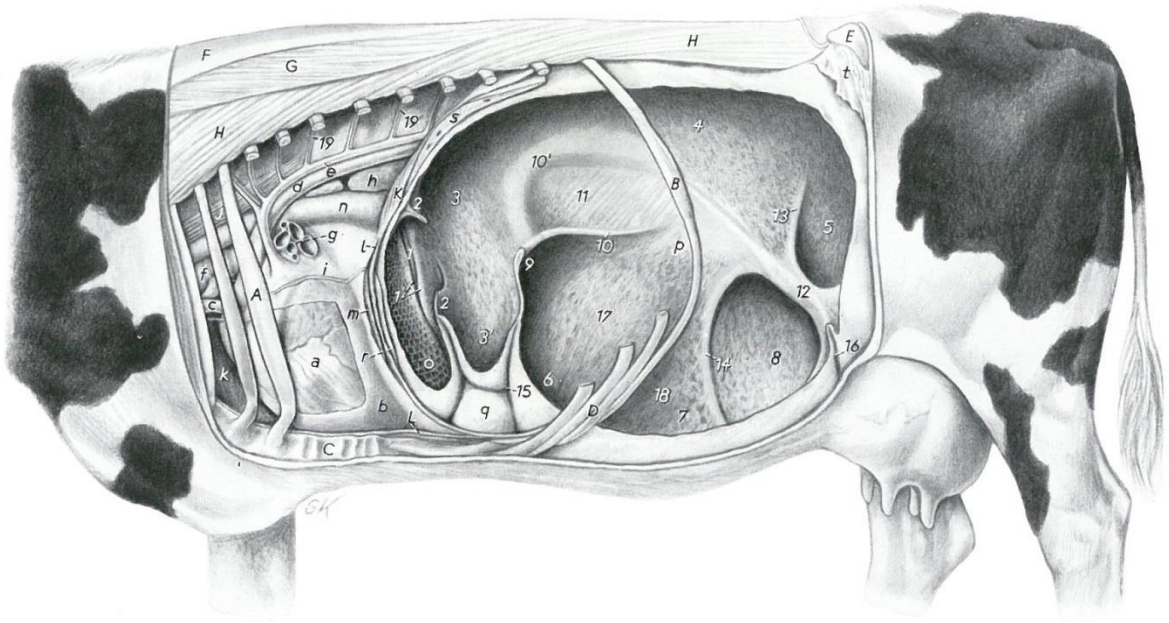



**Figure 1:** Anatomy of the thoracic and abdomal organs from the left side of an adult cow. Copied from Schummer et al. (1975). A. 4th rib, B. 13th rib, C. sternum, D. costal arch, E. *tuber coxae*, F. *funiculus & lamina nuchae*, G. *m. spinalis et semispinalis dorsi et cervicis,* H. *m. longissimuslumborum et thoracis*, J. *m. longus colli*, K. diaphragm, L. *m. transversus thoracis*, a. hart, b. pericard, c. *truncus brachiocephalicus communis*, d. aorta, e. *v. azygos sin.*, f. trachea, g. lung, h. *ln. mediastenalis caudalis longissimus*, i. *n. phrenicus dext.*, k. and l. pre- and postcardial mediastinum, m. lung, n. oesophagus, o. reticulum, p. rumen, q. abomasum, r. liver, s. spleen, t. fat, 1. *sulcus ventriculi*, 2. *plica ruminoreticularis*, 3. *atrium ruminis*, 4. *saccus dorsalis*, 5. *saccus caecus caudodorsalis*, 6. *recessus ruminis*, 7. *saccus ventralis*, 8. *saccus caecus caudoventralis*, 9. *pila cranialis*, 10. *pila longitudinalis dextra*, 10´. *pila accessoria dextra*, 11. *insula ruminis*, 12. *pila caudalis*, 13. *pila coronaria dorsalis*, 14. *pila coronaria ventralis*, 15. *sulcus cranialis*, 16. *sulcus caudalis*, 17. omasal bulge, 18. abomasal bulge, 19. *aa. & vv. intercostales*.

## 1.3. The rumen microbial ecosystem

Upon birth the rumen is only slightly larger than the abomasum and since during the first few weeks milk constitutes the main nutrient source, its fermentation activity is minimal (Mizrahi, 2013). It is only with the ingestion of solid feed that the ingested material does not pass anymore through the eosophagal groove directly into the abomasum, but is fermented and predigested in the rumen (Dehority, 2002). However, studies have shown that the colonization of the rumen with microorganisms occurs as soon as the animal is in contact with the outer world, weeks before the rumen actually becomes functional (Fonty et al., 1987, Jami et al., 2013). Anaerobic species have been found two days after birth and colonization with cellulolytic and methanogenic prokaryotes seems to occur within the first week of life. Concurrently a rapid decline in aerobic and facultative anaerobic bacteria and protozoa can be observed (Fonty et al., 1987). Different data further suggest that for the rapid establishment of the cellulolytic microflora some contact with the mother or other cattle is needed (Bryant et al., 1958). After a few weeks, the rumen has distended markedly in comparison to the abomasum and the transition from milk to forage has been completed (Warner et al., 1956). The calf now possesses a fully functional forestomach system containing an own microbial ecosystem (Li et al., 2012a, Jami et al., 2013).

The microorganisms constituting the rumen microbiome are members of the bacteria, archaea, fungi and protozoa (Hobson and Stewart, 1997, Mizrahi, 2013). The prokaryotes are the most dominant inhabitants of this ecosystem with an estimated 200 species (Golder, 2014) and $10^{10}$ cells per gram rumen content, representing approximately 0.3 % of the total rumen content (Hungate, 1966, Russell, 2002). It has been estimated that ¾ of the rumen bacteria are bound to feed-particles (and some to the rumen epithelium), whereas approximately ¼ is free floating (Russell, 2002). The rumen protozoa constitute the second largest group and are encountered at a concentration of $10^4$-$10^7$ organisms per ml rumen content (Hungate, 1966). Even though their total numbers are much lower compared to the bacteria, they are estimated to account for half of the biomass in the rumen (Russell, 2002). This is attributed to their large size (20-200 µm) compared to the bacteria (0.5-10 µm) (Hungate, 1966, Mackie et al., 2013). Furthermore, also some anaerobic fungi with an average size of their zoospores of 6-10 µm, sporangia of 100 µm, and mycelium of 450 µm have been characterized as being part of the rumen microbial ecosystem (Russell, 2002, Krause et al., 2013, Mackie et al., 2013). It has been estimated that they can contribute up to 8 % of the total biomass (Russell, 2002). These different microorganisms compete for the plant feed resource, but have also shown to interact with and life from each other (Weimer, 2015). In this line different ways of interaction such as inhibition, predation, commensalism and synergism have been described (Mizrahi 2013). Their main substrate are cellulose, hemicellulose, pectine, starch, fructans, organic acids, and

proteins and according to their functional attributed the microorganisms can be assigned to different groups such as cellulolytics, amylolytics, proteolytics, etc. (Henderson et al., 2015).

### 1.3.1. The Eubacteria

Culture and PCR-based techniques have identified and investigated features of different rumen bacteria such as the *Ruminococci*, *Fibrobacter succinogenes*, the *Butyrivibrios*, the *Prevotella* species, *Selemonas ruminantium*, *Streptococcus bovis*, *Megasphaera elsdenii*, *Ruminobacter amylophilus*, *Anaerovibrio lipolytica*, *Succinomonas amylolytica*, *Succinvibrio dextrinosolvens*, the *Spirochetes*, and different obligate amino acid fermenting bacteria (Russell, 2002). It has however been estimated that only approximately 10 % of the rumen microbiome has ever been cultured (Russell, 2002, Krause et al., 2013, Morgavi et al., 2013, Creevey et al., 2014, Henderson et al., 2015). Furthermore, different recent studies indicate that the ability to cultivate a given species does not correlate with its functional importance in the ecosystem (Morgavi et al., 2013). With the upcoming of the non-culture-based methods (summarized in chapter 1.5) new insights into the rumen microbiome and the dynamics of its inhabitants have been gained in the last few years (Golder, 2014, Weimer, 2015). A study by Henderson et al. (2015) has shown that members of the genera *Prevotella*, *Butyrivibrio*, and *Ruminococcus*, unclassified *Lachnospiraceae*, *Ruminococcaceae*, *Bacteroidales* and *Clostridiales* constitute the “core microbiome” of ruminants. This is also in line with different other studies (Fouts et al., 2012, Creevey et al., 2014) investigating the rumen microbiome of cattle, but depending on the DNA extraction methods, PCR primers and sequencing platform used, results differ slightly (Henderson et al., 2013). An overview of the bacterial rumen microbiome is given in Figure 2. Modern sequencing methods have also illustrated that there is no automatic parallel between common taxonomic grouping and microbial phenotype or function. Bacteria that are phylogenetically related may exhibit different functions and metabolic characteristics (Morgavi et al., 2013). Additionally, it has been discovered that the host itself seems to have a strong influence on its rumen microbiome, most likely through behavioral and genetic attributes influencing the eating and ruminal fermentation pattern (discussed in detail in chapter 1.4, Henderson et al. (2015), Weimer (2015)). These two findings highlight the importance of further characterizing and investigating the functional properties and interrelations of and within this ecosystem.

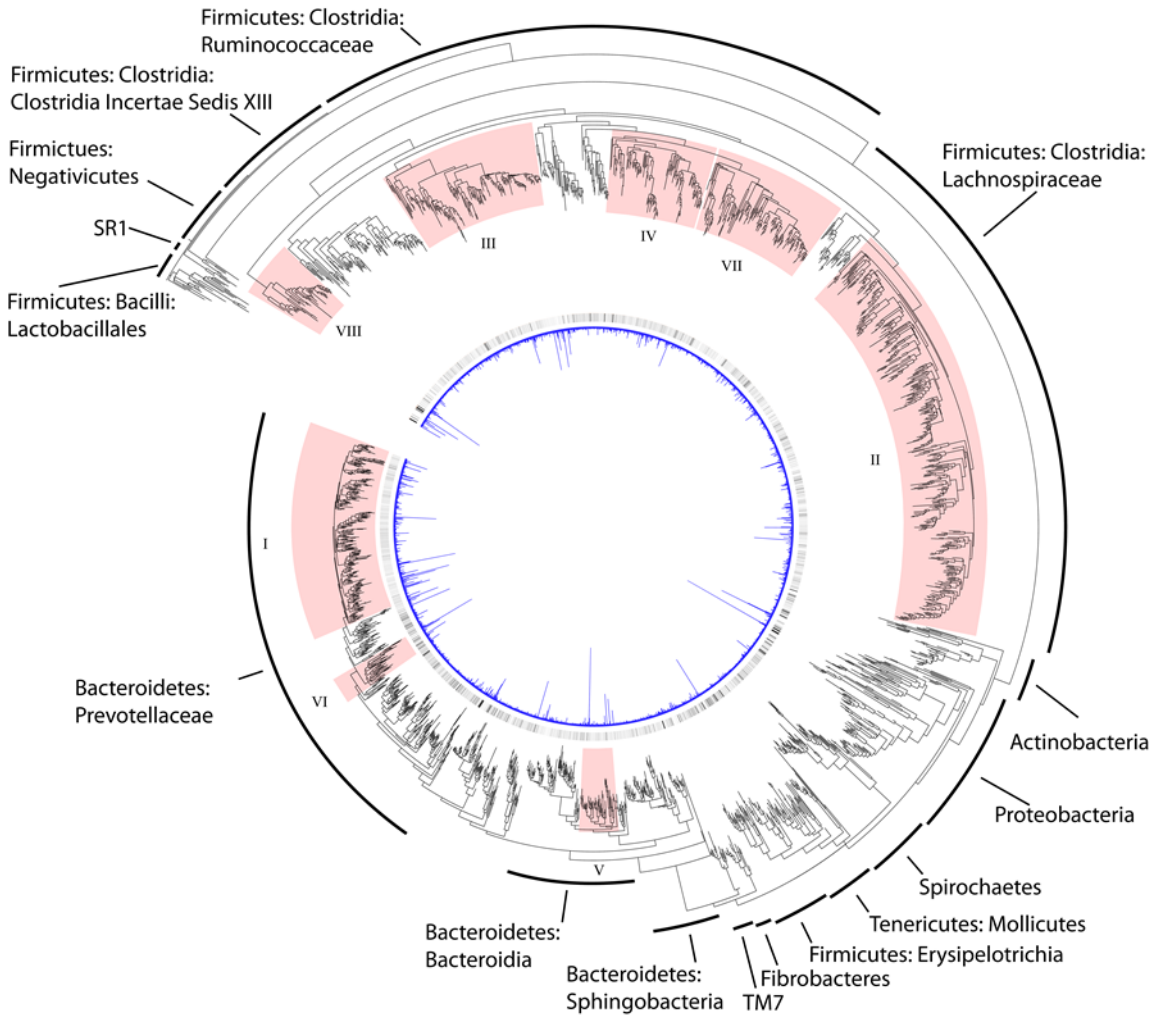



**Figure 2.** An inverted circular phylogenetic tree of rumen bacteria identified as part of a meta-analysis copied from Creevey et al. (2014). The blue graph in the middle represents the average scaled proportion of each species from across the different datasets (7 datasets) analyzed. The colour gradient surrounding represents the prevalence of each species across all datasets analyzed (dark = most prevalent, light = least prevalent). The major groups of bacteria that are represented in the tree are indicated. The clades that are most abundant in the rumen are indicated in red and numbered from I to VIII in order of abundance**.** The data is subjected to the creative commons terms: *https://creativecommons.org/licenses/by/3.0/*.

### 1.3.2. The Archaea

Contrary to the bacteria, the archaea constitute a well investigated group of microorganisms within the rumen, mainly due to their relevance in methanogenesis (Boadi et al., 2004, Hook et al., 2010, Patra, 2012). An estimated 60 % of rumen archaea species have been cultured and fall into named species (Henderson et al., 2015). Research has shown that the different archaeal groups are remarkably similar in all regions of the world and 90 % of the archaeal population in the rumen consists of members *Methanobrevibacter gottschalkii*, *Methanobrevibacter ruminantium*, *Methanospaera sp.* and two *Methanomassiliicoccaceae*-affiliated groups (Henderson et al., 2015). The methanogens depend on other microorganisms to convert complex organic matter into substrates for methanogenesis (Mackie et al., 2013). In a process called syntrophic hydrogen transfer methanogens receive hydrogen, and use it to reduce carbon dioxide to methane (Krause et al., 2013, Mizrahi, 2013). The function of the methanogens as hydrogen sink in the rumen is extremely important, since the accumulation of hydrogen slows down the fermentation rate and efficiency due to an accumulation of reducing equivalents (Krause et al., 2013). However, not only has the methane produced by ruminants a high relevance in the production of greenhouse gasses, but also represents a loss of feed energy of 2-12 % for the animal (Henderson et al., 2015, Weimer, 2015). Therefore, different research projects have focused on possibilities to mitigate methane production in the rumen (discussed in detail in chapter 1.4, Boadi et al. (2004), Hook et al. (2010), Patra (2012), Krause et al. (2013), Henderson et al. (2015)).

### 1.3.3. The Fungi

The rumen fungi have only been discovered as recently as 1973 (Hobson and Stewart, 1997; Mackie et al. 2013). Before they were described as flagellated protozoa and the cultivation of one of these polyflagellated organisms constituted an important step in rumen microbial ecology, as well as fungal phylogeny, since it had been previously thought that fungi were obligate aerobes (Russell, 2002, Mackie et al., 2013, Mizrahi, 2013). Presently five genera have been described, the *Neocallimastix*, *Caecomyces*, *Piromyces*, *Orpinomyces*, *Ruminomyces* (Hobson and Stewart, 1997). Different studies suggest that their overall effect on ruminal fermentation is minor (Mizrahi, 2013). Since they are characterized by a long life cycle, they do only occur in large numbers when the animal feeds on low-quality forage and they are able to reside within the rumen due to an increased retention time of the rumen content (Hobson and Stewart, 1997, Mizrahi, 2013). However, it is thought that the penetration of the plant cell wall by the fungal rhizoids increases the lignocellulose accessibility for other rumen microorganisms, possibly playing an important role in diets characterized by a poor forage quality and a high fiber content (Hobson and Stewart, 1997, Russell, 2002, Mizrahi, 2013).

### 1.3.4. The Protozoa

The protozoa that can be encountered in the rumen are ciliated and mainly anaerobic (Hungate, 1966, Russell, 2002, Mizrahi, 2013). Some of them have been reported to scavenge oxygen, which is thought to be beneficial to maintain the anaerobic milieu (Mizrahi, 2013). The ruminal protozoal community exhibits strong host individuality (Henderson et al., 2015, Weimer, 2015). However, a recent study of Henderson et al. (2015) suggests that greater ubiquity than assumed might be possible. The rumen protozoa are often divided into the *Holotrichia* and *Entodiniomorpha*. The holotrichs are characterized by cilia spread over the entire body, whereas the entodiniomorphs only have cilia in discrete regions (Russell, 2002, Mackie et al., 2013). This classification has recently been altered and several different ways of classification are encountered in the literature (Hobson and Stewart, 1997, Mackie et al., 2013, Mizrahi, 2013). Examples of different rumen protozoa are given in Figure 3. Studies have shown that the ruminal protozoa prey on bacteria and are only able to survive in their presence (Fondevila and Dehority, 2001a, b, Mizrahi, 2013). They are known to attach to feed particles and migrate into the rumen fluid upon the arrival of new feed, resulting in a diurnal appearance in the ruminal fluid (illustrated in Figure 4 in chapter 1.4, Krause et al. (2013), Künzel et al. (2016)). Furthermore, defaunation studies have shown that the rumen protozoa are not essential to the host and that the productivity of the host is even increased without the protozoa being present (Hungate, 1966, Hegarty et al., 2008, Williams and Coleman, 2012). Under these conditions a different bacterial community was observed, as well as a different VFA, ammonia, and fatty acid profile, and a reduction in methane production (Ozutsumi et al., 2005, Belanche et al., 2011, Mosoni et al., 2011, Sultana et al., 2011). The latter can be attributed to the attachment of a part of the methanogenic archaea to the surface of the protozoa, which are estimated to contribute to 9-25 % of the methane production (Vogels et al., 1980, Newbold et al., 1995). Due to this close relationship, different studies have investigated the possibility to decrease ruminal methane production through the inhibition of rumen protozoa replication (Patra, 2012).

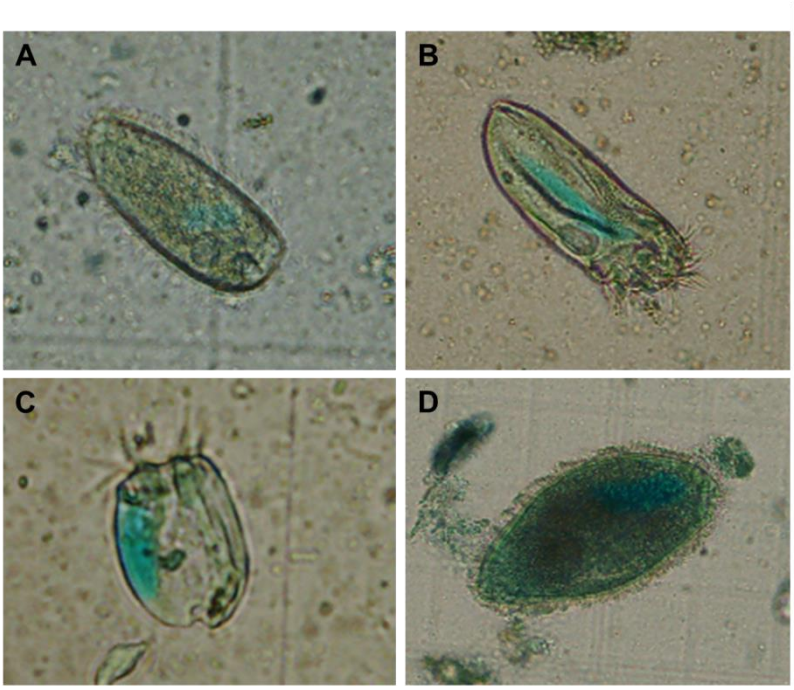



**Figure 3.** Example of rumen protozoa of the taxa *Dasytricha* (A), *Entodiniomorpha* (B and C) and *Isotrichia* (D). Methylgreen staining. Scale: 10x20 (A, B, D) and 10x40 (C). With courtesy of Denise Kaltenbach.

During the last few years several reviews (Krause et al., 2013, Mackie et al., 2013, Mizrahi, 2013, Morgavi et al., 2013, McCann et al., 2014a, Weimer, 2015) and books (Hobson and Stewart, 1997, Russell, 2002) have been published on the rumen microbial ecosystem. They give an elaborate overview on the different microbial species described and their interrelations investigated. Especially the excellent textbook by James B. Russell (2002) needs to be mentioned in this context. A review by McCann et al. (2014a) gives an overview of the physiological interrelation between the rumen microbiome and its host, the different techniques, as well as an elaborate summary of the different studies that have used modern techniques to characterize influences on the rumen microbiome. In 2015 Weimer published a review on the redundancy, resilience and host specificity of the ruminal microbiota, discussing the implications on ruminal fermentation modulation in the light of the current knowledge. He, as well as other authors, claim the need for more effort to go into characterizing the metabolism, roles and interrelations of the different rumen bacteria, with the aim of enhancing animal productivity and reducing methane emissions (Bath et al., 2013, Morgavi et al., 2013, Henderson et al., 2015, Weimer, 2015).

## 1.4. Factors influencing the rumen microbiome

### 1.4.1. Spatial and temporal differences within the rumen

The rumen prokaryotes can be divided into the liquid- (**LAAB**), particle- (**PAAB**), and epithelium (**EAAB**) associated or “epimural” bacteria and archaea (Cho et al., 2006, McCann et al., 2014a). Due to their close spatial association and constantly ongoing interchange the LAAB and PAAB communities exhibit a relatively high similarity (Sadet et al., 2007). As expected, within the group of the PAAB different fibrolytic bacteria were identified, whereas in the LAAB, for example, more members of the *Prevotella* genus are found, due to their affinity to fast fermentable carbohydrates (Koike and Kobayashi, 2009, Kong et al., 2010, Pitta et al., 2010, McCann et al., 2014a, Singh et al., 2015). The EAAB however are known to be a very distinct community, with several bacteria taxa, such as the *Proteobacteria*, that are only found in small(er) quantities in the other two groups (Sadet-Bourgeteau et al., 2010, Petri et al., 2013a). It has been suggested that the EAAB are associated with fermentation end-products, VFA absorption, maintaining an anaerobe environment, recycling of endogenous nitrogen and tissue (Cheng et al., 1979, Wallace et al., 1979, McCann et al., 2014a). It has been further hypothesized whether this microbial community may remain more consistent through dietary changes compared to the LAAB and PAAB (Sadet et al., 2007, McCann et al., 2014a).

The rumen fermentation activity is a direct result of the composition of the ingested feed and the animal behavior (feed intake and rumination pattern) (Leedle et al., 1982). As the feed intake pattern, also the rumen microbiome composition exhibits diurnal variations, which are mirrored in diurnal variations of the different fermentation variables, such as pH, and VFA and ammonia concentrations (illustrated in Figure 4, Leedle et al. (1982), McCann et al. (2014a)). This variation was shown for, as mentioned earlier, the protozoa, but also the different rumen bacteria species (Warner, 1962, Leedle et al., 1982, Li et al., 2009, Welkie et al., 2010), and was mainly observed in the LAAB, and less in the PAAB (Welkie et al., 2010).

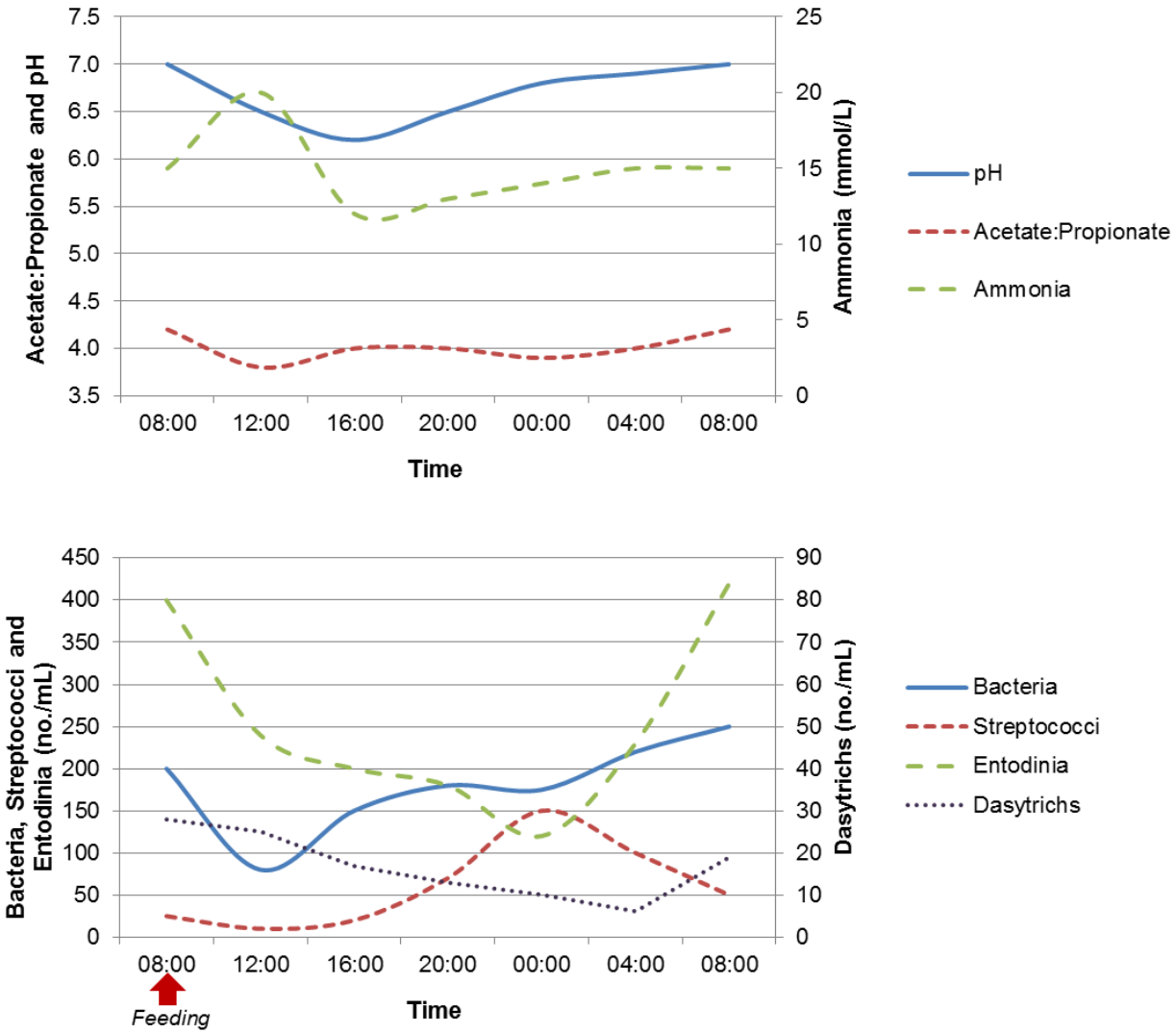



**Figure 4.** Schematic illustration of diurnal variation of a selection of ruminal fermentation variables (top) and microorganisms (bottom) in rumen fluid using data from Leedle et al. (1982) and Warner (1962) (measurements taken at times indicated in the x-lane). After the ingestion of fresh feed an increase in fermentation rate can be observed, leading to an increase in VFA production, causing a decrease in pH, and an increased propionate and ammonia production. Primary a decrease in microorganisms in the rumen fluid can be observed due to their attachment to the new feed particles. Thereafter an increase is observed caused by either replication or detachment from feed particles (Leedle et al., 1982). In this example, the cow was only fed restrictively once per day. In case of multiple feedings or grazing, different diurnal patterns are observed (Bargo et al., 2002, Taweel et al., 2004, Abrahamse et al., 2009).

### 1.4.2. The host effect

As mentioned in the previous chapter (1.3.1), the rumen microbiome exhibits a high host specificity (Jami and Mizrahi, 2012b). It is thought that this can be attributed to acquired behavioral (feed intake and rumination pattern, as partially defined by the time budgeting and management) as well as genetic attributes (e.g. ruminal VFA absorption potential and contraction rate, Weimer (2015)). One of the first and very intriguing experiment illustrating this host specificity was a near-total rumen content exchange study by Weimer et al. (2010b). In this study, the rumen content of two cows consuming the same diet, but exhibiting very different ruminal fermentation profiles were exchanged. Within 24 h, the fermentation characteristics (pH and VFA concentrations) returned to levels before transfer. Moreover, it was shown that bacterial community returned to their prior structure as well, within 14 for one and 61 days for the other cow. Especially in experiments with a small sample size, often larger inter-animal variations than treatment effects can be observed (McCann et al., 2014a). An example of inter-animal variation of the prokaryote rumen microbiome is given in Figure 5.

In this context, different studies were able to correlate certain phenotypical traits, such as age, feed efficiency and breed, with the rumen microbiome (Guan et al., 2008, Hernandez-Sanabria et al., 2010, Hernandez-Sanabria et al., 2012, Jami et al., 2013, Lima et al., 2015, Myer et al., 2015). For example, Hernandez-Sanabria et al. (2012) were able to illustrate a positive correlation between *Succinivibrio sp.* and efficient steers, whereas the occurrence *Robinsoniella sp.* was correlated with a high residual-feed intake (**RFI**), and therefore inefficient animals. Further, several studies also suggest an important role of the different *Prevotella* species in cattle feed efficiency (Carberry et al., 2012, Hernandez-Sanabria et al., 2012, McCann et al., 2014b).

### 1.4.3. Feed composition

To cover requirements of high yielding dairy cows and maximize animal production in dairy as well as beef cattle, the energy input is maximized (Coulon and Rémond, 1991, Owens et al., 1998). However, to maintain ruminal health the ration needs to contain sufficient fiber (Erdman, 1988, Allen, 1997, Maekawa et al., 2002). Therefore, many studies have focused on the differences in the concentrate:roughage ratio and the influence on animal and rumen health, and production (Zebeli et al., 2012, Dieho, 2016). It has been shown that under the conditions of a high-grain diet the rumen microbial diversity and fibrolytic bacteria, such as *Butyrivibrio fibriosolvens* and *Fibrobacter succinogenes* decrease, whereas amylolytic bacteria (e.g.

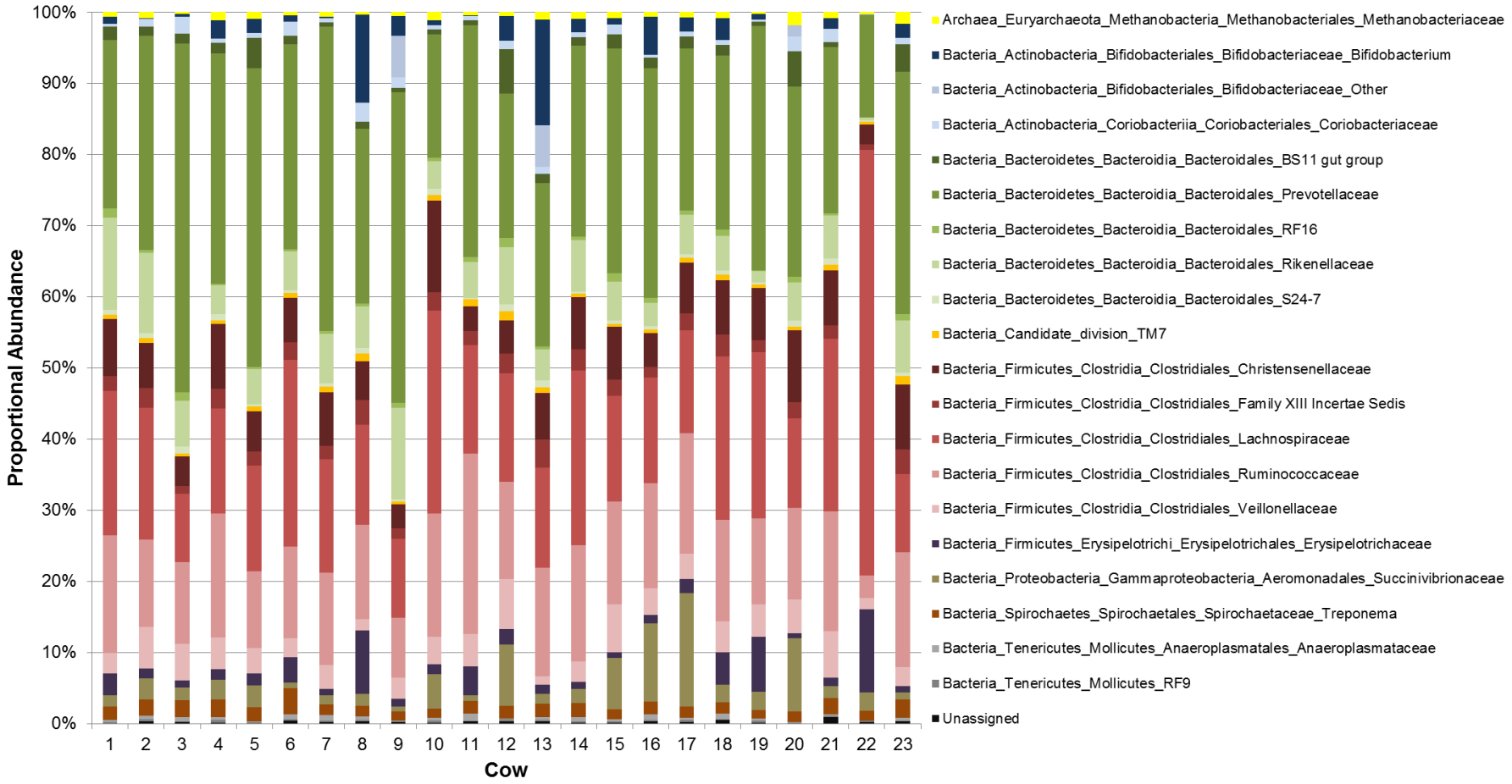



**Figure 5.** Example of inter-animal variation of rumen prokaryote families in dairy cows receiving an identical ration (total mixed ration, consisting of corn and grass silage, and concentrate). Data from the control groups of the experiment are described in chapter 4.

*Streptococcus bovis*), lactic acid utilizers (e.g. *Megasphaera elsdenii*) and members of the *Prevotella* genus increase (Fernando et al., 2010, McCann et al., 2014a). Different other studies also support the observation that an increased microbial diversity can be observed when less digestible diets are fed (Fernando et al., 2010, Pitta et al., 2010, McCann et al., 2014a, Lima et al., 2015, Dieho et al., 2016b). An illustration of the changes observed in the rumen microbiome on phylum-level under the influence of different forage:concentrate ratios is given in Figure 6 Aside a silage- and concentrate-based feeding strategy, a lot of farms, especially in temperate climate zones, implement forage-based systems (Dillon et al., 2005). De Menezes et al. (2011) have investigated the differences between a pasture and TMR-based diet, illustrating 10.5 % dissimilarity between the bacterial populations of the two rations fed. This difference could mainly be attributed to differences in the *Bacteroidetes* and *Firmicutes* population. Further, also a larger difference between the LAAB and PAAB were observed for the TMR-based diet. In this study, as well as in an experiment by Nakano et al. (2013), in which the influence of the transition to a pasture-based diet on the rumen microbiome was investigated, the *Prevotella* genus was found to be more abundant under grazing conditions, suggesting an important role in pasture fermentation.

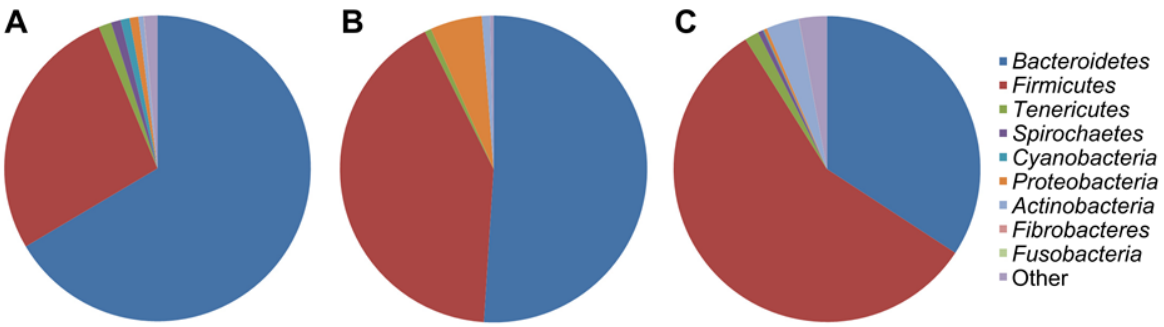



**Figure 6.** Variation in phyla composition of rumen microbiome of dairy cattle. A. 45:55 forage:concentrate TMR, B. 30:70 forage:concentrate TMR, C. subacute ruminal acidosis (SARA) conditions. Copied from McCann et al. (2014a), data from Zhang et al. (2014), Jami and Mizrahi (2012a) and Mao et al. (2013).

### 1.4.4. Ration changes

Different steps in dairy cattle production require the adaptation of the rumen microbiome and its host to new nutritional situations (McCann et al., 2014a). The first and probably also most extreme change, as mentioned in the previous chapter (1.3), is the transition from milk to a solid-based diet, where the rumen develops to a fully functional fermentation chamber over the course of several weeks (Sweeney et al., 2010, Jami et al., 2013). A more sudden change is encountered during the transition from a dry-period, to a lactation-ration upon calving (Ingvartsen, 2006, Mulligan and Doherty, 2008). However, modern production systems have adapted with a better understanding of rumen physiology, and dry rations as well as fresh-cow rations have been graduated to increase the energy concentrations step-wise, and to give the rumen microbiota, as well as epithelium, time to adapt (NRC et al., 2001, Ingvartsen, 2006). In a very recent publication, Dieho et al. (2016b) illustrate the alterations in the rumen microbiome between 50 days antepartum (**a.p.**) and 80 days postpartum (**p.p.**) in dairy cows either undergoing a rapid or gradual increase of concentrate allowance after calving. They observed gradual increases or decreases between 10 days a.p., and 3, and 9 days p.p. for different prokaryotes and the protozoa, concluding that the rumen microbiome follows alterations in substrate composition rapidly (a selection of bacterial taxa exhibiting a significant treatment, time or treatment×time effect is illustrated in Figure 7).

Different studies indicate that the stabilization of the rumen microbiome can be observed within 24 h, but can also still be incomplete after 3 weeks, depending on the group of microorganisms observed and extent of the ration change itself (Hackmann, 2015). Nakano et al. (2013) for example only observed a stabilization of the rumen microbiome after 28 d following turn out to pasture, without an adaption period granted. This is in line with the common acknowledged theorem that the larger the change from the previous diet, and maybe even housing system, involving behavioral adaptations and eliciting neophobia (e.g. grazing), the longer the adaptation period will last (Huhtanen and Hetta, 2012, Grant et al., 2015). Up to now only little research has focused on the influence of dietary changes on the rumen microbiome, using NGS methods, and further research is needed to reveal the differences between certain diets fed, as well as the time needed for the microbiota to stabilize (Hackmann, 2015, Schären, 2016).

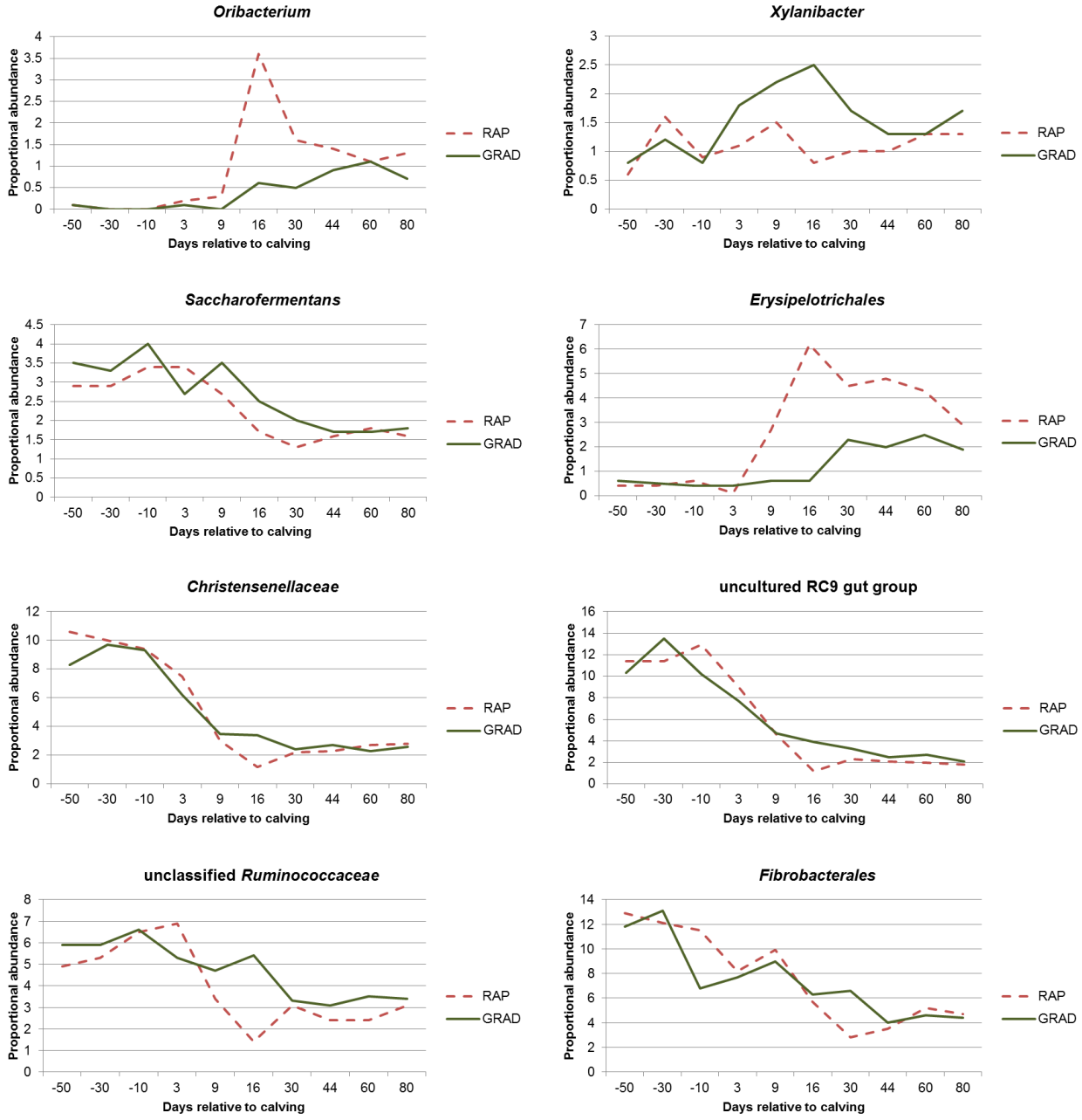



**Figure 7.** Relative abundance of selected bacterial taxa (% of total community, measured at days a.p. and p.p. indicated in the x-lane) during the pretreatment and treatment period with a rapid (RAP; 1.0 kg of DM/d; n = 6) and a gradual (GRAD; 0.25 kg of DM/d; n = 6) rate of increase of concentrate allowance p.p. Data from Dieho et al. (2016b).

### 1.4.5. Feed additives

To enhance animal production and health, or alter the composition of the animal product (e.g. milk and beef), different feed additives have been developed and are commercially available (Hutjens, 1991). Since they alter the ration composition, it is also likely that they are influencing the rumen microbial structure. This was confirmed in several studies showing a correlation between dietary fatty acids (**FA**), the rumen microbiome and the final product (milk and meat) (Lourenço et al., 2010, Shingfield et al., 2012, Weimer, 2014). It has, for example, been shown that some microbes are sensitive to polyunsaturated fatty acids (**PUFA**) (Maia et al., 2007), a group of FA that are commonly encountered in high concentrations in fresh grass (Kelly et al., 1998, Schroeder et al., 2004). The PUFA are biohydrogenated in the rumen into mainly trans vaccenic acid, which is then converted into the most predominant conjugated linoleic acids (**CLA**), rumenic acid, in the mammary gland (Fernandez and Rodriguez, 2012). The milk of grazing cows therefore contains larger amounts of unsaturated FA and trans-FA (CLA and vaccenic acid) compared to milk of cows receiving a silage- and concentrate-based diet (Kelly et al., 1998, Dewhurst et al., 2006, Kalač and Samková, 2010, Vahmani et al., 2013).

Active dry yeast is a feed additive that is regularly used to counteract pH drop and lactate accumulation due to the feeding of high-grain diets (Fonty and Chaucheyras-Durand, 2006, Chaucheyras-Durand et al., 2008). Is has been shown to improve fiber-degradation by stimulation of growth and/or activity of fibrolytic bacteria (Chaucheyras-Durand et al., 2008). Pinloche et al. (2013) have shown, using NGS methods, that the feeding of the probiotic yeast *Saccharomyces cerevisiae* leads to a shift in the fibrolytic groups (*Fibrobacter* and *Ruminococcus*) as well as the lactate utilizing bacteria (*Megasphaera* and *Selenomonas*).

As mentioned in the previous chapter (1.3.2) different strategies to mitigate methane production in cattle have been investigated. As for the feed additives, different lipid sources such as different oils (e.g. coconut, soybean, linseed oil and sunflower oil), nut seeds and shells (e.g. canola, cotton and soybean seeds, cashew nut shell), crystalline fat, as well as different phytochemicals (e.g. saponins, tannins, and essential oils), fumaric acid and ionophore antibiotics have shown to decrease rumen methane production most likely through alterations in the rumen microbiome (Boadi et al., 2004, Hook et al., 2010, Benchaar and Greathead, 2011, Patra, 2012, Leahy et al., 2013). Investigating the effect of two different methane-mitigating diets (addition of grape marc or a combination of lipids and tannin) Ross et al. (2013) were able to identifiy potential biomarkers in the rumen microbiome for low-methane-emitting cattle. In a review by Patra (2012) the effects of animal and dietary interventions, as well as the direct suppression of rumen methanogens by different chemical compounds, ionophores, fat, plant secondary compounds, defaunation, immunization,

bacteriocins, bacteriophage therapy and different alternate hydrogen sinks are elaborately discussed.

The commercial use of monensin however, is not mainly due to its antimethanogenic effect, but can be attributed to its feed efficiency enhancing and ketosis-preventing characteristics (Russell and Strobel, 1989, Duffield, 2000, McGuffey et al., 2001, Ipharraguerre and Clark, 2003). Ionophore antibiotics, such as monensin, attach to bacteria and protozoa and in case of bacteria the ionophore gets solubilized into the lipid bilayer of the cell membrane and causes an exchange of intracellular $K^+$ and extra-cellular protons, leading to cell death due to the acidification of the cytoplasm (McGuffey et al., 2001). Since ionophores exert their effect through alterations in the cellular membrane, monensin sensitivity is cell wall constitution and thickness dependent (Russell and Houlihan, 2003, Russell and Strobel, 2005). Earlier studies suggested that mainly Gram-positive bacteria are affected by monensin (Russell and Houlihan, 2003). More recent studies however suggest no clear cut between Gram-positive and Gram-negative bacteria (Russell and Strobel, 2005, Kim et al., 2014). Culture-based, *in vitro* fermentation and *in vivo* studies have shown that monensin causes an increase in propionate producers (elaborately reviewed in Golder (2014), but tracing its activity to specific microbial groups has been a challenge and systematic studies using NGS methods are lacking (Weimer and Stevenson, 2008). Since propionate is converted in the liver to glucose, the enhanced propionate production caused by monensin has a positive influence on the energy status of the animal (Ipharraguerre and Clark, 2003, Duffield et al., 2004). The application of monensin is easy (mixed into the feed or applied as bolus) and its positive effects on animal production and health, especially during the transition period in high yielding dairy cows, are well known since several decades (approved by the Federal Food and Drug Administration in the USA for the use in confined cattle to improve feed efficiency in 1975, McGuffey et al. (2001)). However, the use of antibiotics in animal production as production enhancers and pro- and methaphylactic therapy is seen critically, due to the upcoming of antibiotic resistances and the large-scale use of antibiotics to compensate inadequate design and management of housing-systems (Joshi and Herdt, 2006, Seal et al., 2013). Therefore, the European Union banned the use of antibiotics as feed additives (Cogliani et al., 2011). Monensin, however, was recently launched as a controlled-release capsule (**CRC**) and may be prescribed by a veterinary if a cow is overconditioned during the transition period and therefore in risk of incurring a clinical ketosis and fatty liver syndrome after onset of lactation (Drong et al., 2016).

Different compounds have been investigated that could possibly exert similar effects as monensin on the rumen microbiome, without falling under legal restrictions or harming the animal (Castillo et al., 2004, Fandiño et al., 2008, Geraci et al., 2012). In this line, a considerable amount of research has focused on different essential oils, such as garlic, dill,

thyme, ginger, coriander, eucalyptus, etc. (Calsamiglia et al., 2007, Benchaar et al., 2008a, Hart et al., 2008, Benchaar and Greathead, 2011, Patra, 2011). Some studies have shown production enhancing effects (Benchaar et al., 2006a, Kung et al., 2008, Giannenas et al., 2011), whereas many others could not confirm this (Benchaar et al., 2003, Benchaar et al., 2006b, Benchaar et al., 2007, Yang et al., 2007, Benchaar et al., 2008b, Tassoul and Shaver, 2009). The reason in this inconsistency most likely lies in the variation in dosage and chemical structure of the essential oil used, as well as ration composition and animal physiology among studies (Calsamiglia et al., 2007, Patra, 2011). Future studies should address the effects of individual essential oils and blends of them with varying proportions in order to design essential oil preparations useful for a health-based ruminal nutrition (Calsamiglia et al., 2007). Further, studies are needed to characterize the underlying changes in the rumen microbiome using modern sequencing techniques (Patra and Yu, 2012).

### 1.4.6. Subacute Ruminal Acidosis (SARA)

The subacute ruminal acidosis or **SARA** is a nutritional disorder commonly observed in dairy cattle production (Krause and Oetzel, 2006, Plaizier et al., 2008). It is a complex disease, involving different predisposing factors such as high-energy diets which are concurrently low in physically effective fiber, inadequate adaptation of the rumen microbiota and epithelium, as well as different management factors (e.g. feed bunk, housing, and group management) that promote irregular feeding pattern (Golder, 2014). The repeatedly moderately depressed ruminal pH (lower than 5.0-5.5) is thought to disturb the osmolality and damage the rumen epithelium (Krause and Oetzel, 2006), causing influx of bacteria and lipopolysaccharides (**LPS**) into the system (Gozho et al., 2005, Emmanuel et al., 2008, Li et al., 2012b, Plaizier et al., 2012), resulting in diarrhea, laminitis, inflammation, and lung- and liver-abscesses (Enemark et al., 2002, Kleen et al., 2003, Plaizier et al., 2008). The estimated prevalence lies at 10.0 to 26.7 % (reviewed in Golder (2014)). This disease complex has mainly been described and investigated in silage- and concentrate-based, so called partially- or total mixed rations (**PMR** or **TMR**), which are predominantly fed in conventional confinement systems (Calsamiglia et al., 2012, Golder, 2014).

With the upcoming of the NGS methods several research groups have investigated the alterations occurring in the rumen microbiome under SARA conditions (Khafipour et al., 2009a, b, Khafipour et al., 2009c, Weimer et al., 2010a, Hook et al., 2011, Khafipour et al., 2011, Li et al., 2012b, Plaizier et al., 2012, Mao et al., 2013, Petri et al., 2013b, Golder, 2014, McCann et al., 2016) (Figure 5). In 2009 Khafipour et al. have illustrated that the consequences of SARA are substrate dependent. They provoked three different SARA conditions in cows, either using grain (severe or mild) or alfalfa-pellets, and showed that the severe-grain induced SARA was dominated by *Streptococcus bovis* and *Escherichia coli*, whereas the mild grain-induced SARA

was dominated by *Megasphaera elsdenii*, and alfalfa pellet-induced SARA was dominated by *P. albanensis* (Khafipour et al., 2009a, b, Khafipour et al., 2009c). These differences can most likely be attributed to the fast replication cycle of *S. bovis*, a main lactic acid producing bacteria, and the slow replication cycle of the lactic acid consuming bacteria *M. elsdenii*. These results suggest that during a grain induced SARA there is a built up in lactic acid due to high amounts and fast fermentation of starch by *S. bovis* and too slow elimination by *M.eldenii*. In milder forms of SARA mainly *M. elsdenii* was present, probably due to a higher degree of microbial balance in the rumen. In both SARA models an increase in ruminal LPS concentrations was observed but an immune response in the peripheral blood has only been observed in grain induced SARA (Khafipour et al., 2009a, b, Khafipour et al., 2009c). Further research revealed that the population structure of *E.coli* during the grain induced SARA changes into a more pathogenic type (Khafipour et al., 2011), leading to the conclusion that low rumen pH and high osmolarity alone are not responsible for triggering the immune system during SARA (Khafipour et al., 2011). In this line of thoughts Calsamiglia et al. (2012) stated that SARA should be renamed into “high-concentrate syndrome”, claiming that two events, namely a high proportion of concentrate in the diet and a low ruminal pH are confounded.

### 1.4.7. Ruminal detoxification

It has been already known for a longer time that the ability of different ruminal microorganisms to detoxify different toxic compounds has been acknowledged (Reiser and Fu, 1962, Allison et al., 1992, Duncan and Milne, 1992, Smith, 1992). For example, it is known that ruminants are able to tolerate higher concentrations of different mycotoxins compared to monogastric animals (Binder et al., 1997, Yiannikouris and Jouany, 2002, Upadhaya et al., 2010). Also, the rumen microbiome is known to adapt to the exposure of certain toxins (Carlson and Breeze, 1984, Domínguez-Bello, 1996). Most likely, this phenomenon can also explain the loss of effect of certain essential oils over time (Benchaar and Greathead, 2011).

A very intriguing discovery made by Jones and Lowry (1984) and Jones and Megarrity (1986) was that the ability of goats living in Australia to tolerate *Leucaena*, an arboreal legume containing mimosine, could be induced by inoculating them with ruminal content from animals from Indonesia and Hawaii that had adapted to the plant. It was later discovered that this detoxifying capability could be attributed to the ruminal bacterium *Synergesties jonesii*, which was isolated and found to degrade 3-hydroxy-4-[1H]-pyridone (3,4-DHP), the toxic microbial product of mimosine (Allison et al., 1992, Hess et al., 2000).

## 1.5. Techniques to characterize the rumen microbiome

Since the rumen is a strictly anaerobic milieu, the culturing and investigation of the microorganisms inhabiting this ecosystem has been a challenge for a long time (Russell, 2002). Only with the invention of different cleverly devised methods, such as the roll-tube culturing method and the RUSITEC system (rumen simulation technique), different scientists were able to describe and investigate interrelations between the different members of the rumen microbiome (Hungate and Macy, 1973, Krause et al., 2013, Morgavi et al., 2013, McCann et al., 2014a). However, as mentioned earlier only an estimated 10 % of the rumen microflora has been cultured and described (Russell, 2002, Krause et al., 2013, Morgavi et al., 2013, Creevey et al., 2014, Henderson et al., 2015). It was only with the upcoming of the PCR technique and discovery that the different species can be distinguished by the differences in their rRNA gene that a more precise estimation of the diversity of the rumen microbiome became possible (McCann et al., 2014a). The rumen protozoa can be differentiated using the inter-species variation in the 18S rRNA gene, whereas for the fungi the 5S and 18S, and for the prokaryotes the 16S rRNA gene is used (Russell, 2002, Mackie et al., 2013, Henderson et al., 2015).

To investigate the quantitative relationship among rumen prokaryotes quantitative real-time PCR (**RT-qPCR**) is performed. For this method, a primer for each species of interest needs to be designed and a PCR conducted (Tajima et al., 2001, Stevenson and Weimer, 2007, Fernando et al., 2010). It is a very sensitive method to gain insights on the abundance of certain prokaryotes species. However, its informative value is bound to the species investigated. To gain insights on general differences, alterations, and influences on the rumen microbiome, the DNA fingerprinting and amplicon sequencing methods are more adequate tools (Golder, 2014).

The different methods have been summarized and thoroughly described by several authors in the past (Boon et al., 2002, Dohrmann et al., 2004, Di Bella et al., 2013, Golder, 2014, McCann et al., 2014a, Van Dijk et al., 2014). Therefore, the focus of this chapter will mainly lie on the DNA fingerprinting method **SSCP** (single-strand conformation polymorphism) and the sequencing method Illumina MiSeq, both used in the studies included in this thesis.

### 1.5.1. The 16S rRNA gene amplification

Carl Woese and George E. Fox were the first to use the uniqueness of the 16S rRNA gene in prokaryote species for phylogenetic classification in the 1970´s (Woese et al., 1975, Fox et al., 1977, Woese et al., 1990, Fox et al., 1992). The 16S rRNA gene possesses nine different hypervariable regions (V1-V9) which are responsible for the differences among species (Chakravorty et al., 2007). Using specific PCR primers specific parts of these hypervariable regions can be amplified. Currently different primers are used spanning the hypervariable regions V1, V2-3, V1-V3, V1-V4, V4, V3-V5, V4-V7, V5-V6, V5-V8, and V6-7 (Yu and Morrison, 2004, Kim et al., 2011a, Di Bella et al., 2013).

A schematic illustration of the different steps performed during the 16S rRNA gene amplification is presented in Figure 8. Firstly, the genomic DNA (**gDNA**) needs to be extracted. The gDNA extraction is performed on a sample that has been ultracentrifuged to concentrate the microorganisms or even directly on a rumen liquid sample (Dohrmann et al., 2004, Meibaum et al., 2012, Henderson et al., 2013, Riede et al., 2013). There are many different gDNA extraction protocols available (Henderson et al., 2013) but they generally all include different cell wall disruption (e.g. using a ribolyser) and digestion (e.g. using lysozyme), as well as DNA purification (e.g. using phenol-chloroform-isoamylalcohol) steps (Henderson et al., 2013). Nowadays several commercial kits are available for different matrices.

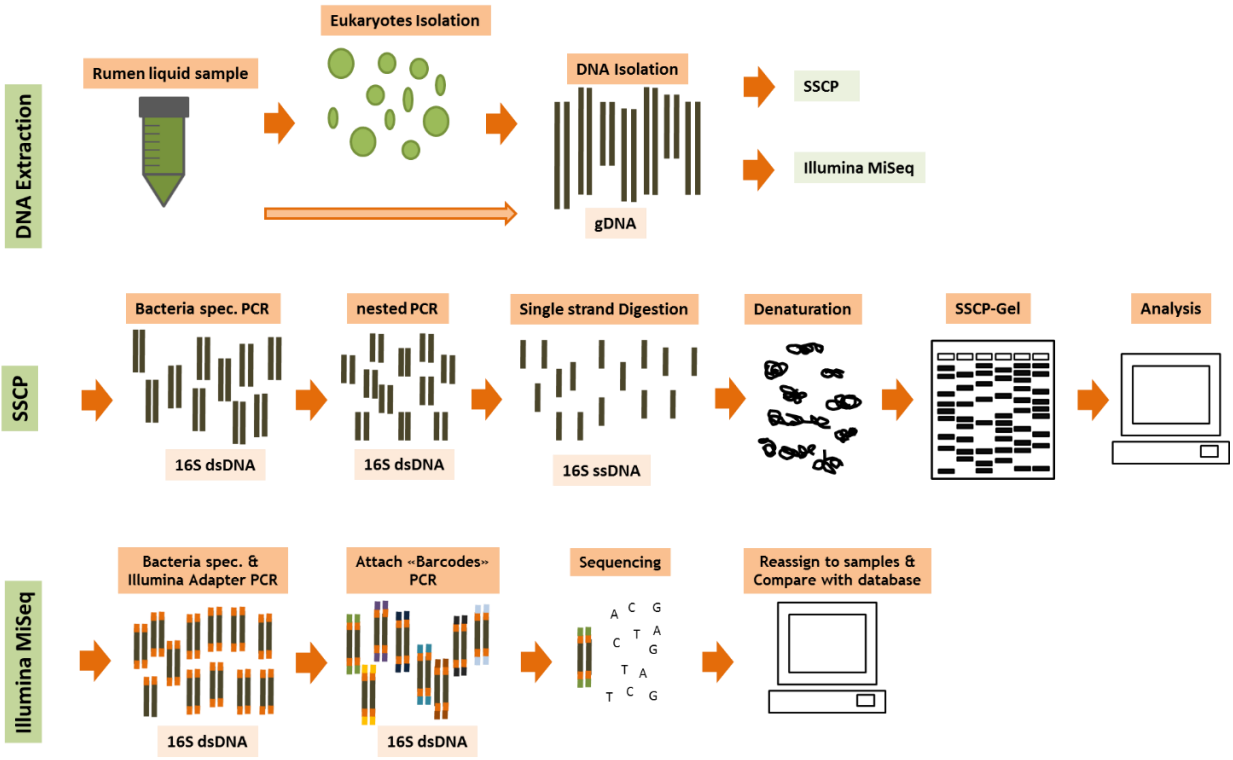



**Figure 8.** Schematic illustration of the 16S rRNA gene amplification and subsequent SSCP or Illumina MiSeq amplicon sequencing analysis.

After the gDNA has been extracted and purified the 16S rRNA genes are amplified using the PCR method (Boon et al., 2002, Dohrmann et al., 2004). Depending on the taxa of interest a primer-pair can be chosen that either covers a very broad range of taxa or only a very specific group of prokaryotes (e.g. *Clostridia* or *Archaea*, Klindworth et al. (2013)). In case of many sequencing platforms adapters to enable recognition by the sequencing device are added during this PCR step (example for the Illumina platform in Figure 8, Di Bella et al. (2013)). To increase the output a two-step amplification using a second, a so called, nested-PCR can be performed (Dohrmann et al., 2004). During the nested-PCR smaller amplicons are generated, whose templates lie within the amplicons of the primary PCR. This step is for example included in the protocol of the DNA fingerprinting method SSCP (Figure 8).

### 1.5.2. DNA fingerprinting methods

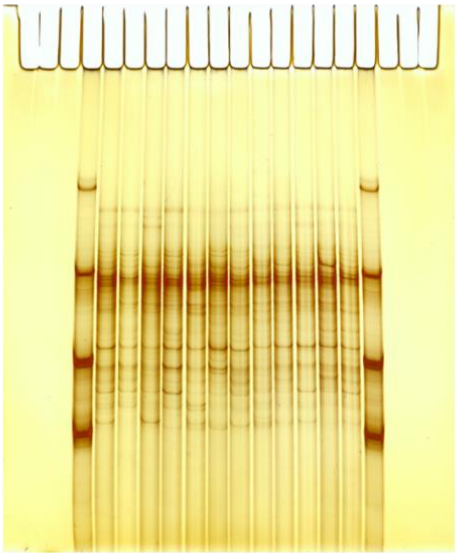
DNA fingerprinting methods are gel-based methods that allow estimating the similarity or dissimilarity of different microbial populations, relying on the ribosomal gene polymorphism in prokaryotes. They include the SSCP, **DGGE** (denaturing gradient gel electrophoresis), **TGGE** (temperature gradient gel electrophoresis), and **T-RFLP** (terminal restriction fragment length polymorphism) method (Boon et al., 2002, Morgavi et al., 2013, Golder, 2014). The SSCP method will be further described to illustrate the principles of the DNA fingerprinting methods (Figure 8).

For the SSCP method the double stranded 16S rRNA gene DNA (**dsDNA**) needs to be subjected to a digestion step using an exonuclease to obtain the single-stranded DNA (**ssDNA**). Thereafter the ssDNA is washed and prior to the gel-electrophoresis a denaturing step is performed by submerging the sample tubes in a hot water bath (Dohrmann et al., 2004). This causes a unique folding of each ssDNA fragment, based on the structure of its 16S rRNA gene. This folding is responsible for the different bands on the gel. The obtained gel is stained and scanned for analysis (Dohrmann et al., 2004). Using a software, the differences between the gel lanes and a (dis)similarity matrix are computed. Using this (dis)similarity matrix different plots such as dendrograms or PCA plots can be created and statistical analysis can be performed (Boon et al., 2002, Dohrmann et al., 2004, Riede et al., 2013). In

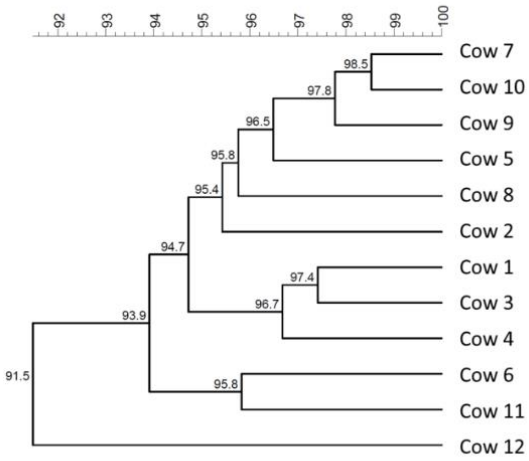
Figure 9 a SSCP gel and the corresponding similarity matrix and dendrogram are illustrated.

If further information on the prokaryotes species involved is warranted the lanes of interest on the gel can be excised, cloned and sequenced (Delbes et al., 2000, Dohrmann et al., 2004, Ercolini, 2004).

A.



C.




B.

| | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| Cow 7 | 100.00 | | | | | | | | | | |
| Cow 10 | 99.71 | 100.00 | | | | | | | | | |
| Cow 9 | 99.46 | 99.23 | 100.00 | | | | | | | | |
| Cow 5 | 97.95 | 98.13 | 98.23 | 100.00 | | | | | | | |
| Cow 8 | 97.77 | 97.30 | 98.83 | 97.18 | 100.00 | | | | | | |
| Cow 2 | 97.94 | 97.48 | 98.45 | 96.89 | 96.97 | 100.00 | | | | | |
| Cow 1 | 96.89 | 97.27 | 97.34 | 98.65 | 94.77 | 95.87 | 100.00 | | | | |
| Cow 3 | 94.47 | 95.26 | 94.89 | 96.89 | 91.53 | 93.26 | 99.31 | 100.00 | | | |
| Cow 4 | 97.71 | 97.67 | 98.03 | 97.63 | 96.14 | 96.82 | 98.55 | 96.98 | 100.00 | | |
| Cow 6 | 95.61 | 95.80 | 95.45 | 95.73 | 95.40 | 90.39 | 94.99 | 93.04 | 96.15 | 100.00 | |
| Cow 11 | 96.45 | 96.35 | 96.32 | 95.73 | 97.31 | 91.84 | 93.57 | 90.97 | 94.85 | 98.51 | 100.00 |
| Cow 12 | 93.87 | 93.27 | 92.42 | 87.87 | 92.15 | 89.42 | 84.29 | 79.81 | 86.70 | 88.67 | 91.39 | 100.00 |

**Figure 9.** Example of a SSCP-gel (A.) and the corresponding similarity matrix (B.) and dendrogram (C., the numbers at the nodes indicate the similarity between the respective samples). Data from the experiment presented in chapter 3 (groups not indicated).

### 1.5.3. Sequencing methods

Currently a selection of different platforms is available that perform high-throughput sequencing. For microbiome analysis, currently mainly the 454 GS FLX Titanium (also called pyrosequencing), the Illumina MiSeq, and the Ion Torrent system are used (Di Bella et al., 2013, Golder, 2014, McCann et al., 2014a). The Illumina MiSeq system is characterized by a high throughput and its inexpensiveness (Claesson et al., 2010, Di Bella et al., 2013, Golder, 2014).

Prior to sequencing each dsDNA fragment needs to be equipped with an adapter that allows the sequencing platform to recognize the amplicons (as mentioned in the previous section, Figure 8, Mardis (2008), Di Bella et al. (2013)). In the Illumina MiSeq system a second PCR is conducted, the so called “library preparation”, in which the dsDNA fragments of each sample are equipped with nucleotide barcodes that allow the re-assigning of each amplicon to the corresponding sample after sequencing, the so called de-multiplexing procedure (Mardis, 2008). With this tool, several different samples can be mixed and sequenced concomitantly.

After sequencing the retrieved nucleotide code needs to be de-multiplexed and the adapters are removed in a process called “trimming”, using specifically designed software (Martin, 2011). Also, overlapping paired-end reads are merged (the so called “stitching”) and overlapping sequences of two normally independent amplicons are detected and removed (“chimera detection, identification and removal”, Edgar et al. (2011)). Thereafter the amplicons are clustered to form **OTU** (operational taxonomic units) based on 97 % sequence similarity with known prokaryotes species, and taxonomic assignment is performed using a reference database (e.g. SILVA, Quast et al. (2013)). The bioinformatical analysis is divided into the alpha- and beta-diversity. The alpha-diversity analysis includes the calculation of microbial diversity indices for every sample. Examples of diversity indices are the number of species observed, the Chao1 and Shannon index. They are are different estimates of richness and evenness of a microbial population and enable the comparison between samples and treatment groups in relation to species diversity (Caporaso et al., 2011). The beta-diversity analysis comprises the comparison of the different samples in relation to their microbial community. It may include cluster analyses (e.g. PCoA plots) or a statistical analysis on the proportional abundance of the different taxa (Caporaso et al., 2011).

### 1.5.4. Outlook

At the present different new tools are being developed to allow a broader insight on the different members present and a more functional investigation of the rumen microbiome (Golder, 2014, Malmuthuge and Guan, 2017, Tapio et al., 2017). Metagenomic sequencing (also called shotgun metagenomics) is currently being adopted (Jovel et al., 2016, Ranjan et al., 2016). It involves the sequencing of the entire DNA present in a sample and assigning it to the different organisms. In this method, no additional PCR step is included to select for a certain region in the genome, minimizing pre-selection of microorganisms through primers. The routine use of this method is thought to give better insights into the abundance and role of rare species in the rumen microbiome (Marco, 2011). Further, the description of the functional activity of rumen micro-organisms is being adopted using gene expression analysis (so called metatranscriptomics, Hess et al. (2011), Qi et al. (2011), Poulsen et al. (2013), Solden et al. (2017), Li and Guan (2017), Wallace et al. (2017)). This method will allow a better understanding of the interrelation between the abundance of different bacteria species and the observed characteristics on rumen fermentation and animal level (Golder, 2014, Malmuthuge and Guan, 2017).

# 2. Aims of Study

The adaptability of the rumen microbiome to new nutritional situations is a key feature in ruminant survival strategy. Different studies and reviews describe the high redundancy and resilience of the rumen microbiome allowing the fermentation and nutrient extraction from a wide range of feedstuffs (chapter 1.4.4). They further highlight the strong host effect and that many questions concerning the temporal, spatial and microbial dynamics involved are still unanswered (chapter 1.3 and 1.4). The aim of this thesis was therefore to investigate different factors influencing the rumen microbiome and their interplay. Three different studies were performed, each examining a different aspect:

The adaptation to a new diet

For the first aspect samples were collected from three different sites in the rumen (liquid, fiber mat and epithelium) at three points in time, in a trial involving the transition from a silage- and concentrate-based to pasture in spring (chapter 3). It was hypothesized that the prokaryote community at the three different sites would be differently affected, since earlier studies suggest that the epithelial-associated microbiome remains more consistent during ration changes, compared to the liquid- and particle-associated (chapter 1.4.1).

The influence of anti-ketogenic feed additives

To investigate the influence of anti-ketogenic feed additives on the rumen microbiome, rumen liquid samples were collected during a trial performed to investigate the influence of monensin and essential oils on health, production and rumen fermentation of transition dairy cows (chapter 4). The aim of this study was to characterize the underlying microbial changes and to test whether essential oils elicit effects similar to monensin.

The interrelations with phenotypical traits of the host

Different studies have shown a significant correlation between different rumen microbial species and phenotypical traits, such as feed efficiency or milk production (chapter 1.4.2). To investigate the underlying physiological interrelations a large dataset containing production, behavior, rumen fermentation, metabolic, and immunological variables from dairy cows in early lactation was analyzed.

## 3. Alterations in the rumen liquid-, particle- and epithelium-associated microbiota of dairy cows during the transition from a silage- and concentrate-based ration to pasture in spring

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State of publication: published April 2017 in *Frontiers of Microbiology*

Author contributions:

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- Trial and project design: MS, UM, SD, GB
- Trial implementation and sample collection: MS
- Sample Analysis: MS, KK, SR
- Data Analysis: MS, MG, SR
- Data Interpretation: MS, SR, MG, JH, TU, GB, UM, SD
- Writing of manuscript: MS
- Revision of manuscript: MS, KK, SR, MG, UM, JH, TU, GB, SD

# Alterations in the rumen liquid-, particle- and epithelium-associated microbiota of dairy cows during the transition from a silage- and concentrate-based ration to pasture in spring

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Published May 2017 in *Frontiers of Microbiology*

Volume 8, Article 744

http://dx.doi.org/10.3389/fmicb.2017.00744

## Abstract

In spring dairy cows are often gradually transitioned from a silage- and concentrate-based ration (total mixed ration, TMR) to pasture. Rumen microbiota adaptability is a key feature of ruminant survival strategy. However, only little is known on the temporal and spatial microbial alterations involved. This study aims to investigate how the rumen liquid (LAAB), particle (PAAB) and epithelium (EAAB) associated archaea and bacteria are influenced by this nutritional change. A 10-wk trial was performed, including 10 rumen-fistulated dairy cows, equally divided into a pasture- and a confinement- group (PG and CG). The CG stayed on a TMR-based ration, while the PG was gradually transitioned from TMR to pasture (wk 1: TMR-only, wk 2: 3 h/day on pasture, wk 3 & 4: 12 h/day on pasture, wk 5-10: pasture-only). In wk 1, wk 5 and wk 10 samples of solid and liquid rumen contents, and papillae biopsies were collected. The DNA was isolated, and PCR-SSCP and 16S rRNA gene amplicon sequencing analysis were performed. Cluster analysis revealed a higher similarity between LAAB and PAAB, compared to the EAAB, characterized by higher species diversity. At all three locations, the microbiota was significantly influenced by the ration change, opposite the generally

acknowledged hypothesis that the EAAB remain more consistent throughout dietary changes. Even though the animals in the PG were already on a full-grazing ration for 4-6 days in wk 5, the microbiota at all three locations was significantly different compared to wk 10, suggesting an adaptation period of several days to weeks. This is in line with observations made on animal level, showing a required time for adaptation of 2-3 weeks for production and metabolic variables. A large part of the rumen prokaryote species remained unaltered upon transition to pasture and exhibited a strong host influence, supporting the hypothesis that the rumen microbiota consists of a core and a variable microbiota. For the effect of the location as well as the ration change either very similar or opposite trends among member species of common taxa were observed, demonstrating that microbes that are phylogenetically close may still exhibit substantially different phenotypes and functions.

**Keywords:** dairy cow nutrition, rumen microbiota, pasture, ration change, PCR-SSCP, amplicon sequencing.

## Introduction

In temperate climate zones, dairy cows often receive a silage and concentrate-based ration (total mixed ration, TMR) during winter time and are then gradually transitioned to a pasture-based ration in spring. Since the two systems (confinement and pasture) do not only differ substantially in ration composition, but also how feed is acquired, considerable metabolic as well as behavioral adaptations are required upon this nutritional change (Osuji, 1974; Kolver, 2003). The adaptability of rumen microbiota is a key feature of ruminant physiology and survival strategy (Russell and Rychlik, 2001; McCann et al., 2014a; Zanton, 2015). It has been shown that whenever cows undergo a ration change rumen microbiota needs between one day and sometimes even longer than three weeks to adapt and stabilize, depending on the group of bacteria, archaea, fungi or protozoa, the extent of diet change and the behavioral adaptation required (Hackmann, 2015). De Menezes et al. (2011) have shown in a cross-over design, with two weeks for diet adaptation, that the liquid and solid rumen bacterial and archaeal community of TMR and pasture fed dairy cows differs significantly. Furthermore, Nakano et al. (2013) showed that rumen microbiota needs 3-4 weeks to adapt to a pasture-based ration when no gradual adaptation to the new nutritional situation is granted. In both studies Prevotellaceae were more prevalent on pasture and a possible key role of this bacterial family in reducing methane production and in transitioning cows to a pasture-based ration was suggested (de Menezes et al., 2011; Nakano et al., 2013; McCann et al., 2014a). However, further data on time required for adaptation of rumen microbiota during the gradual transition

from a TMR to a pasture-based ration, and the prokaryotes playing a key role during this nutritional change are lacking.

Aside the particle- and liquid-associated, a third rumen bacterial community has been described. The epithelium-associated or “epimural” microbiota has been investigated in few studies and it has been suggested that it is associated with fermentation end-products, volatile fatty acid (VFA) absorption, maintaining an anaerobe environment, recycling of endogenous nitrogen and tissue (Cheng et al., 1979; Wallace et al., 1979; McCann et al., 2014a). It has been further speculated whether this microbial community may remain more consistent through dietary changes compared with the particle- and liquid-associated bacterial community (Sadet et al., 2007; McCann et al., 2014a).

In previous publications, we described the alterations in production and rumen variables during a gradual transition from a TMR- to a pasture-based ration (Schären et al., 2016a; Schären et al., 2016b). Primarily a decrease in rumen fermentation activity during the initial phase of transition was observed, most likely due to a decreased intake of fermentable organic matter. After 2-3 weeks on a full-grazing ration an increase in rumen fermentation activity occurred indicated by a decrease in mean daily pH and acetate/propionate ratio as well as an increase in daily pH variation and total VFA concentrations. This was also mirrored in the development of different other rumen (increase of VFA absorption capacity and rumen papillae surface area), performance (stabilization of milk yield and increase in BW) and metabolic (serum non-esterified fatty acid concentrations) variables. We suggested a behavioral and metabolic adaptation after 2-3 weeks on a full-grazing ration leading to an increased intake of fermentable organic matter and therefore rumen fermentation activity and stabilization of milk production. Since rumen microbiota plays a key role in adaptation to a new ration we hypothesized that the effects of a transition from a TMR- to a pasture-based ration observed in other performance, rumen and metabolic variables would also be mirrored in the different rumen archaea and bacteria communities. To investigate whether the archaea and bacteria communities are differently affected by this ration change, polymerase-chain-reaction-single-strand-conformation-polymorphism (PCR-SSCP)- and amplicon sequencing-analysis were performed on samples of the liquid and solid fraction, as well as rumen papillae.

## Material and Methods

Experimental work was conducted at the experimental station of the Friedrich-Loeffler-Institute (FLI) in Brunswick, Germany. The experiment was carried out in accordance with the German Animal Welfare Act approved by the LAVES (Lower Saxony State Office for Consumer Protection and Food Safety, Germany; approval number: 33.09-42502-04-11/0444).

**Experimental design and treatments**

A 10-wk trial (wk1-10) was performed from April 21st until June 27th 2014 including 10 rumen-fistulated German Holstein cows (182 ± 24 days in milk, 23.5 ± 3.5 kg milk/d; parity: 4.5 ± 2.2; mean ± SD; at the beginning of the trial). The full trial included 60 dairy cows (166 ± 23 days in milk and 23.5 ± 3.7 kg milk/day; parity: 1.9 ± 1.6; mean ± SD; at the beginning of the trial); the experimental design, treatments, rations, climate data, animal performance, urine variables, clinical chemistry and total blood counts have been reported previously (Schären et al., 2016a). The rumen fermentation, VFA absorption characteristics and morphology variables assessed in the fistulated animals, as well as their performance data have been separately described (Schären et al., 2016b). The experimental work and data of the present paper have been exclusively conducted and collected in these 10 fistulated animals. At the beginning of the trial the animals were randomly assigned to either a pasture group (PG, n = 5) or a confinement group (CG, n = 5). The CG stayed in a confinement system and received a TMR throughout the whole trial (35 % corn silage, 35 % grass silage, 30 % concentrate; DM basis), whereas the PG was transitioned from a TMR- to a pasture-based ration (wk 1: TMR-only, wk 2: TMR and 3 h/d on pasture, wk 3 and 4: TMR and 12 h/d on pasture, wk 5-10: pasture and 1.75 kg DM concentrate/d offered in 2 equal meals in troughs after morning and evening milking). A continuous grazing system was implemented on ryegrass dominated pasture. The cows were milked two times per day at 0530 h and 1500 h and the TMR was fed daily at approximately 1100 h. Individual TMR and water intake was continuously recorded in the confinement system using electronic weighing throughs (Insentec, B.V., Markenesse, The Netherlands). Dry matter intake (DMI) on pasture was estimated using the n-alkane method in wk 7 and wk 9 (described in detail in Schären et al. (2016a)).

**Sample collection**

Sample collection took place at three points in time: at the beginning of the trial (TMR-only, wk1), during the transitional period (PG being 4-6 days on a full-grazing ration, wk 5) and at the end of the trial (PG being 6 weeks on a full-grazing ration, wk 10). All animals were sampled within three days of the particular week, between 0730 h and 1430 h. Firstly, a sample of approximately 200 g rumen solid content was collected at the height of the rumen fistula aperture (pool sample of grab-samples collected from cranial to caudal in the upper half of the rumen fiber mat). Thereafter a 250 mL rumen fluid sample was collected from the ventral site of the rumen (saccus ventralis) using a manual pump. Both samples were stored at -20 °C within 30 min. Subsequently the total rumen content was evacuated, transferred into insulated barrels and the rumen was washed twice (2 x 10 L water, 39 °C). The rumen papillae were then collected at the most ventral site of the ventral rumen sac (saccus ventralis; approximately 5 cm adjacent to the pila coronaria ventralis) using a biopsy forceps (Lloyd-Davis biopsy

forceps 35cm, Zepf Instruments, Tuttlingen, Germany). Papillae were immediately washed with a 0.9 % NaCl solution, stored in 2 mL cryo tubes (Cryo-Pure Tubes, Sarstedt AG & Co, Nürmbrecht, Germany) and shock frozen using liquid nitrogen. Papillae samples were stored at -80 °C until analysis. After the papillae collection, a VFA absorption test was performed and the rumen content was reintroduced (detailed protocol in Schären et al. (2016b)).

**DNA extraction**

***Rumen liquid content.*** The separation of the liquid-associated microbes from feed particles and the subsequent DNA extraction have been described by Meibaum et al. (2012) and Schären et al. (2017) (exact protocol). Briefly, several centrifugation steps were performed (once 5 min at 600 g (4 °C) to remove feed particles and debris, and four times during 20 min at 27'000 g (4° C); between each centrifugation step the pellet was re-suspended in 40 mL 0.9 % NaCl) and the concentrated samples were liquid shock frozen under the form of droplets for storage at -80 °C. After a centrifugation step (13'000 g, 5 min, 4 °C) the supernatant was discarded and the sample was re-suspended in 1 x tris(hydroxymethyl)-aminomethane-HCl, EDTA (both 10 mM, pH 8.0) and NaCl (150 mM), and a DNA extraction was performed including a mechanical lysis of the cells by bead beating method (Fast Prep, MP Biomedicals, Eschwege, Germany; in two sequences of acceleration, 6.0 m/s and 4.5 m/s, 40 sec. each). This was followed by different incubation steps including lysozyme and RNaseA (30 min at 37 °C), sodiumdodecylsulphate and proteinase K (1 h at 37 °C), and 4 M NaCl and cetyltrimethylammoniumbromide (65 °C during 10 min). To purify the mixture from proteins phenol-chloroform-isoamylalcohol was added, the mixture was centrifuged (7 min, 13'000 g, 4 °C), the supernatants were discarded, chloroform-isoamylalcohol was added, centrifuged again (10 min, 13'000 g, 4 °C) and the supernatant was then kept for further processing. As a final step the samples were further purified using the peqGold Tissue-Kit (peqlab, Erlangen, Germany) according to manufacturer's guidelines. The genomic DNA (gDNA) samples were then stored at 4 °C until further processing.

***Rumen solid content.*** To remove all liquid-associated bacteria from the sample several washing steps were performed per sample (10 g sample, 4-5 washing steps with each 1 L 0.9 % NaCl, using a 4 mm sieve, until washing solution was clear). Thereafter the fiber particles were transferred into a 50 mL vessel, immersed in sterile 0.9 % NaCl solution and sonicated in an ultrasonic-bath during 30 min to detach the particle associated bacteria. Thereafter the sample was sieved (4 mm sieve), centrifuged at 27´000 g during 20 min (4 °C), the supernatant discarded and the pellet was resuspended in 1000 µl 0.9 % NaCl. For DNA extraction and purification 200 µl of the microbe-pellet and the peqGold Tissue-Kit was used (according to manufacturer's guidelines; 1. Incubation: 150 µL TE-Puffer, 50 µL lysozyme, 30 min at 30 °C on thermoshaker; 2. Incubation: 400 µL DNA lysis puffer, 20 µL proteinase K, 15 µL RNaseA,

60 min at 50 °C on thermoshaker). The gDNA samples were then stored at 4 °C until further processing.

***Rumen papillae.*** Rumen papillae samples were thawed on ice and 120 mg of each sample were washed twice with 1000 µL sterile 0.9 % NaCl. Thereafter DNA extraction (400 µL DNA lysis buffer, 20 µL proteinase K, 15 µL RNaseA, 50 min at 60 °C on thermoshaker) and purification was performed using the pegGold Tissue-Kit according to manufacturer's guidelines and samples were then stored at 4 °C until further processing.

**PCR-SSCP analysis**

After DNA extraction a two-step amplification (initial and nested PCR) of the bacteria specific 16S rRNA gene regions and a single-strand digestion step were performed (protocol and primers described in detail in Meibaum et al. (2012)). To compare the bacterial populations at the three different locations in the rumen, as well as the change over time in both groups, 12 different SSCP-gels were created: 6 gels comparing the liquid- (LAB), particle- (PAB) and epithelium- (EAB) associated bacteria at one particular point in time in the PG or CG, 6 gels comparing the LAB, PAB or EAB at the three points in time (wk 1, wk 5 and wk 10) in the PG or CG. Gel-electrophoresis was carried out at 300 V during 22.5 h at 20 °C (described in detail in Dohrmann et al. (2004)). The gels were digitalized and analyzed using ScanMaker (i800, Mikrotek, Willich, Germany) and GelComparII (Applied Maths, Sint-Martens-Latem, Belgium) as described in Meibaum et al. (2012). For graphical illustration two dimensional principal co-ordinates analysis (PCO) plots based on dissimilarities were created with the cmdscale() command in the R Guide 3.0.2 software package (R-Core-Team, 2013).

**Prokaryotic 16S rRNA gene amplification, Illumina MiSeq sequencing and bioinformatics**

For sequencing gDNA samples were sent to Microsynth AG (Balgach, Switzerland). A primer pair with 97.7 % / 98.4 % (forward primer) and 96.9 % / 96.5 % (reverse primer) coverage (one mismatch) for archaea and bacteria, respectively, was chosen for 16S sequencing library preparation: A519F (S-D-Arch-0519-a-S-15): CAGCMGCCGCGGTAA and 802R (S-D-Bact-0785-b-A-18): TACNVGGGTATCTAATCC (Klindworth et al., 2013). Due to the additional inclusion of the archaea in this approach (in comparison to the PCR-SSCP analysis), samples will be referred to as liquid- (LAAB), particle- (PAAB) and epithelium- (EAAB) associated bacteria and archaea. For 16S rDNA amplification the HiFi HotStart PCR Kit (Kapa Biosystems, Wilmington, MA, USA) was used with following PCR conditions: initial denaturation (95 °C, 180 sec), denaturation (98 °C, 20 sec), annealing (50.8 °C, 30 sec) and elongation (72 °C, 30 sec) with 30 cycles, and a final elongation step (72 °C, 5 min). Further, the Illumina Nextera Libraries were prepared according to the manufacturers instruction

(Illumina, San Diego, USA). Sequencing was performed on the Illumina MiSeq Sequencing System using the Illumina MiSeq reagent Kit v2 (2 x 250bp). Sequence data were demultiplexed and trimmed using the Illumina MiSeq v2.5.1.3. reporter and cutadapt v1.8.1 software package (Martin, 2011). Read stitching was performed using FLASH v1.2.11 (Magoč and Salzberg, 2011) and only stitched reads with an average quality score (whole read) of 25 or higher were used for downstream analysis. Further, de novo Chimera detection, identification and removal was done using the Uchime v4.2 (Edgar et al., 2011) and Usearch v8.1.1861 (Edgar, 2010) software package. The taxonomic assignment and the OTU clustering (based on 97 % sequence similarity) were performed using Uclust (Edgar, 2010) and QIIME v1.9.1 (Caporaso et al., 2010), respectively. Only matches with a minimum sequence similarity of ≥ 90 % and a score 0.67 or 1.00 in the greengenes database were used. Singeltons were removed from the dataset to reduce bias introduced by sequencing errors. As a reference database for the taxonomic assignment the SILVA rRNA database v111 was chosen (Quast et al., 2013). For downstream analysis only OTUs with a relative abundance of at least 0.1% were considered. Alpha diversity analysis was performed and PCO plots were created using QIIME. Robustness of clusters displayed in PCO plots was ensured by jackknife resampling (10fold).

**Statistical analysis**

All statistical analyses were performed using the R 3.0.2 software package. In case of the SSCP-gels a PERMANOVA was performed using the adonis() function in the R software package vegan (Oksanen et al., 2015). To evaluate the alterations in similarity of samples on the SSCP gels over the course of the trial (comparing each sample in wk 5 and wk 10 to its reference sample of wk 1 (within the same cow)) a repeated measures ANOVA using the aov() function was performed. Alpha diversity variables (chao1 index, observed species and Shannon index) were analyzed via a PERMANOVA using the aovp() function of the lmPerm software package (Wheeler, 2010). Beta-diversity was evaluated based on the weighted UniFrac distances via a PERMANOVA using the adonis() function in the software package vegan. For species level comparison a PERMANOVA model using the aovp() function in the software package lmPerm was performed. The model included Group, Time and Location and their interactions as well as the Cow and a Cow×Time interaction as fixed factor and the Cow as random factor. Results were considered significant at $P \leq 0.05$ and a trend declared at $0.05 < P \leq 0.10$.

## Results

### SSCP analysis

Cluster analysis displayed a clustering of LAB, PAB and EAB in both ration types and all three points in time, with a higher similarity among LAB and PAB, compared to EAB samples (Figure 1). LAB and PAB had an average similarity of 82 ± 8 % and differ strongly from the EAB with a similarity of 39 ± 11 % EAB compared to LAB, and 37 ± 10 % EAB compared to PAB (mean ± SD). For illustrational purposes, the dendrogram and SSCP-gel of the comparison of the samples collected in wk 5 in the CG are depicted in Figure 2. Within the different bacteria communities, a significant influence of time was only observed for the LAB in the PG and the EAB in CG (Figure 3). However, when comparing the samples of the different bacteria populations in wk 5 and wk 10 to their reference sample in wk 1, a significant greater decrease in similarity over time in all three bacteria populations was observed for the PG compared to the CG (Figure 4).

### 16S rRNA gene amplicon analysis

For the LAAB, PAAB and EAAB a total number of 2,151 ± 312, 2,615 ± 338, and 662 ± 161 different OTUs were detected, respectively. Filtering (exclusion of OTUs with a relative abundance < 0.1%) resulted in a total number of 177 different OTUs with an average of 167 ± 5 (LAAB), 162 ± 4 (PAAB), and 74 ± 8 (EAAB) different OTUs per sample (mean ± SD, Table 1), with an average of 12,882 ± 3,389, 11,853 ± 3,433 and 2,129 ± 815 reads per sample (after filtering, mean ± SD), respectively. Four samples from the EAAB were excluded due to an extremely low reads count (wk1 CG, 2x wk 1 PG and wk 5 PG with 942, 590, 859 and 801 reads per sample). Most OTUs could be taxonomically classified to the family level, while their genus or species level affiliation were “uncultured bacterium or archaeon" in many cases (Table 1). One OTU was assigned to an archaeal and 176 OTUs to bacterial taxa.

***Alpha diversity.*** Alpha diversity analysis revealed a lower chao1 and Shannon index as well as lower observed species count in the EAAB compared to the LAAB and PAAB with an average of 73 (30; median (IQR)) compared to 169 (6) and 162 (4) observed species ($P < 0.001$), a chao1 index of 108 (33) compared to 173 (5) and 168 (7) ($P < 0.001$), and a Shannon index of 3.0 (0.2) compared to 4.3 (0.2) and 4.6 (0.2) ($P < 0.001$), respectively (Figure 5). The LAAB further exhibited a higher observed species count ($P < 0.001$) as well as chao1 index ($P = 0.004$) compared to the PAAB, whereas the PAAB had a higher Shannon index ($P < 0.001$). In the PG a significantly lower observed species count in the EAAB compared to the CG was observed ($P = 0.035$). Further, no significant treatment effects were observed. In two diversity variables (observed species and Shannon index) a significant Cow effect was observed.

***Beta diversity.*** Beta diversity analysis revealed a significant Location ($P < 0.001$), Group ($P < 0.001$), Time ($P = 0.035$) and Cow ($P < 0.001$) effect, as well as a significant Location×Group ($P = 0.011$) and Group×Time ($P = 0.036$) interaction. PCO plots show a clustering of the LAAB, PAAB and EAAB, with a higher similarity between LAAB and PAAB, compared to the EAAB samples (Figure 6). Taxonomic classification at family level showed broad differences in community composition between the LAAB, PAAB and EAAB (Figure 7). In the LAAB members of the Prevotellaceae (25 %), Lachnospiraceae (18 %), Ruminococcaeceae (16 %), Christensenellaceae (12 %), Veillonellaceae (6 %), Rikenellaceae (4 %), Erysipelotrichaceae (4 %), Coriobacteriaceae (3 %) and the uncultured BS11 gut group (Bacteroidales, 2 %) contributed to 90 % of the relative abundance of 16S rRNA genes. In the PAAB a similar pattern was observed with members of the Ruminococcaceae (28 %), Lachnospiraceae (23 %), Prevotellaceae (18 %), Veillonellaceae (8 %), Christensenellaceae (4 %), Rikenellaceae (3 %), Spirochaetaceae (2 %), uncultured BS11 gut group (2 %), Erysipelotrichaceae (2 %) and Succinivibrionaceae (2 %) accounting for 90 % of the relative abundance, whereas in the group of the EAAB members of the families Lachnospiraceae (26 %), Family XIII Incertae Sedis (Clostridiales, 18 %), Desulfobulbaceae (15 %), Cardiobacteriaceae (5 %), Comamonadaceae (11 %), Campylobacteraceae (5 %), Ruminococcaceae (5 %) and Rikenellaceae (4 %) were the dominant community members.

Statistical analysis on OTU level showed a significant location effect (i.e. LAAB, PAAB and EAAB) for all 177 OTUs, except for two members of Prevotella genus and one member of Succinivibrionaceae family (Table 1 and Appendix 1). OTUs within particular families either exhibited a similar distribution pattern (Bifidobacteriaceae, Coriobacteriaceae, Desulfobulbaceae, Succinivibrionaceae and Spirochaetaceae), a generally similar distribution pattern with few exceptions [Prevotella, Fibrobacteraceae, and members of the order RF9 (Mollicutes)] or a very diverse distribution pattern among the three locations [uncultured BS11 gut group (Bacteroidales), uncultured RC9 gut group (Rikenellaceae), uncultured S24-7 (Bacteroidales), members of the Candidate division TM7 phylum, Christensenellaceae, Family XIII Incertae Sedis (Clostridiales), Lachnospiraceae, Ruminococcaceae, Veillonellaceae and Erysipelotrichales]. Analysis on OTU level also revealed that the predominant role of the family Lachnospiraceae in the EAAB can be attributed to mainly four OTUs (two of the genus Butyrivibrio and two further unclassified OTUs).

Similar to the location, also for the effect of the ration change from TMR to pasture in the PG variable trends within taxonomic groups were observed (Table 1). In some taxa either a decrease [Bifidobacteriaceae, uncultured RC9 gut group (Rikenellaceae), uncultured S24-7 (Bacteroidales), uncultured SHA-109 (Cyanobacteria), Christensenellaceae, Acetitomaculum (Lachnospiraceae), Succiniclasticum (Veillonellaceae), further unclassified members of the

Erysipelotrichaceae, Suttonella (Cardiobacteriaceae) and uncultured RF9 (Mollicutes)], an increase of at least one member species [Atopobium, Incertae Sedis (Lachnospiraceae), Oribacterium, Pseudobutyrivibrio, Shuttleworthia, Incertae Sedis (Ruminococcaceae), Anaerovibrio, Selenomonas, Catenibacterium, Comamonas and Desulfobulbus], or no alterations [Methanobrevibacter, uncultured BS11 gut group (Bacteroidales), uncultured Candidate division SR1 and TM7, Fibrobacter, uncultured Family XIII Incertae Sedis (Clostridiales), Saccharofermentans, Sharpea, Campylobacter, Succinivibrionaceae and Treponema] in proportional abundances were observed, whereas others exhibited mixed effects (Prevotellaceae, Butyrivibrio and Roseburia, Ruminococcus and other further unclassified Ruminococcaceae).

For most OTUs that showed an increase or decrease in proportional abundance over the course of the trial in the PG, a difference between wk 1, wk 5 and wk 10 was observed, mostly exhibiting a gradual increase or decrease over the three points in time (C1-3, F1, F17, F21, F23, G6, K, O1, O3, O16, O24, O25, O27, O28, O30, O35, O41, O42, O47, P1, P2, P4, P7, P20, P22, Q2, Q3, Q4, R1, S and T2 in Table 1 and Appendix 1). A few OTUs, however, exhibited a V-shaped evolution over the course of wk 1, wk 5 and wk 10 (M7, O29, O42, P6, Q1, R3, W and Y1). And for a few OTUs that differed in abundance between wk 1 and wk 10 in the PG, no alterations between wk 5 and wk 10 were observed (B3, G2, G7, H1, O4, O11, P4, P24, P27, Q1, Q2, Q4 and R3).

In the OTUs where a group effect was observed, the proportional abundance was altered at either all (B3, C1-C3, F21, G7, H1, K, O1, O3, O4, O11, O16, O24, O25, O27, O29, O30, O47, P1, P4, P7, P22, P27, Q1-4, R1, R3, S, T2 and W in Table 1 and Appendix 1) or only at a part of the locations (F1, F17, F23, G2, G6, M7, O28, O35, O41, O42, P2, P6, P20, P24 and Y1) where the OTUs exhibited a significant abundance (Table 1).

In case of 28 OTUs a significant or a trend for a Cow or Cow×Time effect was observed. To illustrate the alterations in proportional abundance of each species on cow level, plots including this aspect have been added to the appendix (Appendix 2).

## Discussion

### General differences between the LAAB, PAAB and EAAB

The DNA fingerprinting as well as amplicon sequencing approach revealed distinct differences between the three locations, with a higher similarity between the PAAB and LAAB, compared to the EAAB. This is in line with an earlier study using PCR-DGGE (Sadet et al., 2007) and can be explained by the close spatial relationship and constant interchange between the two

communities due to the constant ongoing fiber colonization and degradation. As described in previous publications members of the Prevotellaceae constituted the most dominant family in the LAAB (Kong et al., 2010; Pitta et al., 2010; Singh et al., 2015), whereas in the PAAB a higher abundance of members of taxa were found that are associated with fiber digestion such as Ruminococcaceae and Fibrobacter (Koike and Kobayashi, 2009). This was to be expected, since a key role of the PAAB in the degradation of fiber can be assumed (Kong et al., 2010; McCann et al., 2014a). When analyzing the distribution across the three locations of the individual OTUs, no clear pattern at a higher level than species was observed for most taxa. Some species in a given taxa were detected in all or two communities, whereas others could exclusively be found in one. This finding illustrates that observed effects on phylum, class, order, family or genus level do not necessarily account for all of its member species, demonstrating that microbes that are phylogenetically close may still exhibit substantially different phenotypes and functions (Morgavi et al., 2013). This discrepancy between taxonomic classification (or genomic commonality) and phenotype has been reported and criticized earlier and can most likely be attributed to differences in gene expression as a result of environmental influences (Achenbach and Coates, 2000; Kampfer and Glaeser, 2012). Weimer (2015) notes that the genetic capability of different degradative functions may reside within a single bacterial strain, but that it is dependent on the presence of potential competitors and symbionts if a particular degradative capability is carried out. Kampfer and Glaeser (2012) therefore suggest revising the polyphasic approach (integration of genotype and phenotype) in prokaryotic taxonomy. Regarding rumen microbiota research, different authors have noted that future studies should focus on the characterization of the functional properties of the rumen microbial ecosystem, aside the different microbial species (Morgavi et al., 2013).

When comparing our results with different studies comparing the LAAB and PAAB, we observed similar as well as different results concerning the abundance of different taxa (Cho et al., 2006; Brulc et al., 2009; Kong et al., 2010; Pei et al., 2010; Pitta et al., 2010; de Menezes et al., 2011; Kim and Yu, 2012; Singh et al., 2015). Further, similar to our results de Menezes et al. (2011) have shown a higher species diversity in the LAAB compared to the PAAB, whereas the results of Kong et al. (2010), Pitta et al. (2010) and Sadet et al. (2007) show the opposite. We suggest that these observed differences among studies can be ascribed to differences in ration composition (Henderson et al., 2015), time the animals received the ration prior to sampling (Hackmann, 2015), number of animals sampled (Weimer, 2015), sample collection (Li et al., 2009), microorganism and DNA isolation (Henderson et al., 2013), microbiota analysis method (DNA fingerprinting vs. amplicon sequencing, Sadet et al. (2007)), sequencing platform and depth (Klindworth et al., 2013) (also discussed in Schären et al. (2017)).

In the EAAB we observed a much less diverse microbiota, with very different species compared to the PAAB and LAAB, which is in line with other studies (Cho et al., 2006; Sadet et al., 2007). Our results are fairly similar to the observations made by Petri et al. (2013) with the Lachnospiraceae, uncultured Family XIII Incertae Sedis (Clostridiales), Ruminococcaceae, Prevotellaceae, Desulfobulbaceae, Erysipelotrichaceae, and Rikenellaceae constituting the core microbiota of the EAAB in their trial. These findings illustrate a mixture of Gram-positive and Gram-negative bacteria, which is in contrast to the findings of older culture- (Wallace et al., 1979) and electron-microscopy-based studies (McCowan et al., 1978; Cheng et al., 1979), describing a mainly Gram-positive community. Earlier studies described the EAAB as being possibly associated with fermentation end-products, VFA absorption, oxygen consumption, urea digestion and initiated breakdown of dead epithelial tissue (Cheng et al., 1979; Wallace et al., 1979; McCann et al., 2014a). The hypothesis of an oxygen scavenging function of the EAAB is supported by the finding that some of the OTUs, that were detected in our trial as being mainly or only present in the EAAB, have been assigned to taxa that were earlier described as being aerobic (Erysipelotrichaceae, Comamonas and Suttonella) or microaerophilic (Campylobacter) (Garrity et al., 2006; Vos et al., 2009). Further, a part of the OTUs have been assigned to taxa that have been described as being asaccharolytic (Campylobacter and Mogibacterium), nitrate reducing (Comamonas and Campylobacter), complex organic compound degrading (Comamonas), putrescine fermenting (Anaerovorax) and sulfur compounds reducing (Desulfobulbus) (Garrity et al., 2006; Vos et al., 2009). Further research involving cultivation-independent techniques is needed to elucidate the relevance of these functional properties in the rumen and the interrelations between the different microbial species and their host.

**Effects of the ration change from TMR to pasture**

The DNA fingerprinting as well as the beta-diversity analysis of the amplicon sequencing approach showed that at all three locations the microbiota was significantly influenced by the ration change. However, the hypothesis that the EAAB remain more consistent throughout dietary changes (Sadet et al., 2007; McCann et al., 2014a) was not confirmed. This result is opposite to the findings of Sadet-Bourgeteau et al. (2010) illustrating only minor alterations in the EAAB using a DNA-fingerprinting (PCR-DGGE) method in a trial involving wethers that were consecutively fed forage and different mixed concentrate forage diets. The authors however admitted that this method may not be sensitive enough to detect subtler changes in the community (the PCR-DGGE technique has an relative abundance limit of 1 %, it is therefore likely that alterations in less abundant taxa are underestimated (Sadet et al., 2007)). Contrary to this study, applying a more severe dietary influence, Petri et al. (2013) observed significant alterations in various taxa of the EAAB in a trial involving the transition from a forage

to a high grain diet, an acidosis-challenge, and a recovery period, which is in line with our results. Additionally, an earlier study by McCowan et al. (1980) has shown that the distribution pattern of the epithelium adherent bacterial population is diet dependent. This aspect should be included in future studies.

Both analysis techniques (DNA fingerprinting and amplicon sequencing) showed that even though the animals in the PG were already on a full-grazing ration for 4-6 days in wk 5, the microbiota at all three locations was significantly different from that in wk 10. During this trial we also analyzed the ruminal protozoal counts on a weekly basis and observed a gradual increase in holotrich protozoa concentrations from wk 5 on (Künzel et al., 2016). After wk 7 a plateau was observed, suggesting also an adaptation in this period. These findings further agree with the observations made on animal level. We observed alterations in different production, metabolic and rumen variables, all pointing towards a decreased rumen fermentation activity in the first weeks on a full-grazing ration due to a decreased DMI. Thereafter, most likely due to a behavioral and metabolic adaptation, DMI and rumen fermentation activity increased again, causing a decrease in energy deficit and stabilization of various variables in wk 8-10 of the trial (Schären et al., 2016a; Schären et al., 2016b). Taken together this data illustrates that the adaption of the cow's rumen microbiota and metabolism to a pasture-based ration most likely required 2-3 weeks in our trial. This is in line with a study of Nakano et al. (2013) showing a stabilization of the rumen microbiota of steers 28 days after being switched onto a full-grazing ration. However, in future trials weekly or even daily sampling should be involved to monitor microbial changes in the rumen upon a ration change more closely and to investigate the delay with which metabolic and production variables follow alterations in the rumen microbiota.

Similar to de Menezes et al. (2011) and Nakano et al. (2013) we observed an increase in most OTUs assigned to Prevotella when cows were transitioned to a pasture-based ration. It has been suggested that members of Prevotella grow rapidly whenever readily fermentable carbohydrates are available (Tajima et al., 2001; Bekele et al., 2010; Pitta et al., 2010). Since fresh grass contains high amounts of water-soluble carbohydrates this could explain their increase in relative abundance. Further, de Menezes et al. (2011) hypothesized whether the increased propionate production on the pasture-based diet was related to the increased abundance of Prevotellaceae and Veillonellaceae. Also in our trial we observed a lower acetate proportion in wk 9 and 10 as well as lower acetate/propionate ratio in wk 9 in the PG (Schären et al., 2016b), along with an increase of these two taxa, supporting this hypothesis. However, we did not observe several alterations described by these other two studies, such as a higher relative abundance of the Fibrobacteraceae on a TMR-based ration, an increase in the abundance of the Erysipelotrichaceae in the LAAB and the Lachnospiraceae in the PAAB, no

alterations in the rumen protozoal community (de Menezes et al., 2011) or an increase in OTUs assigned to the *Butyrivibrio* species (Nakano et al., 2013). Similar to the location effect we suggest that the differences between studies can be attributed to the different rations fed and the time the animals received the rations prior to sampling (14 and 28 d in study of de Menezes et al. (2011) and Nakano et al. (2013)) as well as methodological aspects. Further, most studies so far summarized the effects on a higher taxonomic level than the species, possibly mingling effects in some cases. Our results have shown that the location as well as treatment effect can either be very similar throughout member species of a taxa, or exhibit opposite trends. This is in line with a study of Bekele et al. (2010) suggesting the existence of diet-specific members of Prevotella. Future studies should include this aspect by further characterizing the different member species and differentiating functional and taxonomic interrelations.

In the current study a filtering step was applied, in which all OTUs with a relative abundance of <0.1 % were excluded. This was done to guarantee a solid differentiation between artefact and true organism. This however also implies that alterations in low abundant species (members of the so called “rare biosphere”) were not captured. It is generally acknowledged that the more dominant species most likely contribute to the key functions in rumen fermentation (Henderson et al., 2015). However, only little is known on the function and relevance of the low abundance members and future studies should involve their identification and functional characterization (Morgavi et al., 2013). Further, due to the different physical properties of the samples three different DNA extraction methods were used. Henderson et al. 2013 have shown that depending on the method applied, the abundance of different taxa varies. The authors for example describe an increase in the abundance of the Bacteroidetes phylum and a concurrent decrease in the Firmicutes phylum, when a non-mechanical lysis procedure is used. It was suggested that this can be attributed to their cell wall constitution (Gram-negative vs. Gram-positive). In our data, however, no apparent divergence towards the phylum Bacteroidetes was observed in the samples treated with a non-mechanical procedure (PAAB and EAAB samples). Henderson et al. 2013 also describe an increase in the Fibrobacteres in non-mechanical DNA extraction methods. In our study, we observed a higher abundance of Fibrobacteres in the PAAB, compared to the LAAB, indicating a possible influence of the DNA extraction method in this context. However, as described above, these findings are also in line with the literature, describing the Fibrobacteres as fiber digesting bacteria. We therefore conclude that the possibility of a certain bias due to the different DNA extraction methods applied cannot fully be excluded, and that future studies should involve more uniform DNA extraction methods whenever possible, but its implications might be neglectable in this case. Further, the main focus of this manuscripts lies on the alterations in

the three different communities over time. Since the comparisons are performed within sample types, these results are not affected by the different DNA extraction protocols.

**The effect of the individual cow**

Different studies have shown that the cow itself as an individual has a significant influence on its rumen microbiota, most likely through behavioral and physiological processes, such as rumination, salivation, absorption and passage of VFA in the rumen, thereby controlling the ruminal chemistry (Sadet-Bourgeteau et al., 2010; de Menezes et al., 2011; Petri et al., 2013; Weimer, 2015). Several of these effects were also confirmed in our trial. The alterations over time in the PG compared to the CG would only emerge properly in the SSCP gels, when samples were compared to their own reference sample collected in wk 1 from the same cow. In the alpha-diversity analysis of the amplicon sequencing data, a significant Cow effect was observed, illustrating that certain cows seem to possess a more diverse rumen microbiota than others. Further, the beta-diversity analysis revealed a significant Cow or Cow×Time effect in 16 % of OTUs. Several recent studies illustrate that the rumen microbiota of dairy cows and steers can be linked to different phenotypic characteristics such as milk production and composition (Jami et al., 2014; Lima et al., 2015), feed efficiency (Guan et al., 2008; Zhou et al., 2009; Hernandez-Sanabria et al., 2010; Zhou et al., 2010; Carberry et al., 2012; Hernandez-Sanabria et al., 2012; Rius et al., 2012; McCann et al., 2014b; Myer et al., 2015), and breed (Guan et al., 2008). These and our results suggest that acquired animal behavior through environmental conditions as well as genetics may play a role in the rumen microbiota composition (Henderson et al., 2015).

The significant Cow effect, as well as the finding that a large part of the detected OTUs at all three locations remained unaltered in their abundance upon the ration change, are in line with the generally acknowledged assumption that the rumen microbiota consists of a core and a variable microbiota, but that individual taxa abundances may vary greatly across diets and animals (Jami and Mizrahi, 2012; Wu et al., 2012; Henderson et al., 2015).

In summary, our data illustrated that the LAAB, PAAB and EAAB are three distinct prokaryote communities, differing in species diversity and composition. The LAAB and PAAB exhibit a higher species diversity and similarity, compared to the EAAB. Where the latter can most likely be attributed to the constant interchange between the two communities due to the ongoing fiber colonization and degradation. Many bacteria species found in the EAAB have earlier been described as possessing functional properties in culture, of which their relevance in rumen fermentation and metabolism is yet to be elucidated. The ration change from TMR to pasture influenced the microbial composition in all three locations significantly, contrary to the earlier stated hypothesis that the EAAB remain more consistent throughout dietary changes. Our data

further illustrates that the time for adaptation from TMR to pasture most likely requires several days to weeks. However, future studies should include more frequent sampling. Further, the hypothesis that the rumen microbiota consists of a core and a variable microbiota, exhibiting a strong host influence was confirmed, but future studies should include the description of rare prokaryote species as well. For the effect of location as well as the ration change either very similar or opposite trends among member species of common taxa were observed. This finding highlights the importance of functional aside genomic characterization, and supports earlier studies suggesting that the genotype as well as phenotype should be included in taxonomic classification (polyphasic approach).

## Acknowledgements

The study was supported by the Ministry for Science and Culture of Lower Saxony (MWK) within the collaborative research project SAM, Analysis of Dairy Production: Grazing versus Indoor Housing of Dairy Cows, Support Code: ZN 2864. The authors want to thank the coworkers at the Institute of Animal Nutrition and the experimental station of the Friedrich-Loeffler-Institute (FLI, Brunswick, Germany) for caring for the experimental animals, assisting with experimental measurements as well as performing the analyses. Special thanks go to the members of the laboratory staff of the Department of Physiology (University of Veterinary Medicine Hannover, Germany) for their support while performing the microbiota analyses. The support and guidance of Kasper Dieho and Jan Dijkstra during the establishment of the protocol for rumen papillae biopsy collection is greatly acknowledged. Many thanks go to Inga Ruddat and Martin Beyerbach (Institute for Biometrics, University of Veterinary Medicine, Hannover, Germany) for excellent bioinformatical support. The authors also want to thank Christian Hundsrucker and Pascal Weigold from Microsynth AG, as well as Andres Buser for their outstanding consultancy.

## Supplementary material

The Supplementary Material for this article can be found online at:
*http://journal.frontiersin.org/article/10.3389/fmicb.2017.00744/full#supplementary-material*

The sequencing data has been submitted at the European Nucleotide Archive (ENA) under the following accession number: PRJEB19414 and can be accessed using following link: *http://www.ebi.ac.uk/ena/data/view/PRJEB19414.*

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## Tables and Illustrations

**Table 1.** Summary of detected OTUs, location and treatment effects[1]

| Boxplot Nr.[2] | OTU Nr.[3] | Taxonomy[4] | | | | | Location Effect[5] | | | | Treatment Effect[6] | | | | Cow Effect[7] |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | Phylum | Class | Order | Family | Genus | *P*-value | EAAB | LAAB | PAAB | *P*-value | EAAB | LAAB | PAAB | |
| **A.** | EF112194 | Euryarchaeota | Methanobacteria | Methanobacteriales | Methanobacteriaceae | Methanobrevibacter | *** | + | +++ | - | - | | | | - |
| **B1.** | EU779121 | Actinobacteria | Actinobacteria | Bifidobacteriales | Bifidobacteriaceae | u.b. | *** | - | ++ | + | * | | | | - |
| **B2.** | AB559503 | Actinobacteria | Actinobacteria | Bifidobacteriales | Bifidobacteriaceae | Bifidobacterium | *** | - | ++ | ++ | - | | | | * |
| **B3.** | AM277978 | Actinobacteria | Actinobacteria | Bifidobacteriales | Bifidobacteriaceae | Bifidobacterium | ** | - | + | ++ | † | | d | d | * |
| **C1.** | EU469015 | Actinobacteria | Coriobacteriia | Coriobacteriales | Coriobacteriaceae | Atopobium | *** | - | ++ | - | ** | | ii | | * |
| **C2.** | AB270014 | Actinobacteria | Coriobacteriia | Coriobacteriales | Coriobacteriaceae | Atopobium | *** | - | ++ | - | ** | | ii | | - |
| **C3.** | New.Ref.OTU | Actinobacteria | Coriobacteriia | Coriobacteriales | Coriobacteriaceae | Atopobium | *** | - | ++ | - | ** | | ii | | - |
| **D.** | EF445233 | Bacteroidetes | Bacteroidia | Bacteroidales | u.b. | | *** | - | ++ | ++ | - | | | | - |
| **E1.** | AB185544 | Bacteroidetes | Bacteroidia | Bacteroidales | BS11 gut group | u.b. | *** | ++ | ++ | +++ | - | | | | - |
| **E2.** | EF686531 | Bacteroidetes | Bacteroidia | Bacteroidales | BS11 gut group | u.b. | *** | ++ | ++ | ++ | - | | | | - |
| **E3.** | EU773647 | Bacteroidetes | Bacteroidia | Bacteroidales | BS11 gut group | u.b. | *** | - | +++ | ++ | - | | | | - |
| **E4.** | AY244965 | Bacteroidetes | Bacteroidia | Bacteroidales | BS11 gut group | u.b. | *** | - | ++ | ++ | - | | | | * |
| **F1.** | AB009235 | Bacteroidetes | Bacteroidia | Bacteroidales | Prevotellaceae | Prevotella | *** | - | ++ | ++ | * | | | d | - |
| **F2.** | EU259377 | Bacteroidetes | Bacteroidia | Bacteroidales | Prevotellaceae | Prevotella | *** | + | +++ | ++ | - | | | | - |
| **F3.** | EF445293 | Bacteroidetes | Bacteroidia | Bacteroidales | Prevotellaceae | Prevotella | *** | - | ++ | ++ | - | | | | - |
| **F4.** | AB009192 | Bacteroidetes | Bacteroidia | Bacteroidales | Prevotellaceae | Prevotella | *** | + | +++ | +++ | - | | | | - |
| **F5.** | New.Ref.OTU | Bacteroidetes | Bacteroidia | Bacteroidales | Prevotellaceae | Prevotella | *** | - | ++ | ++ | - | | | | - |
| **F6.** | AB269981 | Bacteroidetes | Bacteroidia | Bacteroidales | Prevotellaceae | Prevotella | *** | - | +++ | ++ | - | | | | - |
| **F7.** | EF445210 | Bacteroidetes | Bacteroidia | Bacteroidales | Prevotellaceae | Prevotella | *** | - | + | ++ | - | | | | - |
| **F8.** | EU844726 | Bacteroidetes | Bacteroidia | Bacteroidales | Prevotellaceae | Prevotella | *** | + | - | ++ | - | | | | - |
| **F9.** | GQ327024 | Bacteroidetes | Bacteroidia | Bacteroidales | Prevotellaceae | Prevotella | *** | - | ++ | ++ | - | | | | † |
| **F10.** | EF436359 | Bacteroidetes | Bacteroidia | Bacteroidales | Prevotellaceae | Prevotella | *** | - | ++ | ++ | - | | | | - |
| **F11.** | EU719305 | Bacteroidetes | Bacteroidia | Bacteroidales | Prevotellaceae | Prevotella | - | - | - | - | - | | | | - |
| **F12.** | AB185608 | Bacteroidetes | Bacteroidia | Bacteroidales | Prevotellaceae | Prevotella | *** | - | ++ | ++ | - | | | | - |
| **F13.** | AY244946 | Bacteroidetes | Bacteroidia | Bacteroidales | Prevotellaceae | Prevotella | *** | + | +++ | +++ | - | | | | - |
| **F14.** | AF018469 | Bacteroidetes | Bacteroidia | Bacteroidales | Prevotellaceae | Prevotella | *** | - | ++ | ++ | - | | | | * |
| **F15.** | GQ327306 | Bacteroidetes | Bacteroidia | Bacteroidales | Prevotellaceae | Prevotella | *** | - | ++ | ++ | - | | | | - |
| **F16.** | AB034102 | Bacteroidetes | Bacteroidia | Bacteroidales | Prevotellaceae | Prevotella | *** | - | +++ | +++ | * | | | | - |
| **F17.** | AB270138 | Bacteroidetes | Bacteroidia | Bacteroidales | Prevotellaceae | Prevotella | *** | + | +++ | +++ | * | | ii | | - |
| **F18.** | AB270130 | Bacteroidetes | Bacteroidia | Bacteroidales | Prevotellaceae | Prevotella | *** | + | - | ++ | - | | | | - |

| Boxplot Nr.[2] | OTU Nr.[3] | Taxonomy[4] | | | | | Location Effect[5] | | | | Treatment Effect[6] | | | | Cow Effect[7] |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | Phylum | Class | Order | Family | Genus | *P*-value | EAAB | LAAB | PAAB | *P*-value | EAAB | LAAB | PAAB | |
| **F19.** | GQ327214 | Bacteroidetes | Bacteroidia | Bacteroidales | Prevotellaceae | Prevotella | *** | - | ++ | ++ | - | | | | - |
| **F20.** | EU719226 | Bacteroidetes | Bacteroidia | Bacteroidales | Prevotellaceae | Prevotella | *** | - | ++ | ++ | - | | | | - |
| **F21.** | AF001777 | Bacteroidetes | Bacteroidia | Bacteroidales | Prevotellaceae | Prevotella | † | - | ++ | ++ | ** | i | ii | i | - |
| **F22.** | AB269968 | Bacteroidetes | Bacteroidia | Bacteroidales | Prevotellaceae | Prevotella | *** | - | +++ | +++ | - | | | | - |
| **F23.** | GU302536 | Bacteroidetes | Bacteroidia | Bacteroidales | Prevotellaceae | Prevotella | *** | - | ++ | ++ | * | | ii | | - |
| **F24.** | New.Ref.OTU | Bacteroidetes | Bacteroidia | Bacteroidales | Prevotellaceae | Prevotella | *** | - | ++ | ++ | ** | | | | - |
| **F25.** | New.Ref.OTU | Bacteroidetes | Bacteroidia | Bacteroidales | Prevotellaceae | Prevotella | *** | - | ++ | ++ | - | | | | - |
| **F26.** | EU381920 | Bacteroidetes | Bacteroidia | Bacteroidales | Prevotellaceae | u.b. | *** | - | ++ | ++ | - | | | | † |
| **F27.** | EU461494 | Bacteroidetes | Bacteroidia | Bacteroidales | Prevotellaceae | u.b. | *** | - | ++ | ++ | - | | | | - |
| **G1.** | AB494890 | Bacteroidetes | Bacteroidia | Bacteroidales | Rikenellaceae | RC9 gut group | *** | + | + | +++ | - | | | | - |
| **G2.** | DQ394621 | Bacteroidetes | Bacteroidia | Bacteroidales | Rikenellaceae | RC9 gut group | *** | ++ | ++ | ++ | * | dd | dd | | - |
| **G3.** | EU842535 | Bacteroidetes | Bacteroidia | Bacteroidales | Rikenellaceae | RC9 gut group | * | ++ | ++ | ++ | - | | | | - |
| **G4.** | GU304085 | Bacteroidetes | Bacteroidia | Bacteroidales | Rikenellaceae | RC9 gut group | *** | +++ | ++ | - | - | | | | - |
| **G5.** | AM183042 | Bacteroidetes | Bacteroidia | Bacteroidales | Rikenellaceae | RC9 gut group | *** | + | + | ++ | - | | | | - |
| **G6.** | AB494915 | Bacteroidetes | Bacteroidia | Bacteroidales | Rikenellaceae | RC9 gut group | *** | - | +++ | ++ | † | | dd | | - |
| **G7.** | GU302529 | Bacteroidetes | Bacteroidia | Bacteroidales | Rikenellaceae | RC9 gut group | *** | ++ | ++ | - | * | dd | d | | - |
| **G8.** | New.Ref.OTU | Bacteroidetes | Bacteroidia | Bacteroidales | Rikenellaceae | RC9 gut group | *** | - | ++ | ++ | - | | | | - |
| **H1.** | EU470196 | Bacteroidetes | Bacteroidia | Bacteroidales | S24-7 | u.b. | *** | + | ++ | ++ | * | dd | | dd | - |
| **H2.** | EU843773 | Bacteroidetes | Bacteroidia | Bacteroidales | S24-7 | u.b. | *** | - | - | ++ | - | | | | - |
| **I.** | EU381782 | Candidate division SR1 | u.b. | | | | *** | ++ | - | +++ | - | | | | - |
| **J1.** | EU462203 | Candidate division TM7 | u.b. | | | | *** | - | + | ++ | - | | | | - |
| **J2.** | GQ327541 | Candidate division TM7 | u.b. | | | | *** | - | - | ++ | - | | | | - |
| **J3.** | EU474584 | Candidate division TM7 | u.b. | | | | *** | +++ | ++ | ++ | - | | | | - |
| **J4.** | EU381496 | Candidate division TM7 | u.b. | | | | *** | + | ++ | ++ | - | | | | - |
| **K.** | GU303955 | Cyanobacteria | SHA-109 | u.b. | | | *** | - | ++ | ++ | *** | | | d | - |
| **L1.** | EF190826 | Fibrobacteres | Fibrobacteria | Fibrobacterales | Fibrobacteraceae | Fibrobacter | *** | - | + | ++ | - | | | | - |
| **L2.** | EU381811 | Fibrobacteres | Fibrobacteria | Fibrobacterales | Fibrobacteraceae | Fibrobacter | *** | + | + | ++ | - | | | | * |
| **L3.** | EU381936 | Fibrobacteres | Fibrobacteria | Fibrobacterales | Fibrobacteraceae | Fibrobacter | *** | - | + | ++ | - | | | | - |
| **M1.** | EF436353 | Firmicutes | Clostridia | Clostridiales | Christensenellaceae | u.b. | *** | - | +++ | - | - | | | | - |
| **M2.** | AB270057 | Firmicutes | Clostridia | Clostridiales | Christensenellaceae | u.b. | *** | ++ | +++ | - | - | | | | - |
| **M3.** | AB185717 | Firmicutes | Clostridia | Clostridiales | Christensenellaceae | u.b. | *** | - | ++ | ++ | - | | | | - |
| **M4.** | AB270004 | Firmicutes | Clostridia | Clostridiales | Christensenellaceae | u.b. | *** | + | +++ | ++ | † | | | | - |
| **M5.** | EU468616 | Firmicutes | Clostridia | Clostridiales | Christensenellaceae | u.b. | *** | - | ++ | - | - | | | | - |
| **M6.** | AY854343 | Firmicutes | Clostridia | Clostridiales | Christensenellaceae | u.b. | *** | - | ++ | + | * | | | | - |
| **M7.** | AB185553 | Firmicutes | Clostridia | Clostridiales | Christensenellaceae | u.b. | *** | ++ | +++ | +++ | ** | | d | | - |
| **M8.** | AB494899 | Firmicutes | Clostridia | Clostridiales | Christensenellaceae | u.b. | *** | ++ | +++ | ++ | - | | | | - |

| Boxplot Nr.[2] | OTU Nr.[3] | Taxonomy[4] | | | | | Location Effect[5] | | | | Treatment Effect[6] | | | | Cow Effect[7] |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | Phylum | Class | Order | Family | Genus | *P*-value | EAAB | LAAB | PAAB | *P*-value | EAAB | LAAB | PAAB | |
| **M9.** | AB185594 | Firmicutes | Clostridia | Clostridiales | Christensenellaceae | u.b. | * | ++ | + | ++ | - | | | | - |
| **N1.** | EU843488 | Firmicutes | Clostridia | Clostridiales | Family XIII Incertae Sedis | Anaerovorax | *** | +++ | - | + | - | | | | - |
| **N2.** | New.Ref.OTU | Firmicutes | Clostridia | Clostridiales | Family XIII Incertae Sedis | Incertae Sedis | *** | +++ | + | - | - | | | | - |
| **N3.** | EU842492 | Firmicutes | Clostridia | Clostridiales | Family XIII Incertae Sedis | Incertae Sedis | *** | +++ | - | - | - | | | | - |
| **N4.** | EU842291 | Firmicutes | Clostridia | Clostridiales | Family XIII Incertae Sedis | Mogibacterium | *** | ++ | ++ | ++ | - | | | | - |
| **N5.** | AY854273 | Firmicutes | Clostridia | Clostridiales | Family XIII Incertae Sedis | Mogibacterium | *** | - | ++ | ++ | - | | | | * |
| **N6.** | FJ682205 | Firmicutes | Clostridia | Clostridiales | Family XIII Incertae Sedis | Mogibacterium | *** | +++ | ++ | - | - | | | | - |
| **O1.** | AB494822 | Firmicutes | Clostridia | Clostridiales | Lachnospiraceae | Acetitomaculum | *** | - | ++ | - | *** | | dd | | - |
| **O2.** | AB185642 | Firmicutes | Clostridia | Clostridiales | Lachnospiraceae | Acetitomaculum | *** | - | +++ | - | - | | | | - |
| **O3.** | AM039826 | Firmicutes | Clostridia | Clostridiales | Lachnospiraceae | Butyrivibrio | *** | - | ++ | ++ | ** | | i | ii | - |
| **O4.** | New.Ref.OTU | Firmicutes | Clostridia | Clostridiales | Lachnospiraceae | Butyrivibrio | *** | +++ | - | - | *** | dd | | | - |
| **O5.** | AB494805 | Firmicutes | Clostridia | Clostridiales | Lachnospiraceae | Butyrivibrio | *** | + | ++ | +++ | - | | | | - |
| **O6.** | AB494848 | Firmicutes | Clostridia | Clostridiales | Lachnospiraceae | Butyrivibrio | *** | - | ++ | ++ | - | | | | - |
| **O7.** | EF445238 | Firmicutes | Clostridia | Clostridiales | Lachnospiraceae | Butyrivibrio | *** | - | + | ++ | - | | | | † |
| **O8.** | AB034052 | Firmicutes | Clostridia | Clostridiales | Lachnospiraceae | Butyrivibrio | *** | - | +++ | +++ | - | | | | - |
| **O9.** | GU303299 | Firmicutes | Clostridia | Clostridiales | Lachnospiraceae | Butyrivibrio | *** | +++ | + | - | - | | | | - |
| **O10.** | AB494833 | Firmicutes | Clostridia | Clostridiales | Lachnospiraceae | Butyrivibrio | *** | - | ++ | ++ | - | | | | - |
| **O11.** | FJ032568 | Firmicutes | Clostridia | Clostridiales | Lachnospiraceae | Butyrivibrio | *** | - | + | ++ | ** | | d | d | - |
| **O12.** | EU843345 | Firmicutes | Clostridia | Clostridiales | Lachnospiraceae | Butyrivibrio | *** | - | ++ | ++ | - | | | | - |
| **O13.** | GU124460 | Firmicutes | Clostridia | Clostridiales | Lachnospiraceae | L. Incertae Sedis | *** | - | ++ | ++ | - | | | | * |
| **O14.** | AF001722 | Firmicutes | Clostridia | Clostridiales | Lachnospiraceae | L. Incertae Sedis | *** | - | ++ | +++ | - | | | | - |
| **O15.** | AB269976 | Firmicutes | Clostridia | Clostridiales | Lachnospiraceae | L. Incertae Sedis | *** | - | + | ++ | - | | | | * |
| **O16** | EU381578 | Firmicutes | Clostridia | Clostridiales | Lachnospiraceae | L. Incertae Sedis | *** | - | + | ++ | * | | i | ii | - |
| **O17.** | AB494761 | Firmicutes | Clostridia | Clostridiales | Lachnospiraceae | L. Incertae Sedis | *** | - | ++ | ++ | - | | | | - |
| **O18.** | New.Ref.OTU | Firmicutes | Clostridia | Clostridiales | Lachnospiraceae | L. Incertae Sedis | *** | - | ++ | ++ | - | | | | - |
| **O19.** | New.Ref.OTU | Firmicutes | Clostridia | Clostridiales | Lachnospiraceae | L. Incertae Sedis | *** | - | ++ | ++ | - | | | | † |
| **O20.** | EF436345 | Firmicutes | Clostridia | Clostridiales | Lachnospiraceae | L. Incertae Sedis | *** | - | ++ | - | - | | | | - |
| **O21.** | DQ237938 | Firmicutes | Clostridia | Clostridiales | Lachnospiraceae | L. Incertae Sedis | *** | - | +++ | ++ | - | | | | * |
| **O22.** | EF436445 | Firmicutes | Clostridia | Clostridiales | Lachnospiraceae | L. Incertae Sedis | *** | - | ++ | - | - | | | | - |
| **O23.** | New.Ref.OTU | Firmicutes | Clostridia | Clostridiales | Lachnospiraceae | L. Incertae Sedis | *** | - | ++ | ++ | - | | | | - |
| **O24.** | GU303078 | Firmicutes | Clostridia | Clostridiales | Lachnospiraceae | Oribacterium | *** | - | - | ++ | * | | | ii | - |
| **O25.** | DQ085079 | Firmicutes | Clostridia | Clostridiales | Lachnospiraceae | Pseudobutyrivibrio | *** | - | ++ | ++ | ** | | ii | i | - |
| **O26.** | AB494919 | Firmicutes | Clostridia | Clostridiales | Lachnospiraceae | Pseudobutyrivibrio | *** | - | ++ | ++ | - | | | | - |
| **O27.** | FJ032427 | Firmicutes | Clostridia | Clostridiales | Lachnospiraceae | Roseburia | ** | - | ++ | ++ | * | | ii | | - |
| **O28.** | EU842536 | Firmicutes | Clostridia | Clostridiales | Lachnospiraceae | Roseburia | *** | - | ++ | ++ | ** | | dd | | - |
| **O29.** | AF371623 | Firmicutes | Clostridia | Clostridiales | Lachnospiraceae | Roseburia | * | - | ++ | ++ | † | | ii | dd | - |

| Boxplot Nr.[2] | Taxonomy[4] | | | | | | Location Effect[5] | | | | Treatment Effect[6] | | | | Cow Effect[7] |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | OTU Nr.[3] | Phylum | Class | Order | Family | Genus | P-value | EAAB | LAAB | PAAB | P-value | EAAB | LAAB | PAAB | |
| **O30.** | JF797351 | Firmicutes | Clostridia | Clostridiales | Lachnospiraceae | Shuttleworthia | * | - | ++ | ++ | ** | | i | ii | - |
| **O31.** | AF001734 | Firmicutes | Clostridia | Clostridiales | Lachnospiraceae | u.b. | *** | - | - | ++ | - | | | | * |
| **O32.** | EU845282 | Firmicutes | Clostridia | Clostridiales | Lachnospiraceae | u.b. | *** | + | ++ | ++ | - | | | | - |
| **O33.** | EU773612 | Firmicutes | Clostridia | Clostridiales | Lachnospiraceae | u.b. | *** | + | ++ | ++ | - | | | | - |
| **O34.** | EU843817 | Firmicutes | Clostridia | Clostridiales | Lachnospiraceae | u.b. | *** | - | ++ | ++ | - | | | | - |
| **O35.** | EU381579 | Firmicutes | Clostridia | Clostridiales | Lachnospiraceae | u.b. | *** | + | ++ | ++ | ** | | i | | *** |
| **O36.** | AB270112 | Firmicutes | Clostridia | Clostridiales | Lachnospiraceae | u.b. | *** | - | ++ | ++ | - | | | | - |
| **O37.** | AB494866 | Firmicutes | Clostridia | Clostridiales | Lachnospiraceae | u.b. | *** | - | + | ++ | - | | | | - |
| **O38.** | FJ032551 | Firmicutes | Clostridia | Clostridiales | Lachnospiraceae | u.b. | *** | - | ++ | ++ | - | | | | - |
| **O39.** | AY854272 | Firmicutes | Clostridia | Clostridiales | Lachnospiraceae | u.b. | *** | - | ++ | ++ | - | | | | - |
| **O40.** | EU381488 | Firmicutes | Clostridia | Clostridiales | Lachnospiraceae | u.b. | *** | - | ++ | ++ | - | | | | - |
| **O41.** | AF001717 | Firmicutes | Clostridia | Clostridiales | Lachnospiraceae | u.b. | *** | - | + | ++ | * | | | dd | ** |
| **O42.** | EU719231 | Firmicutes | Clostridia | Clostridiales | Lachnospiraceae | u.b. | *** | - | ++ | ++ | * | | d | | - |
| **O43.** | AB494778 | Firmicutes | Clostridia | Clostridiales | Lachnospiraceae | u.b. | *** | - | ++ | +++ | - | | | | - |
| **O44.** | AB270116 | Firmicutes | Clostridia | Clostridiales | Lachnospiraceae | u.b. | *** | - | + | ++ | - | | | | - |
| **O45.** | AB269996 | Firmicutes | Clostridia | Clostridiales | Lachnospiraceae | u.b. | *** | +++ | + | - | - | | | | - |
| **O46.** | GU304496 | Firmicutes | Clostridia | Clostridiales | Lachnospiraceae | u.b. | *** | +++ | - | - | - | | | | * |
| **O47.** | AB494806 | Firmicutes | Clostridia | Clostridiales | Lachnospiraceae | u.b. | *** | - | ++ | ++ | ** | | ii | ii | - |
| **P1.** | AB270001 | Firmicutes | Clostridia | Clostridiales | Ruminococcaceae | Incertae Sedis | *** | - | ++ | + | ** | | ii | i | ** |
| **P2.** | EF686593 | Firmicutes | Clostridia | Clostridiales | Ruminococcaceae | Ruminococcus | *** | - | ++ | ++ | - | | d | | - |
| **P3.** | EF436321 | Firmicutes | Clostridia | Clostridiales | Ruminococcaceae | Ruminococcus | *** | - | ++ | +++ | - | | | | - |
| **P4.** | EU469842 | Firmicutes | Clostridia | Clostridiales | Ruminococcaceae | Ruminococcus | *** | - | ++ | ++ | * | | ii | ii | - |
| **P5.** | AB494882 | Firmicutes | Clostridia | Clostridiales | Ruminococcaceae | Ruminococcus | *** | + | ++ | +++ | - | | | | - |
| **P6.** | EU381458 | Firmicutes | Clostridia | Clostridiales | Ruminococcaceae | Ruminococcus | *** | - | ++ | ++ | * | | ii | | - |
| **P7.** | AAQK01009861 | Firmicutes | Clostridia | Clostridiales | Ruminococcaceae | Ruminococcus | *** | - | +++ | ++ | ** | | dd | dd | - |
| **P8.** | EU381848 | Firmicutes | Clostridia | Clostridiales | Ruminococcaceae | Ruminococcus | *** | + | ++ | ++ | - | | | | † |
| **P9.** | GQ327231 | Firmicutes | Clostridia | Clostridiales | Ruminococcaceae | Saccharofermentans | *** | + | ++ | +++ | † | | | | - |
| **P10.** | EF686527 | Firmicutes | Clostridia | Clostridiales | Ruminococcaceae | Saccharofermentans | *** | - | ++ | ++ | - | | | | - |
| **P11.** | AB494824 | Firmicutes | Clostridia | Clostridiales | Ruminococcaceae | Saccharofermentans | *** | ++ | ++ | +++ | - | | | | - |
| **P12.** | AY854346 | Firmicutes | Clostridia | Clostridiales | Ruminococcaceae | Saccharofermentans | *** | + | - | ++ | - | | | | - |
| **P13.** | EU381703 | Firmicutes | Clostridia | Clostridiales | Ruminococcaceae | Saccharofermentans | *** | - | ++ | ++ | - | | | | * |
| **P14.** | GQ327304 | Firmicutes | Clostridia | Clostridiales | Ruminococcaceae | Saccharofermentans | *** | - | ++ | ++ | - | | | | - |
| **P15.** | AB034038 | Firmicutes | Clostridia | Clostridiales | Ruminococcaceae | Saccharofermentans | *** | - | ++ | ++ | * | | | | - |
| **P16.** | EU468242 | Firmicutes | Clostridia | Clostridiales | Ruminococcaceae | u.b. | *** | - | ++ | ++ | - | | | | - |
| **P17.** | AB494879 | Firmicutes | Clostridia | Clostridiales | Ruminococcaceae | u.b. | *** | + | +++ | +++ | - | | | | - |
| **P18.** | EU344218 | Firmicutes | Clostridia | Clostridiales | Ruminococcaceae | u.b. | *** | - | ++ | +++ | - | | | | - |

| Boxplot Nr.[2] | Taxonomy[4] | | | | | | Location Effect[5] | | | | Treatment Effect[6] | | | | Cow Effect[7] |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | OTU Nr.[3] | Phylum | Class | Order | Family | Genus | *P*-value | EAAB | LAAB | PAAB | *P*-value | EAAB | LAAB | PAAB | |
| **P19.** | EU381706 | Firmicutes | Clostridia | Clostridiales | Ruminococcaceae | u.b. | *** | + | + | ++ | - | | | | - |
| **P20.** | AB270149 | Firmicutes | Clostridia | Clostridiales | Ruminococcaceae | u.b. | *** | - | ++ | ++ | * | | | ii | - |
| **P21.** | EU381964 | Firmicutes | Clostridia | Clostridiales | Ruminococcaceae | u.b. | *** | - | + | ++ | - | | | | * |
| **P22.** | AB494900 | Firmicutes | Clostridia | Clostridiales | Ruminococcaceae | u.b. | *** | - | - | ++ | * | | | dd | † |
| **P23.** | EU381950 | Firmicutes | Clostridia | Clostridiales | Ruminococcaceae | u.b. | *** | +++ | ++ | +++ | - | | | | - |
| **P24.** | AF001762 | Firmicutes | Clostridia | Clostridiales | Ruminococcaceae | u.b. | *** | +++ | ++ | ++ | * | dd | | | * |
| **P25.** | AF001761 | Firmicutes | Clostridia | Clostridiales | Ruminococcaceae | u.b. | *** | + | - | ++ | - | | | | - |
| **P26.** | AB009186 | Firmicutes | Clostridia | Clostridiales | Ruminococcaceae | u.b. | *** | - | - | ++ | - | | | | - |
| **P27.** | EU842742 | Firmicutes | Clostridia | Clostridiales | Ruminococcaceae | u.b. | *** | - | ++ | ++ | † | | d | i | * |
| **P28.** | AB185556 | Firmicutes | Clostridia | Clostridiales | Ruminococcaceae | u.b. | *** | + | +++ | +++ | - | | | | * |
| **P29.** | EU381629 | Firmicutes | Clostridia | Clostridiales | Ruminococcaceae | u.b. | *** | - | + | ++ | - | | | | - |
| **P30.** | AY854363 | Firmicutes | Clostridia | Clostridiales | Ruminococcaceae | u.b. | *** | + | ++ | +++ | - | | | | - |
| **P31.** | AB185810 | Firmicutes | Clostridia | Clostridiales | Ruminococcaceae | u.b. | * | - | ++ | ++ | - | | | | † |
| **P32.** | DQ394677 | Firmicutes | Clostridia | Clostridiales | Ruminococcaceae | u.b. | *** | - | + | ++ | - | | | | - |
| **P33.** | AB009189 | Firmicutes | Clostridia | Clostridiales | Ruminococcaceae | u.b. | *** | ++ | - | ++ | - | | | | - |
| **Q1.** | AB009216 | Firmicutes | Clostridia | Clostridiales | Veillonellaceae | Anaerovibrio | *** | - | ++ | ++ | ** | | ii | i | - |
| **Q2.** | AB034139 | Firmicutes | Clostridia | Clostridiales | Veillonellaceae | Selenomonas | *** | - | ++ | ++ | ** | | ii | ii | - |
| **Q3.** | GQ327079 | Firmicutes | Clostridia | Clostridiales | Veillonellaceae | Selenomonas | ** | - | + | ++ | * | | i | i | - |
| **Q4.** | AY244976 | Firmicutes | Clostridia | Clostridiales | Veillonellaceae | Succiniclasticum | *** | +++ | +++ | +++ | * | d | dd | dd | - |
| **Q5.** | EU843672 | Firmicutes | Clostridia | Clostridiales | Veillonellaceae | Succiniclasticum | *** | + | - | ++ | - | | | | - |
| **R1.** | AB210825 | Firmicutes | Erysipelotrichia | Erysipelotrichales | Erysipelotrichaceae | Catenibacterium | * | + | ++ | ++ | ** | i | ii | ii | - |
| **R2.** | FJ032444 | Firmicutes | Erysipelotrichia | Erysipelotrichales | Erysipelotrichaceae | Sharpea | *** | - | + | ++ | - | | | | - |
| **R3.** | EU458717 | Firmicutes | Erysipelotrichia | Erysipelotrichales | Erysipelotrichaceae | u.b. | *** | - | +++ | ++ | * | | dd | dd | - |
| **R4.** | EU381583 | Firmicutes | Erysipelotrichia | Erysipelotrichales | Erysipelotrichaceae | u.b. | *** | - | ++ | ++ | - | | | | - |
| **R5.** | EU381506 | Firmicutes | Erysipelotrichia | Erysipelotrichales | Erysipelotrichaceae | u.b. | *** | +++ | ++ | ++ | - | | | | - |
| **S.** | New.Ref.OTU | Proteobacteria | Betaproteobacteria | Burkholderiales | Comamonadaceae | Comamonas | *** | +++ | - | - | ** | ii | | | - |
| **T1.** | EU844167 | Proteobacteria | Deltaproteobacteria | Desulfobacterales | Desulfobulbaceae | Desulfobulbus | *** | +++ | - | - | - | | | | † |
| **T2.** | New.Ref.OTU | Proteobacteria | Deltaproteobacteria | Desulfobacterales | Desulfobulbaceae | Desulfobulbus | *** | +++ | - | - | † | ii | | | - |
| **T3.** | GU303056 | Proteobacteria | Deltaproteobacteria | Desulfobacterales | Desulfobulbaceae | Desulfobulbus | *** | +++ | - | - | - | | | | - |
| **U.** | DQ174169 | Proteobacteria | Epsilonproteobacteria | Campylobacterales | Campylobacteraceae | Campylobacter | *** | +++ | - | - | † | | | | - |
| **V1.** | EF445274 | Proteobacteria | Gammaproteobacteria | Aeromonadales | Succinivibrionaceae | u.b. | - | + | ++ | + | - | | | | - |
| **V2.** | EU381934 | Proteobacteria | Gammaproteobacteria | Aeromonadales | Succinivibrionaceae | u.b. | *** | + | +++ | +++ | - | | | | - |
| **W.** | New.Ref.OTU | Proteobacteria | Gammaproteobacteria | Cardiobacteriales | Cardiobacteriaceae | Suttonella | *** | +++ | - | - | ** | dd | | | - |
| **X1.** | AB270123 | Spirochaetes | Spirochaetes | Spirochaetales | Spirochaetaceae | Treponema | *** | + | ++ | ++ | - | | | | - |
| **X2.** | AF001693 | Spirochaetes | Spirochaetes | Spirochaetales | Spirochaetaceae | Treponema | *** | + | +++ | +++ | - | | | | † |
| **Y1.** | EF445251 | Tenericutes | Mollicutes | RF9 | u.b. | | *** | + | ++ | ++ | * | | dd | | - |

| Boxplot Nr.[2] | Taxonomy[4] | | | | | | Location Effect[5] | | | | Treatment Effect[6] | | | | Cow Effect[7] |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | OTU Nr.[3] | Phylum | Class | Order | Family | Genus | *P*-value | EAAB | LAAB | PAAB | *P*-value | EAAB | LAAB | PAAB | |
| **Y2.** | EU381563 | Tenericutes | Mollicutes | RF9 | u.b. | | *** | + | ++ | ++ | - | | | | - |
| **Y3.** | EU381558 | Tenericutes | Mollicutes | RF9 | u.b. | | *** | + | + | ++ | - | | | | † |
| **Y4.** | AF001770 | Tenericutes | Mollicutes | RF9 | u.b. | | *** | + | - | ++ | * | | | | - |

[1]Cows were divided into a pasture and confinement group (PG, CG, n = 5). The CG stayed on a TMR based ration during the entire trial while the PG was slowly introduced to a pasture-based ration: wk 1: TMR, wk 2: TMR and 3 h pasture/d, wk 3 and 4: TMR and 12 h pasture/d, wk 5-10: pasture and 1.75 kg DM concentrate/d. Samples of the rumen liquid- (LAAB), particle- (PAAB) and epithelium-(EAAB) associated archaea and bacteria were collected in wk 1, wk 5 and wk 10 and 16S rRNA gene amplification and sequencing was performed.

[2]Boxplots with statistics included in appendix.

[3]OTU = Operational Taxonomic Unit, New.Ref.OTU = New Reference OTU

[4]u.a. = unculturable archeon, u.b. = unculturable bacterium, all OTUs were classified as “unculturable bacterium or archeon” at species level, therefore only taxonomic classification up to the genus level is shown.

[5]*P*-value: symbols indicate a significant difference in OTU abundance between locations (*** $P < 0.001$, ** $P < 0.01$, * $P < 0.05$, † $P < 0.10$). Symbols describe the proportional abundance of the OTU at the three different locations (+++ > 1%, ++ > 0.1 %, + < 0.1 %, - not detected).

[6]Influence of the ration change from TMR to pasture on proportional abundance in the PG (comparison wk 1 and wk 10). *P*-value: symbols indicate a significant difference for a Group×Time or Group×Time×Location interaction (*** $P < 0.001$, ** $P < 0.01$, * $P < 0.05$, † $P < 0.10$), dd/ ii = decrease/increase by > 2x or > 1 % in proportional abundance when decreasing to/increasing from 0, d/i = a decrease/increase by < 2x or < 1 % in proportional abundance when decreasing to/increasing from 0.

[7]Symbols indicates a significant Cow or Cow×Time effect (*** $P < 0.001$, ** $P < 0.01$, * $P < 0.05$, † $P < 0.10$).

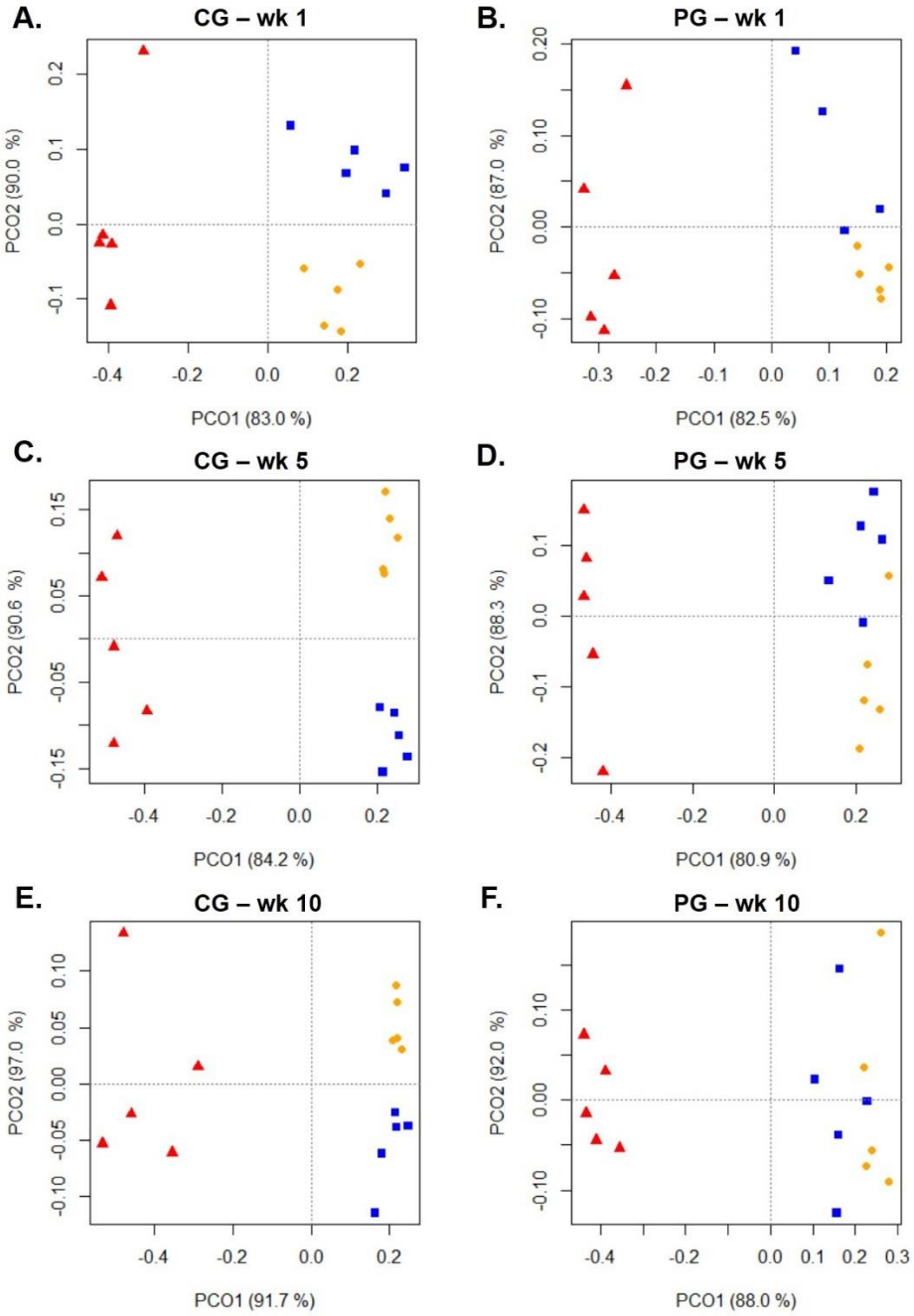


**Figure 1.** Two dimensional PCO-plots from SSCP-gels of rumen liquid (LAB, orange dot), particle (PAB, blue square) and epithelium (EAB, red triangle) associated bacteria of the confinement group (CG) and pasture group (PG) in wk 1, wk 5 and wk 10 (explained variance indicated in % on x- and y-axis, n = 5). The CG stayed on a TMR based ration during the entire trial while the PG was slowly introduced to a pasture-based ration: wk 1: TMR, wk 2: TMR and 3 h pasture/d, wk 3 and 4: TMR and 12 h pasture/d, wk 5-10: pasture and 1.75 kg DM concentrate/d. Significance: A: $P = 0.001$, B: $P = 0.001$, C: $P = 0.001$, D: $P = 0.001$, E: $P = 0.001$, F: $P = 0.002$.

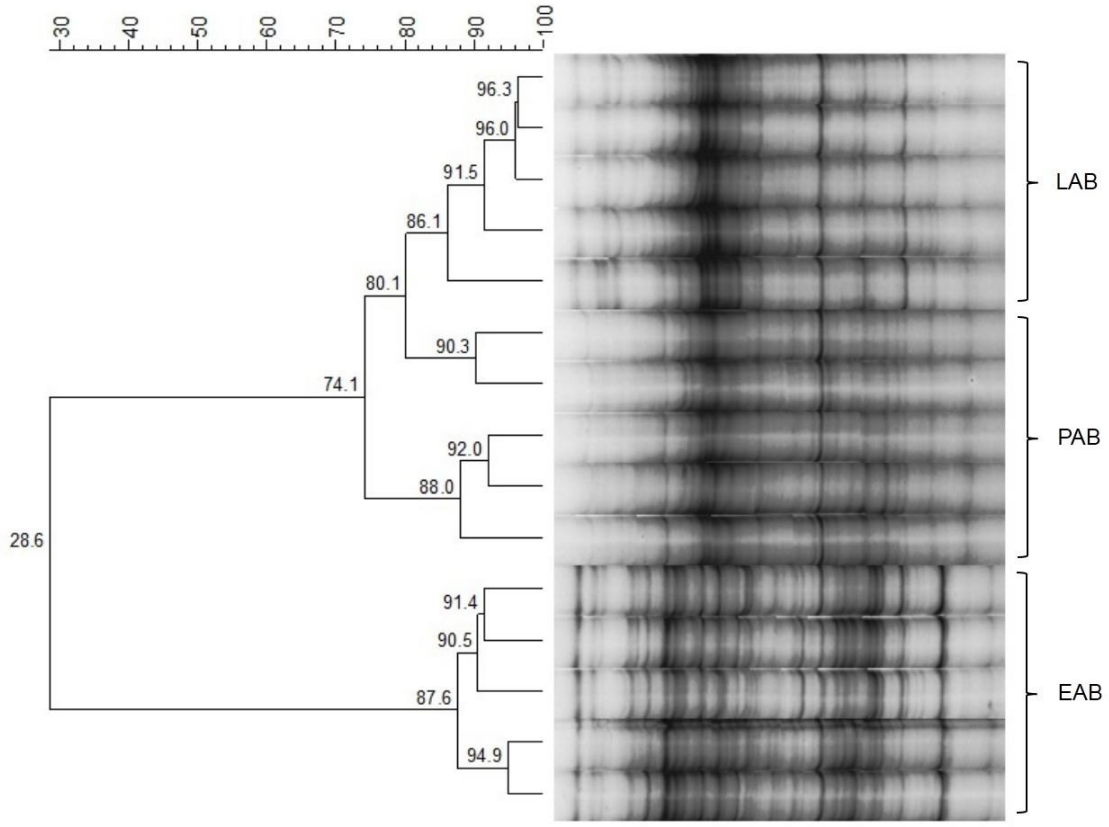


**Figure 2.** Example of SSCP-Gel and dendrogram of rumen liquid (LAB), particle (PAB) and epithelium (EAB) associated bacteria at one point in time during the trial (wk 5, confinement group, n = 5). Numbers indicate similarity (in %) between samples/clusters.

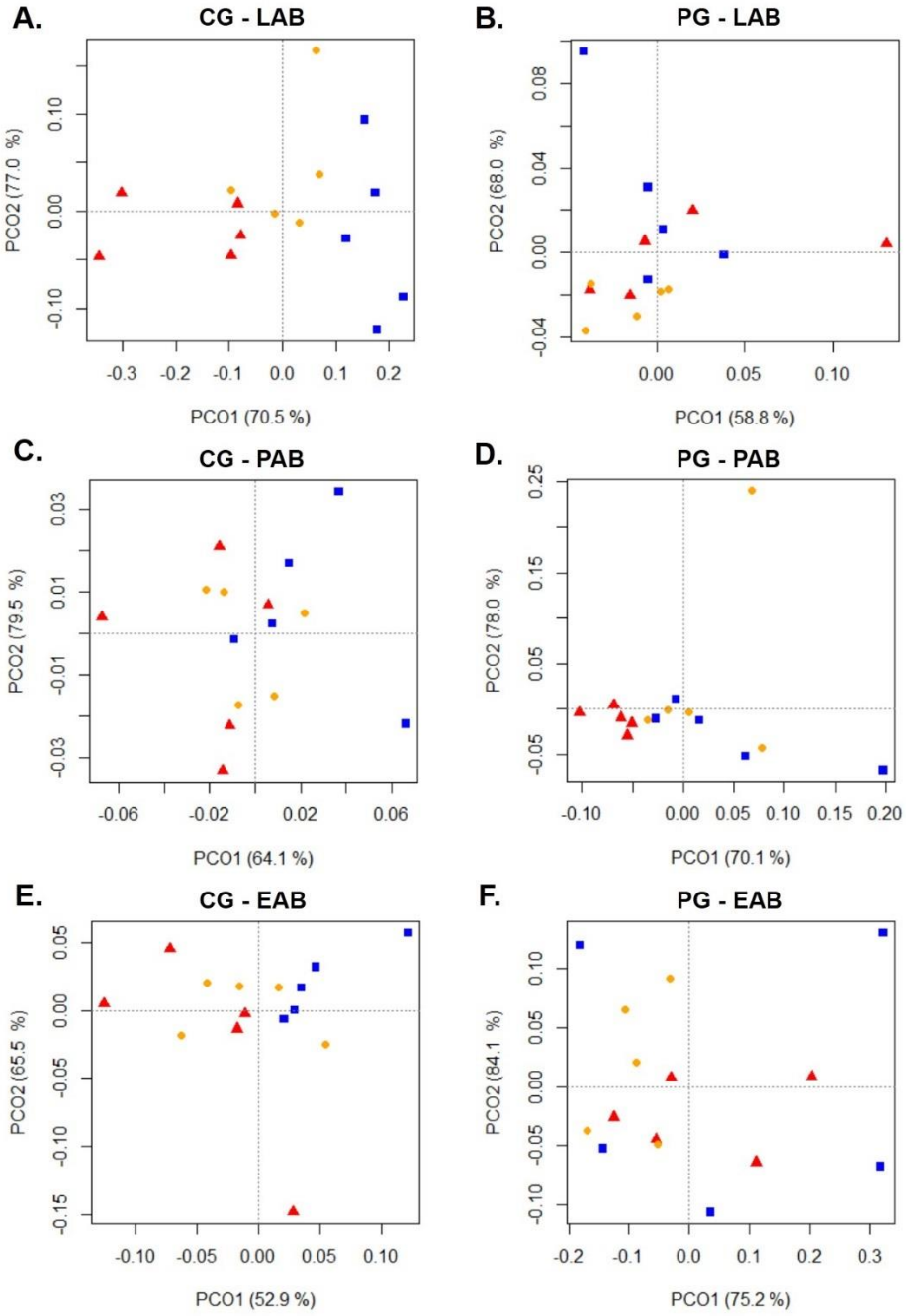


**Figure 3.** Two dimensional PCO-plots from SSCP-gels of rumen liquid (LAB), particle (PAB) and epithelium (EAB) associated bacteria illustrating changes over the course of the trial (wk 1 = red triangle, wk 5 = orange dot, wk 10 = blue square) in the confinement group (CG) and pasture group (PG; n = 5; explained variance indicated in % on x- and y-axis). The CG stayed on a TMR based ration during the entire trial while the PG was slowly introduced to a pasture-based ration: wk 1: TMR, wk 2: TMR and 3 h pasture/d, wk 3 and 4: TMR and 12 h pasture/d, wk 5-10: pasture and 1.75 kg DM concentrate/d. Significance: A: *P* = 0.141, B: *P* = 0.001, C: *P* = 0.080, D: *P* = 0.328, E: *P* = 0.013, F: *P* = 0.115.

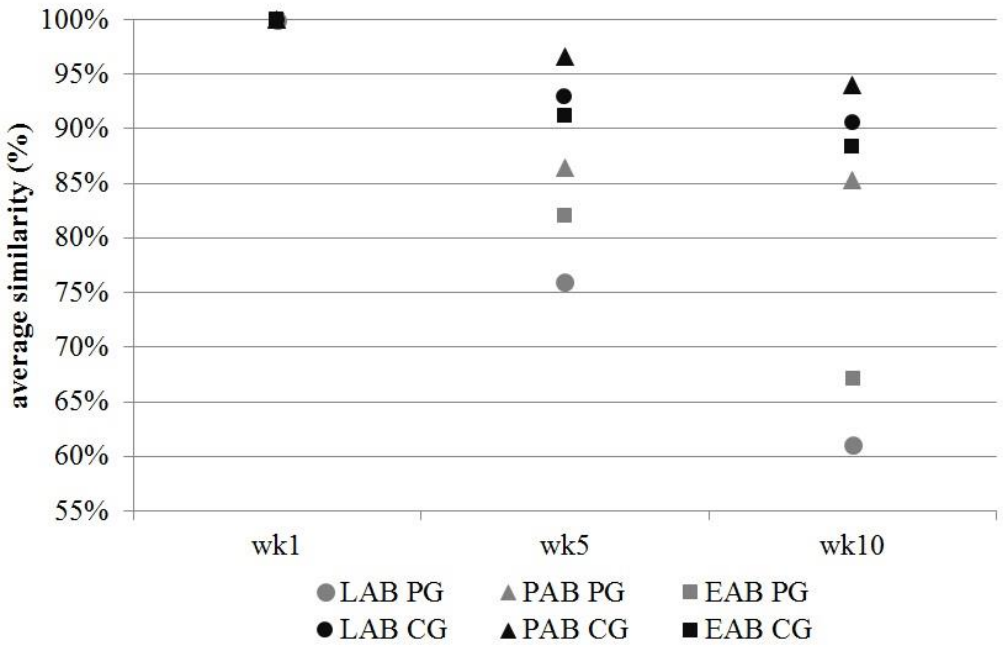


**Figure 4.** Change in bacterial communities of the liquid- (LAB), particle- (PAB) and epithelium-associated bacteria (EAB) over time expressed in average similarity (in %) of samples compared to their reference sample in wk 1. CG = confinement group, PG = pasture group (n = 5). A significant greater decrease in similarity over time in all three bacteria populations was observed for the PG compared to the CG ($P_{EAB}$ = 0.002, $P_{LAB}$ = 0.008; $P_{PAB}$ = 0.003; $SD_{EAB}$ = 11 %; $SD_{LAB}$ = 8 %, $SD_{PAB}$ = 16 %). The CG stayed on a TMR based ration during the entire trial while the PG was slowly introduced to a pasture-based ration: wk 1: TMR, wk 2: TMR and 3 h pasture/d, wk 3 and 4: TMR and 12 h pasture/d, wk 5-10: pasture and 1.75 kg DM concentrate/d.

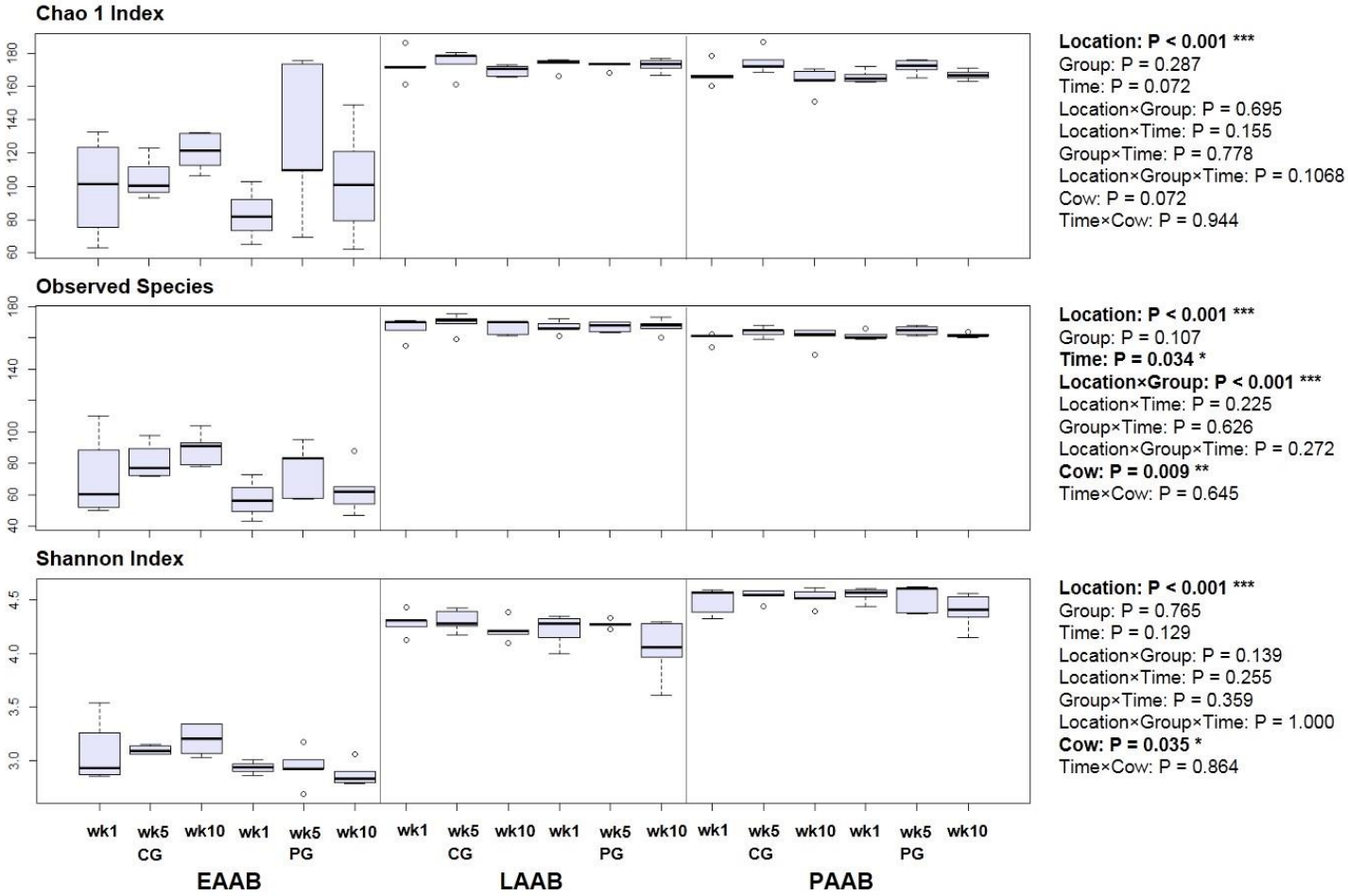


**Figure 5.** Boxplots of diversity variables of rumen liquid (LAAB), particle (PAAB) and epithelium (EAAB) associated archaea and bacteria in wk 1, wk 5 and wk 10 of the pasture (PG) and confinement (CG, n = 5) group. The CG stayed on a TMR based ration during the entire trial while the PG was slowly introduced to a pasture-based ration: wk 1: TMR, wk 2: TMR and 3 h pasture/d, wk 3 and 4: TMR and 12 h pasture/d, wk 5-10: pasture and 1.75 kg DM concentrate/d.

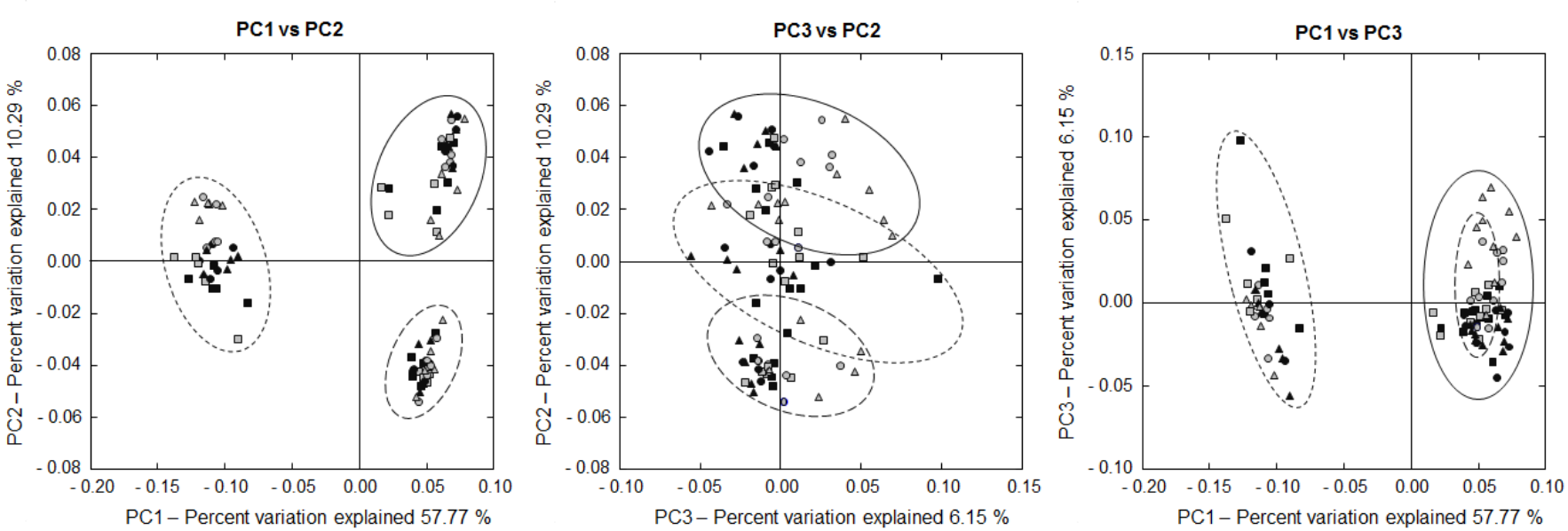


**Figure 6.** Two dimensional PCO plots of 16S rRNA sequencing results (based on weighted UniFrac distances) of rumen liquid (LAAB, solid line), particle (PAAB, large dashed line) and epithelium (EAAB, small dashed line) associated archaea and bacteria in wk 1 (square), wk 5 (round) and wk 10 (triangle) of the pasture (PG, grey) and confinement (CG, black, n = 5) group. The CG stayed on a TMR based ration during the entire trial while the PG was slowly introduced to a pasture-based ration: wk 1: TMR, wk 2: TMR and 3 h pasture/d, wk 3 and 4: TMR and 12 h pasture/d, wk 5-10: pasture and 1.75 kg DM concentrate/d.

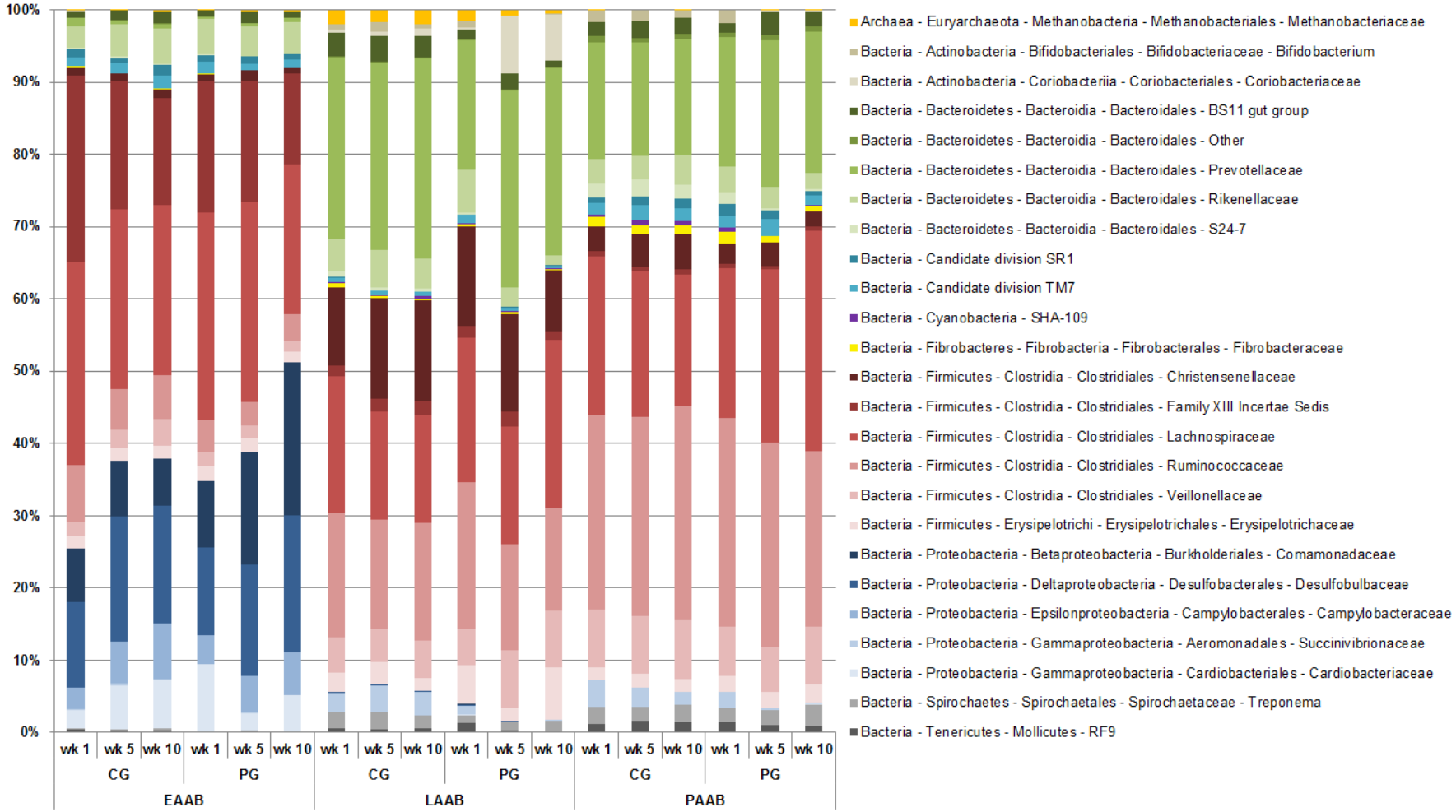


**Figure 7.** Differences in relative abundances (expressed as percentages) of OTUs derived from 16S rRNA gene sequencing on family level of rumen liquid (LAAB), particle (PAAB) and epithelium (EAAB) associated archaea and bacteria families in wk 1, wk 5 and wk 10 of the pasture (PG) and confinement (CG, n = 5) group. The CG stayed on a TMR based ration during the entire trial while the PG was slowly introduced to a pasture-based ration: wk 1: TMR, wk 2: TMR and 3 h pasture/d, wk 3 and 4: TMR and 12 h pasture/d, wk 5-10: pasture and 1.75 kg DM concentrate/d.

# 4. Differential effects of monensin and a blend of essential oils on rumen microbiota composition of transition dairy cows

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State of publication: published April 2017 in the *Journal of Dairy Science*

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# Differential Effects of Monensin and a Blend of Essential Oils on Rumen Microbiota Composition of Transition Dairy Cows

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Published April 2017 in the *Journal of Dairy Science*

Volume 100, Issue 4, Pages 2765-2783

http://dx.doi.org/10.3168/jds.2016-11994

## Abstract

In response to oral application monensin alters the rumen microbiota, thereby increasing ruminal propionate production and energy availability in the animal. Data from different studies indicate that the susceptibility of rumen bacteria to monensin is mainly cell wall dependent but tracing its activity to specific microbial groups has been a challenge. Several studies have shown a similar effect for essential oils, but results are inconsistent. To investigate the influence of monensin and a blend of essential oils (BEO, containing thymol, guaïacol, eugenol, vanillin, salicylaldehyde and limonene) on the rumen microbiome rumen liquid samples were collected orally on day 56 postpartum from cows that had either received a monensin controlled-release capsule 3 weeks antepartum, a diet containing a BEO from 3 weeks antepartum onwards, or a control diet (n = 12). The samples were analyzed for pH, volatile fatty acid, ammonia and LPS concentrations and protozoal counts. A 16S rRNA gene fingerprinting analysis (PCR-SSCP) and sequencing revealed that the essential oils treatment had no effect on the rumen microbiota, whereas monensin decreased the bacterial diversity.

Twenty-three bacterial species-level operational taxonomic units (OTU) were identified for which monensin caused a significant decrease in their relative abundance, all belonging to the phyla *Bacteroidetes* (uncultured BS11 gut group and BS9 gut group) and *Firmicutes* (*Lachnospiraceae*, *Ruminococcaceae* and *Erysipelotrichaceae*). Ten bacterial OTU, belonging to the phyla *Actinobacteria* (*Coriobacteriaceae*), *Bacteroidetes* (*Prevotella*), *Cyanobacteria* (SHA-109) and *Firmicutes* (*Lachnospiraceae* and *Ruminococcaceae*), increased in relative abundance due to the monensin treatment. These results confirm the hypothesis that varying effects depending on cell wall constitution and thickness might apply for monensin sensitivity rather than a clear cut between Gram-negative and Gram-positive bacteria. No effect of monensin on the archaea population was observed, confirming the assumption that reported inhibition of methanogenesis is most likely caused through a decrease in substrate availability, rather than by a direct effect on the methanogens. The data supports the hypothesis that the observed increased ruminal molar propionate proportions due to monensin may be caused by a decrease in abundance of non- and moderate propionate producers and an increase in abundance of succinate and propionate producers.

***Key words:*** rumen microbiota, monensin, essential oils, sequencing

## Introduction

At the onset of lactation a dairy cow's metabolism is confronted with a massive increase in energy demand which cannot be met by a simultaneous increase in feed intake. Therefore, a physiological tissue mobilization and a decrease in body condition are observed. However, in case of an extreme negative energy balance, excessive tissue mobilization occurs and the metabolizing capacity of the liver is exceeded, leading to metabolic disorders such as ketosis and fatty liver syndrome (Duffield, 2000; Bobe et al., 2004).

Monensin is an ionophore antibiotic which is used in ketosis prevention and as a production enhancer in dairy cows (Ipharraguerre and Clark, 2003). When added to the diet, it increases the ruminal propionate production through alterations in the rumen microbiota, causing an increased hepatic gluconeogenesis and thereby increasing the energy supply to the animal (Russell and Strobel, 1989; Ipharraguerre and Clark, 2003). The use of antibiotics as feed additives has been banned in the EU, but recently monensin was launched as an intraruminally applied controlled-release capsule (CRC) indicated for overconditioned transition dairy cows (only upon veterinary prescription) (Calsamiglia et al., 2007; Drong et al., 2016a). It has been proposed that monensin preferentially inhibits Gram-positive bacteria, but tracing its activity to specific microbial groups has been a challenge and systematic studies are lacking (Weimer and Stevenson, 2008). Furthermore, it has been shown that monensin decreases the methane

emissions from ruminants (Boadi et al., 2004). Different studies suggest that monensin does not act directly on rumen methanogens, but rather through the limitation of substrate availability through the inhibition of other rumen microorganisms. Functional relationships among microbes have however not yet been identified (Hook et al., 2009).

Essential oils have gained a lot of attention in the last decennia, since several studies indicated an effect on rumen fermentation similar to monensin (Calsamiglia et al., 2007). However, up to now results are not consistent concerning the effects of essential oils on rumen fermentation and animal performance, most likely due to variation in dosage and chemical structure of the essential oil used, as well as ration composition and animal physiology among studies (Calsamiglia et al., 2007; Patra, 2011). Patra and Yu (2012) showed in an in vitro study using denaturing gradient gel electrophoresis analysis and quantitative real-time PCR that rumen bacterial and archaeal diversity is decreased by different essential oils and stated that sequencing studies should be performed to further investigate these alterations in species composition in detail.

In previous publications we described the influence of monensin and a specific blend of essential oils (**BEO**) on performance, energy metabolism and rumen fermentation, as well as on immunological, hematological and biochemical variables in transition dairy cows (Drong et al., 2016b; a). Increased rumen molar propionate proportions, decreased subclinical and clinical ketosis prevalence, and an influence on liver health and immune system in monensin supplemented cows was observed, whereas the supplementation of a BEO failed to elicit any effect. In the current study we aimed at characterizing the underlying compositional changes in the rumen microbiota to verify several of the hypotheses regarding the effects of monensin and BEO. Therefore, the microbiota of ruminal liquid samples collected at day 56 postpartum (p.p.) were analyzed using single strand conformation polymorphism (SSCP) analysis and next generation Illumina MiSeq amplicon sequencing. The detected differences in bacterial and archaeal community composition are discussed in the light of the current understanding of monensin and BEO effects.

## Material and Methods

Experimental work was conducted at the experimental station of the Institute of Animal Nutrition (Friedrich-Loeffler-Institute) in Brunswick, Germany. The experiment was carried out in accordance with the German Animal Welfare Act approved by the LAVES (Lower Saxony State Office for Consumer Protection and Food Safety, Germany).

### Experimental Design, Sample Collection and Analysis of Performance and Metabolic Variables

A trial involving 60 pluriparous German Holstein cows was performed from August 2013 until February 2014 to investigate the effect of monensin and essential oils on performance, energy metabolism and immunological parameters of transition dairy cows. The experimental design, rations, performance, energy metabolism and rumen fermentation variables have been previously published in Drong et al. (2016a). Variables illustrating the impact of these feed additives on immunity have been described in Drong et al. (2016b).

Briefly, the cows were allocated 6 weeks antepartum (**a.p.**) in either a low (2.77 ± 0.14, LC, n = 15, parity: 1.7 ± 0.9, mean ± SD) or high (3.95 ± 0.08, n = 45) body condition score (**BCS**) group (5-point scale according to Edmonson et al. (1989)). The cows in the high BCS group were then further divided into a control group (HC, n = 15, parity: 2.5 ± 1.4) and two treatment groups receiving either monensin (MO, n = 15, parity: 2.6 ± 1.3) or BEO (EO, n = 15, parity: 2.4 ± 1.6). During the dry period the LC cows received a ration consisting of 80 % roughage (50 % maize silage, 50 % grass silage) and 20 % concentrate based on DM content. After calving a TMR was fed with an initial concentrate feed proportion of 30 %, which was increased stepwise to 50 % of the daily ration within 2 weeks (details in Drong et al. (2016a)). The high conditioned animals (group HC, MO and EO) were oversupplied with energy during the dry period (concentrate feed proportion of 60 %) and subjected to a decelerated increase in concentrate feed proportion p.p. (from 30 % to 50 % in 3 instead of 2 weeks) to stimulate p.p. lipolysis and induce a ketogenic metabolic state (Schulz et al., 2014). In the EO group a BEO (CRINA® ruminants, DSM, Basel, Switzerland) containing thymol (25-35 %), guaïacol (10-15 %), eugenol (5-10 %), vanillin (10-20 %), salicylaldehyde (5-10 %) and limonene (20-35 %) on an organic carrier (as described in the patent, Rossi (1999)) was administered through the pelleted concentrate (target: 1 g/cow/d) from day 21 a.p. onwards. Each cow in the MO group received a monensin CRC (Kexxtone, Elanco®, Bad Homburg, Germany) at day 21 a.p. releasing 335 mg monensin/d for a period of 95 d.

The cows were milked two times per day at 0530 h and 1530 h and the TMR was fed ad libitum and offered fresh daily at approximately 1100 h. Individual TMR intake was continuously recorded using electronic balance troughs (Insentec, B.V., Markenesse, The Netherlands).

Body weight (**BW**) was assessed twice daily after milking and BCS was recorded weekly. Morning and evening milk samples were collected at 2 days per week (Monday evening & Tuesday morning; Thursday evening & Friday morning) and stored at 4 °C until analysis. Milk samples were analyzed for fat, protein, lactose and urea concentrations using an infrared milk analyzer (Milkoscan FT 6000, Foss Electric A/S, Hillerød, Denmark).

Blood samples were collected from a *Vena jugularis externa* in a 10 mL evacuated serum separating blood tube, centrifuged immediately thereafter (Heraeus Varifuge® 3.0R, Heraeus, Osterode, Germany; 2300 *g*, 15 °C, 15 min) and stored at -80 °C before chemical analysis for glucose, BHB, fatty acids, urea, albumin, total protein, cholesterol, total bilirubin and triglyceride concentrations, and aspartate transaminase (**AST**), γ-glutamyltransferase (**γ-GT**) and glutamate dehydrogenase (**GLDH**) activity using an automatic clinical chemistry analyzer (Eurolyser CCA180, Eurolab, Austria, described in detail in Schären et al. (2016a)).

To investigate the influence of these two feed additives on rumen microbiota and fermentation variables, rumen fluid samples (ca. 750 mL) were collected from 48 animals (n = 12) at day 56 p.p. using an oral rumen tube and a hand vacuum pump. Immediately after collection, pH was measured using a glass electrode (model: pH 525; WTW, Weilheim, Germany) and samples for microbiota analysis were immediately stored at -20 °C. For protozoal density assessment 15 mL of rumen fluid were mixed with 15 mL of a methylgreen-formalin solution and stored at 4 °C. Protozoa were counted using a Fuchs-Rosenthal chamber under an optical microscope and differentiated into entodiniomorpha and holotrichia (Ogimoto and Imai, 1981). Samples for ammonia (**$NH_3$-N**), volatile fatty acids (**VFA**), LPS and protozoa concentration were cooled to 4 °C until further processing approximately 1-2 h after sample collection. Volatile fatty acids were determined according to Koch et al. (2006) using a gas chromatograph (Gaschromatograph 5890 II, Hewlett Packard®, Böblingen, Germany) and $NH_3$-N was determined using steam distillation according to the Kjeldahl method (DIN38406-E5-2, Anonymous (1998)). To assess LPS concentrations rumen fluid samples were centrifuged, filtered, heated and stored at -20 °C. For analysis samples were diluted and measured spectrophotometrically using the Limulus amebocyte lysate (LAL) assay (Kinetic-QCLTM, Lonza, Walkersville, MD, USA; following the manufacturer´s instructions) and a microplate reader with incubation chamber (Infinite M200, Tecan Group Ltd., Männedorf, Switzerland) and then evaluated using the MagellanTM Data Analysis Software (Tecan Group Ltd., Männedorf, Switzerland; detailed protocol in Schären et al. (2016b) and Gozho et al. (2005)).

The data presented in the current work have exclusively been collected in these 48 cows at day 56 ± 3 p.p. (for performance data means of these 7 days were calculated). Due to technical issues at that time, several rumen liquid samples for fermentation variable analysis were lost

during storage and analysis (especially in LC group). It was therefore decided to only present the results of the HC (n = 9), MO (n = 12) and EO (n = 10) group.

**DNA Extraction**

Firstly, several centrifugation steps were performed on the rumen liquid samples to concentrate the bacteria and archaea, and to remove feed particles and debris. The samples were thawed at room temperature, 80 mL were then centrifuged for 5 min at 600 g (4 °C) and the supernatant was kept for further processing. Thereafter the supernatants were centrifuged four times for 20 min at 27'000 g (4° C) and between each centrifugation step the pellet was re-suspended in 40 mL 0.9 % NaCl. After the last centrifugation step the pellet was re-suspended in 1000 µL 0.9 % NaCl, single droplets were shock frozen in liquid nitrogen and stored at -80 °C. DNA extraction was adapted from Meibaum et al. (2012). At first 240 µL of the microbe-pellet were thawed on ice, centrifuged (13'000 *g*, 5 min, 4 °C) and the supernatant was discarded. Thereafter the sample was re-suspended in 550 µL 1 x tris(hydroxymethyl)-aminomethane-HCl, EDTA (both 10 mM, pH 8.0) and NaCl (150 mM) and a mechanical lysis of the cells was performed by bead beating method (Ribolyser Cell Disrupter, Hybaid Ltd., Ashford, United Kingdom) in two sequences of acceleration (6.0 m/s and 4.5 m/s, 40 sec. each). After centrifugation (13'000 *g*, 15 min, 4 °C) the supernatant was incubated with 50 µL lysozyme (100 mg/mL) and 10 µL RNaseA (20 mg/ml) during 30 min at 37 °C. This was followed by an incubation step with 15 µL 20 % sodiumdodecylsulphate and 10 µL proteinase K (20 mg/mL) during 1 h at 37 °C. The final incubation step using 125 µL 4 M NaCl and 80 µL 10 % cetyltrimethylammoniumbromide was performed at 65 °C during 10 min. To purify the mixture from proteins 780 µL of phenol-chloroform-isoamylalcohol were added, the mixture was centrifuged (7 min, 13'000 g, 4 °C), the supernatants were discarded, 780 µL chloroform-isoamylalcohol was added, centrifuged again (10 min, 13'000 *g*, 4 °C) and the supernatant was then kept for further processing. To further purify the DNA, two washing steps were performed using the peqGold Tissue-Kit (peq lab, Erlangen, Germany) according to manufacturer's guidelines. The gDNA samples were then stored at 4 °C until further processing.

**PCR-SSCP Analysis**

After DNA extraction a two-step amplification (initial and nested PCR) of either bacterial or archaeal 16S rRNA genes and a single-strand digestion step was performed (protocol and primer described in detail in Meibaum et al. (2012)). The 48 samples were then divided onto 4 SSCP gels (12 samples per gel, n = 3) for bacteria as well as for archaea and gel-electrophoresis was carried out at 300 V during 22.5 h at 20 °C (described in detail in Dohrmann et al. (2004)). The gels were digitalized and analyzed using ScanMaker (i800, Mikrotek, Willich, Germany) and GelComparII (Applied Maths, Sint-Martens-Latem, Belgium) as described in Meibaum et al. (2012). For graphical illustration two dimensional PCO plots based on dissimilarities were created with the cmdscale() command in the R 3.0.2 software package (R Foundation for Statistical Computing, Vienna, Austria, (R-Core-Team, 2013)) and for a descriptive illustration all comparisons across the four gels were summarized in a boxplot.

**Prokaryotic 16S rRNA Gene Amplification, Illumina MiSeq Sequencing and Bioinformatics**

For sequencing, gDNA samples were sent to Microsynth AG (Balgach, Switzerland). A primer pair with 97.7 % / 96.9 % (forward primer) and 98.4 % / 96.5 % (reverse primer) coverage (one mismatch) for archaea and bacteria, respectively, was chosen for 16S sequencing library preparation: A519F (S-D-Arch-0519-a-S-15): CAGCMGCCGCGGTAA and 802R (S-D-Bact-0785-b-A-18): TACNVGGGTATCTAATCC (Klindworth et al., 2013). For 16S rRNA gene amplification the HiFi HotStart PCR Kit (Kapa Biosystems, Wilmington, MA, USA) was used with following PCR conditions: initial denaturation (95 °C, 180 sec), denaturation (98 °C, 20 sec), annealing (50.8 °C, 30 sec) and elongation (72 °C, 30 sec) with 30 cycles, and a final elongation step (72 °C, 5 min). Furthermore, the Illumina Nextera Libraries were prepared according to the manufacturer's instruction (Illumina, San Diego, USA). Sequencing was performed on the Illumina MiSeq Sequencing System using the Illumina MiSeq reagent Kit v2 (2 x 250bp). Sequence data were de-multiplexed and trimmed using the Illumina MiSeq v2.5.1.3. reporter and cutadapt v1.8.1 software package (Martin, 2011). Read stitching was performed using FLASH v1.2.11 (Magoč and Salzberg, 2011) and only stitched reads with an average quality score (whole read) of 25 or higher were used for downstream analysis. Furthermore, de novo Chimera detection, identification and removal was done using the Uchime v4.2 (Edgar et al., 2011) and Usearch v8.1.1861 (Edgar, 2010) software package. The operational taxonomic units (**OTU**) clustering (based on 97 % sequence similarity) and the taxonomic assignment was performed using Uclust (Edgar, 2010) and QIIME v1.9.1 (Caporaso et al., 2010), respectively. As a reference database for the taxonomic assignment the SILVA rRNA database v111 was chosen (Quast et al., 2013). Alpha diversity analysis was performed

and PCoA plots were created using QIIME. Robustness of clusters displayed in PCoA plots was ensured by jackknife resampling (10fold).

### Statistical Analysis

All statistical analyses were performed using the R 3.0.2 software package. If variables were recorded more than once a week, means were calculated per cow and week (day 56 ± 3 p.p.) prior to statistical evaluation. To obtain a normal distribution, rumen LPS concentrations were logarithmically transformed prior to statistical analysis. For normal distributed data a one-way ANOVA with Tukey post-hoc test and for non-normal distributed data a Nemenyi-Damico-Wolfe-Dunn test (joint ranking (Hollander and Wolfe, 1999); modified to function from the R software package coin (Hothorn et al., 2006)) was performed. In case of the SSCP-gels a PERMANOVA was performed using the adonis() function in the R software package vegan (Oksanen et al., 2015). Beta diversity of sequencing results was tested using the anosim() function within the R package vegan on weighed UniFrac distances. Results were considered significant at $P < 0.05$ and a trend was declared at $0.05 < P < 0.10$.

## Results

### Animal Performance

No significant differences between groups for DMI, milk production variables (milk yield, milk protein and fat content and milk urea concentration), and BW and condition score were observed (Table 1).

### Rumen Fermentation

In comparison with EO, monensin increased the molar propionate proportion, and decreased the molar acetate proportion, resulting in a decreased acetate/propionate ratio compared with the HC and EO group (Table 2). The monensin treatment also increased the LPS concentration (comparison EO vs. MO: $P = 0.108$) and the concentration of the holotrich protozoa compared with the HC group. No significant differences among groups were observed for pH, total VFA and $NH_3$-N concentrations, butyrate, valerate and isovalerate proportions, and entodiniomorpha and total protozoa counts.

**Metabolic variables**

In the HC group higher serum BHB and protein concentrations were measured compared to the MO group (Table 3). All other serum variables (glucose, fatty acids, triglycerides, cholesterol, albumin, AST, γ-GT, GLDH, bilirubin and urea) did not differ significantly among groups.

**SSCP Analysis**

For the archaea no clustering was observed of any of the groups on PCO plots and the PERMANOVA revealed no significant differences among groups for any of the gels (Figure 1). Also in the boxplots, summarizing the different comparisons across all four gels, no differences between comparisons are visible (Figure 3A).

For the bacteria, PERMANOVA revealed for two of the four SSCP gels a significant difference between groups (Figure 2A and B). A clear clustering of the MO samples was observed on the first gel (Figure 2A) and on the second gel two of the MO samples differ clearly from the other samples (Figure 2B). Also, on each of these two gels, two samples of the EO group are clearly separated from the main cluster with the control animals. For the other two gels no clustering and no significant difference among groups was observed (Figure 2C and D). In the boxplots, summarizing the different comparisons across all four gels, a lower dissimilarity among MO samples compared to other groups and comparisons is visible (Figure 3B).

**16S rRNA Gene Amplicon Analysis**

Illumina MiSeq sequencing resulted in 12’206 ± 3’424 reads (after filtering, mean ± SD) per sample. In total a number of 177 different species-level OTUs were identified, with an average of 167 ± 6 (mean ± SD) different OTUs per sample. Most OTUs could be taxonomically classified to the family level, while their genus or species level affiliation were “uncultured bacterium or archaeon” in many cases.

Alpha diversity analysis revealed a decreased species diversity in the MO group, expressed in a lower number of observed OTU in the MO group (compared to all other groups, approx. 162 vs. 170 OTU) and a lower Shannon index (compared with HC and LC group, comparison MO vs. EO: $P = 0.224$, Table 4).

Beta diversity analysis showed a significant difference between groups ($P < 0.001$) and PCoA plots exhibited a clustering of the MO samples (Figure 4). Analysis of the taxonomic composition on family level revealed a decrease of the abundance of the members of the uncultured BS11 gut group (phylum *Bacteroidetes*), *Rikenellaceae*, *Lachnospiraceae* and unassigned OTU, and an increase in abundance of members of the *Coriobacteriaceae*,

*Prevotellaceae*, S24-7 (phylum *Bacteroidetes*) and SHA-109 (phylum *Cyanobacteria*) families in the MO group (Figure 5). Within the family of the *Lachnospiraceae* monensin caused a decrease of the genus *Butyrivibrio* ($P < 0.001$), *Pseudobutyrivibrio* ($P = 0.068$) and *Incertae Sedis* ($P = 0.005$), whereas the genus *Oribacterium* ($P < 0.001$) was increased (data not shown). Of the archaeal population the only OTU detected was classified as a member of the *Methanobrevibacter* genus and its abundance was not influenced by the MO treatment. No difference was observed between the EO and control groups (HC and LC) in archaea or bacteria abundance on any taxonomic level.

In the MO group twenty-three bacterial OTU were identified where the MO treatment caused a decrease in relative abundance. Those were mainly belonging to the phyla *Bacteroidetes* and *Firmicutes* (Table 5). The members of the families *Rikenellaceae* (uncultivated RC9 gut group) and *Lachnospiraceae* (genera *Butyrivibrio* and *Pseudobutyrivibrio*) accounted for 74 % of the decrease in relative abundance with a difference of 3.5 and 4.0 % to the means of the LC, HC and EO group (total of 10.1 %), respectively. In case of ten bacterial OTU from the phyla *Actinobacteria*, *Bacteroidetes*, *Cyanobacteria* and *Firmicutes* the monensin treatment resulted in an increase in abundance (total difference of 5.2 % compared to the mean of LC, HC and EO groups), with the families *Lachnospiraceae* and *Ruminococcaceae* being the most important contributors, accounting for 24 and 48 % of the total difference, respectively. When taking into account cell wall constitution and fermentation characteristics (illustrated in Table 6), no clear-cut effect of monensin on Gram-stain negative and positive bacteria was observed.

## Discussion

In a previous manuscript we described the production and rumen fermentation characteristics as affected by monensin and a BEO in transition dairy cows. Monensin caused an increase in rumen molar propionate proportions, reducing the occurrence of subclinical or clinical ketosis (Drong et al., 2016a). Furthermore, also an influence of monensin on liver health and the immune response after vaccination was observed (Drong et al., 2016b). The supplementation of a BEO did not result in alterations on rumen fermentation, production or immunological level.

We hypothesized that the observed effects would also be mirrored in the rumen microbial community. Therefore, samples of rumen liquid were collected orally at day 56 p.p. for 16S rRNA gene analysis. To give an illustration of the metabolic status of the animals at the time, the production data of day 56 ± 3 p.p. and clinical chemistry variables assessed at day 56 p.p. were summarized. No difference between groups was observed, which is in line with the observation that monensin mainly elicited an effect during the first two weeks p.p., when the negative energy balance was most pronounced (Drong et al., 2016a). The positive effect of

monensin on rumen propionate production, thereby enhancing the energy availability for the animal, was confirmed on the level of the fermentation (highest propionate proportion and lowest acetate/propionate ratio), as well as metabolic variables (lowest serum BHB concentrations in MO group).

**Effect of Essential Oils**

Also on rumen microbial level, no influence of the EO treatment was observed. In vitro and in vivo studies applying similar daily dosages of the same BEO have come to similar results. Benchaar et al. (2003) (in vitro, dairy cow rumen fluid), Benchaar et al. (2006) (in vivo, dairy cows), Benchaar et al. (2007) (in vivo, dairy cows), and Giannenas et al. (2011) (in vivo, dairy ewes) reported no influence on ruminal total viable or cellulolytic bacteria counts, or protozoa numbers. Only Giannenas et al. (2011) reported a decrease in hyper-ammonia-producing (HAP) bacteria counts, which is in line with in vitro experiments of McIntosh et al. (2003) showing that the growth of certain HAP bacteria is inhibited by a BEO. McIntosh et al. (2003) further observed an adaptation to essential oils in several bacteria strains. This was also confirmed by Cardozo et al. (2004) in a continuous culture fermentation study including different essential oils. The aspects of rumen microbiota adaptation and the discrepancy between *in vitro* and *in vivo* conditions concerning the effect of essential oils have been discussed by Benchaar and Greathead (2011), stating that observations in short-term experiments or in vitro experiments, with in vivo unachievable high concentrations of essential oils, may lead to inaccurate conclusions. In the trial presented in this manuscript the BEO was fed during approximately 80 days prior to sampling. A possible explanation for the absence of effects of a BEO supplementation could therefore be the long exposure period and subsequent adaptation of the rumen microbiota.

**Comparison of DNA based Microbiota Analysis Techniques**

The influence of monensin on the rumen bacteria community could be demonstrated by both methodical approaches on microbial diversity, i.e., at sequencing and SSCP level. However, results were much less clear on the latter. Only on two of the four gels the monensin effect was clearly visible and statistically significant. But the descriptive illustration of the different comparisons across all four gels revealed a much lower dissimilarity among MO samples, which was confirmed by the sequencing results. The difference in results could possibly be attributed to the lower amount of observations on each gel (n = 3) compared to the sequencing analysis (n = 12) due to a high inter-animal variation (Weimer, 2015). On the other hand, when comparing the PCoA plots of the sequencing results with the PCO plots of the PCR-SSCP analysis no pattern concerning samples and outliers emerges (data not shown). A second aspect that could explain the discrepancy between results of these two methods is the use of

different primers for the 16S rRNA gene amplification (Klindworth et al., 2013). However, all primers used in the current trial exhibit a high coverage and the sequencing results showed that the alterations due to the MO treatment were substantial enough to be captured with the primers used for the PCR-SSCP analysis. We suggest the limited resolution inherent to this gel-based method and the higher amount of available data points for statistical analysis in the amplicon sequencing approach as being the most likely explanation for the lower sensitivity of the PCR-SSCP method (Kisand and Wikner, 2003; Shendure and Ji, 2008). The output of the PCR-SSCP method only consisted of dissimilarities between samples, whereas the amplicon sequencing method rendered the proportional abundance of approx. 170 different OTU per sample. These results illustrate that the underlying method used may influence the results of microbiome studies significantly (Weimer, 2015). They further indicate that amplicon sequencing seems to be the more reliable method and constitutes a promising method for routine screening in the future (Shendure and Ji, 2008; Caporaso et al., 2012).

**Effect of Monensin on Rumen Microbiota**

Ionophores accumulate in cell membranes and act as antiporters by increasing the influx of sodium and protons, depleting the cell of potassium (Russell and Houlihan, 2003). Therefore, ionophore resistance is mainly correlated with differences in cell envelope structure (Russell and Houlihan, 2003). Different studies have pointed out that rather than a clear cut between Gram-negative and Gram-positive bacteria a model of varying effect depending on cell wall constitution and thickness applies (Callaway et al., 1999; Russell and Houlihan, 2003; Weimer et al., 2008), which was also confirmed in our study. We further observed higher LPS concentrations in ruminal fluid in the MO compared to the HC group. This is in line with hypothesis that monensin selects for bacteria groups with less permeable cell walls (Beveridge, 1999; Weimer et al., 2008).

The 16S rRNA gene sequencing revealed 23 bacterial OTU all belonging to the phyla *Bacteroidetes* and *Firmicutes* that exhibited a decrease in relative abundance under the influence of monensin. In contrast the abundance of 10 bacterial OTU from the phyla *Actinobacteria*, *Bacteroidetes*, *Cyanobacteria* and *Firmicutes* was increased. These results illustrate that monensin significantly alters the composition of what has been recently described as the core microbiome and decreases its diversity (Creevey et al., 2014; Weimer, 2015). Our results are generally in line with a study of Weimer et al. (2008) in which the influence of monensin feeding and withdrawal on populations of individual bacterial species was investigated in two cows receiving a high-starch ration using real-time PCR. They observed an increase in the genus *Prevotella*, a decrease in a species of the *Butyrivibrio* genus and no alterations in a species of the genus *Ruminobacter*, *Selenomonas*, the family *Succinivibrionaceae* and four species of the domain *Archaea*. They further observed a decline

in the species *Megasphaera elsdenii* and no alterations in the species *Eubacterium ruminantium*, two bacteria species that were not detected in our study. Similar alterations were observed in a study of Kim et al. (2014a) and (2014b), investigating the effect of monensin supplementation on ruminal bacterial communities of dairy and feedlot cattle, respectively, using next generation sequencing. However, the results of these studies differ from ours on various levels. For example, Kim et al. (2014b) observed, similar to our results, on family and genus level an increase in the relative abundance of the family *Lachnospiraceae* and genus *Prevotella*, as well as a decrease in the family *Ruminococcaceae*, but further also a decrease in abundance in the genera *Oscillobacter*, *Ruminococcus*, *Succiniclasticum*, *Syntrophococcus*, and *Sharpea*, which were either not detected (*Oscillobacter* and *Sharpea*) or not altered (*Ruminococcus*, *Succiniclasticum* and *Syntrophococcus*) in our study. In contrast, we observed alterations in abundance of different bacteria on phylum (*Cyanobacteria*), and family and species (BS11 gut group, *Rikenellaceae* (RC9 gut group), *Lachnospiraceae* (*Pseudobutyrivibrio* and *Oribacterium)*) level, which were not described by Kim et al. (2014b). Kim et al. (2014a) observed alterations in the phyla *Firmicutes*, *Bacteroidetes*, *Actinobacteria* and *Cyanobacteria*, similar to our results. Kim et al. (2014a) also observed various alterations in different other bacteria taxa that were either not detected (e.g. genus *Moryella*, family *Porphyromonadaceae* and order *Streptophyta*) or unaltered in our study (e.g. phylum TM7, genera *Succiniclasticum* and *Syntrophococcus*). The differences between the results of these two studies and our study can most likely be attributed to several factors, such as the different rations fed (ground corn and long alfalfa hay (Kim et al., 2014a) and 60 % dried distillers grain, corn and corn-silage (Kim et al., 2014b) vs. TMR consisting of corn- and grass-silage, and concentrate; (Henderson et al., 2015)), the different DNA extraction methods (Henderson et al., 2013), primers and sequencing system used (targeting of V1-V3 vs. V4-V5 hypervariable region, 454 GS FLX Titanium vs. Illumina MiSeq system (Klindworth et al., 2013)) and the lower amount of reads per sample (6,268 and 7,616 vs. 12,206 reads per sample). Furthermore, the samples in the study of Kim et al. (2014a) and Kim et al. (2014b) were collected from eight and six cannulated animals, respectively, and pooled per group for sequencing, whereas in our case of each group samples from 12 animals were analyzed separately. Also the length of exposure (21 d and 'information missing' vs. 80 d) as well as the daily dosage (13 mg/kg and 33 mg/kg vs. 19 mg/kg DM; 268 mg/d and 274 mg/d vs. 335 mg/d) could play a role in the observed differences. Several studies showed that the sensitivity of different bacteria species and strains to monensin varies with its concentration and that certain bacteria strains are able to increase their resistance over time, most likely by altering their cell wall structure (Russell and Houlihan, 2003; Weimer et al., 2011). Furthermore, it is known that the rumen microbiota of the liquid and fiber rumen content differs and it is speculated whether monensin acts differently on the two fractions due to its feed-particle binding properties

(Callaway et al., 1999; Cho et al., 2006; Wischer et al., 2013). In the study of Kim et al. (2014b) it is not clear whether the sample was a mixture of both fractions, or whether the samples contained only liquid rumen content. However, Kim et al. (2014a) differentiated between the rumen liquid and fiber associated bacteria, and their results (obtained from the analysis of six pooled samples) point towards a confirmation of this hypothesis. In this train of thoughts Kim et al. (2014a) also mentioned that the form under which monensin is supplied may play a role in the response of the microbial community. Summarizing these different methodological aspects, we can state that future studies should involve metagenome sequencing to avoid selection of certain taxa by 16S rRNA gene primers, a prolonged trial period with repeated measurements to involve adaptational aspects, different rations and forms under which monensin is supplied, as well as the analysis of the influence on different rumen microbial communities at the different locations in the rumen (the liquid, fiber and epithelium associated microbiota (Cho et al., 2006)).

The rumen propionate production enhancing effect of monensin has been discovered already decades ago, but tracing the underlying mode of action has been a challenge (Weimer et al., 2008). Callaway et al. (1999) stated that *Butyrivibrio fibrosolvens* is an important acetate and butyrate producer and the ability of monensin to inhibit bacteria of the *Butyrivibrio* genus might result in an increased propionate production. In our trial, most bacterial taxa that were significantly decreased in their abundance due to monensin have been described as moderate or non-propionate producers, which is in line with this hypothesis. Additionally, we observed an increase in abundance of members of two of the taxa that have earlier been described as succinate and propionate producers (*Prevotella* and *Ruminococcaceae*; Koike and Kobayashi (2009); Krieg et al. (2009); Vos et al. (2009); Watanabe et al. (2010)). Since succinate is rapidly converted into propionate by succinate-decarboxylating bacteria in the rumen (Koike and Kobayashi, 2009), this might be an additional indication on how monensin alters the rumen fermentation profile.

Unfortunately, most ruminal bacteria species have not been cultured and functional properties of and interrelations between different microbial species in vivo are poorly understood. New cultivation-independent techniques, however, promise new insights into rumen microbiota dynamics (Morgavi et al., 2013). For example, Solden et al. (2016) have shown by metagenome sequencing and shotgun proteomics that members of the yet uncultivated *Bacteroides* BS11 gut group, that was monensin sensitive in our study (Table 5 and 6), is likely a hemicellulose fermenter that produces acetate and butyrate.

Earlier studies suggested that monensin causes a decreased crude protein degradation in the rumen through an inhibitory effect on HAP bacteria (Wischer et al., 2013). A decrease in rumen liquid $NH_3$-N concentrations and HAP bacteria have been described under the influence of

monensin *in vitro* (Yang and Russell, 1993a; Eschenlauer et al., 2002) as well as *in vivo* (Yang and Russell, 1993b; Ruiz et al., 2001). In the current study we did not observe lower $NH_3$-N concentrations in the rumen of the monensin supplemented animals and to our knowledge none of the bacteria that were decreased under the influence of monensin has previously been described as high amino acid fermenters. However, Lana and Russell (1997) found that monensin may increase or decrease rumen $NH_3$-N concentrations, depending on the basal diet fed, but decreases the deamination rate consistently. Therefore, future studies should include this aspect additionally.

Appuhamy et al. (2013) showed in a meta-analysis that the addition of monensin to the diet results in a reduction of the methane production of 2 and 15 % in dairy cows and beef cattle, respectively (average: 5.4 %). It has been widely acknowledged that monensin does not act onto methanogenic bacteria directly, but most likely through suppression of other rumen microorganisms that convert more complex organic matter into substrates for methanogenesis (Hook et al., 2009; Mackie et al., 2013). This was also confirmed in our study, in the PCR-SSCP as well as sequencing approach, where no alteration in the relative abundance of the archaeal taxa was observed. Using the hydrogen provided by other bacteria, methanogens in general produce methane from a variety of carbon substrates such as carbon dioxide, methanol, methylamines or acetate (Valdez-Vazquez and Poggi-Varaldo, 2009). The amount of hydrogen generated in the rumen is directly influenced by the VFA pattern of fermentation. Hydrogen is produced during the process of glycolysis, as well as during the final synthesis of VFA in the rumen. The production of 1 mole acetate results in 2 moles of hydrogen, whereas the production of 1 mole propionate only renders 1 mole of hydrogen (Czerkawski, 1986). The shift of the acetate:propionate ratio caused by monensin will therefore lead to a decrease in methane, as demonstrated by Wischer et al. (2013). Other possible and suggested pathways leading to a reduction in methane production such as an increase in bacteria species that compete for hydrogen (e.g. sulfate reducers and acetogens, Morvan et al. (1996); Valdez-Vazquez and Poggi-Varaldo (2009); Mackie et al. (2013)) or a decrease in hydrogen production through the inhibition of protozoa (Russell and Strobel, 1989) are not supported by the data of the current study. We observed no effects on total protozoal counts and entodiniomorph protozoa, and highest holotrich protozoa concentrations in the MO group alongside with a high variation between animals. This is in line with different studies observing a variable and transient effect of monensin on rumen protozoa (Dennis et al., 1986; Arakaki et al., 2000; Benchaar et al., 2006; Sylvester et al., 2009).

## Conclusion

The results of this trial show that the intraruminal application of monensin decreases the rumen microbiome diversity by acting onto the core microbiome, whereas the applied BEO (containing

thymol, guaïacol, eugenol, vanillin, salicylaldehyde and limonene) failed to elicit any effects. It was confirmed that rather than a clear-cut between Gram-negative and Gram-positive bacteria a model of varying effects depending on cell wall constitution and thickness applies in monensin sensitivity. These data support the hypothesis that the observed increased ruminal molar propionate proportions due to monensin may be caused by a decrease in abundance of non- and moderate propionate producers and an increase in abundance of succinate producers, such as the *Prevotella* genus. It was further confirmed that the decreased methane production observed under monensin supplementation most likely cannot be ascribed to a direct effect of monensin onto the methanogens, but rather to a decrease in substrate availability for methanogenesis by acting onto other rumen microorganisms. By the application of two different methods (DNA fingerprinting vs. sequencing), as well as comparison with other publications implementing different methods to define alterations in the rumen microbiome, it was illustrated that results can vary and the power of amplicon sequencing for screening purposes was emphasized. To further investigate the mode of action of monensin and to characterize the resulting alterations in the rumen microbiome, techniques to investigate alterations on a functional level, such as metagenomics, metatranscriptomics or metaproteomics should be applied. Furthermore, the aspects of adaptation and difference between fiber, liquid and rumen epithelium associated bacteria should be considered in future studies.

## Acknowledgements

The authors want to thank Inga Ruddat and Martin Beyerbach (Institute for Biometrics, University of Veterinary Medicine, Hanover, Germany) for excellent bioinformatical support. Many thanks go to the coworkers at the Institute of Animal Nutrition and the experimental station of the Institute of Animal Nutrition, Friedrich-Loeffler-Institute (FLI) in Braunschweig, Germany, for caring for the experimental animals, assisting with experimental measurements as well as performing the analyses.

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## Tables and Illustrations

**Table1.** Effect of monensin and a blend of essential oils on performance and efficiency in transition dairy cows at day 56 postpartum (p.p.) [1]

| Variable[2] | Group[3] | | | | Significance[4] |
|---|---|---|---|---|---|
| | LC | HC | MO | EO | |
| DMI (kg/d) | 18.9 ± 0.5 | 17.3 ± 0.9 | 17.2 ± 0.4 | 16.5 ± 0.9 | 0.171 |
| Milk yield (kg/d) | 33.4 ± 1.5 | 28.3 ± 1.5 | 32.7 ± 1.5 | 29.8 ± 1.8 | 0.124 |
| Milk protein content (%) | 2.87 ± 0.05 | 2.73 ± 0.04 | 2.69 ± 0.05 | 2.70 ± 0.09 | 0.163 |
| Milk fat content (%) | 4.22 ± 0.17 | 4.19 ± 0.20 | 3.73 ± 0.19 | 4.47 ± 0.24 | 0.105 |
| Milk urea (ppm) | 103 ± 7 | 74 ± 8 | 94 ± 11 | 105 ± 8 | 0.083 |
| Body weight (kg) | 603 ± 14 | 612 ± 15 | 582 ± 10 | 596 ± 15 | 0.484 |
| BCS (scale 1-5) | 2.60 ± 0.07 | 2.98 ± 0.15 | 2.71 ± 0.14 | 2.98 ± 0.09 | 0.073 |

[1] Average of day 56 ± 3 p.p.

[2] Means ± SE

[3]Animals were divided into a low condition (LC) and a high condition control (HC), monensin (MO) and essential oil (EO) group 6 weeks antepartum (a.p., n = 12). High conditioned animals (HC, MO, EO) were oversupplied with energy in dry period (60 vs. 20 % concentrate feed proportion) and subjected to a decelerated increase in concentrate feed proportion p.p. (from 30 to 50 % in 3 instead of 2 weeks) to increase p.p. lipolysis and induce a ketogenic metabolic state. The MO group received a monensin controlled release capsule 21 d a.p. and the EO group was fed with concentrate containing a blend of essential oils from 3 weeks a.p. on and during lactation.

[4]One-way ANOVA, Tukey post-hoc test

**Table 2.** Effect of monensin and a blend of essential oils on rumen fermentation variables in transition dairy cows at day 56 postpartum (p.p.) [1]

| | Group[3] | | | |
|---|---|---|---|---|
| **Variable**[2] | **HC** | **MO** | **EO** | **Significance**[4] |
| pH | 7.27 ± 0.06 | 7.21 ± 0.05 | 7.24 ± 0.06 | 0.722 |
| Total VFA (mmol/L) | 59.7 ± 4.7 | 66.6 ± 3.5 | 62.2 ± 5.4 | 0.580 |
| Acetate (mol %) | 64.1 ± 0.8 [ab] | 61.9 ± 0.8 [b] | 65.5 ± 0.9 [a] | 0.017 |
| Propionate (mol %) | 19.1 ± 0.4 [b] | 22.7 ± 0.6 [a] | 19.2 ± 0.7 [b] | < 0.001 |
| Butyrate (mol %) | 14.5 ± 0.5 | 13.2 ± 0.4 | 13.4 ± 0.3 | 0.081 |
| Valerate (mol %) | 0.79 ± 0.10 | 0.57 ± 0.09 | 0.64 ± 0.13 | 0.420 |
| Isovalerate (mol %) | 1.55 ± 0.20 | 1.64 ± 0.09 | 1.20 ± 0.13 | 0.288 |
| Acetate:propionate | 3.38 ± 0.10 [a] | 2.75 ± 0.09 [b] | 3.46 ± 0.16 [a] | < 0.001 |
| $NH_3$-N (mmol/L) | 1.73 ± 0.40 | 3.02 ± 0.47 | 3.40 ± 0.61 | 0.112 |
| LPS ($^{10}$Log(IU/ml)) | 2.92 ± 0.14 [b] | 3.54 ± 0.17 [a] | 3.12 ± 0.08 [ab] | 0.019 |
| Total Protozoa ($10^3$/ml) | 55.7 ± 15.7 | 86.4 ± 12.8 | 73.8 ± 10.6 | 0.309 |
| Entodiniomorpha ($10^3$/ml) | 54.2 ± 14.8 | 83.7 ± 12.8 | 72.0 ± 10.6 | 0.322 |
| Holotrichia ($10^3$/ml) * | 1.31 / 0.31 [b] | 1.41 / 1.59 [a] | 0.94 / 0.94 [ab] | 0.024 |

[1]Oral rumen fluid samples were collected at day 56 ± 1.4 p.p. (mean ± SD).

[2]For normal distributed data the mean ± SE and for non-normal distributed data (*) the median / interquartile range (IQR) is illustrated.

[3]Animals were divided into a low condition (LC) and a high condition control (HC), monensin (MO) and essential oil (EO) group 6 weeks antepartum (a.p., n = 12). High conditioned animals (HC, MO, EO) were oversupplied with energy in dry period (60 vs. 20 % concentrate feed proportion) and subjected to a decelerated increase in concentrate feed proportion p.p. (from 30 to 50 % in 3 instead of 2 weeks) to increase p.p. lipolysis and induce a ketogenic metabolic state. The MO group received a monensin controlled release capsule 21 d a.p. and the EO group was fed with concentrate containing a blend of essential oils from 3 weeks a.p. on and during lactation. Due to technical issues at the time, several samples (especially in LC group) were lost during storage and analysis. It was therefore decided to only present the results of the HC (n = 9), MO (n = 12) and EO (n = 10) group.

[4]For normal distributed data a one-way ANOVA with Tukey post-hoc test and for non-normal distributed data (*) a Nemenyi-Damico-Wolfe-Dunn test (Hollander and Wolfe, 1999) was performed.

[a-b]Values within ration group in a row with different superscript letters differ ($P < 0.05$).

**Table 3.** Effect of monensin and a blend of essential oils on serum clinical chemistry and liver variables in transition dairy cows at day 56 postpartum (p.p.) [1]

| | Group[3] | | | | |
|---|---|---|---|---|---|
| **Variable**[2] | **LC** | **HC** | **MO** | **EO** | **Significance**[4] |
| BHB (mmol/L) | 0.67 ± 0.07 [ab] | 1.48 ± 0.10 [a] | 0.64 ± 0.08 [b] | 0.92 ± 0.12 [ab] | 0.018 |
| Glucose (mg/dL) | 61.6 ± 2.3 | 62.0 ± 2.4 | 61.9 ± 2.5 | 58.0 ± 2.4 | 0.626 |
| Fatty acids (mmol/L) | 0.48 ± 0.10 | 0.47 ± 0.07 | 0.41 ± 0.04 | 0.50 ± 0.07 | 0.870 |
| Triglycerides (mg/dL) | 10.6 ± 0.5 | 11.8 ± 0.7 | 10.8 ± 0.4 | 11.0 ± 0.9 | 0.650 |
| Cholesterol (mg/dL) | 204 ± 9 | 185 ± 11 | 193 ± 12 | 186 ± 12 | 0.636 |
| Albumin (g/L) | 36.8 ± 0.6 | 37.6 ± 1.1 | 34.4 ± 0.7 | 35.7 ± 1.1 | 0.091 |
| Total Protein (g/L) | 73.9 ± 2.6 [ab] | 77.2 ± 1.9 [a] | 68.0 ± 1.5 [b] | 70.9 ± 2.7 [ab] | 0.044 |
| AST (IU/L) | 65.7 ± 5.4 | 85.5 ± 8.9 | 61.3 ± 3.0 | 72.0 ± 6.2 | 0.061 |
| γ-GT (IU/L) * | 31.3 / 9.9 | 34.5 / 15.5 | 31.5 / 16.9 | 34.1 / 40.1 | 0.947 |
| GLDH (IU/L) * | 10.3 / 9.2 | 12.3 / 7.5 | 9.3 / 13.0 | 9.8 / 12.8 | 0.822 |
| Bilirubin (mg/dL) | 0.17 ± 0.02 | 0.16 ± 0.03 | 0.15 ± 0.03 | 0.19 ± 0.04 | 0.799 |
| Urea (mg/dL) | 16.3 ± 0.8 | 13.9 ± 1.3 | 19.9 ± 1.9 | 16.5 ± 0.9 | 0.083 |

[1]Blood samples were collected at day 56 ± 1.4 p.p. (mean ± SD).

[2]For normal distributed data the mean ± SE and for non-normal distributed data (*) the median / interquartile range (IQR) is illustrated. AST = aspartate transaminase, γ-GT = γ-glutamyltransferase, GLDH = glutamate dehydrogenase.

[3]Animals were divided into a low condition (LC) and a high condition control (HC), monensin (MO) and essential oil (EO) group 6 weeks antepartum (a.p., n = 12). High conditioned animals (HC, MO, EO) were oversupplied with energy in dry period (60 vs. 20 % concentrate feed proportion) and subjected to a decelerated increase in concentrate feed proportion p.p. (from 30 to 50 % in 3 instead of 2 weeks) to increase p.p. lipolysis and induce a ketogenic metabolic state. The MO group received a monensin controlled release capsule 21 d a.p. and the EO group was fed with concentrate containing a blend of essential oils from 3 weeks a.p. on and during lactation.

[4]For normal distributed data a one-way ANOVA with Tukey post-hoc test and for non-normal distributed data (*) a Nemenyi-Damico-Wolfe-Dunn test (Hollander and Wolfe, 1999) was performed.

[a-b]Values within ration group in a row with different superscript letters differ ($P < 0.05$).

**Table 4.** Effect of monensin and a blend of essential oils on rumen microbiome diversity[1]

| | Group[3] | | | | |
|---|---|---|---|---|---|
| **Variable**[2] | **LC** | **HC** | **MO** | **EO** | **Significance**[4] |
| Chao1 | 172.4 / 4.3 | 171.3 / 2.2 | 166.8 / 5.7 | 171.0 / 7.5 | 0.323 |
| Observed species | 170.5 / 4.0 [a] | 171.0 / 4.5 [a] | 162.5 / 4.0 [b] | 170.5 / 8.5 [a] | 0.015 |
| Shannon index | 4.43 / 0.16 [a] | 4.41 / 0.23 [a] | 4.20 / 0.13 [b] | 4.39 / 0.37 [ab] | 0.020 |

[1]Oral rumen fluid samples were collected at day 56 ± 1.4 postpartum (p.p., mean ± SD).

[2]Median / Interquartile Range (IQR)

[3] Animals were divided into a low condition (LC) and a high condition control (HC), monensin (MO) and essential oil (EO) group 6 weeks antepartum (a.p., n = 12). High conditioned animals (HC, MO, EO) were oversupplied with energy in dry period (60 vs. 20 % concentrate feed proportion) and subjected to a decelerated increase in concentrate feed proportion p.p. (from 30 to 50 % in 3 instead of 2 weeks) to increase p.p. lipolysis and induce a ketogenic metabolic state. The MO group received a monensin controlled release capsule 21 d a.p. and the EO group was fed with concentrate containing a blend of essential oils from 3 weeks a.p. on and during lactation.

[4] Nemenyi-Damico-Wolfe-Dunn test (Hollander and Wolfe, 1999)

[a-b]Values within ration group in a row with different superscript letters differ ($P < 0.05$).

**Table 5.** Relative abundance of rumen bacteria affected by monensin treatment[1]

| | | Taxonomy | | | | | Group[3] | | | | | Significance[4] | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | OTU Nr[2] | Phylum | Class | Order | Family | Genus | LC | HC | MO | EO | Global | LC:EO | HC:EO | MO:EO | LC:HC | MO:LC | MO:HC |
| Decreased | AB185544 | Bacteroidetes | Bacteroidia | Bacteroidales | BS11 gut group | u. b. | 0.16 | 0.30 | 0.05 | 0.27 | 0.001 | 0.999 | 0.653 | 0.001 | 0.552 | 0.001 | 0.076 |
| | AY244965 | Bacteroidetes | Bacteroidia | Bacteroidales | BS11 gut group | u. b. | 0.27 | 0.91 | 0.06 | 0.23 | 0.000 | 0.745 | 0.945 | 0.027 | 0.966 | 0.000 | 0.003 |
| | EU773647 | Bacteroidetes | Bacteroidia | Bacteroidales | BS11 gut group | u. b. | 0.63 | 0.59 | 0.22 | 0.47 | 0.034 | 0.990 | 0.997 | 0.082 | 0.958 | 0.034 | 0.119 |
| | EF686531 | Bacteroidetes | Bacteroidia | Bacteroidales | BS11 gut group | u. b. | 0.33 | 0.29 | 0.04 | 0.39 | 0.002 | 0.952 | 0.973 | 0.017 | 0.762 | 0.083 | 0.002 |
| | DQ394621 | Bacteroidetes | Bacteroidia | Bacteroidales | Rikenellaceae | RC9 gut group | 1.32 | 0.55 | 0.42 | 1.13 | 0.003 | 0.142 | 0.925 | 0.003 | 0.415 | 0.655 | 0.027 |
| | AB494890 | Bacteroidetes | Bacteroidia | Bacteroidales | Rikenellaceae | RC9 gut group | 0.37 | 0.37 | 0.03 | 0.43 | 0.000 | 1.000 | 0.999 | 0.000 | 0.996 | 0.000 | 0.000 |
| | AY244944 | Bacteroidetes | Bacteroidia | Bacteroidales | Rikenellaceae | RC9 gut group | 0.43 | 0.27 | 0.02 | 0.25 | 0.000 | 1.000 | 0.918 | 0.001 | 0.941 | 0.001 | 0.000 |
| | AB494915 | Bacteroidetes | Bacteroidia | Bacteroidales | Rikenellaceae | RC9 gut group | 1.05 | 0.59 | 0.28 | 1.64 | 0.000 | 0.499 | 1.000 | 0.001 | 0.435 | 0.116 | 0.000 |
| | EU719222 | Bacteroidetes | Bacteroidia | Bacteroidales | Rikenellaceae | RC9 gut group | 0.33 | 0.15 | 0.03 | 0.20 | 0.000 | 0.532 | 0.960 | 0.000 | 0.813 | 0.054 | 0.002 |
| | GU302529 | Bacteroidetes | Bacteroidia | Bacteroidales | Rikenellaceae | RC9 gut group | 0.76 | 0.70 | 0.14 | 0.66 | 0.004 | 0.999 | 0.998 | 0.011 | 0.990 | 0.018 | 0.004 |
| | EU719287 | Bacteroidetes | Bacteroidia | Bacteroidales | Rikenellaceae | RC9 gut group | 0.36 | 0.23 | 0.01 | 0.20 | 0.000 | 0.987 | 0.891 | 0.001 | 0.984 | 0.000 | 0.000 |
| | N.R.OTU | Bacteroidetes | Bacteroidia | Bacteroidales | Rikenellaceae | RC9 gut group | 0.44 | 0.39 | 0.05 | 0.48 | 0.000 | 0.836 | 0.949 | 0.000 | 0.990 | 0.001 | 0.000 |
| | AB034052 | Firmicutes | Clostridia | Clostridiales | Lachnospiraceae | Butyrivibrio | 1.78 | 1.71 | 0.69 | 2.06 | 0.000 | 0.473 | 0.796 | 0.000 | 0.947 | 0.065 | 0.009 |
| | GQ327740 | Firmicutes | Clostridia | Clostridiales | Lachnospiraceae | Butyrivibrio | 0.24 | 0.30 | 0.13 | 0.34 | 0.027 | 0.996 | 0.672 | 0.027 | 0.814 | 0.057 | 0.358 |
| | AB494792 | Firmicutes | Clostridia | Clostridiales | Lachnospiraceae | Butyrivibrio | 0.36 | 0.22 | 0.12 | 0.18 | 0.009 | 0.994 | 0.894 | 0.100 | 0.970 | 0.049 | 0.009 |
| | AB494805 | Firmicutes | Clostridia | Clostridiales | Lachnospiraceae | Butyrivibrio | 2.49 | 1.59 | 0.50 | 2.02 | 0.000 | 0.577 | 0.916 | 0.000 | 0.198 | 0.045 | 0.000 |
| | AM039826 | Firmicutes | Clostridia | Clostridiales | Lachnospiraceae | Butyrivibrio | 0.31 | 0.22 | 0.00 | 0.40 | 0.000 | 0.763 | 1.000 | 0.000 | 0.739 | 0.001 | 0.000 |
| | AB494919 | Firmicutes | Clostridia | Clostridiales | Lachnospiraceae | Pseudobutyrivibrio | 1.08 | 1.19 | 0.56 | 1.01 | 0.055 | 1.000 | 0.999 | 0.073 | 0.995 | 0.054 | 0.090 |
| | AB494866 | Firmicutes | Clostridia | Clostridiales | Lachnospiraceae | u. b. | 0.29 | 0.26 | 0.09 | 0.19 | 0.002 | 0.807 | 0.451 | 0.246 | 0.946 | 0.025 | 0.002 |
| | EF686527 | Firmicutes | Clostridia | Clostridiales | Ruminococcaceae | Saccharofermentans | 0.36 | 0.38 | 0.15 | 0.37 | 0.013 | 1.000 | 0.999 | 0.026 | 0.999 | 0.024 | 0.013 |
| | EU344218 | Firmicutes | Clostridia | Clostridiales | Ruminococcaceae | u. b. | 0.33 | 0.39 | 0.12 | 0.35 | 0.002 | 0.729 | 0.999 | 0.066 | 0.801 | 0.002 | 0.037 |
| | EU381706 | Firmicutes | Clostridia | Clostridiales | Ruminococcaceae | u. b. | 0.22 | 0.19 | 0.04 | 0.21 | 0.000 | 1.000 | 0.995 | 0.002 | 0.993 | 0.002 | 0.000 |
| | EU381583 | Firmicutes | Erysipelotrichi | Erysipelotrichales | Erysipelotrichaceae | u. b. | 0.62 | 0.49 | 0.00 | 0.57 | 0.000 | 0.958 | 0.999 | 0.000 | 0.918 | 0.000 | 0.000 |
| | N.R.OTU | Unassigned | | | | | 0.20 | 0.28 | 0.00 | 0.22 | 0.000 | 0.892 | 1.000 | 0.001 | 0.919 | 0.000 | 0.000 |
| Increased | N.R.OTU | Actinobacteria | Coriobacteriia | Coriobacteriales | Coriobacteriaceae | u.b. | 0.11 | 0.04 | 0.70 | 0.09 | 0.000 | 1.000 | 0.981 | 0.000 | 0.976 | 0.000 | 0.002 |
| | EU381847 | Bacteroidetes | Bacteroidia | Bacteroidales | Prevotellaceae | Prevotella | 0.24 | 0.23 | 0.64 | 0.19 | 0.003 | 0.926 | 0.885 | 0.003 | 1.000 | 0.031 | 0.035 |
| | N.R.OTU | Bacteroidetes | Bacteroidia | Bacteroidales | Prevotellaceae | Prevotella | 0.29 | 0.23 | 0.43 | 0.19 | 0.001 | 0.812 | 0.253 | 0.001 | 0.803 | 0.038 | 0.293 |
| | GU303955 | Cyanobacteria | SHA-109 | u. b. | | | 0.06 | 0.09 | 0.51 | 0.07 | 0.001 | 0.978 | 1.000 | 0.001 | 0.982 | 0.007 | 0.001 |
| | AB185771 | Firmicutes | Clostridia | Clostridiales | Lachnospiraceae | Incertae Sedis | 0.05 | 0.12 | 0.47 | 0.12 | 0.000 | 0.972 | 0.609 | 0.007 | 0.329 | 0.033 | 0.000 |
| | EU843661 | Firmicutes | Clostridia | Clostridiales | Lachnospiraceae | Oribacterium | 0.16 | 0.07 | 0.38 | 0.09 | 0.000 | 0.894 | 0.512 | 0.009 | 0.145 | 0.000 | 0.323 |
| | EU842536 | Firmicutes | Clostridia | Clostridiales | Lachnospiraceae | Roseburia | 0.29 | 0.30 | 0.55 | 0.26 | 0.031 | 0.971 | 1.000 | 0.031 | 0.985 | 0.110 | 0.036 |
| | AF001717 | Firmicutes | Clostridia | Clostridiales | Lachnospiraceae | u. b. | 0.16 | 0.16 | 0.57 | 0.12 | 0.006 | 0.874 | 0.954 | 0.006 | 0.994 | 0.074 | 0.030 |
| | AB270001 | Firmicutes | Clostridia | Clostridiales | Ruminococcaceae | Incertae Sedis | 0.37 | 0.12 | 0.61 | 0.17 | 0.000 | 0.933 | 0.777 | 0.002 | 0.395 | 0.000 | 0.050 |
| | AB185556 | Firmicutes | Clostridia | Clostridiales | Ruminococcaceae | u. b. | 1.36 | 1.34 | 3.25 | 1.63 | 0.000 | 0.958 | 0.923 | 0.008 | 1.000 | 0.001 | 0.000 |

[1]Oral rumen fluid samples were collected at day 56 ± 1.4 postpartum (p.p., mean ± SD). Median in %.

[2]All species were classified as “unculturable bacteria” (u.b.) at species level. N.R. OTU = New Reference OTU

[3]Animals were divided into a low condition (LC) and a high condition control (HC), monensin (MO) and essential oil (EO) group 6 weeks antepartum (a.p., n = 12). High conditioned animals (HC, MO, EO) were oversupplied with energy in dry period (60 vs. 20 % concentrate feed proportion) and subjected to a decelerated increase in concentrate feed proportion p.p. (from 30 to 50 % in 3 instead of 2 weeks) to increase p.p. lipolysis and induce a ketogenic metabolic state. The MO group received a monensin controlled release capsule 21 d a.p. and the EO group was fed with concentrate containing a blend of essential oils from 3 weeks a.p. on and during lactation.

[4] Nemenyi-Damico-Wolfe-Dunn test (Hollander and Wolfe, 1999)

**Table 6.** Characteristics of taxa influenced by monensin treatment

| | Taxonomy | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | Phylum | Class | Order | Family | Genus | Cell wall structure | Fermentation characteristics | Reference |
| Decreased | Bacteroidetes | Bacteroidia | Bacteroidales | BS11 gut group | | Gram stain negative | Butyrate and acetate production, hemicellulose fermentation, | Krieg et al. (2009), Solden et al. (2016) |
| | Bacteroidetes | Bacteroidia | Bacteroidales | Rikenellaceae | RC9 gut group | Gram-stain negative | Unknown, other members of the *Rikenellaceae* produce acetate and succinic acid, some also propionate (e.g. *Rikenella*) | Krieg et al. (2009) |
| | Firmicutes | Clostridia | Clostridiales | Lachnospiraceae | Butyrivibrio | Gram-stain negative, but structurally Gram-positive, lack trilamellar outer membrane | Butyrate is major end product, formate, acetate, lactate, no propionate production, fiber degraders (hemicellulose, xylans, starch and pectines) | Vos et al. (2009) |
| | Firmicutes | Clostridia | Clostridiales | Lachnospiraceae | Pseudobutyrivibrio | Gram-stain negative | Formate, butyrate, lactate and acetate, no propionate | Vos et al. (2009) |
| | Firmicutes | Clostridia | Clostridiales | Ruminococcaceae | Saccharofermentans | Gram-stain positive | Unknown. Other members of the *Ruminococcaceae* produce succinate (e.g. *Ruminococcus*) | Vos et al. (2009), Koike and Kobayashi (2009) |
| | Firmicutes | Erysipelotrichi | Erysipelotrichales | Erysipelotrichaceae | | Gram-stain positive | Weakly fermentative, acid but no gas produced from carbohydrates | Vos et al. (2009), |
| Increased | Actinobacteria | Coriobacteriia | Coriobacteriales | Coriobacteriaceae | | Gram-stain positive | Acetate, lactate and ethanol | Whitman et al. (2012) |
| | Bacteroidetes | Bacteroidia | Bacteroidales | Prevotellaceae | Prevotella | Gram-negative | Mainly succinate and acetate, highly active hemicellulolytic and proteolytic | Krieg et al. (2009), Thoetkiattikul et al. (2013) |
| | Cyanobacteria | SHA-109 | | | | Unknown. Cyanobacteria exhibit an overall gram-negative structure, but the peptidoglycan layer is considerably thicker than that of most gram-negative bacteria | Unknown | Hoiczyk and Hansel (2000) |
| | Firmicutes | Clostridia | Clostridiales | Lachnospiraceae | Incertae Sedis | Unknown | Unknown | |
| | Firmicutes | Clostridia | Clostridiales | Lachnospiraceae | Oribacterium | Gram-stain positive, may stain Gram-negative | Acetate and lactate | Vos et al. (2009) |
| | Firmicutes | Clostridia | Clostridiales | Lachnospiraceae | Roseburia | Gram-negative to Gram-variable staining reaction | $H_2$, $CO_2$, and large amounts of butyrate from fermentation of glucose and acetate | Vos et al. (2009) |
| | Firmicutes | Clostridia | Clostridiales | Ruminococcaceae | Incertae Sedis | Gram-stain positive | Unknown. Other members of the *Ruminococcaceae* produce succinate (e.g. *Ruminococcus*) | Vos et al. (2009), Koike and Kobayashi (2009) |

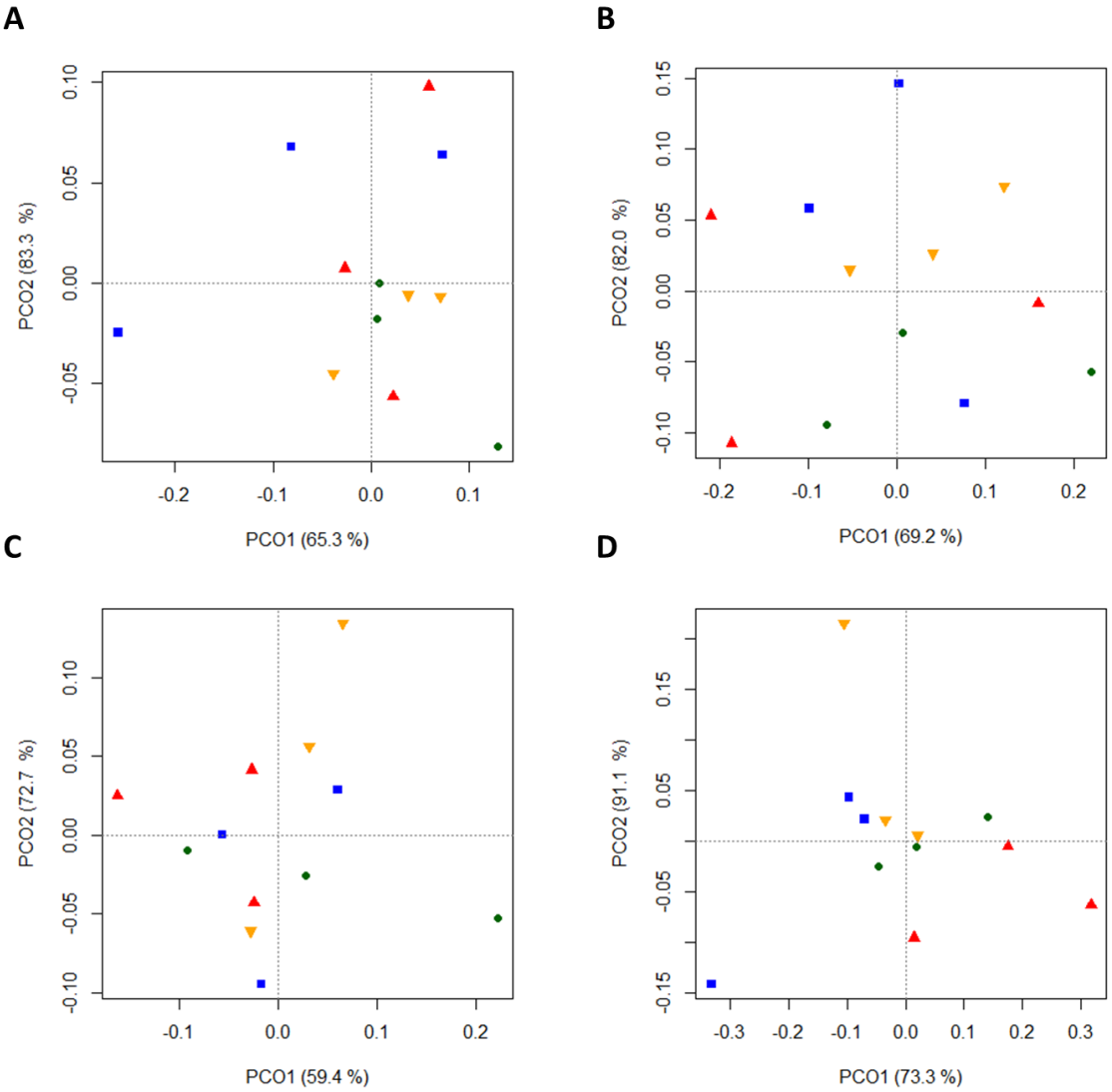



**Figure1.** Two dimensional PCO-plots from SSCP-gels of rumen liquid associated archaea (explained variance indicated in % on x- and y-axis). Animals were divided into a low condition (LC, blue/square) and a high condition control (HC, orange/downward triangle), monensin (MO, green/dot) and essential oil (EO, red/upward triangle) group 6 weeks antepartum (a.p., n = 12). High conditioned animals (HC, MO, EO) were oversupplied with energy in dry period (60 vs. 20 % concentrate feed proportion) and subjected to a decelerated increase in concentrate feed proportion postpartum (p.p., from 30 to 50 % in 3 instead of 2 weeks) to increase p.p. lipolysis and induce a ketogenic metabolic state. The MO group received a monensin controlled release capsule 21 d a.p. and the EO group was fed with concentrate containing a blend of essential oils from 3 weeks a.p. on and during lactation. Rumen liquid samples were collected at day 56 ± 1.4 p.p. (mean ± SD) from 48 animals (n = 12). The samples were then divided onto 4 SSCP gels (A-D, 12 samples per gel, n = 3). Significance: A: $P = 0.087$, B: $P = 0.561$, C: $P = 0.753$, D: $P = 0.175$.

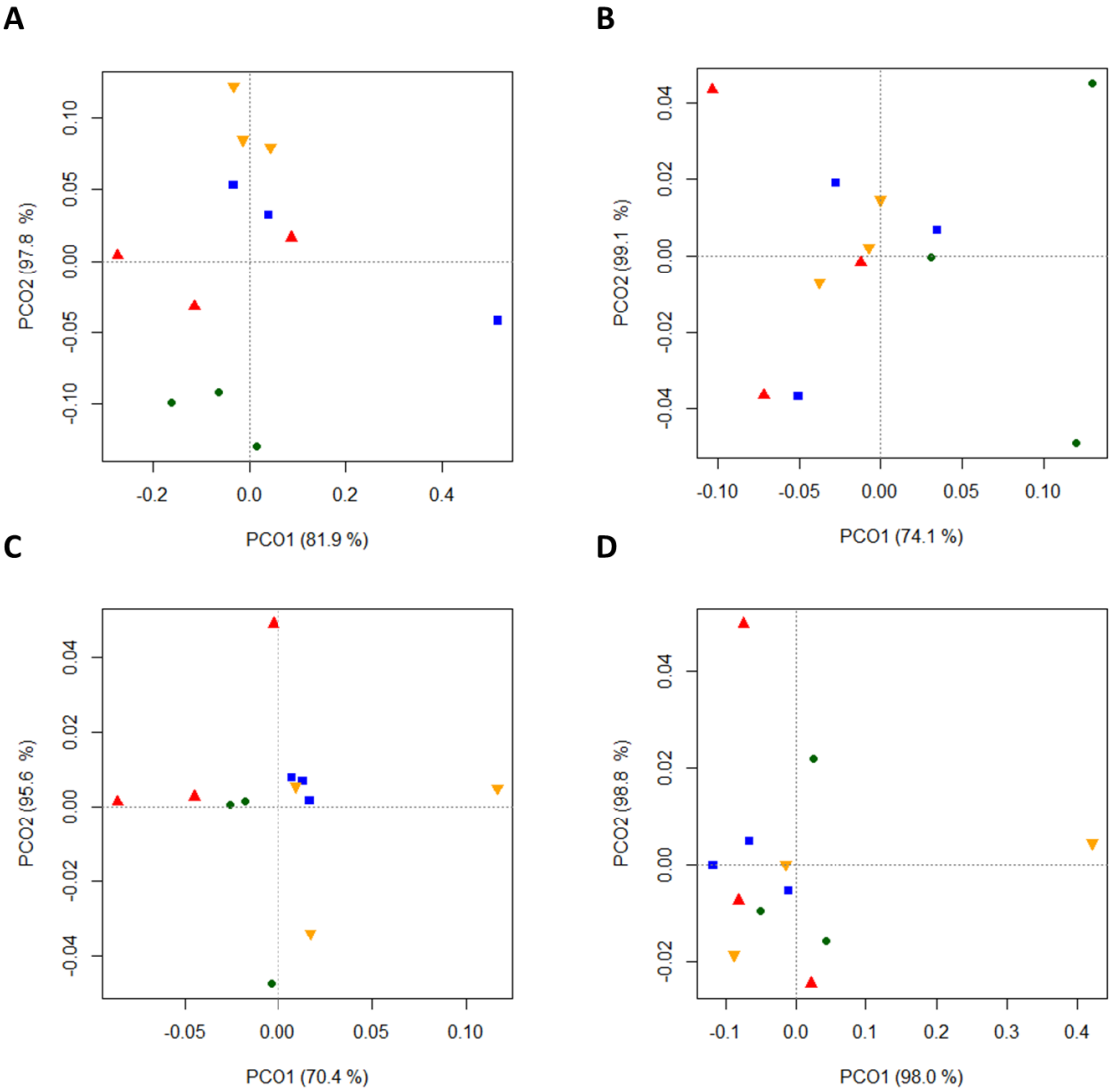



**Figure 2.** Two dimensional PCO-plots from SSCP-gels of rumen liquid associated bacteria (explained variance indicated in % on x- and y-axis). Animals were divided into a low condition (LC, blue/square) and a high condition control (HC, orange/downward triangle), monensin (MO, green/dot) and essential oil (EO, red/upward triangle) group 6 weeks antepartum (a.p., n = 12). High conditioned animals (HC, MO, EO) were oversupplied with energy in dry period (60 vs. 20 % concentrate feed proportion) and subjected to a decelerated increase in concentrate feed proportion postpartum (p.p., from 30 to 50 % in 3 instead of 2 weeks) to increase p.p. lipolysis and induce a ketogenic metabolic state. The MO group received a monensin controlled release capsule 21 d a.p. and the EO group was fed with concentrate containing a blend of essential oils from 3 weeks a.p. on and during lactation. Rumen liquid samples were collected at day 56 ± 1.4 p.p. (mean ± SD) from 48 animals (n = 12). The samples were then divided onto 4 SSCP gels (A-D, 12 samples per gel, n = 3). Significance: A: P = 0.042, B: P = 0.029, C: P = 0.082, D: P = 0.091.

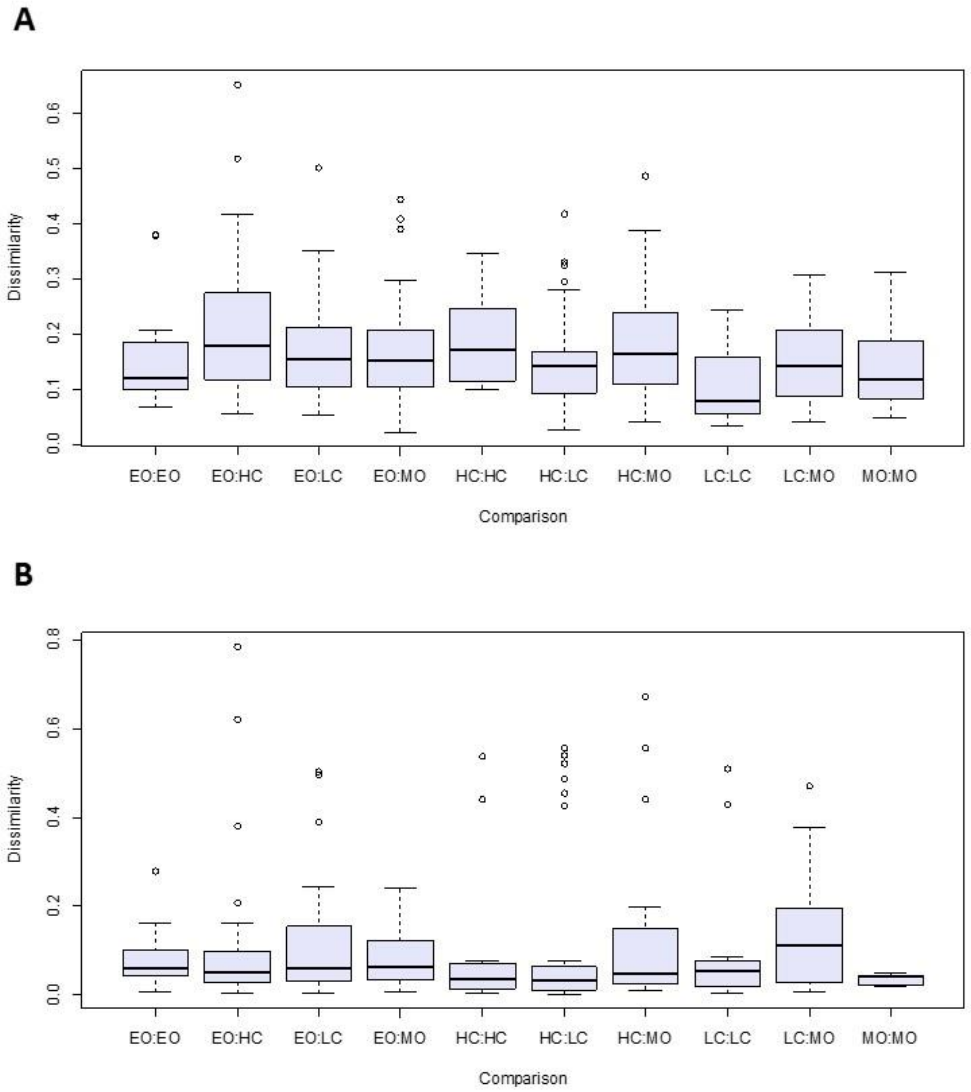



**Figure 3.** Boxplots summarizing the different comparisons across all 4 SSCP gels of rumen liquid associated archaea (**A**) and bacteria (**B**). Animals were divided into a low condition (LC) and a high condition control (HC), monensin (MO) and essential oil (EO) group 6 weeks antepartum (a.p., n = 12). High conditioned animals (HC, MO, EO) were oversupplied with energy in dry period (60 vs. 20 % concentrate feed proportion) and subjected to a decelerated increase in concentrate feed proportion postpartum (p.p., from 30 to 50 % in 3 instead of 2 weeks) to increase p.p. lipolysis and induce a ketogenic metabolic state. The MO group received a monensin controlled release capsule 21 d a.p. and the EO group was fed with concentrate containing a blend of essential oils from 3 weeks a.p. on and during lactation. Rumen liquid samples were collected at day 56 ± 1.4 p.p. (mean ± SD) from 48 animals (n = 12). The samples were then divided onto 4 SSCP gels (12 samples per gel, n = 3) for archaea and bacteria respectively.

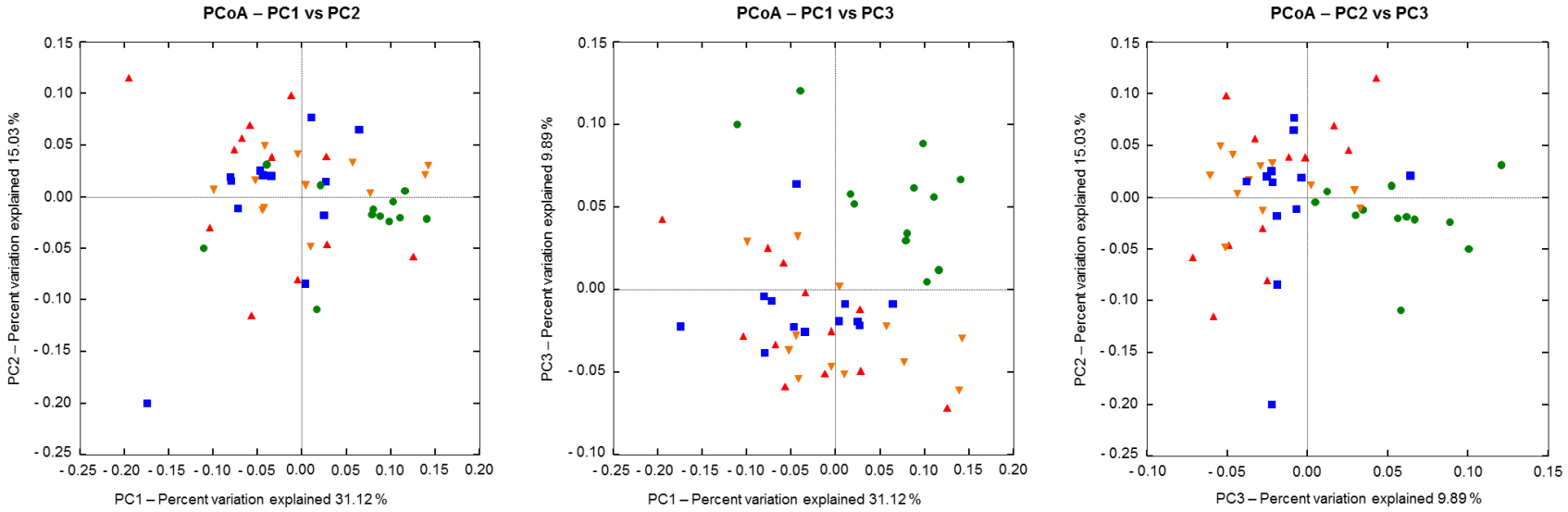
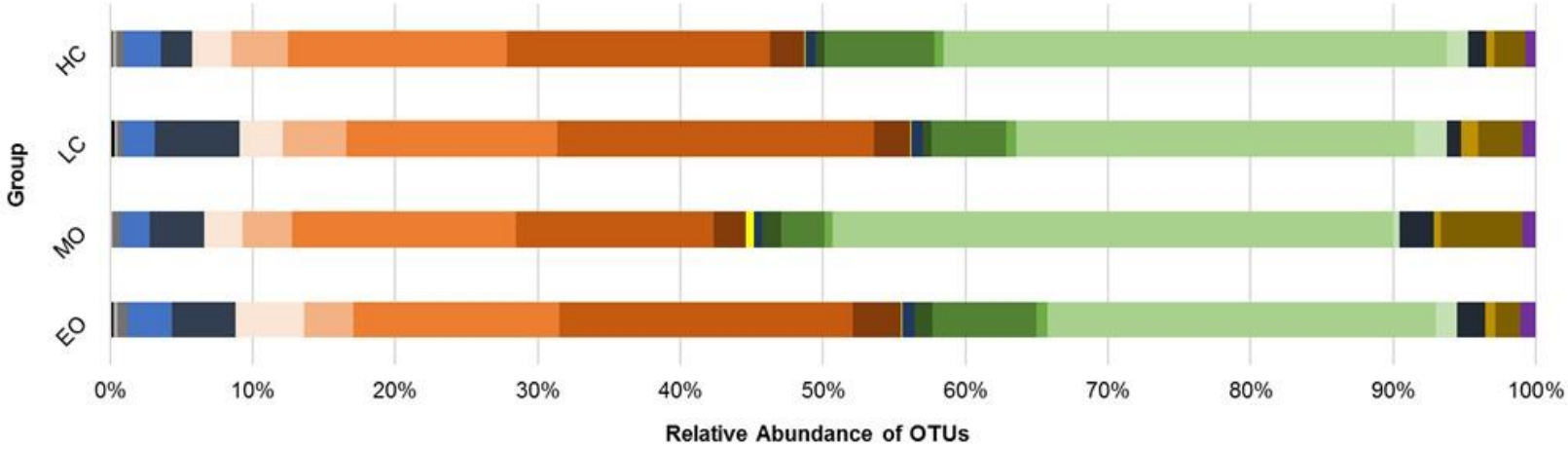



**Figure 4.** Two dimensional PCoA plots of 16S rRNA sequencing results of rumen liquid associated archaea and bacteria. Animals were divided into a low condition (LC, blue/square) and a high condition control (HC, orange/downward triangle), monensin (MO, green/dot) and essential oil (EO, red/upward triangle) group 6 weeks antepartum (a.p., n = 12). High conditioned animals (HC, MO, EO) were oversupplied with energy in dry period (60 vs. 20 % concentrate feed proportion) and subjected to a decelerated increase in concentrate feed proportion postpartum (p.p., from 30 to 50 % in 3 instead of 2 weeks) to increase p.p. lipolysis and induce a ketogenic metabolic state. The MO group received a monensin controlled release capsule 21 d a.p. and the EO group was fed with concentrate containing a blend of essential oils from 3 weeks a.p. on and during lactation. Rumen liquid samples were collected at day 56 ± 1.4 p.p. (mean ± SD) from 48 animals (n = 12).




| Taxonomy | Global | *P*-value EO:LC | EO:HC | EO:MO | LC:HC | MO:LC | MO:HC |
|---|---|---|---|---|---|---|---|
| Archaea - Euryarchaeota - Methanobacteria - Methanobacteriales – Methanobacteriaceae | 0.846 | - | - | - | - | - | - |
| Bacteria - Actinobacteria - Bifidobacteriales - Bifidobacteriaceae - Bifidobacterium | 0.141 | - | - | - | - | - | - |
| Bacteria - Actinobacteria - Bifidobacteriales - Bifidobacteriaceae - uncultured and other | 0.229 | - | - | - | - | - | - |
| Bacteria - Actinobacteria - Coriobacteriia - Coriobacteriales - Coriobacteriaceae | 0.025 | 0.610 | 0.913 | 0.425 | 0.937 | 0.024 | 0.108 |
| Bacteria - Bacteroidetes - Bacteroidia - Bacteroidales - BS11 gut group | 0.001 | 0.839 | 0.998 | 0.029 | 0.915 | 0.001 | 0.012 |
| Bacteria - Bacteroidetes - Bacteroidia - Bacteroidales - Prevotellaceae | 0.050 | 1.000 | 0.316 | 0.049 | 0.316 | 0.049 | 0.818 |
| Bacteria - Bacteroidetes - Bacteroidia - Bacteroidales - RF16 | 0.779 | - | - | - | - | - | - |
| Bacteria - Bacteroidetes - Bacteroidia - Bacteroidales - Rikenellaceae | 0.000 | 0.807 | 1.000 | 0.001 | 0.778 | 0.026 | 0.000 |
| Bacteria - Bacteroidetes - Bacteroidia - Bacteroidales - S24-7 | 0.064 | 0.948 | 0.978 | 0.227 | 0.999 | 0.063 | 0.082 |
| Bacteria - Candidate division TM7 | 0.440 | - | - | - | - | - | - |
| Bacteria - Cyanobacteria - SHA-109 | 0.001 | 0.970 | 1.000 | 0.001 | 0.982 | 0.006 | 0.001 |
| Bacteria - Firmicutes - Clostridia - Clostridiales - Family XIII Incertae Sedis | 0.342 | - | - | - | - | - | - |
| Bacteria - Firmicutes - Clostridia - Clostridiales - Lachnospiraceae | 0.007 | 0.948 | 0.825 | 0.006 | 0.991 | 0.043 | 0.085 |
| Bacteria - Firmicutes - Clostridia - Clostridiales - Ruminococcaceae | 0.981 | - | - | - | - | - | - |
| Bacteria - Firmicutes - Clostridia - Clostridiales - Veillonellaceae | 0.476 | - | - | - | - | - | - |
| Bacteria - Firmicutes - Erysipelotrichi - Erysipelotrichales - Erysipelotrichaceae | 0.151 | - | - | - | - | - | - |
| Bacteria - Proteobacteria - Gammaproteobacteria - Aeromonadales - Succinivibrionaceae | 0.183 | - | - | - | - | - | - |
| Bacteria - Spirochaetes - Spirochaetales - Spirochaetaceae - Treponema | 0.193 | - | - | - | - | - | - |
| Bacteria - Tenericutes - Mollicutes - Anaeroplasmatales - Anaeroplasmataceae | 0.192 | - | - | - | - | - | - |
| Bacteria - Tenericutes - Mollicutes - RF9 | 0.729 | - | - | - | - | - | - |
| Unassigned | 0.000 | 0.908 | 1.000 | 0.001 | 0.911 | 0.000 | 0.000 |

.**Figure 5.** Differences in relative abundances (expressed as percentages) of OTU derived from 16S rRNA gene sequencing on family level. Animals were divided into a low condition (LC) and a high condition control (HC), monensin (MO) and essential oil (EO) group 6 weeks antepartum (a.p., n = 12). High conditioned animals (HC, MO, EO) were oversupplied with energy in dry period (60 vs. 20 % concentrate feed proportion) and subjected to a decelerated increase in concentrate feed proportion postpartum (p.p., from 30 to 50 % in 3 instead of 2 weeks) to increase p.p. lipolysis and induce a ketogenic metabolic state. The MO group received a monensin controlled release capsule 21 d a.p. and the EO group was fed with concentrate containing a blend of essential oils from 3 weeks a.p. on and during lactation. Rumen liquid samples were collected at day 56 ± 1.4 p.p. (mean ± SD) from 48 animals (n = 12). For statistical analysis a Nemenyi-Damico-Wolfe-Dunn test was performed (Hollander and Wolfe, 1999) (Hollander and Wolfe, 1999) (Hollander and Wolfe, 1999) (Hollander and Wolfe, 1999) (Hollander and Wolfe, 1999) (Hollander and Wolfe, 1999) (Hollander and Wolfe, 1999) (Hollander and Wolfe, 1999) (Hollander and Wolfe, 1999) (Hollander and Wolfe 1999).

# 5. Interrelations between the rumen microbiota and production, behavioral, rumen fermentation, metabolic and immunological attributes of dairy cows

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State of publication: under revision at the *Journal of Dairy Science* (December 2017)

Contribution of authors:

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- Trial and project design: SD, UM, SK, JF, MS
- Trial implementation and sample collection: MS
- Sample analysis: MS
- Data analysis and interpretation: MS, JF, SK, UM, JH
- Writing of manuscript: MS
- Revision of manuscript: JF, SK, UM, JH, GB, SD

# Interrelations between the rumen microbiota and production, behavioral, rumen fermentation, metabolic and immunological attributes of dairy cows

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Submitted August 2017 at the *Journal of Dairy Science* (currently under revision)

## Abstract

Different studies have shown a strong correlation between the rumen microbiome and a range of production traits (e.g. feed efficiency, milk yield and components) in dairy cows. Underlying dynamics concerning cause and effect are, however, still widely unknown and warrant further investigation. The aim of the current study was to describe possible functional interrelations and pathways using a large set of variables describing the production, the metabolic and immunological state as well as the rumen microbiome and fermentation characteristics of dairy cows in early lactation (n = 36, day 56 ± 3d in milk). It was further hypothesized that the feed intake associated behavior may influence the ruminal fermentation pattern and a set of variables describing these individual animal attributes was included. Principal component analysis (PCA) as well as Spearman´s Rank correlations were conducted including a total of 265 variables. The attained plots describe several well-known associations between metabolic, immunological and production traits. Main drivers of variance within the dataset included milk production and efficiency, and rumen fermentation and microbiome diversity attributes, whereas behavioral, metabolic and immunological variables did not exhibit any strong interrelations with the other variables. The previously well documented strong correlation of production traits with distinct prokaryote groups was confirmed. This mainly included a negative correlation of OTU ascribed to the Prevotella genus with milk and fat yield and feed

efficiency. A central role of the animals´ feed intake behavior in this context could however not be affirmed. Furthermore, different methodological and interpretability aspects concerning the microbiome analysis by 16S rRNA gene sequencing, such as the discrepancy between taxonomic classification and functional communality, as well as the comparability with other studies, are discussed. It is concluded that, to further investigate the driving force that causes the difference between efficient and inefficient animals, studies including more sophisticated methods to describe phenotypical traits of the host (e.g. rumen physiology, metabolic and genetic aspects) as well as the rumen microbiome (e.g. Metagenome, -transcriptome, -proteome and Metabolome analysis) are needed.

***Key words:*** rumen microbiota, feed efficiency, behavior, immunology

## Introduction

The cow, being a ruminant, lives in a symbiotic relationship with her rumen microbiota. By ingesting feed, she delivers new substrates to the microorganisms, which in return produce valuable nutrients through fermentation and form a nutrient source themselves (Mizrahi, 2013). Early studies showed that the rumen microbial composition is very much determined by the feed composition, as well as feed intake pattern of the host (Bryant and Burkey, 1953; Warner, 1962; Mackie et al., 1978; Leedle et al., 1982). It was further described that especially the rumen protozoal population exhibits a host individuality (Kofoid and MacLennan, 1933; Eadie, 1962). With the advent of DNA fingerprinting and sequencing techniques this finding was extended to the rumen prokaryotes (Li et al., 2009; Kong et al., 2010; Welkie et al., 2010; Jami and Mizrahi, 2012). The current understanding of rumen microbial dynamics is that the rumen microbiota consists of a core and a variable microbiota (Wu et al., 2012; Creevey et al., 2014; Henderson et al., 2015). The core microbiota is found across a wide geographical range and consists of different taxa that increase or decrease in their abundance according to the diet fed (Henderson et al., 2015). It therefore constitutes a key element in the survival strategy of ruminants, by allowing fast and appropriate adaptation to new diets (redundancy and resilience: Weimer (2015), Solden et al. (2016b), Dieho et al. (2017a), Schären et al. (2017b)). It is thought that the variable or individual microbiota is a result of inter-animal variation in behavioral and genetic attributes, as well as environmental influences (Mizrahi, 2013; McCann et al., 2014a; Henderson et al., 2015; Weimer, 2015; Malmuthuge and Guan, 2017). Different studies have shown interrelations between production variables, such as feed efficiency (Guan et al., 2008; Zhou et al., 2009; Hernandez-Sanabria et al., 2010; Zhou et al., 2010; Carberry et al., 2012; Hernandez-Sanabria et al., 2012; Rius et al., 2012; McCann et al., 2014b; Myer et al., 2015; Jewell et al., 2015; Shabat et al., 2016; Li and Guan, 2017) and milk production

and composition (Jami et al., 2014; Lima et al., 2015), and the rumen microbiota. However, the underlying dynamics concerning cause and effect still need to be elucidated (Weimer, 2015; Malmuthuge and Guan, 2017).

Therefore, the aim of the current study was to investigate the associations between the rumen microbiota and a large set of variables describing the production, as well as the metabolic and immunological state of dairy cows in early lactation, plus behavioral attributes, attempting to describe possible functional interrelations and pathways.

## Material and Methods

Experimental work was conducted at the experimental station of the Institute of Animal Nutrition (Friedrich-Loeffler-Institute) in Brunswick, Germany. The experiment was carried out in accordance with the German Animal Welfare Act approved by the LAVES (Lower Saxony State Office for Consumer Protection and Food Safety, Germany).

### Experimental Design

The data was collected in a trial investigating the influence of monensin and a blend of essential oils on production, rumen fermentation and metabolic variables (Drong et al., 2016a), immunology (Drong et al., 2016b), and rumen microbiome (Schären et al., 2017a) of transition dairy cows. Sixty German Holstein cows were divided into a low (n=15) and a high condition group (n=45) at the beginning of the dry period according to their body condition score (BCS). The animals in the high condition group were further divided into a control group and two treatment groups (either receiving a blend of essential oils or a monensin controlled release bolus, n=15). The animals of the low condition group were then fed a normal transition ration (80 % roughage (50 % maize silage, 50 % grass silage) and 20 % concentrate based on DM content) during the dry period, and after calving a TMR with an initial concentrate feed proportion of 30 %, which was increased stepwise to 50 % of the daily ration within 2 weeks (details in Drong et al. (2016a)). The animals in the high condition group were exposed to a ketogenic ration by an oversupply with energy during the dry period (concentrate feed proportion of 60 %) and a subsequently decelerated increase in concentrate feed proportion p.p. (from 30 % to 50 % in 3 instead of 2 weeks, animal model described in Schulz et al. (2014)). Production data, blood, liver and rumen fermentation samples were collected troughout the trial at different points in time and showed that monensin increased the energy availability in the animal by increasing the ruminal propionate production, whereas the blend of essential oils failed to elicit any positive effect (Drong et al., 2016a; b). At day 56 postpartum (**p.p.**) oral rumen liquid samples were collected from 48 animals (n=12) to investigate the underlying microbial alterations. The PCR-single-strand-conformation-polymorphism (SSCP) and 16S rRNA gene amplicon sequencing analysis revealed alterations in the rumen

microbiota due to the monensin treatment, and corresponding with the results on animal level no effect of the blend of essential oils was observed (Schären et al., 2017a). At that stage of lactation (day 56 p.p.) no significant difference between the control and the essential oil groups on production, metabolic and rumen fermentation level was observed (except higher serum protein concentrations in the high condition control group), whereas the monensin treated animals exhibited lower serum BHB as well as higher rumen propionate concentrations (Schären et al., 2017a). Because the present study aimed to investigate the interrelations between phenotypic traits of dairy cows and their rumen microbiota at a normal physiological state, it was decided to exclude the animals of the monensin group in the current analysis. The animals in the two control and the essential oils group (n = 36) were considered as being representative for dairy cows at this stage of lactation with an average milk production of 30.7 ± 6.0 kg/d (fat %: 4.3 ± 0.7, protein %: 2.8 ± 0.2, means ± SD), DMI of 17.7 ± 2.9 kg/d, a BCS of 2.9 ± 0.4, and all measured metabolic variables in physiological ranges (e.g. serum BHB of 0.86 ± 0.36 mmol/L and fatty acids of 0.48 ± 0.29 mmol/L) (Schären et al., 2017a).

**Sample Collection and Analysis**

All variables presented in the manuscript were assessed in samples collected at day 56 p.p., a detailed description of sample collection and analysis has been published in Drong et al. (2016a; 2016b) and Schären et al. (2017a). The production data (milk yield, milk components and body weight (**BW**)) were summarized for the period day 56 ± 3 d p.p. and have previously been published in Schären et al. (2017a).

***Production and behavior variables.*** The cows were milked twice per day (0530 h and 1530 h) and the BW was recorded thereafter. The TMR was fed ad libidum (offered fresh daily at approximately 1100 h) and individual intakes were continuously recorded using electronic balance troughs (Insentec, B.V., Marknesse, The Netherlands). Additionally, a small amount of concentrate (2.18 ± 0.73 kg DM/cow/d, depending on the individual daily TMR intake to adjust to a concentrate feed proportion of 50 %) was fed using automated feeding stations (Insentec, B.V., Markenesse, The Netherlands). The cows were able to access the first half of their daily ration at midnight, the second half at 0800 h. The concentrate was fed in 100 g portions (a new portion was fed whenever the cow remained in the station, until ration limit was reached). The number and sizes of the ingested portions were recorded for TMR [number of meals per day (accessions when no feed was ingested excluded) = **TMR Freq**, average TMR intake per meal = **TMR Size**, variation in meal size = SD of TMR Size = **TMR SD**, average duration of a meal (time eating) = **TMR TE**, number of times cow accessed a weighing trough without eating (zero counts) = **TMR ZC**, average time spent at weighing trough when no feed was ingested (time zero) = **TMR TZ**], concentrate intake [**CI**, total CI from feeding station per day = **CI Tot**, number of meals per day at feeding station (accessions when no concentrate

was ingested excluded) = **CI Freq**, average CI per meal = **CI Size**, variation in meal size = SD of CI Size = **CI SD**, average duration of meals = **CI TE**, how often the cow was standing in the feeding station without concentrate allowance/not eating = **CI ZC**, average time spent in feeding station when no feed was ingested = **CI TZ**] and water intake [**WI**, times cow accessed water trough to drink (accessions when no water was ingested excluded) = **WI Freq**, average WI per drinking = **WI Size**, variation in amount of water ingested = SD of TMR Size = **WI SD**, average duration of drinking = **WI TD**, number of times cow accessed a water trough without drinking = **WI ZC**, average time spent at water trough when no water was ingested = **WI TZ]**. The BCS was assessed according to a 5-point scale (Edmonson et al., 1989). Milk samples were collected twice per week (weighed means of Monday evening & Tuesday morning and Thursday evening & Friday morning, stored at 4 °C until analysis). Milk component analysis included fat, protein, lactose and urea concentrations using an infrared milk analyzer (Milkoscan FT 6000, Foss Electric A/S, Hillerød, Denmark) and weighted daily means were calculated.

From the different production variables three feed efficiency estimators were calculated. For the first variable, the daily DMI was divided by the daily milk yield (**FE1** = inverse of gross feed efficiency = DMI/milk yield). For the second variable, the daily protein intake was divided by the daily milk protein production (**FE2** = protein intake/protein yield). The two variables exhibited a mean of 0.56 and 2.74 with an SD of 0.09 and 0.42, respectively. Also, the residual energy intake (**REI**) was calculated for each animal according to Hurley et al. (2016). Therefore, a piecewise regression construction was applied including different possible energy sinks to estimate the estimated net energy intake ($NEIntake_{Estimated}$), resulting in the following equation ($r^2 = 0.614$):

$$NEIntake_{Estimated} = -85.24+(1.45*MilkKG)+(38.40*MilkProtein\%)+(0.16*BW)+(-12.23*BCS)$$

Thereafter, the actual net energy intake ($NEIntake_{Actual}$) was calculated from energy content of the diet multiplied by the individual DMI. The individual REI was then calculated by subtraction of the two latter variables: $REI = NEIntake_{Estimated} - NEIntake_{Actual}$. The REI exhibited a mean of -3.06 with a SD of 13.04.

***Blood variables.*** Blood samples were collected from a *Vena jugularis externa* in a serum separating, an EDTA, and a heparin containing blood tube.

A complete blood count of each sample was performed within 2 h after sampling using an automated hematology analyzer [Celltac alpha MEK-6450, Nihon Kohden Corporation, Tokyo, Japan; including red blood cell count (**RBC**) and associated variables (MCV, MCH, MCHC), hemoglobin (**HGB**), hematocrit (**HCT**), white blood cell population (lymphocyte (**LY**), monocyte

(**MO**), eosinophile (**EO**) and granulocyte (**GR**) count, and the respective proportions (**LY%**, **MO%** and **GR%**)), Schären et al. (2016a)].

The serum was separated and stored at -80 °C before chemical analysis using an automatic clinical chemistry analyzer (Eurolyser CCA180, Eurolyser Diagnostica GmbH, Salzburg, Austria; including serum glucose, beta-hydroxy-butyrate (**BHB**), fatty acids (**FA**), urea, albumin, total protein, cholesterol, aspartate transaminase (**AST**), γ-glutamyltransferase (**γ-GT**), total bilirubin, glutamate dehydrogenase (**GLDH**) and triglyceride, Schären et al. (2016a)). Kynurenine (**Kyr**) and tryptophan (**Trp**) concentrations were determined via HPLC (Shimadzu, Kyoto, Japan; as described in detail in Drong et al. (2017)) and their ratio (**Kyr:Trp**) calculated.

Peripheral blood mononuclear cells (**PBMC**) were isolated from whole blood samples (heparinized blood) by gradient centrifugation and stored at -80 °C until analysis (Renner et al., 2011; Drong et al., 2016b). Cell metabolic activity and Concavalin A (**ConA**)-stimulated proliferation of PBMC were evaluated using the Alamar Blue (**AB**) assay (described in detail in Drong et al. (2016b)). The proliferation of PBMC (stimulation index ex vivo) was then calculated by the ratio between fluorescence of ConA-stimulated PBMC (**$AB_{stim}$**) and non-stimulated PBMC (**$AB_{unstim}$**): **SI** = (Fluorescence of ConA - Fluorescence of $AB_{stim}$)/(Fluorescence of $AB_{unstim}$) (Drong et al., 2016b). T-cell phenotyping was performed on whole blood samples by monoclonal antibodies staining (for CD4 and CD8, or isotype controls) and subsequent flow cytometry analysis (FACSCantoII, BD Bioscience, San Jose, CA, USA). The lymphoid populations were then identified according to their side- and forward-scattering properties and an estimated number of T-cells of each phenotype as well as their ratio (**$CD4^+$/$CD8^+$**) were calculated using the percentages obtained by the flow cytometer (Drong et al., 2016b). The capacity of polymorphonuclear leukocytes (**PMN**) and leukocytes to release reactive oxygen species (**ROS**) was measured using an assay based on the oxidation of the nonfluorescent dihydrorhodamine 123 (**DHR**) to the fluorescent metabolite rhodamine 123 (**R123**) by quantifying the mean fluorescence intensity (**MFI**, flow cytometry (FACSCantoII)), resulting in the basal amount of PMN and lymphocytes that oxidase DHR (**$R123\%_{unstim}$** and **R123+ Lym%**) and the amount of radicals that are produced per cell on average (**$R123\ MFI_{unstim}$** and **R123+ Lym MFI**). To elicit and quantify the maximum oxidative burst capacity the PMN were additionally stimulated in parallel with phorbol-12-mystristate-13-acetate (**PMA**), resulting in the population of PMN performing an oxidative burst (**$R123\%_{stim}$**) and the mean capacity per cell for the oxidative burst (**$R123\ MFI_{stim}$**) (Drong et al., 2016b).

**Rumen fermentation variables.** Rumen liquid samples (ca. 750 mL) were collected in the morning after milking (prior to feeding) orally using an oral rumen tube and a hand vacuum pump. Immediately after collection, pH was measured using a glass electrode (model: pH 525; WTW, Weilheim, Germany). Until further processing (approximately 1-2 h after sample

collection), samples for ammonia (**$NH_3$-N**), volatile fatty acids (VFA, total VFA concentration = VFA, acetate (**C2**), propionate (**C3**), butyrate (**C4**), isovalerate (**iC5**), valerate (**C5**), and their respective proportions (**C2%**, **C3%**, **C4%**, **iC5%**, **C5%**), and samples for LPS concentration were cooled to 4 °C. Volatile fatty acids were determined according to Koch et al. (2006) using a gas chromatograph (Gaschromatograph 5890 II, Hewlett Packard®, Böblingen, Germany) and $NH_3$-N was determined using steam distillation according to the Kjeldahl method (DIN38406-E5-2, Anonymous (1998)). Rumen lipopolysaccharide (**LPS**) concentrations were measured spectrophotometrically after centrifugation, heating and storage (-20 °C), using the *Limulus* amebocyte lysate (LAL) assay (Kinetic-QCL™, Lonza, Walkersville, MD, USA; following the manufacturer´s instructions) as described in Schären et al. (2016b) and Gozho et al. (2005)). Due to technical issues at the day of sample collection and storage, 11 rumen liquid samples for fermentation variable analysis were lost. The dataset therefore includes fermentation variables of n = 25 instead of the total 36 animals.

**Rumen microbiome analysis.** Rumen liquid samples for protozoal density assessment were fixed 1:1 with methylgreen-formalin solution (stored at 4 °C) directly after sample collection and protozoa were counted using a Fuchs-Rosenthal chamber under an optical microscope and differentiated into entodiniomorpha and holotrichia (Ogimoto and Imai, 1981). The rumen liquid samples for microbial analysis were stored at -20 °C and DNA extraction was performed as described in Schären et al. (2017a). Prokaryotes were separated from feed and debris trough several centrifugation steps (1 x 5 min at 600 g, 4 x 20 min at 27'000 g (4 °C) with resuspension between centrifugation steps in 0.9 % NaCl) and liquid shock frozen as little droplets. DNA extraction involved mechanical lysis by a bead beating method, incubation steps with lysozyme, sodiumdodecylsulfate, proteinase K and cetyltrimethylammoniumbromide, a protein purification step with (phenol)-chloroform-isoamylalcohol, and washing steps using the peqGold Tissue-Kit (peq lab, Erlangen, Germany). Samples were stored at 4 °C until further analysis. For sequencing, gDNA samples were sent to Microsynth AG (Balgach, Switzerland). For 16S sequencing library preparation the primers A519F (S-D-Arch-0519-a-S-15) and 802R (S-D-Bact-0785-b-A-18) were chosen (Klindworth et al., 2013) and amplified using the HiFi HotStart PCR Kit (Kapa Biosystems, Wilmington, MA, USA). Illumina Nextera Libraries were prepared according to the manufacturer's instruction (Illumina, San Diego, USA) and sequencing was performed on the Illumina MiSeq Sequencing System using the Illumina MiSeq reagent Kit v2 (2 x 250bp). De-multiplexing (using the Illumina MiSeq v2.5.1.3. reporter and cutadapt v1.8.1 software package (Martin, 2011)), read stitching (using FLASH v1.2.11 (Magoč and Salzberg, 2011)), de novo Chimera detection, identification and removal (using the Uchime v4.2 (Edgar et al., 2011) and Usearch v8.1.1861 (Edgar, 2010)), operational taxonomic units (**OTU**) clustering (based on 97 % sequence similarity, using Uclust (Edgar, 2010)), and taxonomic assignment (using QIIME v1.9.1 (Caporaso et al., 2010) and the SILVA

rRNA database v111 (Quast et al., 2013)) was performed. Singeltons were removed from the dataset to reduce bias introduced by sequencing errors. For downstream analysis, only OTU with a relative abundance of at least 0.1% were considered to guarantee a solid differentiation between artifact and true organism. The amplicon sequencing resulted in 12'206 ± 3'424 reads (after filtering, mean ± SD) per sample. In total, a number of 177 different species-level OTUs were identified (167 ± 6 (mean ± SD) different OTUs per sample). A list of the detected OTU, their corresponding taxonomic classification and proportional abundance is given in Table 1. Most OTUs could be taxonomically classified to the family level, while their genus or species level affiliation were "uncultured bacterium or archaeon" in many cases.

**Statistical Analysis**

All statistical analyses were performed in STATISTICA 12.0 (StatSoft, Inc. 2014, Tulsa, OK, USA). If variables were recorded more than once a week, means were calculated per cow and week (day 56 ± 3 p.p.) prior to statistical evaluation. To obtain a normal distribution, rumen LPS concentrations were logarithmically transformed prior to statistical analysis. Correlation coefficients between different variables were estimated using Spearman´s Rank correlation and results were considered significant at $P < 0.05$. Principal component analysis (**PCA**) was performed including all measured variables. Thereafter, for some variables (which clustered due to their close biological interrelation) only one representative variable was chosen, to obtain a clearly arranged graph. This was the case for following variables (representative variable chosen, followed by deleted variables in brackets): DMI (Starch Intake, Concentrate Intake, Protein Intake, $NEIntake_{Actual}$), HCT (HGB, MCV, MCH, MCHC), and LY, MO, GR (LY%, MO%, GR%, respectively).

## Results and Discussion

All data points were summarized in one "master-table" and a Spearman´s Rank correlation with all 265 variables was conducted, resulting in a table with 70,225 correlations (Appendix 10, available for download in supplements). Because the interpretation and the deduction of biological dynamics from such a table is difficult, one main PCA plot ("master-plot") was created, to gain a better overview of the data and the different interrelations (Figure 1, interactive document available for download in supplements). The "master-plot" is interpreted as follows: each dot represents one variable (grouped by color). The closer two variables are, the higher they are positively associated. The farther away two variables are, the more negatively they are associated. The more variables are located towards the middle of the plot, the less they contribute to the variation within the data and the smaller is their association with other variables (in this particular PCA plot). The "master-plot" was then divided into subjective

clusters being based on the spatial similarity and biological interpretations of the variables captured within them (Figure 1, Cluster 1-7). Their significance and interpretation are discussed in the following chapters. The “master-plot” was further used as a base to plot the significant correlations detected by the Spearman´s Rank correlations. For every single variable, a copy of the “master-plot” was created in which only the labels of those variables that correlate significantly with the respective main-variable are shown (positive correlations in green, negative correlations in orange, examples see Figures 2-7, all plots available for download in supplements: Appendix 1-8). To systematically guide the reader trough the data within the “master-plot”, the production variables and the different associated (well-known) biological interrelations are used as a starting point. From there on the associated data and thematic clusters (Cluster 1-7) will be discussed in an integrative manner in the following sections.

**Production Variables and Feed Efficiency**

In the lower left-hand corner of Figure 1, a cluster containing several variables related to milk production quantity (milk, protein and fat yield, and FCM) are observed (Cluster 1). These variables are also closely associated with the metabolic variables cholesterol and albumin, most likely due to their role in milk protein synthesis (example of plot for fat yield in Figure 2) (Busato et al., 2002; Kida, 2003). The protein% and protein yield are further associated with the blood tryptophan concentrations (Trp) which can be ascribed to an increased protein metabolism in animals with higher feed intake and milk production (plot available for download in Appendix 2 – Milk Production), rather than an altered immune status (alterations in the Kyr:Trp ratio may indicate an influence on immune reactivity (Laich et al., 2002)). Furthermore, as expected milk urea and serum urea correlate positively and are closely associated with rumen $NH_3$-N concentrations located within Cluster 1 (Appendix 2 and Figure 1).

Opposite to Cluster 1 a second, very defined, cluster can be identified in the upper right corner of the plot (Cluster 2) with the efficiency variables FE1 and FE2 in its center. The allocation of Cluster 1 and Cluster 2 in one axis, but in an opposite manner, can most likely be attributed to the fact that animals with a high milk production are generally more efficient (lower FE1 and FE2) since they need proportionally less energy for maintenance (Vandehaar, 1998). Both of these efficiency variables correlate significantly with the third one, the REI, which by itself, however, correlates with only a few other variables and prokaryotes, and due to its location within the plot (within Cluster 7, closer to the center) only accounts for a small part of the variation in the dataset. Aside from the efficiency variables FE1 and FE2 Cluster 2 is mainly composed of OTU belonging to the genus *Prevotella* (Label: E) and the variable F/B (the Firmicutes/Bacteroidetes ratio), due to the dominant abundance of *Prevotella* within the Bacteroidetes phylum. Also in the Spearman´s Rank correlations the two efficiency variables

FE1 and FE2 show a strong association with several *Prevotella* species (example for FE1 in Figure 3). These observations are very much in line with the data presented by Jami et al. (2014), showing a strong positive correlation between the F/B and fat yield, and Myer et al. (2015), observing a significant positive correlation between the abundance of Firmicutes and the feed efficiency of steers. Also, Jewell et al. (2015), Lima et al. (2015), Carberry et al. (2012) and McCann et al. (2014b) show a significant negative correlation between the abundance of Prevotellaceae or *Prevotella* and feed efficiency or milk production. Additionally, Jewell et al. (2015) illustrate that the association of the individual OTU (belonging to the *Prevotella* genus) may be positive or negative, illustrating possible differences in functional properties between members of a common genus (discussed in the section "taxonomic classification and functional commonality").

Moreover, similar to studies of Li and Guan (2017), Lima et al. (2015), Myer et al. (2015), McCann et al. (2014b), Shabat et al. (2016) and Jami et al. (2014), our dataset revealed only few positive correlations of different prokaryotes with feed efficiency and other production traits. Generally, the described associations are quite different across all named studies, and also our data does not line up consistently with what had been described before. We suggest that reasons for this may lie in different methodological aspects (discussed in the last section) and the different rations fed in the respective studies (Carberry et al., 2012; Henderson et al., 2015; Schären et al., 2017a).

**Behavior Variables and Lactation Effect**

As Myer et al. (2015) and Malmuthuge and Guan (2017) stated, it is not clear whether the observed patterns in the above described microbial groups are actively contributing to differences in feed efficiency and production, or if other host factors are the driving cause for the observed alterations in the microbiome. We therefore hypothesized that the animal feed intake behavior (e.g. meal size, duration and frequency) could play a central role in this context by influencing the ruminal fermentation pattern and included several behavior variables in the PCA analysis. All of these variables are, however, located in Cluster 7 and do not exhibit any clear association with the feed efficiency traits or microbial groups. Even variables that may predict dominance or social rank, such as the time spent at the concentrate feeding station or water bunk without any feed or water intake (Wierenga, 1990; Grant and Albright, 1995; Olofsson, 1999), only correlate with a few other variables (see Appendix 4 – Behavior). However, the recorded behavioral attributes only included aspects of feed and water intake. Because also the rumination and resting time may have a large influence on the rumen environment (Gao and Oba, 2014), these aspects should be included in future studies.

The number of meals per day decreased and the time spent eating and size of the meals increased with the lactation number, which indicates that older cows seemed to spend less time at the feed bunk while consuming larger quantities in one meal and highlights their social dominance (Grant and Albright, 1995). At this point it should be noted, that the study did not include any first lactation animals (heifers) and the observed effects can be attributed to age dependent alterations observed from the second lactation onwards. The lactation number also correlated negatively with several immunological variables, such as haptoglobin, SI and R123 $MFI_{stim}$, and positively with serum protein concentrations and HCT (Figure 4). We suggest that this can be attributed to the age dependent alterations in the immune system such as a lower reactivity and higher immunoglobulin content with increasing age (Stoop et al., 1969; Weiskopf et al., 2009; Tienken et al. 2015; Bühler et al. 2017). Besides, a small cluster of bacteria species (at the line between the squares B1 and B2 in Figure 4) are also positively associated with the lactation number. These bacteria are part of a larger cluster (formed by Cluster 4 and 6 illustrated in Figure 1) which represents an increase in species diversity, since they include the largest part of prokaryotes and the three diversity indices (number of species, and the Shannon and Chao1 index, detailed discussion in the next section). This is in line with earlier studies describing an increase in microbiome diversity with age (Jami et al., 2013; Jewell et al. 2015; Lima et al., 2015).

In sum, our dataset does not provide any conclusive pattern explaining the clearly emerging correlation between certain microbial species and production traits. We suggest that future studies should involve data collection concerning rumen fermentation variables (e.g. continuous fermentation variable recording, for example using rumen sensor technique, Duffield et al. (2004); Zebeli et al. (2008)) and concomitant bacterial abundance and metatranscriptome, -proteome and metabolome analysis (Jiang et al., 2016; AlZahal et al., 2017; Li and Guan, 2017; Wallace et al., 2017) to gain a more precise understanding of diurnal fermentation patterns and influencing factors. Also, aspects of the rumen wall physiology (absorption rate and capacity) might have a substantial influence on the rumen environment (Gäbel et al., 2002; Aschenbach et al., 2011) and these interrelations should be more deeply investigated using *in vivo* marker based methods for VFA absorption dynamics (Dijkstra et al., 1993; Júnior et al., 2006) and rumen epithelium gene expression analysis (Dieho et al. 2017b; Penner et al., 2009; Penner et al., 2011). Also, different studies have shown that there is a substantial animal genetic component underlying feed efficiency differences (Archer et al., 1999; Arthur et al., 2001; Spurlock et al., 2012) and possible underlying mechanisms such as differences in nutrient partitioning and metabolic rate (Bauman et al., 1985; Rauw et al., 1998; Drackley, 1999; Nkrumah et al., 2006) require further investigation.

**Microbial Diversity and Rumen Fermentation**

Concerning species diversity, the data shows a clear association between increased fermentation rate and a decrease in species diversity, illustrated by the clear opposite allocation of the fermentation variables in Cluster 3 and Cluster 4 (Figure 1). Cluster 3 contains the variables representing the total production of VFA such as VFA, C2, C3, C4, C5 and iC5 as well as the proportions of C3 (C3%) and C5 (C5%), surrounded by only few prokaryotes. These variables are known to increase under an increased fermentation rate, whereas the variables in Cluster 4 will concomitantly decrease (pH, C2/C3, C2%) (Bergman, 1990; Van Houtert, 1993). As described above, Cluster 4, together with Cluster 5 and 6 contain the largest part of the prokaryotes of the dataset, as well as the different species diversity indices. This is in line with various other studies showing a decrease in species diversity whenever the fermentation rate is increased, for example under the influence of an increased concentrate feeding (Bekele et al., 2010; Kong et al., 2010; McCann et al., 2014a; Dieho et al. 2017a, 2017; Tapio et al., 2017). In our dataset, this is most pronouncedly visible in the plot made for the Shannon index (Figure 5), where a clear negative correlation with the total VFA and C3 concentration stands opposite to a positive correlation with a range of prokaryotes in Cluster 4, 5 and 6.

These interrelations are further confirmed by the allocation of the only archaea present in this dataset (*Methanobrevibacter*, abbreviated with the letter A) in Cluster 5 (in the rectangle A3 at the edge of the circle). Methanobacteria rely on the hydrogen produced by other bacteria from a variety of carbon sources to produce methane by reduction of $CO_2$ (Valdez-Vazquez and Poggi-Varaldo, 2009; Patra, 2012). While the production of acetate is connected to the formation of reducing equivalents, propionate is considered a net sink for them (Czerkawski, 1986; Russell 2002). Therefore, under the circumstances of a slow fermentation rate (for example due to a low DMI or high fiber rations) expressed by a higher pH and increased species diversity also an increase in methane production can be observed as a consequence of higher acetate proportion (Popova et al., 2011; Patra, 2012; Shabat et al., 2016; Tapio et al., 2017). Interestingly, the *Methanobrevibacter* are not allocated in the center of Cluster 4, but at the edge of Cluster 5 and are not clearly associated with the other fermentation variables, except for pH (Figure 6). One possible explanation could be that the spot sampling of the rumen fluid is only partially representative for the rumen fermentation pattern throughout the day (Hall et al., 2015; Schären et al., 2016b) or that other variables seem to influence the *Methanobrevibacter* occurrence, which have not been covered with our dataset. This is supported by the relatively solitary position of the *Methanobrevibacter* in our plot. Different studies have shown that the abundance of methanogens is not necessarily altered due to alterations in the substrate availability for methanogenesis, as well as that methanogen

abundance is not necessarily linked to methane production (Hook et al., 2009; Popova et al., 2011; Singh et al., 2013; Schären et al., 2017a), and other complex biochemical interrelations or genetically driven host attributes may be responsible for changes in their abundance (Zhou et al., 2009; 2010; Roehe et al., 2016; Malmuthuge and Guan, 2017). In contrast, others showed a shift in methanogen diversity, but not necessarily total abundance, upon increased fermentation rate (e.g. due to increased concentrate feeding, Liu et al. (2012), Tapio et al. (2017)). Also, different other studies have shown an association between the methanogen abundance and diversity, gene expression, and feed efficiency (Zhou et al., 2009; 2010; Carberry et al., 2014; Roehe et al., 2016; Li and Guan, 2017). Both aspects could not be thoroughly investigated in our study due to the abundance threshold of 0.1 % in the filtering step during the bioinformatics (most archaea or methanogens exhibit a lower abundance and were therefore excluded from our analysis). Considering the importance of understanding the dynamics behind methanogenesis and mitigation strategies, future studies should investigate possible aspects concerning inter-animal variation as well as the genetic variation in the methanogenic community more closely (Zhou et al., 2009; Liu et al., 2012; Leahy et al., 2013; McAllister et al., 2015).

Furthermore, for the different VFA concentrations and proportions no strong correlation pattern can be observed in relation to certain prokaryote groups (plot see Appendix 7 – Rumen Fermentation). Molar acetate proportions correlate positively with serum triglyceride concentrations most likely due to its involvement in the fatty acid metabolism (Bergman, 1990). It is also positively associated with serum BHB concentrations, which may point towards an interrelation between slow rumen fermentation rate and an energy deficit (Bergman, 1990). Mainly OTU belonging to the taxon Firmicutes (*Butyrivibrio*, *Mogibacterium*, *Ruminococcaceae*, Family XIII *Incertae Sedis* and *Saccharofermentans*) exhibit a positive correlation with C2%. Molar butyrate proportions exhibit a positive association with several OTU ascribed to the *Prevotella*, *Bifidobacterium*, *Ruminococcus*, uncultured *Ruminococcaceae*, *Anaerovibrio*, and *Selenomonas* (Appendix 7). Interestingly, none of the members of the taxon *Butyrivibrio* were positively associated with the propionate proportion (C3%), in contrast to older studies appointing this genus a major role in the ruminal propionate production (Paillard et al., 2007). All these findings are only partially in line with other studies (Wang et al., 2012; Sandri et al., 2014; Dieho et al., 2017a) and the reason for observing only these few interrelations between rumen VFA concentrations and proportions could be that in our dataset the basal ration and the concentrate proportion was similar across all animals, therefore (strong) variation among rumen fermentation pattern and rates did most likely not occur. This aspect of variance contribution is discussed in detail in the following section.

**Minor Variance Contributing Variables - Metabolism and Immunology**

The “master-plot” (Figure 1) illustrates that the main drivers for variation in our dataset are milk production (Cluster 1), feed efficiency (Cluster 2), rumen fermentation (Cluster 3 and 4), microbiome diversity (Cluster 4, 5 and 6), and their associated prokaryotes. A large part of variables is however allocated rather near the center of the plot (Cluster 7), indicating that they contribute only little to the variation within the dataset. In this cluster we find, aside from the above mentioned behavioral variables, the largest part of the serum metabolic and immunological variables, as well as the rumen LPS concentrations. Our dataset therefore ascribes them only little influence or interrelation with the variability of the rumen microbiome and also the other variance driving variables. This is contrary to several other studies showing changes in these variables in connection with alterations in the rumen fermentation and microbiome, such as under the influence of a subacute rumen acidosis (SARA) (Gozho et al., 2005; Khafipour et al., 2009; Zebeli et al., 2011). However, it has to be noted, that the 36 animals included in this dataset were all healthy and in a steady metabolic condition (no (sub-)clinical ketosis, etc.) and therefore demonstrated only marginal variation in the health and metabolic variables. Also, the diet fed was not to be expected to elicit any SARA (Zebeli et al., 2008). We therefore suggest that these well documented interrelations would only emerge if the animals were metabolically or immunologically challenged.

In this context, it also needs to be discussed in general whether the role and interrelations we are ascribing in this particular dataset are ubiquitous or whether they possibly just represent a certain, relatively undisturbed, state. Morgavi et al. (2013) state that many basic ecological questions concerning the rumen microbiome have not been answered yet. For example, it is not clear whether numerically dominant rumen microbes also constitute the key members or if key functions might be carried out by rare members (the so called “rare biosphere”, with an abundance < 0.1 %). It might be the case that they only increase their abundance in certain situations (e.g. under the influence of a ration change or toxin degradation) or even carry out key functions despite their low abundance (Morgavi et al., 2013; Shabat et al., 2016). To investigate these subtler interrelations, larger studies across different diets and metabolic states, as well as repeated measurements and the use of different methods which include members of the rare biosphere (e.g. Metagenomics analysis or different filtering threshold within an amplicon sequencing method) are required to attain the variance and depth needed (Fouts et al., 2012; Malmuthuge and Guan, 2017).

**Taxonomic Classification, Functional Communality and Redundancy**

When analyzing the distribution of the OTU of a common taxon on the “master-plot” (Figure 1) it can be observed that some are very closely associated, whereas others are not. This is even more clearly visible when looking at the plots illustrating the Spearman´s Rank correlations of the different OTU with all the other variables included in the dataset (Appendix 8b-l). As an example, 4 different OTU from the *Prevotella* genus are shown in Figure 7 (abbreviated with the Letter E, all OTU of the *Prevotella* genus available in Appendix 8c). The different correlation distribution patterns on the “master-plot” illustrate that the different OTU, even though belonging to a common taxon, have different functional properties. Exemplary for this is the association of only some OTU ascribed to the *Prevotella* species with fat% (Appendix 2 – Milk Production) which is in line with a study of Jewell et al. (2015). Bekele et al. (2010) describe diet specific *Prevotella* species, each occupying a distinct metabolic niche, strongly supporting our data. Furthermore, also various older studies have shown that the genus *Prevotella* exhibits a broad range of fermentation products (Marounek and Duskova, 1999; Krieg et al. 2009; Emerson and Weimer 2017). Other examples in our dataset of taxa containing OTU exhibiting distinct correlation distribution patterns are: *Bifidobacterium*, BS11 gut group, RC9 gut group, candidate division TM7, Christensenellaceae, Family XIII *Incertae Sedis*, several taxa belonging to the Lachnospiraceae (e.g. *Butyrivibrio*), *Ruminococcus*, *Saccharofermentans*, Erysipelotrichaceae, Succinivibrionaceae and *Treponema* (see Appendix 8b-m). These findings are in line with studies showing that microbes that are phylogenetically close may still exhibit substantially different phenotypes and functions (Schären et al., 2017b) and important variation in microbial communities lies at a finer resolution than the genus or higher taxonomic levels (Myer et al., 2015). As discussed in Schären et al. (2017b), this discrepancy between phylogenetic (or genomic) commonality and phenotype can most likely be attributed to differences in gene expression as a result of environmental influences. Different authors have therefore suggested the revision of the polyphasic approach (integration of genotype and phenotype) in prokaryotic taxonomy and repeatedly stated that more effort should go into unravelling functional aspects of the rumen microbiome (Achenbach and Coates, 2000; Kampfer and Glaeser, 2012; Morgavi et al., 2013; Solden et al., 2016a).

An additional aspect in this context, which has not been addressed yet within this manuscript, is the functional redundancy of the different members of the rumen microbiome (Weimer 2015). Taxis et al. 2015 show, by integrating metabolic and metagenomic analyses of a rumen microbiota, that different community metabolic networks may exhibit the same metabolic inputs and outputs but differ in their internal structure. The ability of the different community members to carry out similar metabolic pathways and substitute each other in the different metabolic

niches represents a key feature for the plasticity of the rumen microbiome, however represents a major challenge in unravelling the different functional properties of the single members due to large confounding effects. Again, also this aspect illustrates that new biotechnical as well as computational methods are needed to capture and integrate the different interrelations, dynamics and pathways.

**Methodological Aspects and Remarks**

Concerning the methodological aspects, different studies have shown and discussed that depending on the sampling technique and time (Li et al., 2009; Kong et al., 2010; Welkie et al., 2010), the DNA extraction method and primer choice (Henderson et al., 2013; Klindworth et al., 2013; Albertsen et al., 2015), the sequencing platform (Goodwin et al., 2016) and the bioinformatical settings (Majaneva et al., 2015; Malmuthuge and Guan, 2017) used, the results may vary considerably (Schären et al., 2017a; Schären et al., 2017b). The results presented in this, as well as in other manuscripts, should therefore be interpreted within this context. Besides, the amplicon sequencing analysis applied in this study only covered the largest part of the prokaryotes (due to the primer chosen), however, it did not include a thorough differentiation of the protozoa (which were only evaluated using an optical counting method) nor an evaluation of the fungi or virome. Also, the rumen microbiome is subjected to spatial differences. Several studies have shown that the microbiome associated with feed particles and the rumen epithelium is distinct from the liquid associated microbiome (which is most frequently sampled for rumen microbiome analysis and which was also the focus of the present study), each comprising scarcely investigated characteristics and functions (Cho et al., 2006; Kim and Yu, 2012; Henderson et al. 2013; Malmuthuge and Guan, 2017; Schären et al., 2017b). And finally, it also needs to be considered that the rumen is only the first part of the gastro-intestinal system and that the microbial community in the lower gastro-intestinal tract most certainly also plays an important role in nutrient utilization (Myer et al., 2015). Only little is known about the relevance of these different aspects in the host-microbiome interaction and the upcoming of affordable and powerful new sequencing techniques should be used to attain more insight in these interrelations (McAllister et al., 2015; Jiang et al., 2016; Raszek et al., 2016; Malmuthuge and Guan, 2017; Wallace et al., 2017).

Weimer (2015) describes that a vast amount of studies has been published during the last decade analyzing interrelations between the gut microbiota and different clinical conditions (especially in humans) and eloquently states that we however *"are only occasionally reminded that the "conclusions" of these studies have almost always been based on association, rather than rigorous demonstration of cause and effect"*. This is also a point which needs to be emphasized when analyzing and discussing the dataset of the present study. The principal component together with the correlation analysis have shown that almost all variables in the

dataset are somehow interrelated, it is however often not clear what the driving force behind certain dynamics is (e.g. feed efficiency and microbiome, Malmuthuge and Guan (2017)). As highlighted in the different sections of the discussion, larger datasets including more variation (e.g. age, stage of lactation, diet) as well as greater data depth by using sophisticated methodologies are needed to further unravel these interrelations (McAllister et al., 2015; Solden et al., 2016a; Malmuthuge and Guan, 2017; Wallace et al., 2017).

## Conclusion

Our dataset describes several well-known associations between metabolic and production traits in healthy dairy cows and can therefore be considered a valid basis to investigate interrelations concerning the host-microbiome interaction. It confirms the previously described strong correlation of certain production traits (e.g. feed efficiency and milk production variables) with the rumen microbiome, as well as the association of fermentation rate with microbial diversity. The hypothesis that the feed intake pattern plays a key role in this context by influencing the ruminal fermentation pattern was, however, not confirmed and suggests that another undescribed driving force causes the distinct differences in the rumen microbiome between efficient and inefficient animals. Future studies should therefore include more sophisticated methods to describe phenotypical traits of the host (e.g. rumen physiology, metabolic and genetic aspects) as well as the rumen microbiome (e.g. Metagenome, -transcriptome, -proteome and Metabolome analysis).

## Acknowledgements

Many thanks go to the coworkers at the Institute of Animal Nutrition and the experimental station of the Friedrich-Loeffler-Institute (FLI, Brunswick, Germany) for caring for the experimental animals, assisting with experimental measurements as well as performing the analyses. The authors thank Caroline Drong for her contribution in sample analysis and data structuring. Special thanks go to Erik Bannert for his assistance and stamina in data sorting and analysis. The support of the members of the laboratory staff of the Department of Physiology (University of Veterinary Medicine Hannover, Germany) while performing the microbiota analyses is greatly acknowledged. The authors also want to thank Christian Hundsrucker and Pascal Weigold from Microsynth AG, as well as Andres Buser for their outstanding consultancy. The authors also want to thank Prof. Alexander Starke (University of Leipzig, Germany) for fruitful scientific discussion and for creating the space needed to finalize the manuscript.

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## Tables and Illustrations

**Table 1.** Summary of detected OTU and their proportional abundance

| | | Taxonomy[3] | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| **Label[1]** | **OTU ID[2]** | **Kingdom** | **Phylum** | **Class** | **Order** | **Family** | **Genus** | **%[4]** | **%[5]** |
| A | EF112194 | Archaea | Euryarchaeota | Methanobacteria | Methanobacteriales | Methanobacteriaceae | Methanobrevibacter | 0.857 | |
| B1 | EU779121 | Bacteria | Actinobacteria | Actinobacteria | Bifidobacteriales | Bifidobacteriaceae | u.b. | 0.439 | 3.70 |
| B2 | AB559503 | Bacteria | Actinobacteria | Actinobacteria | Bifidobacteriales | Bifidobacteriaceae | Bifidobacterium | 2.716 | |
| B3 | AM277978 | Bacteria | Actinobacteria | Actinobacteria | Bifidobacteriales | Bifidobacteriaceae | Bifidobacterium | 0.139 | |
| B4 | New.Ref.OTU | Bacteria | Actinobacteria | Actinobacteria | Bifidobacteriales | Bifidobacteriaceae | Bifidobacterium | 0.144 | |
| B5 | HQ842703 | Bacteria | Actinobacteria | Actinobacteria | Bifidobacteriales | Bifidobacteriaceae | Bifidobacterium | 0.266 | |
| C1 | New.Ref.OTU | Bacteria | Actinobacteria | Coriobacteriia | Coriobacteriales | Coriobacteriaceae | u.b. | 0.239 | 1.54 |
| C2 | AB270068 | Bacteria | Actinobacteria | Coriobacteriia | Coriobacteriales | Coriobacteriaceae | Atopobium | 0.196 | |
| C3 | EU469015 | Bacteria | Actinobacteria | Coriobacteriia | Coriobacteriales | Coriobacteriaceae | Atopobium | 0.613 | |
| C4 | AB270014 | Bacteria | Actinobacteria | Coriobacteriia | Coriobacteriales | Coriobacteriaceae | Atopobium | 0.494 | |
| D1 | AB185544 | Bacteria | Bacteroidetes | Bacteroidia | Bacteroidales | BS11 gut group | u.b. | 0.193 | 1.30 |
| D2 | EU773647 | Bacteria | Bacteroidetes | Bacteroidia | Bacteroidales | BS11 gut group | u.b. | 0.475 | |
| D3 | EF686531 | Bacteria | Bacteroidetes | Bacteroidia | Bacteroidales | BS11 gut group | u.b. | 0.263 | |
| D4 | AY244965 | Bacteria | Bacteroidetes | Bacteroidia | Bacteroidales | BS11 gut group | u.b. | 0.369 | |
| E1 | EF445210 | Bacteria | Bacteroidetes | Bacteroidia | Bacteroidales | Prevotellaceae | Prevotella | 0.169 | 30.11 |
| E2 | AB009187 | Bacteria | Bacteroidetes | Bacteroidia | Bacteroidales | Prevotellaceae | Prevotella | 0.363 | |
| E3 | EU845138 | Bacteria | Bacteroidetes | Bacteroidia | Bacteroidales | Prevotellaceae | Prevotella | 0.180 | |
| E4 | EU259377 | Bacteria | Bacteroidetes | Bacteroidia | Bacteroidales | Prevotellaceae | Prevotella | 2.339 | |
| E5 | AY850504 | Bacteria | Bacteroidetes | Bacteroidia | Bacteroidales | Prevotellaceae | Prevotella | 1.282 | |
| E6 | EF445293 | Bacteria | Bacteroidetes | Bacteroidia | Bacteroidales | Prevotellaceae | Prevotella | 0.249 | |
| E7 | AB009192 | Bacteria | Bacteroidetes | Bacteroidia | Bacteroidales | Prevotellaceae | Prevotella | 4.933 | |
| E8 | EU381847 | Bacteria | Bacteroidetes | Bacteroidia | Bacteroidales | Prevotellaceae | Prevotella | 0.326 | |
| E9 | AB269981 | Bacteria | Bacteroidetes | Bacteroidia | Bacteroidales | Prevotellaceae | Prevotella | 3.810 | |
| E10 | EU472961 | Bacteria | Bacteroidetes | Bacteroidia | Bacteroidales | Prevotellaceae | Prevotella | 0.215 | |

| | | Taxonomy[3] | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| **Label[1]** | **OTU ID[2]** | **Kingdom** | **Phylum** | **Class** | **Order** | **Family** | **Genus** | **%[4]** | **%[5]** |
| E11 | GQ326951 | Bacteria | Bacteroidetes | Bacteroidia | Bacteroidales | Prevotellaceae | Prevotella | 0.332 | |
| E12 | GQ326973 | Bacteria | Bacteroidetes | Bacteroidia | Bacteroidales | Prevotellaceae | Prevotella | 0.294 | |
| E13 | GQ327024 | Bacteria | Bacteroidetes | Bacteroidia | Bacteroidales | Prevotellaceae | Prevotella | 0.273 | |
| E14 | EF436359 | Bacteria | Bacteroidetes | Bacteroidia | Bacteroidales | Prevotellaceae | Prevotella | 0.953 | |
| E15 | AB185608 | Bacteria | Bacteroidetes | Bacteroidia | Bacteroidales | Prevotellaceae | Prevotella | 0.960 | |
| E16 | AY244948 | Bacteria | Bacteroidetes | Bacteroidia | Bacteroidales | Prevotellaceae | Prevotella | 0.183 | |
| E17 | AY244946 | Bacteria | Bacteroidetes | Bacteroidia | Bacteroidales | Prevotellaceae | Prevotella | 1.312 | |
| E18 | AF018469 | Bacteria | Bacteroidetes | Bacteroidia | Bacteroidales | Prevotellaceae | Prevotella | 0.205 | |
| E19 | GQ327306 | Bacteria | Bacteroidetes | Bacteroidia | Bacteroidales | Prevotellaceae | Prevotella | 0.484 | |
| E20 | AF018482 | Bacteria | Bacteroidetes | Bacteroidia | Bacteroidales | Prevotellaceae | Prevotella | 0.204 | |
| E21 | AB270138 | Bacteria | Bacteroidetes | Bacteroidia | Bacteroidales | Prevotellaceae | Prevotella | 2.485 | |
| E22 | AY244916 | Bacteria | Bacteroidetes | Bacteroidia | Bacteroidales | Prevotellaceae | Prevotella | 0.368 | |
| E23 | GQ327214 | Bacteria | Bacteroidetes | Bacteroidia | Bacteroidales | Prevotellaceae | Prevotella | 0.212 | |
| E24 | EU719226 | Bacteria | Bacteroidetes | Bacteroidia | Bacteroidales | Prevotellaceae | Prevotella | 0.962 | |
| E25 | EU381791 | Bacteria | Bacteroidetes | Bacteroidia | Bacteroidales | Prevotellaceae | Prevotella | 0.156 | |
| E26 | EU381948 | Bacteria | Bacteroidetes | Bacteroidia | Bacteroidales | Prevotellaceae | Prevotella | 0.177 | |
| E27 | AB034102 | Bacteria | Bacteroidetes | Bacteroidia | Bacteroidales | Prevotellaceae | Prevotella | 1.330 | |
| E28 | AB269968 | Bacteria | Bacteroidetes | Bacteroidia | Bacteroidales | Prevotellaceae | Prevotella | 3.065 | |
| E29 | GU302536 | Bacteria | Bacteroidetes | Bacteroidia | Bacteroidales | Prevotellaceae | Prevotella | 0.681 | |
| E30 | New.Ref.OTU | Bacteria | Bacteroidetes | Bacteroidia | Bacteroidales | Prevotellaceae | Prevotella | 0.270 | |
| E31 | New.Ref.OTU | Bacteria | Bacteroidetes | Bacteroidia | Bacteroidales | Prevotellaceae | Prevotella | 0.293 | |
| E32 | New.Ref.OTU | Bacteria | Bacteroidetes | Bacteroidia | Bacteroidales | Prevotellaceae | Prevotella | 0.286 | |
| E33 | New.Ref.OTU | Bacteria | Bacteroidetes | Bacteroidia | Bacteroidales | Prevotellaceae | Prevotella | 0.156 | |
| E34 | EU381920 | Bacteria | Bacteroidetes | Bacteroidia | Bacteroidales | Prevotellaceae | u.b. | 0.176 | |
| E35 | EU461494 | Bacteria | Bacteroidetes | Bacteroidia | Bacteroidales | Prevotellaceae | u.b. | 0.427 | |
| F | AY244938 | Bacteria | Bacteroidetes | Bacteroidia | Bacteroidales | RF16 | u.b. | 0.618 | |
| G1 | AB494890 | Bacteria | Bacteroidetes | Bacteroidia | Bacteroidales | Rikenellaceae | RC9 gut group | 0.299 | 5.37 |
| G2 | DQ394621 | Bacteria | Bacteroidetes | Bacteroidia | Bacteroidales | Rikenellaceae | RC9 gut group | 0.845 | |

| | | Taxonomy[3] | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| **Label[1]** | **OTU ID[2]** | **Kingdom** | **Phylum** | **Class** | **Order** | **Family** | **Genus** | **%[4]** | **%[5]** |
| G3 | FJ028738 | Bacteria | Bacteroidetes | Bacteroidia | Bacteroidales | Rikenellaceae | RC9 gut group | 0.172 | |
| G4 | EU842535 | Bacteria | Bacteroidetes | Bacteroidia | Bacteroidales | Rikenellaceae | RC9 gut group | 0.855 | |
| G5 | GU304085 | Bacteria | Bacteroidetes | Bacteroidia | Bacteroidales | Rikenellaceae | RC9 gut group | 0.369 | |
| G6 | HQ008599 | Bacteria | Bacteroidetes | Bacteroidia | Bacteroidales | Rikenellaceae | RC9 gut group | 0.197 | |
| G7 | AY244944 | Bacteria | Bacteroidetes | Bacteroidia | Bacteroidales | Rikenellaceae | RC9 gut group | 0.239 | |
| G8 | AB494915 | Bacteria | Bacteroidetes | Bacteroidia | Bacteroidales | Rikenellaceae | RC9 gut group | 0.886 | |
| G9 | EU719222 | Bacteria | Bacteroidetes | Bacteroidia | Bacteroidales | Rikenellaceae | RC9 gut group | 0.174 | |
| G10 | GU302529 | Bacteria | Bacteroidetes | Bacteroidia | Bacteroidales | Rikenellaceae | RC9 gut group | 0.564 | |
| G11 | EU719287 | Bacteria | Bacteroidetes | Bacteroidia | Bacteroidales | Rikenellaceae | RC9 gut group | 0.197 | |
| G12 | GU302534 | Bacteria | Bacteroidetes | Bacteroidia | Bacteroidales | Rikenellaceae | RC9 gut group | 0.236 | |
| G13 | New.Ref.OTU | Bacteria | Bacteroidetes | Bacteroidia | Bacteroidales | Rikenellaceae | RC9 gut group | 0.336 | |
| H | EU470196 | Bacteria | Bacteroidetes | Bacteroidia | Bacteroidales | S24-7 | u.b. | 0.886 | |
| J1 | GQ327541 | Bacteria | Candidate division TM7 | u.b. | u.b. | u.b. | u.b. | 0.193 | 0.68 |
| J2 | EU474584 | Bacteria | Candidate division TM7 | u.b. | u.b. | u.b. | u.b. | 0.266 | |
| J3 | EU381496 | Bacteria | Candidate division TM7 | u.b. | u.b. | u.b. | u.b. | 0.224 | |
| K | GU303955 | Bacteria | Cyanobacteria | SHA-109 | u.b. | u.b. | u.b. | 0.184 | |
| L1 | EF436353 | Bacteria | Firmicutes | Clostridia | Clostridiales | Christensenellaceae | u.b. | 0.657 | 7.41 |
| L2 | AB270057 | Bacteria | Firmicutes | Clostridia | Clostridiales | Christensenellaceae | u.b. | 0.924 | |
| L3 | AB185717 | Bacteria | Firmicutes | Clostridia | Clostridiales | Christensenellaceae | u.b. | 0.277 | |
| L4 | EU468616 | Bacteria | Firmicutes | Clostridia | Clostridiales | Christensenellaceae | u.b. | 0.201 | |
| L5 | AY854343 | Bacteria | Firmicutes | Clostridia | Clostridiales | Christensenellaceae | u.b. | 0.280 | |
| L6 | AB185553 | Bacteria | Firmicutes | Clostridia | Clostridiales | Christensenellaceae | u.b. | 3.642 | |
| L7 | AB494899 | Bacteria | Firmicutes | Clostridia | Clostridiales | Christensenellaceae | u.b. | 0.643 | |
| L8 | AB270004 | Bacteria | Firmicutes | Clostridia | Clostridiales | Christensenellaceae | u.b. | 0.785 | |
| M1 | EU842291 | Bacteria | Firmicutes | Clostridia | Clostridiales | Family XIII Incertae Sedis | Mogibacterium | 0.706 | 2.44 |
| M2 | AY854273 | Bacteria | Firmicutes | Clostridia | Clostridiales | Family XIII Incertae Sedis | Mogibacterium | 0.465 | |
| M3 | FJ682205 | Bacteria | Firmicutes | Clostridia | Clostridiales | Family XIII Incertae Sedis | Mogibacterium | 0.438 | |
| M4 | GU303061 | Bacteria | Firmicutes | Clostridia | Clostridiales | Family XIII Incertae Sedis | Mogibacterium | 0.204 | |

| | | Taxonomy[3] | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| **Label[1]** | **OTU ID[2]** | **Kingdom** | **Phylum** | **Class** | **Order** | **Family** | **Genus** | **%[4]** | **%[5]** |
| M5 | JF797395 | Bacteria | Firmicutes | Clostridia | Clostridiales | Family XIII Incertae Sedis | u.b. | 0.235 | |
| M6 | GU120128 | Bacteria | Firmicutes | Clostridia | Clostridiales | Family XIII Incertae Sedis | u.b. | 0.200 | |
| M7 | AB185628 | Bacteria | Firmicutes | Clostridia | Clostridiales | Family XIII Incertae Sedis | u.b. | 0.195 | |
| N1 | AB494806 | Bacteria | Firmicutes | Clostridia | Clostridiales | Lachnospiraceae | u.b. | 0.807 | 3.07 |
| N2 | New.Ref.OTU | Bacteria | Firmicutes | Clostridia | Clostridiales | Lachnospiraceae | u.b. | 0.235 | |
| N3 | EU773612 | Bacteria | Firmicutes | Clostridia | Clostridiales | Lachnospiraceae | u.b. | 0.227 | |
| N4 | EU381579 | Bacteria | Firmicutes | Clostridia | Clostridiales | Lachnospiraceae | u.b. | 0.205 | |
| N5 | AB494866 | Bacteria | Firmicutes | Clostridia | Clostridiales | Lachnospiraceae | u.b. | 0.208 | |
| N6 | EU466207 | Bacteria | Firmicutes | Clostridia | Clostridiales | Lachnospiraceae | u.b. | 0.159 | |
| N7 | AY854272 | Bacteria | Firmicutes | Clostridia | Clostridiales | Lachnospiraceae | u.b. | 0.287 | |
| N8 | AF001717 | Bacteria | Firmicutes | Clostridia | Clostridiales | Lachnospiraceae | u.b. | 0.254 | |
| N9 | AB494778 | Bacteria | Firmicutes | Clostridia | Clostridiales | Lachnospiraceae | u.b. | 0.682 | |
| O1 | AB494822 | Bacteria | Firmicutes | Clostridia | Clostridiales | Lachnospiraceae | Acetitomaculum | 0.412 | 1.18 |
| O2 | AB185642 | Bacteria | Firmicutes | Clostridia | Clostridiales | Lachnospiraceae | Acetitomaculum | 0.769 | |
| P1 | AB494935 | Bacteria | Firmicutes | Clostridia | Clostridiales | Lachnospiraceae | Butyrivibrio | 0.224 | 5.32 |
| P2 | AM039826 | Bacteria | Firmicutes | Clostridia | Clostridiales | Lachnospiraceae | Butyrivibrio | 0.234 | |
| P3 | EU469259 | Bacteria | Firmicutes | Clostridia | Clostridiales | Lachnospiraceae | Butyrivibrio | 0.267 | |
| P4 | AB494792 | Bacteria | Firmicutes | Clostridia | Clostridiales | Lachnospiraceae | Butyrivibrio | 0.216 | |
| P5 | AB494765 | Bacteria | Firmicutes | Clostridia | Clostridiales | Lachnospiraceae | Butyrivibrio | 0.201 | |
| P6 | AB494805 | Bacteria | Firmicutes | Clostridia | Clostridiales | Lachnospiraceae | Butyrivibrio | 1.630 | |
| P7 | AB034052 | Bacteria | Firmicutes | Clostridia | Clostridiales | Lachnospiraceae | Butyrivibrio | 1.558 | |
| P8 | GQ327740 | Bacteria | Firmicutes | Clostridia | Clostridiales | Lachnospiraceae | Butyrivibrio | 0.255 | |
| P9 | GU303299 | Bacteria | Firmicutes | Clostridia | Clostridiales | Lachnospiraceae | Butyrivibrio | 0.157 | |
| P10 | FJ172809 | Bacteria | Firmicutes | Clostridia | Clostridiales | Lachnospiraceae | Butyrivibrio | 0.209 | |
| P11 | AB494833 | Bacteria | Firmicutes | Clostridia | Clostridiales | Lachnospiraceae | Butyrivibrio | 0.176 | |
| P12 | EU843345 | Bacteria | Firmicutes | Clostridia | Clostridiales | Lachnospiraceae | Butyrivibrio | 0.197 | |
| Q | AY854354 | Bacteria | Firmicutes | Clostridia | Clostridiales | Lachnospiraceae | Howardella | 0.296 | |
| R1 | GU124460 | Bacteria | Firmicutes | Clostridia | Clostridiales | Lachnospiraceae | Incertae Sedis | 0.340 | 3.28 |

| | | Taxonomy[3] | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| **Label[1]** | **OTU ID[2]** | **Kingdom** | **Phylum** | **Class** | **Order** | **Family** | **Genus** | **%[4]** | **%[5]** |
| R2 | AF001722 | Bacteria | Firmicutes | Clostridia | Clostridiales | Lachnospiraceae | Incertae Sedis | 0.444 | |
| R3 | AB185771 | Bacteria | Firmicutes | Clostridia | Clostridiales | Lachnospiraceae | Incertae Sedis | 0.192 | |
| R4 | EU845624 | Bacteria | Firmicutes | Clostridia | Clostridiales | Lachnospiraceae | Incertae Sedis | 0.223 | |
| R5 | EU381578 | Bacteria | Firmicutes | Clostridia | Clostridiales | Lachnospiraceae | Incertae Sedis | 0.194 | |
| R6 | New.Ref.OTU | Bacteria | Firmicutes | Clostridia | Clostridiales | Lachnospiraceae | Incertae Sedis | 0.259 | |
| R7 | DQ237938 | Bacteria | Firmicutes | Clostridia | Clostridiales | Lachnospiraceae | Incertae Sedis | 1.027 | |
| R8 | EF436345 | Bacteria | Firmicutes | Clostridia | Clostridiales | Lachnospiraceae | Incertae Sedis | 0.317 | |
| R9 | EF436445 | Bacteria | Firmicutes | Clostridia | Clostridiales | Lachnospiraceae | Incertae Sedis | 0.282 | |
| S | EU843661 | Bacteria | Firmicutes | Clostridia | Clostridiales | Lachnospiraceae | Oribacterium | 0.176 | |
| T1 | DQ085079 | Bacteria | Firmicutes | Clostridia | Clostridiales | Lachnospiraceae | Pseudobutyrivibrio | 0.221 | 1.18 |
| T2 | AB494919 | Bacteria | Firmicutes | Clostridia | Clostridiales | Lachnospiraceae | Pseudobutyrivibrio | 0.957 | |
| U1 | FJ032427 | Bacteria | Firmicutes | Clostridia | Clostridiales | Lachnospiraceae | Roseburia | 0.921 | 2.68 |
| U2 | AB034119 | Bacteria | Firmicutes | Clostridia | Clostridiales | Lachnospiraceae | Roseburia | 0.254 | |
| U3 | EU842536 | Bacteria | Firmicutes | Clostridia | Clostridiales | Lachnospiraceae | Roseburia | 0.350 | |
| U4 | AF371623 | Bacteria | Firmicutes | Clostridia | Clostridiales | Lachnospiraceae | Roseburia | 1.158 | |
| V | EF445237 | Bacteria | Firmicutes | Clostridia | Clostridiales | Lachnospiraceae | Syntrophococcus | 0.299 | |
| W | AB270001 | Bacteria | Firmicutes | Clostridia | Clostridiales | Ruminococcaceae | Incertae Sedis | 0.316 | |
| X1 | EF686593 | Bacteria | Firmicutes | Clostridia | Clostridiales | Ruminococcaceae | Ruminococcus | 0.658 | 4.85 |
| X2 | EF436321 | Bacteria | Firmicutes | Clostridia | Clostridiales | Ruminococcaceae | Ruminococcus | 0.951 | |
| X3 | EU469842 | Bacteria | Firmicutes | Clostridia | Clostridiales | Ruminococcaceae | Ruminococcus | 0.169 | |
| X4 | DQ673486 | Bacteria | Firmicutes | Clostridia | Clostridiales | Ruminococcaceae | Ruminococcus | 0.305 | |
| X5 | EU468942 | Bacteria | Firmicutes | Clostridia | Clostridiales | Ruminococcaceae | Ruminococcus | 0.176 | |
| X6 | EU381458 | Bacteria | Firmicutes | Clostridia | Clostridiales | Ruminococcaceae | Ruminococcus | 0.279 | |
| X7 | EU474863 | Bacteria | Firmicutes | Clostridia | Clostridiales | Ruminococcaceae | Ruminococcus | 0.174 | |
| X8 | AAQK01009861 | Bacteria | Firmicutes | Clostridia | Clostridiales | Ruminococcaceae | Ruminococcus | 2.132 | |
| Y1 | EF686527 | Bacteria | Firmicutes | Clostridia | Clostridiales | Ruminococcaceae | Saccharofermentans | 0.313 | 1.91 |
| Y2 | GQ327231 | Bacteria | Firmicutes | Clostridia | Clostridiales | Ruminococcaceae | Saccharofermentans | 0.340 | |

| | | Taxonomy[3] | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| **Label[1]** | **OTU ID[2]** | **Kingdom** | **Phylum** | **Class** | **Order** | **Family** | **Genus** | **%[4]** | **%[5]** |
| Y3 | AB494824 | Bacteria | Firmicutes | Clostridia | Clostridiales | Ruminococcaceae | Saccharofermentans | 0.780 | |
| Y4 | EU381703 | Bacteria | Firmicutes | Clostridia | Clostridiales | Ruminococcaceae | Saccharofermentans | 0.258 | |
| Y5 | AB034038 | Bacteria | Firmicutes | Clostridia | Clostridiales | Ruminococcaceae | Saccharofermentans | 0.214 | |
| Z1 | AF371808 | Bacteria | Firmicutes | Clostridia | Clostridiales | Ruminococcaceae | u.b. | 0.153 | 6.86 |
| Z2 | EU468242 | Bacteria | Firmicutes | Clostridia | Clostridiales | Ruminococcaceae | u.b. | 0.237 | |
| Z3 | EU344218 | Bacteria | Firmicutes | Clostridia | Clostridiales | Ruminococcaceae | u.b. | 0.297 | |
| Z4 | AB494879 | Bacteria | Firmicutes | Clostridia | Clostridiales | Ruminococcaceae | u.b. | 1.830 | |
| Z5 | EU381706 | Bacteria | Firmicutes | Clostridia | Clostridiales | Ruminococcaceae | u.b. | 0.164 | |
| Z6 | AB270093 | Bacteria | Firmicutes | Clostridia | Clostridiales | Ruminococcaceae | u.b. | 0.227 | |
| Z7 | EU381964 | Bacteria | Firmicutes | Clostridia | Clostridiales | Ruminococcaceae | u.b. | 0.172 | |
| Z8 | EU381950 | Bacteria | Firmicutes | Clostridia | Clostridiales | Ruminococcaceae | u.b. | 0.503 | |
| Z9 | FJ508236 | Bacteria | Firmicutes | Clostridia | Clostridiales | Ruminococcaceae | u.b. | 0.266 | |
| Z10 | AB185556 | Bacteria | Firmicutes | Clostridia | Clostridiales | Ruminococcaceae | u.b. | 1.909 | |
| Z11 | AY854363 | Bacteria | Firmicutes | Clostridia | Clostridiales | Ruminococcaceae | u.b. | 0.336 | |
| Z12 | AB185810 | Bacteria | Firmicutes | Clostridia | Clostridiales | Ruminococcaceae | u.b. | 0.169 | |
| Z13 | AJ863538 | Bacteria | Firmicutes | Clostridia | Clostridiales | Ruminococcaceae | u.b. | 0.125 | |
| Z14 | AB009189 | Bacteria | Firmicutes | Clostridia | Clostridiales | Ruminococcaceae | u.b. | 0.257 | |
| Z15 | EU842742 | Bacteria | Firmicutes | Clostridia | Clostridiales | Ruminococcaceae | u.b. | 0.214 | |
| AA | AB009216 | Bacteria | Firmicutes | Clostridia | Clostridiales | Veillonellaceae | Anaerovibrio | 0.233 | |
| AB | AB034139 | Bacteria | Firmicutes | Clostridia | Clostridiales | Veillonellaceae | Selenomonas | 0.678 | |
| AC | AY244976 | Bacteria | Firmicutes | Clostridia | Clostridiales | Veillonellaceae | Succiniclasticum | 2.629 | |
| AD | AB210825 | Bacteria | Firmicutes | Erysipelotrichia | Erysipelotrichales | Erysipelotrichaceae | Catenibacterium | 0.569 | |
| AE1 | AB009179 | Bacteria | Firmicutes | Erysipelotrichia | Erysipelotrichales | Erysipelotrichaceae | u.b. | 0.236 | 2.60 |
| AE2 | EU475554 | Bacteria | Firmicutes | Erysipelotrichia | Erysipelotrichales | Erysipelotrichaceae | u.b. | 0.555 | |
| AE3 | New.Ref.OTU | Bacteria | Firmicutes | Erysipelotrichia | Erysipelotrichales | Erysipelotrichaceae | u.b. | 0.209 | |
| AE4 | EU458717 | Bacteria | Firmicutes | Erysipelotrichia | Erysipelotrichales | Erysipelotrichaceae | u.b. | 1.186 | |
| AE5 | EU381583 | Bacteria | Firmicutes | Erysipelotrichia | Erysipelotrichales | Erysipelotrichaceae | u.b. | 0.418 | |

| | | Taxonomy[3] | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| **Label[1]** | **OTU ID[2]** | **Kingdom** | **Phylum** | **Class** | **Order** | **Family** | **Genus** | **%[4]** | **%[5]** |
| AF | AB682235 | Bacteria | Proteobacteria | Alphaproteobacteria | Rhodospirillales | Acetobacteraceae | Acetobacter | 0.170 | |
| AG1 | AB239483 | Bacteria | Proteobacteria | Gammaproteobacteria | Aeromonadales | Succinivibrionaceae | Ruminobacter | 0.269 | 3.81 |
| AG2 | EF445274 | Bacteria | Proteobacteria | Gammaproteobacteria | Aeromonadales | Succinivibrionaceae | u.b. | 0.592 | |
| AG3 | GQ327554 | Bacteria | Proteobacteria | Gammaproteobacteria | Aeromonadales | Succinivibrionaceae | u.b. | 0.624 | |
| AG4 | EU381934 | Bacteria | Proteobacteria | Gammaproteobacteria | Aeromonadales | Succinivibrionaceae | u.b. | 2.322 | |
| AH1 | AB270123 | Bacteria | Spirochaetes | Spirochaetales | Spirochaetaceae | Treponema | u.b. | 0.440 | 1.92 |
| AH2 | AF001693 | Bacteria | Spirochaetes | Spirochaetales | Spirochaetaceae | Treponema | u.b. | 1.035 | |
| AH3 | HM049812 | Bacteria | Spirochaetes | Spirochaetales | Spirochaetaceae | Treponema | Treponema sp. | 0.231 | |
| AH4 | GQ402096 | Bacteria | Spirochaetes | Spirochaetales | Spirochaetaceae | Treponema | Treponema sp. | 0.216 | |
| AI1 | EU871422 | Bacteria | Tenericutes | Mollicutes | Anaeroplasmatales | Anaeroplasmataceae | Anaeroplasma | 0.286 | 0.48 |
| AI2 | New.Ref.OTU | Bacteria | Tenericutes | Mollicutes | Anaeroplasmatales | Anaeroplasmataceae | Anaeroplasma | 0.196 | |
| AJ | EF445251 | Bacteria | Tenericutes | Mollicutes | RF9 | u.b. | u.b. | 0.219 | |
| AK | New.Ref.OTU | Unassigned | | | | | | 0.174 | |

[1] Label used to group and label OTU in PCA plots

[2] OTU = Operational Taxonomic Unit, New.Ref.OTU = New Reference OTU

[3] u.b. = unculturable bacterium, all OTU were classified as “unculturable bacterium or archeon” at species level, therefore only the taxonomic classification up to the genus level is shown.

[4] proportional abundance in % of OTU

[5] proportional abundance in % of OTU group (grouping is indicated by common letter in label)

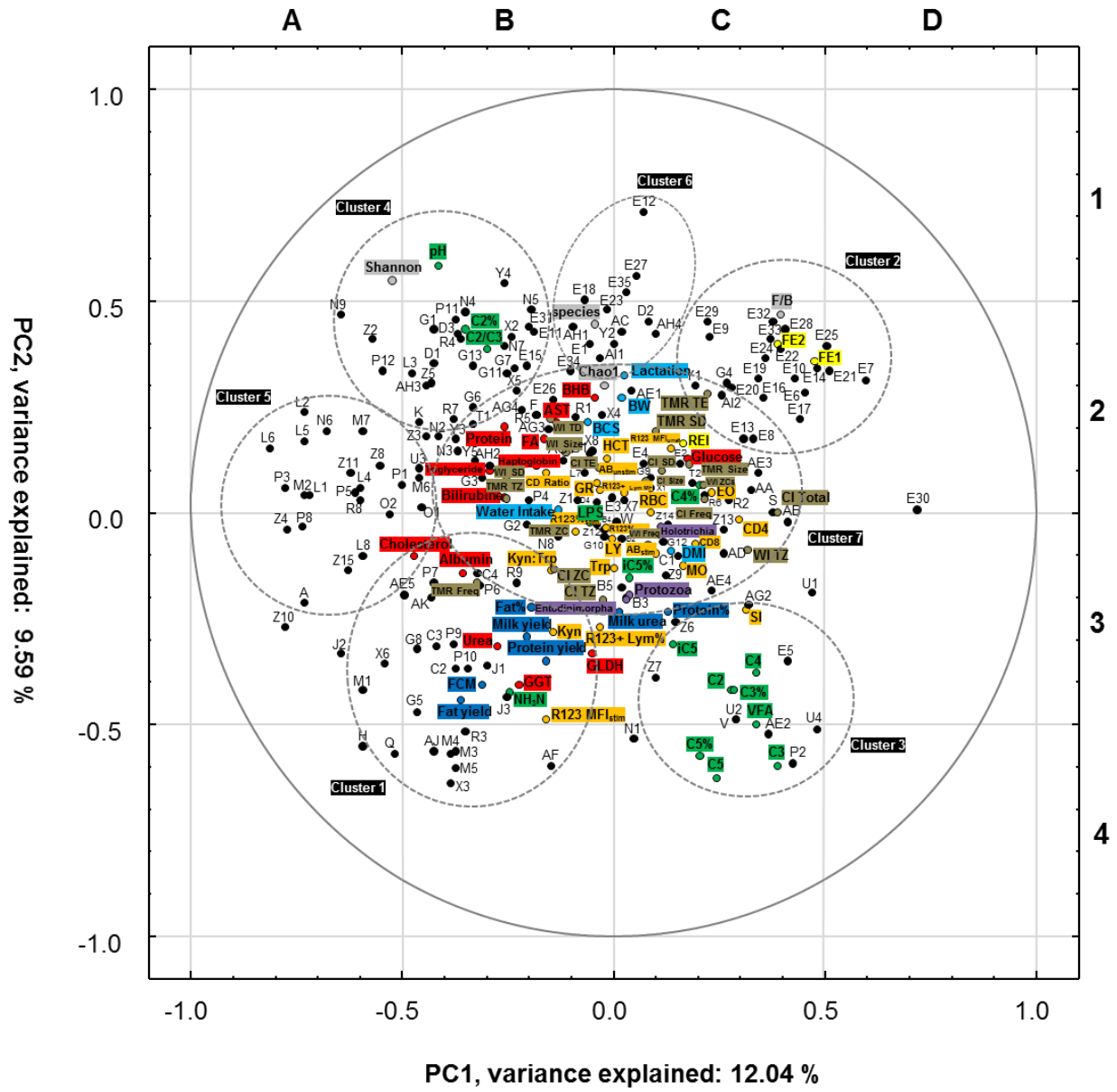


**Figure 1.** Principal component analysis (PCA) plot of different phenotypic variables and rumen microbiome of dairy cows at day 56 ± 3 p.p. (n = 36). Variable grouping and label color: general production (light blue), milk production (dark blue), feed efficiency (yellow), behavior (brown), rumen fermentation (green), immunology (orange), serum metabolic variables (red), rumen microbiome indices (grey), rumen prokaryotes (white) and protozoa (purple). Abbreviations can be found in Material and Methods. Grouping and labelling of prokaryotes in Table 1. Color version available online. Interactive document available for download in supplements.

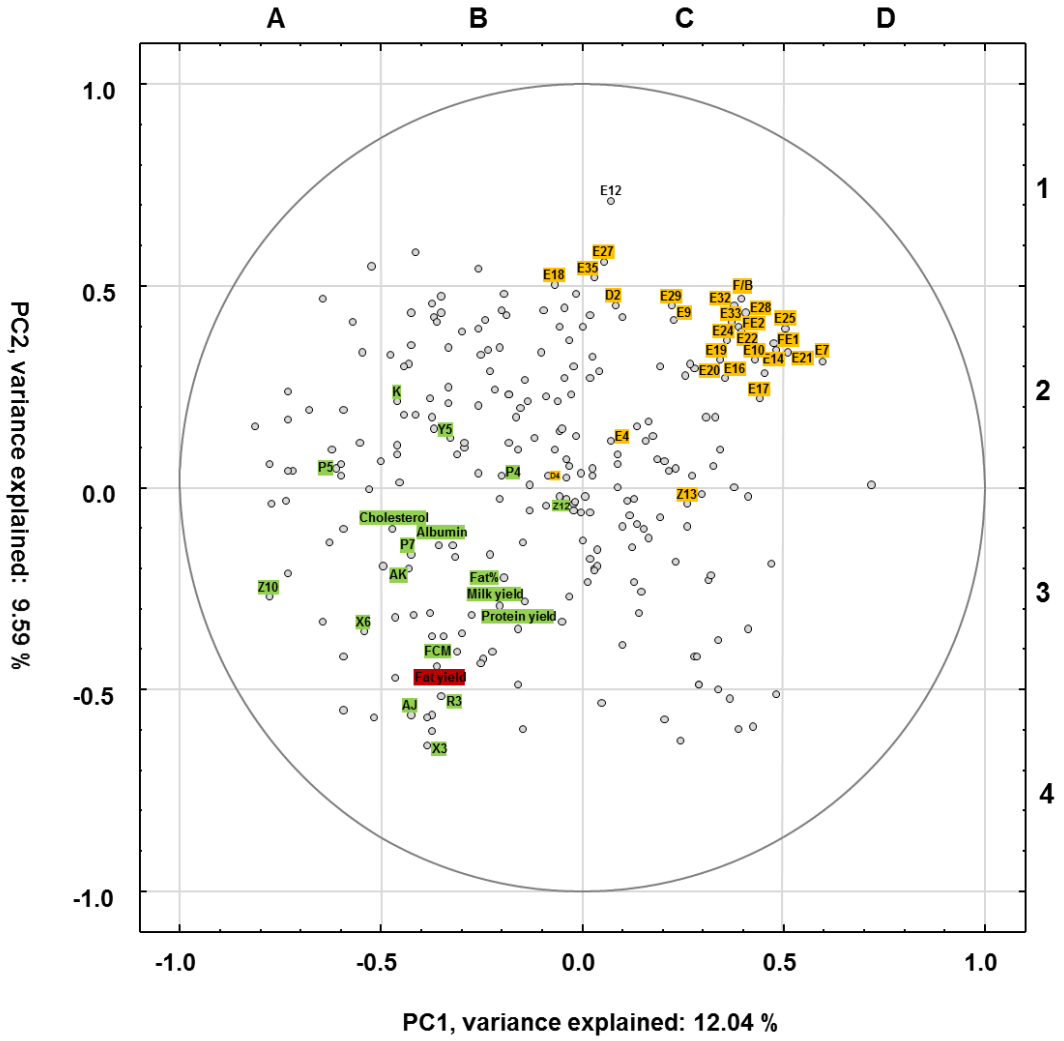


**Figure 2.** Principal component analysis (PCA) plot from Figure 1, only showing the variables that significantly correlate (P < 0.05, Spearman´s Rank correlation) with fat yield (dark red label). Positive correlations in green, negative correlations in orange. Abbreviations can be found in Material and Methods. Grouping and labelling of prokaryotes in Table 1. Color version available online. Similar plots for every variable and prokaryote included in the main PCA available for download in supplements.

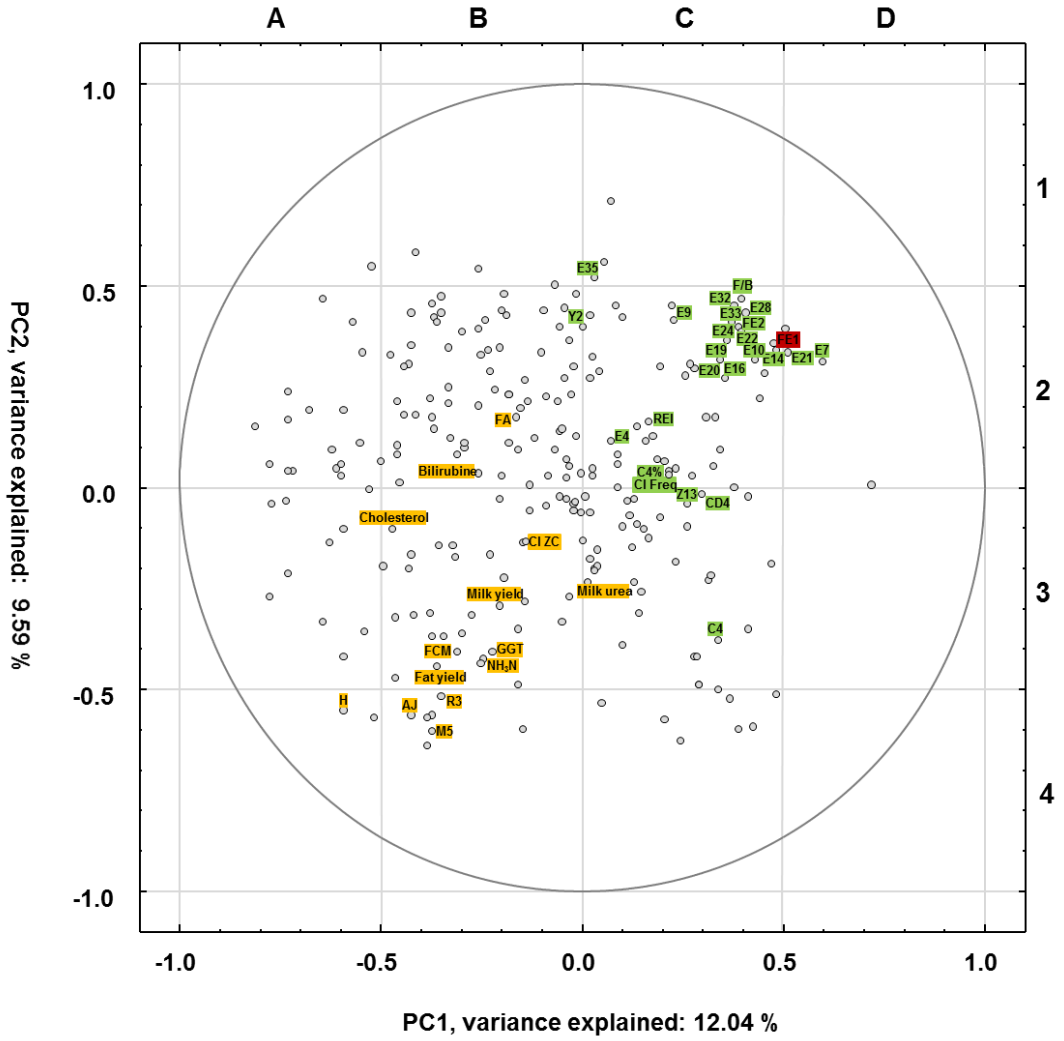


**Figure 3.** Principal component analysis (PCA) plot from Figure 1, only showing the variables that significantly correlate ($P < 0.05$, Spearman´s Rank correlation) with the feed efficiency variable FE1 = dry matter intake / milk yield ratio (dark red label). Positive correlations in green, negative correlations in orange. Abbreviations can be found in Material and Methods. Grouping and labelling of prokaryotes in Table 1. Color version available online. Similar plots for every variable and prokaryote included in the main PCA available for download in supplements.

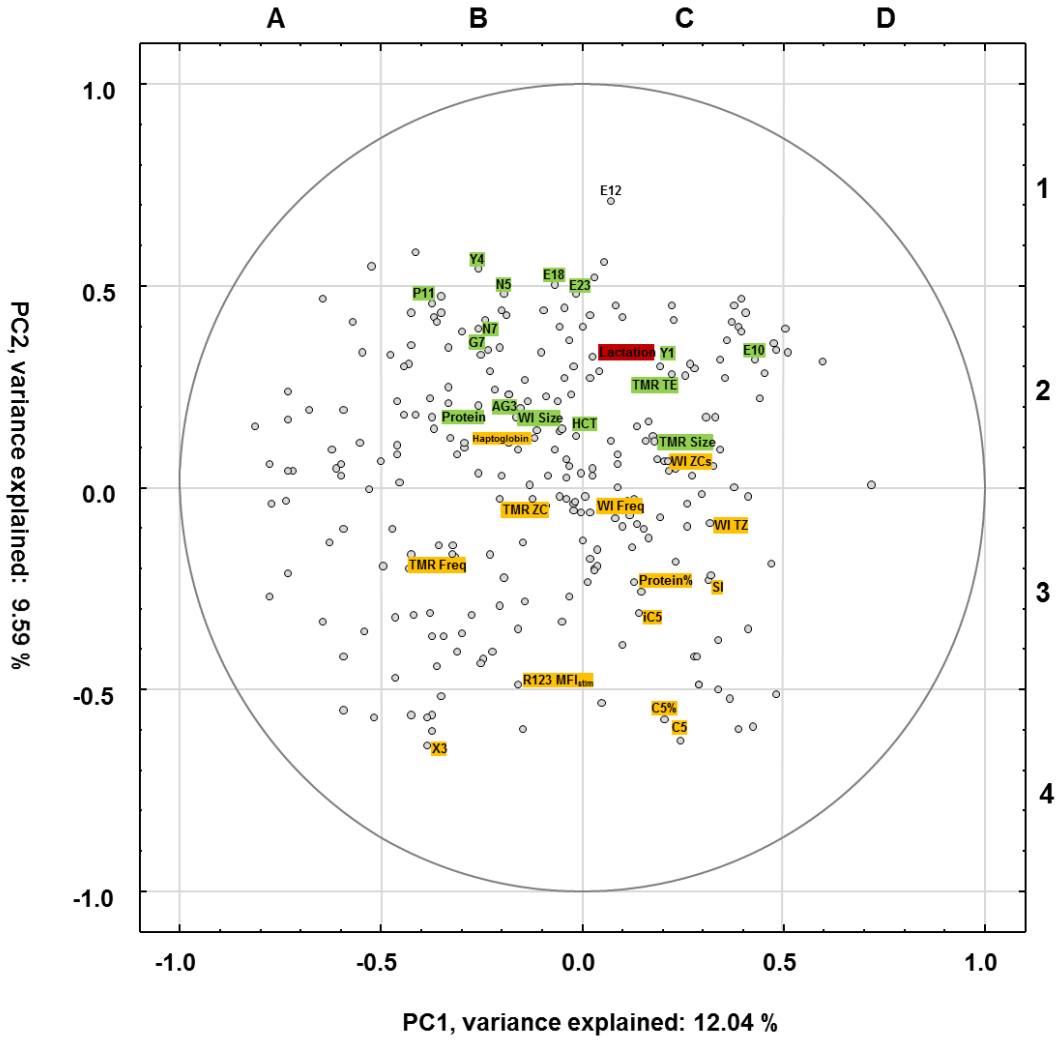


**Figure 4.** Principal component analysis (PCA) plot from Figure 1, only showing the variables that significantly correlate ($P < 0.05$, Spearman´s Rank correlation) with lactation number (Lactation, dark red label). Positive correlations in green, negative correlations in orange. Abbreviations can be found in Material and Methods. Grouping and labelling of prokaryotes in Table 1. Color version available online. Similar plots for every variable and prokaryote included in the main PCA available for download in supplements.

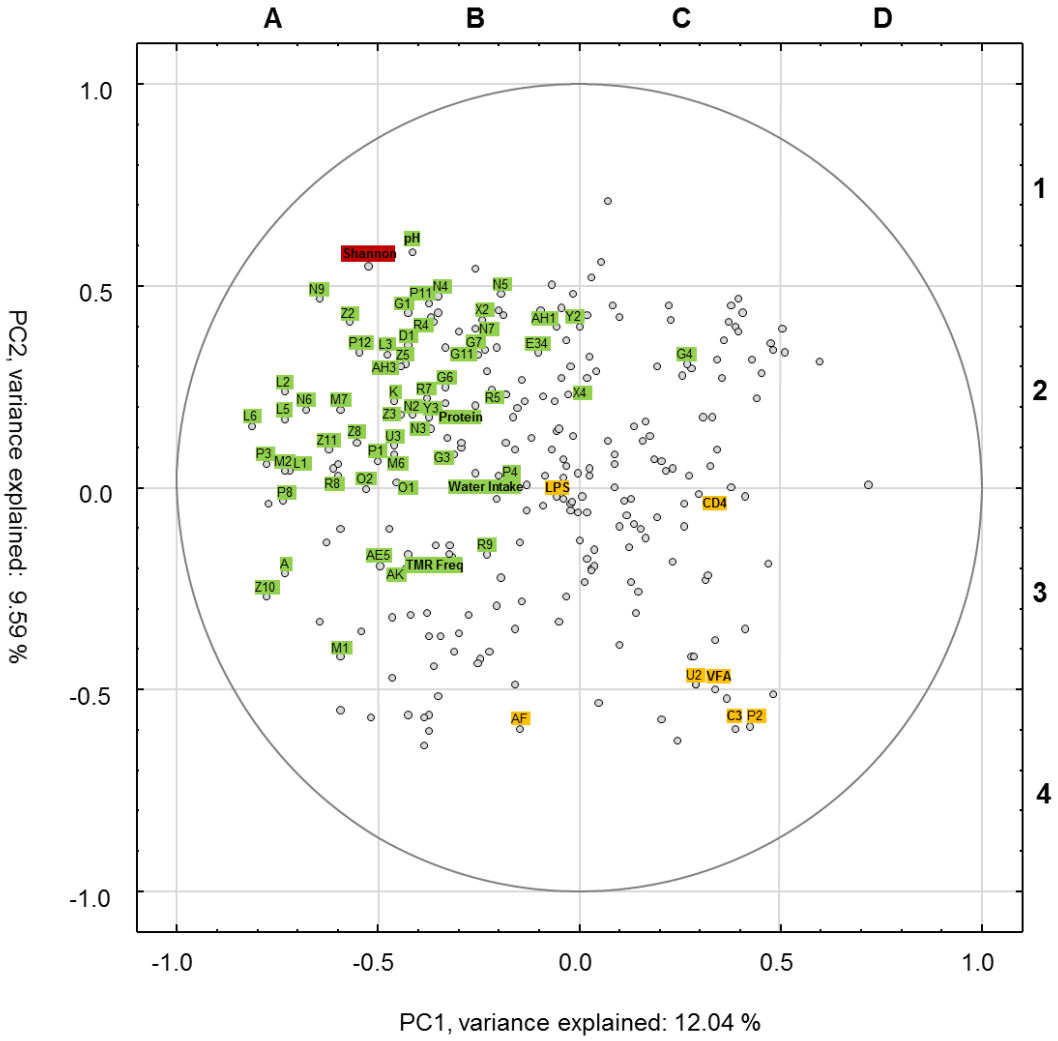


**Figure 5.** Principal component analysis (PCA) plot from Figure 1, only showing the variables that significantly correlate ($P < 0.05$, Spearman´s Rank correlation) with the rumen microbiome diversity variable Shannon index (Shannon, dark red label). Positive correlations in green, negative correlations in orange. Abbreviations can be found in Material and Methods. Grouping and labelling of prokaryotes in Table 1. Color version available online. Similar plots for every variable and prokaryote included in the main PCA available for download in supplements.

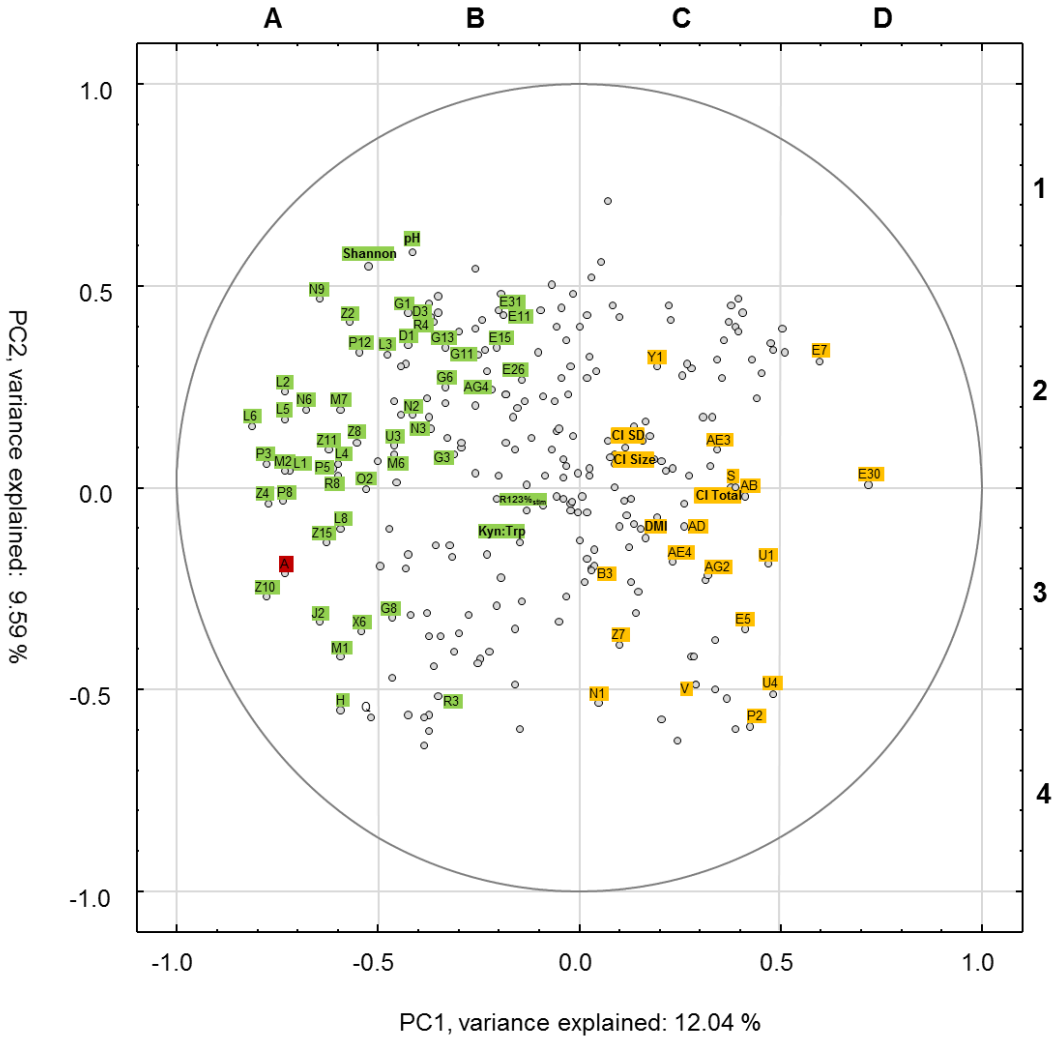


**Figure 6.** Principal component analysis (PCA) plot from Figure 1, only showing the variables that significantly correlate ($P < 0.05$, Spearman´s Rank correlation) with the abundance of the archaeal taxon Methanobrevibacter (A, label description in Table 1, dark red label). Positive correlations in green, negative correlations in orange. Abbreviations can be found in Material and Methods. Grouping and labelling of prokaryotes in Table 1. Color version available online. Similar plots for every variable and prokaryote included in the main PCA available for download in supplements.

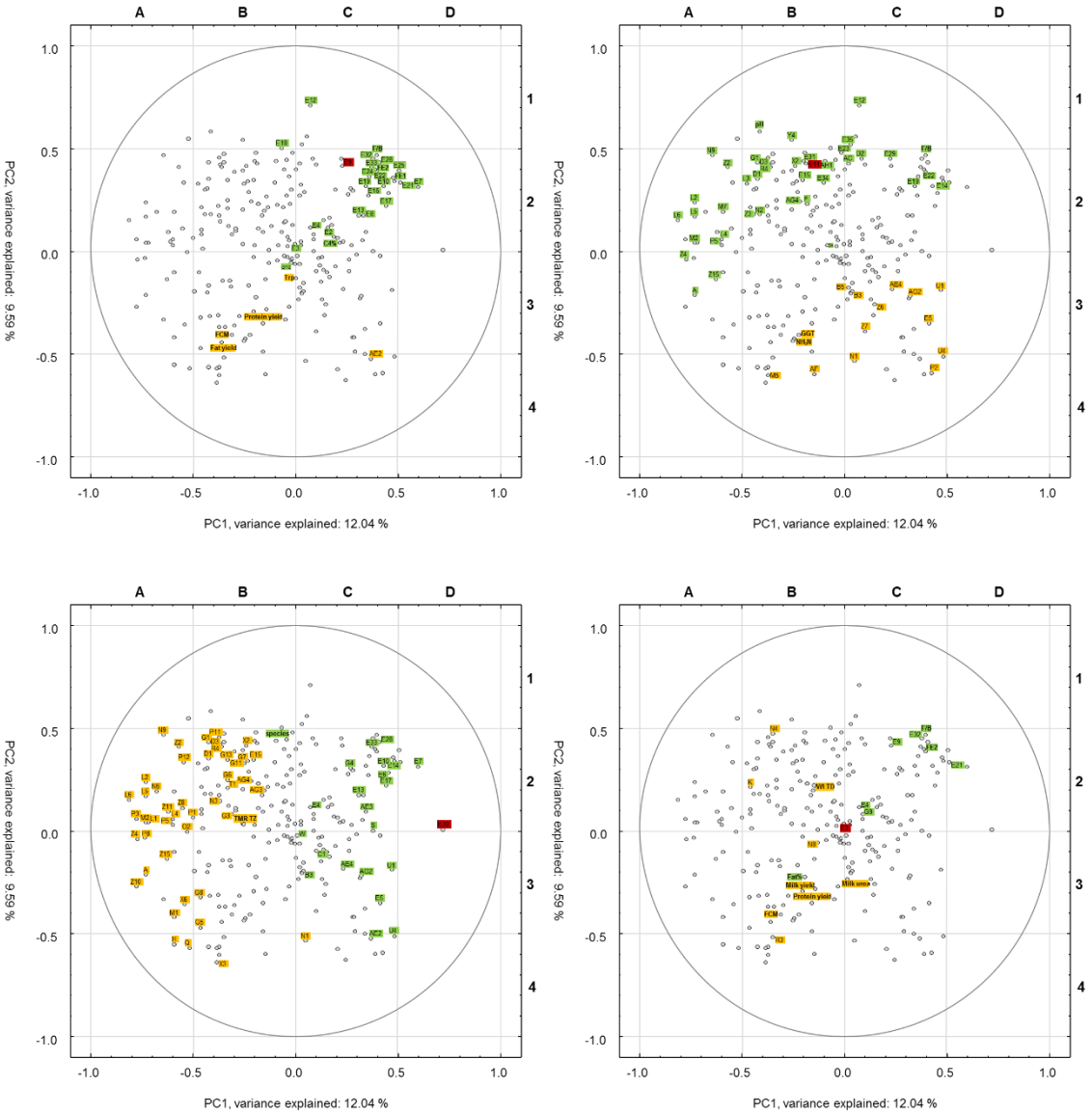


**Figure 7.** Four different principal component analysis (PCA) plots from Figure 1, only showing the variables that significantly correlate ($P < 0.05$, Spearman´s Rank correlation) with a specific OTU ascribed to the *Prevotella* genus (E9 (upper left), E11 (upper right), E30 (lower left), E3 (lower right), label description in Table 1, dark red labels). Positive correlations in green, negative correlations in orange. Abbreviations can be found in Material and Methods. Grouping and labelling of prokaryotes in Table 1. Color version available online. Similar plots for every variable and prokaryote included in the main PCA available for download in supplements.

# 6. Conclusion

The overall aim of the thesis was to investigate the factors influencing the rumen microbiome, their role in rumen metabolism and interplay. Each of the three presented manuscripts (chapter 3-5) reflects upon a different aspect of the rumen microbial plasticity and dynamics.

The first two studies confirmed that the cow possesses a “core and variable microbiome”. It was confirmed that the ration fed has the largest influence of the rumen microbiome compilation. We further illustrated that the concept of the “core microbiome” accounts for the free floating, the feedparticle-associated as well as prokaryotes attached to the rumen wall, and that these three communities are distinctively different. We also showed that the epithelium associated microbiome seems to be affected by a ration change in a similar way as the other two locations. This is a novel finding, since it was previously commonly acknowledeged (but with reliable studies lacking) that the rumen wall associated microbiota would be more consistent throughout dietary changes. Combining the observations made on microbial level with the data gathered of animal level we were able to deduct that changes in the rumen microbiome cannot solely be accounted to the time needed for the different species to adapt the new substrate, but also to temporal aspects in behavioral and physiological alterations of and in the host involved.

In the second study, we then illustrated that the “core microbiome” may be altered, by substances such as monensin, leading to alterations in the metabolism of the host. This study further confirmed the capability of the rumen microbiome to adapt to certain feed additives, such as essential oils, over time, leaving them ineffective.

The data and statistical analysis of the first study further showed a significant “cow” effect in a range of prokaryote species, statistically confirming the concept of the “variable or individual microbiome”. The third study then tried to investigate different aspects responsible for this “individual microbiome”. We were able to confirm several well-known interrelations between the rumen microbiome and production traits (e.g. *Prevotella* abundance and feed efficiency) and showed that the individual feed intake behavior seems to only have a minor influence in this context. The question remains however, what the driving factor between these differences in the rumen microbiome and the linked differences in feed efficiency is.

The three different studies have lead to the indentification of several other topics which warrant further investigation:

- Dynamics between the different members of the rumen microflora (e.g. prokaryotes, protozoa, fungi and the virome)
- Interrelations between microbiome and host phenotypical, metabolic and genetic traits
- The function of the prokaryotes attached to the rumen wall and their interrelations with the host´s metabolism
- The role of the microbial species of low abundance (< 0.1 %), the so called “rare biosphere”
- Interrelations between rumen microbiome and the microbiome of the lower gut

With the upcoming of more sophisticated sequencing methods (e.g. Metagenomics, Metatranskriptomics) the unraveling of these different aspects will become more feasible. To better characterize the hosts metabolism and traits the parallel application of methods recording the rumen fermentation (e.g. sensor technique), the rumen physiology (e.g. VFA absorption capacity tests, gene expression), metabolic rate (e.g. using metabolism chambers) and genetic attributes need to be considered.

All three manuscripts highlight the importance of different methodological aspects concerning rumen microbiome sequencing, and that ours as well as other studies should always be interpreted in the proper context. Especially the finding that a common taxonomic classification of prokaryotes does not necessarily imply functional communality warrants further investigation as well as consideration when interpretating microbiome sequencing results.

## 7. References

*(account for chapters 1. Background, 2. Aims of Study, and 5. Discussion)*


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## 8. Affidavit

I herewith declare that I autonomously carried out the PhD-thesis entitled *"Interrelations between feed, host and rumen microbiota in dairy cows"*.

No third party assistance has been used.

I did not receive any assistance in return for payment by consulting agencies or any other person. No one received any kind of payment for direct or indirect assistance in correlation to the content of the submitted thesis.

I conducted the project at the following institution(s):

*Institute of Animal Nutrition, Friedrich-Loeffler-Institute (FLI), Federal Research Institute for Animal Health, Braunschweig, Germany*

*Department of Physiology, University of Veterinary Medicine Hannover, Germany*

The thesis has not been submitted elsewhere for an exam, as thesis or for evaluation in a similar context.

I hereby affirm the above statements to be complete and true to the best of my knowledge.

Date: _____ Signature: _____

## 9. Acknowledgements

I want to express my deepest gratitude to my supervisors at the Institute of Animal Nutrition (FLI), Prof. Sven Dänicke and Dr. Ulrich Meyer for their scientific guidance, giving me a great scope for development and supporting us through the first months of Till´s life. Without their extensive and kind support the preparation of the different manuscripts, this thesis, as well as my facilely return to work after the maternity leave would not have been possible. I further thank Prof. Gerhard Breves for his scientific input and advice, as well as the joyful time when residing at his institute. His academic work and heritage is greatly acknowledged. I also thank Prof. Jürgen Hummel for the scientific exchange and support during the writing of the manuscripts and thesis. I really enjoyed the scientific discussions with him, due to his friendly, kind and calm nature, and hope we will have more possibilities to collaborate in future.

I would like to show my gratitude to the members of the Institute of Physiology (TiHo Hannover) for their support and guidance during the microbiome sample preparation and analysis (Susanne Riede, Kerstin Kiri, Marion Burmester and Karin Hustedt). A very big thank you goes to Kerstin Kiri who has helped me through the highs and lows of the SSCP analysis and made the stay at the laboratory extremely joyful.

I further would like to thank Christian Hundsrucker and Pascal Weigold from Microsynth AG (Balgach, Switzerland) and Andres Buser for their outstanding consultancy, professionalism and kind help in a bottleneck-phase of this thesis. Without their help, the manuscripts would not have attained such a high quality and I really enjoyed their input.

I send a big thank you to Mark Gardener, who has spontaneously assisted us with his great knowledge in statistical analysis of community ecology. I really enjoyed this collaboration due to his willingness to teach and great patience. I hope we will get the chance one day to meet in person!

I am further indebted to the the co-workers at the Institute of Animal Nutrition, namely the members of the immune-nutrition group (Jana Frahm, Nicola Mickenautsch, Lara Lindner and Elenia Scholz), basic-analytics group (Liane Hüther, Kathrin Hillendahl, Hans Eckardt, Axel Jagow, Johanna Sturm and Susanne Stock), the experimental station (Florian Mühlhaus, Jonas Knäpple, Dirk Wolters, Annerose Junghans, Katrin Kuhrmeier, Karin Rothe, Andreas Thies), administrational staff (Sabine Hartinger, Gudrun Mittelbach, Vanessa Tabatt, Sigrid Herweg and Britta Immenroth), Dirk von Soosten, and many of my fellow doctoral students at the FLI (Caroline Drong, Reka Tienken, Kirsten Schulz, Mohammad Ebrahem, Marc-Alexander Liebholdt, Wendy Liermann, Katrin Stelter, Marleen Paulick, Janine Winkler, Julia Hartwiger,

Rolf Schmitz und Karina Schnabel), as well as Institute of Physiology (Kristin Elfers, Melanie Eger, Patrick Lange and Lisa Marholt), and the external friends and colleagues including Gero Seyfang and the masterstudents Stefanie Ruesink, Sandra Jostmeier and Susanne Künzel and our collaborating partners at Haus Riswick, University of Halle, University of Bonn, University of Hohenheim, TiHo Hannover and the University of Wageningen.

A special thank you goes to Jan Dijkstra and Kasper Dieho. Kasper has become a great friend over the course of this thesis and I always enjoyed his enthusiastic and positive nature. Jan´s passion for critical discussion and outstanding high-quality research was and is very inspiring. He has supported me through the finalization of an earlier manuscript just days before my son was born. I see in him a great mentor and friend.

I also want to thank Prof. Alexander Starke for creating the room and time needed to finalize the last two manuscripts as well as this thesis. I enjoy his creativity and deeply respect his devotion and passion for cattle veterinary medicine as well as the production sector as a whole. I further would like to thank my new colleagues at the clinic for cattle and swine in Leipzig, especially Fanny, Wolf and Romy, for motivating me and always being there for me, whenever I needed a shoulder. They have become great friends and I deeply admire their strength, endurance and passion.

I owe my deepest gratitude to my friends and family for understanding that time was scarce and I was not always an easy companion during the last few months.

I especially want to thank Anja and her family for being such a great help and friends. Anja, you are a wonderful “Tagesmutter” and we feel blessed that Till could stay his first year with you. You supported his sunny nature and playfully managed his temper. You gave me the possibility to go freely to work, knowing Till is in good hands. We all miss you.

I cannot find the words to express my gratitude to my partner Erik. Without his extensive support and his love, I would not be where I am now. He is a wonderful and caring father, motivating me to get the best out of me. Instead of telling me to step back, he tells me to stay strong and fight. Love is not to hold somebodies hand when they are giving up, but to show them how to push her/his own boundaries and that giving up is not an option.

… and of course, Till… he is my sunshine and eternal source of joy and tranquility. Children indeed make you relativize everything.