The characterization and manipulation of the reticulated microbiome in vertebrates

PhD thesis by

Michael Roggenbuck, M.Sc.
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Michael Roggenbuck

Principle supervisor:

Prof. Søren Johannes Sørensen¹

External supervisor:

Prof. Lars Hestbjerg Hansen²

Co-supervisor:

Kenneth Klingenberg Barfod³

¹Department of Biology, Section for Microbiology, University of Copenhagen
²Department of Environmental Science – Environmental microbiology & Biotechnology, Aarhus University
³Statens Serum Institut, Artillerivej 5, 2300 Copenhagen S, Denmark
The first hexagon (left) shows a cartoon drawn by the author of a black and turkey vulture of which the gut and the facial microbial communities are subject of this thesis. The heatmap is the easiest and most direct “finger” print application to visualize the microbial community profile (second hexagon) of the vertebrate host - a method often applied in this study. Potential interactions between the bacterial specimens were predicted analyzing static co-occurrence networks (third hexagon).
Preface

This PhD thesis was self-dependently conducted and fulfills the requirements to obtain the PhD-degree at the Section for Microbiology, at the Department of Biology, University of Copenhagen in Denmark.

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Michael Roggenbuck,

Copenhagen, September 2014

_Damit das Mögliche entstehe, muß immer wieder das Unmögliche versucht werden._ - Hermann Hesse,

_Zen, Brief an Wilhelm Gundert_
Summary

The term *microbiome* - “The ecological community of commensal, symbiotic, and pathogenic microorganisms that literally share our body space” - was first described by Professor Joshua Lederberg of the Rockefeller University. With the beginning of the golden age of High-throughput-Sequencing, it has become more evident that animals and their microbial communities are metabolically and immunologically tightly connected and highly dependent on each other. Today the complex microbial flora is often considered as an organ – with a healthy and a diseased stage. Currently the human microbiome is most intense evaluated. However, mechanistically questions often cannot be studied in humans, therefore animal research is applied.

In the first part of this thesis, the diet intervention on the “total” microbial community of two animal model organisms – mice and lambs - was characterized using 16S rRNA gene amplicon sequencing. We aimed to co-correlate the lung microbial composition of vitamin D supplemented and depleted diet in mice. Vitamin D deficiency has been recognized for its role in chronic allergic diseases, but a link between vitamin D status and the development of asthma has not been established. We focused our study on the lung sampling from bronchoalveolar lavage (BAL) fluids and lung tissue. The BAL fluids are commonly used to determine inflammatory responses and/or the diseased stage of the lung. The microbial flora of the lower respiratory tract in mice has never been described due to the low microbial DNA yield and the high risk of contamination from the upper respiratory tract during sampling – therefore we evaluated different experimental approaches (*Manuscript 1* - published). We consistently amplified microbial DNA from the lung that clusters in bacterial composition apart from other parts of the body, such as the mice intestine. In the second experiment we exposed the mice to vitamin D and ovalbumin (OVA) – a common model to induce asthma-allergic responses in the lung. We investigated the variation between the OVA sensitized and naïve mice under vitamin D supplemented and depleted conditions (*Manuscript 2 – in preparation*). We found that the lung microbial floral composition is significantly affected by the OVA treatment, but responses inconsistent to vitamin D exposure.

The second animal-microbiome model focused on the foregut of young ruminants (lambs). The livestock methane production is a substantial part of the annual anthropogenic greenhouse gas production. There is a large interest in reducing the methane emission since the worldwide meat consumption will double until 2050. Interestingly calves and lambs emit little methane in the early phase of life since the major foregut methanogenesis in herbivores is associated to fiber rich diet of adult ruminants. Therefore we designed a “colostrum”-like diet (maternally produced fat-rich newborn feed) and changed the development of the rumen in lambs from birth until the age of 6 months. The diet reduced the methane production of 87% compared to the hay fed control group and resulted in the development of a rudimentary rumen (*Manuscript 3 - published*). The fat-rich diet significantly reduced the relative counts of methane producing bacteria (methanogens) in the ruminal fluid and decreased the methanogenic diversity. However, the diet manipulation increased the frequency of a single methanogenic species (*Methanobrevibacter* sp.) in the ruminal solid...
fraction compared to the control group (Hay fed). Additionally we evaluated the complex microbial community of the undeveloped rumen system. Alternative hydrogen oxidations pathways (alternatives to methanogenesis) were predicted based on significantly elevated species previously described by cultivation studies. Finally we estimated the interactions of Methanobrevibacter sp. with static co-correlation and co-exclusion models (Manuscript 4 – in preparation).

The second part of this thesis was of a more descriptive nature. Wild animals can highlight new microbial-host relations with health or environmental impact. Vultures are carrion feeders. As forensic studies revealed, vultures can wait up to 48 hours of decay prior preying, possibly because the advanced decomposition softens the tissue and eases the birds to feed large carrians. However, feeding on carcass is associated to the potential exposure of bacterial toxins, with severe outcome such as avian botulism. As Ley et al (2008) described – diet is the key driver of microbial composition of the digestive system in vertebrates. Therefore we raised the question of what microbes would be found in the vulture gut community. We observed that 50 wild vulture hindgut samples (large intestine), of the species Coragyps atratus and Cathartes aura. Their hindgut microbiome were highly similar and dominated by Clostridium sp (putative C. perfringens) and Fusobacterium sp (putative F. nucleatum) with a low species diversity (Manuscript 5 - submitted). We speculated that the vulture digestive system has co-evolved with these two microbes. To address this question we compared our results from the wild with birds in captivity from the Copenhagen zoo. We evaluated zoo vultures as well as carnivore, herbivore and omnivore birds. The carnivorous birds, including the vultures, were fed fresh meat. However, despite of the similar diet between bird types, Zoo-vulture feces contained a more statistically similar microbial composition to wild vultures, than compared to other fresh meat fed carnivorous birds. In this study we propose for first time the term carrionovore – as “animals that prey on carcasses”.

As part of a larger rumen screening approach we have also analyzed the microbial flora of giraffes (Manuscript 6 - accepted) and wallabies (marsupial) foreguts (in progress of analysis). Giraffes are herbivores that prefer leaves and fruits with higher crude protein and lignin content compared to the common livestock animals of cattle and sheep fed with hay. The purpose of this study was to uncover new microbes previously undescribed. Our results reveal that the major part of the giraffe rumen environment contains mostly sequences not assigned to any known genera.
Resumé af thesis

Microbiomet er "det økologiske samfund bestående af kommensale, symbiotiske og patogene mikroorganismer, som vi deler vores krop med" blev for nylig beskrevet af professor Joshua Lederberg, Rockefeller University.

Med starten af "the golden age" med High-throughput sekventering, er det blevet mere evident, at dyr og deres mikrobielle samfund er metabolisk og immunologisk tæt forbundne og meget afhængige af hinanden. I dag betragtes den komplekse mikrobielle flora ofte som et organ - med en sund og en syg fase. I øjeblikket er det menneskelige microbiom det mest intensivt undersøgte. Men på grund af etik kan sundhedsrelaterede spørgsmål ikke altid undersøges hos mennesker og det er nødvendigt at anvende forskning på dyr i stedet for.


I det andet kapitel i denne thesis er fokuseret på manipulation af foregut hos unge drøvtyggere (lam). Produktionen metan fra husdyr er en væsentlig del af den årlige, menneskeskabte drivhusgas produktion og da det verdensomspændende kødforbrug vil blive fordoblet frem til 2050, er der stor interesse i at reducere udledningen af metan. Interessant nok udsender kalve og lam meget lidt metan i den tidlige fase af deres liv og den store foregut methanogenese hos planteædere er associeret med fiber rig kost hos voksne drøvtyggere. Derfor har vi udviklet en "colostrum"-lignende diæt (moder produceret fedt-rig føde til de nyfødte) og hæmmede udviklingen af vommen hos lam fra fødslen indtil en alder på 6 måneder, hvilket resulterede i en reduceret produktion på 87% metan i forhold til en høfodret kontrolgruppe (Manuskript 3 - offentliggjort). Fedt-rig kost reducere signifikant det relative antal af methanogenere i mavevæsken og den metanogenes diversitet faldt, men en enkelt methanogen art (Methanobrevibacter sp.) blev meget hyppigere I den ruminale faste fraktion i forhold til kontrollen. Desuden undersøgte vi det komplekse, mikrobielle samfund af det uudviklede vomsystem, forudsagde alternative hydrogen oxidations reaktioner (alternative veje til methanogenese) baseret på signifikant flere arter, tidligere beskrevet fra

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Introduction
Introduction

1. Co-adaptation of vertebrate and gut microbes.

Prokaryotes are ubiquitous (Finlay BJ, 2002) and have evolved approximately 3.4 billion years ago (Figure 1). Eukaryotic multicellular organisms evolved approx. 1.2 billion years ago (Knoll AH, 2014). This substantial long period of co-presence between both domains has resulted in strong co-adaptation. One example of this adaptation is given in figure 2. Despite the large number of known microbial phyla (recent approximates, list more than 52 cultured and uncultured bacterial phyla, Rappé & Giovannoni 2003) on earth, the two bacterial taxa of Bacteroidetes and Firmicutes appear to prevail vertebrates feces, largely different compared to the environmental samples.

Figure 1. The putative 3.4 billion years old archaeal fossil at the Strelley pool formation in Western Australia visualized with high-resolution transmission electron microscopy. Arrow indicates raptured cells (Figure was adapted from Wacey et al [1])

The adaptation of prokaryotes to their eukaryotic hosts has also been experimentally shown. For instance Lactobacillus reuteri, a vertebrate gut specialist was found to diverge into host specific subspecies when introduced into gnotobiotic rodents (Frese et al, 2011). The same study also investigated if L. reuteri isolated from different vertebrate digestive system such as humans, chicken, rats, pigs and mice would colonize the gnotobiotic rodents. The authors reported that only L. reuteri strains isolated from mice and rat managed to colonize the rodent guts within a period of 2 weeks. Thus, the mice and rat isolated L. reuteri strains were already adapted to the rodent environment enabling the successful colonization.
Figure 2. Relative microbial phyla distribution of 462 vertebrate and environmental samples published by Ley et al [2]. The group of “Other human” includes samples taken from, mouth, ear, skin, vagina and vulva.

The co-adaptation of eukaryotes to their microbes becomes evident when comparing the digestive systems of herbivore and carnivore/omnivore vertebrates. The diet of herbivores is plant material, but vertebrates are not genetically equipped to split the (β)1-4 glycosidic bonds of cellulose, the primary component of plants (Beguin & Aubert, 1994). Microbes, however can utilize difficult or non-digestible food, converting complex carbohydrates as cellulose into volatile fatty acids (acetate, butyrate, propionate, lactate) the major energy source of herbivores. This has presumably driven the evolution of the multi-chambered forestomach in herbivore mammals that elongates the fermentation process. The anoxic microbial food degradation in the carn-/omnivore animals (Hofmann RR, 1988; Ley et al, 2008) occurs in the hindgut and is of shorter retention time compared to the fore-stomach (Macfarlane & Macfarlane, 2010, Ley et al, 2008).

2. The microbiome – a non-described organ

Besides the primary support of digestion, gut commensals and their metabolites are known to contribute to the maturation of the epidermal mucosa, stimulates the immune system and supply essential vitamins such as vitamin K, biotin and cobalamin (e.g. Rakoff-Nahoum et al, 2004, Furusawa et al, 2013 LeBlanc et al, 2013). Evidently the gut microbial community has a substantial impact on the health of the host. In fact, the human gut contains with 100 trillion microbial cells and
250000 bacterial genes, a 10-fold larger gene catalogue compared to the approx. 25000 human genes (Ley et al, 2006) that substantially adds to the host genetic toolbox. Therefore the gut microbiome* has recently been considered as an organ with a healthy and pathologic stage, where damages of the smaller fraction can affect the entire community and the host eventually (Baquero & Nombela, 2012).

Other animal-microbial relationships as in lung, skin and vagina are not or less comprehensively described. For instance the impact of microbes of the lung physiology is an open field of discussion since the lung was hypothesized to be sterile (Baughman et al, 1987 and Thorpe et al, 1987). However recent observations characterized by culture independent sequencing revealed that healthy and diseased pulmonary alveoli (Figure 3) - the side of major gas exchange in the lung – contained bacteria (Beck et al, 2012, Lozupone et al, 2013, Huang et al, 2011 and Hilty et al, 2010). Nevertheless these results are contentious because the samples were taken via the upper respiratory tract and potentially reflect either mouth or nose community (depending on route of sampling). However, alveoli contain macrophages and T-cells indicating the need for defense (Figure 3) most likely due to the continuous exposure of microbes (Cheung et al, 2000). As found in the gut and skin (Naik et al, 2012), commensal bacteria could have beneficial, and developmental effects of lung immune system (Dickson et al, 2014).

*Microbiome - The ecological community of commensal, symbiotic, and pathogenic microorganisms that literally share our body space (Lederberg & McCray, 2001)


Different from “normal” organs, the microbial flora can in theory be manipulated with potential beneficial outcome. There is a large environmental, health and commercial related interest in changing the microbiota associated to animals. For example, bacterial fiber degraders enrich hydrogen in the foregut of herbivores. The methanogenic archaeb such as Methanobrevibacter use the hydrogen as energy source and reduce CO₂ to CH₄ (Leahy et al, 2010). The enteric methane production was estimated to account for up to 40 % of the annual anthropogenic produced greenhouse gas (Gerber et al, 2013). Considering that the world meat consumption is estimated to double until 2050 (Alexandratos et al, 2006), the methane emitted by livestock will substantially support the enrichment of greenhouse gases in the environment. Thus research focuses currently on designing alternative strategies to reduce the methane emission in foregut fermenters by changing the microbial community.

In health sciences, the manipulation of the gut microbial flora is of central interest. For example, humans exposed to the virulent version of Clostridium difficile that failed treatment by antibiotics, were recovered by fecal transplantation (“replacement” of an unhealthy microbiota with a healthy one – called fecal transplant - removing the stage of disease) (Gough et al, 2011). In premature births, babies have a much higher incident of necrotizing enterocolitis with a high death rate. But by altering the emerging gut flora of the infants with Lactobacillus, the incident rate drops markedly (Yang et al, 2014).
Furthermore, the Center for Disease Control and Prevention (CDC, 2013) reported 38.9 million asthma patients in the US in 2011 with an increase of 28% from 2001-2011. Previous reports show an associated variation of the lung microbiota with asthma. For example, *Haemophilus* was enriched in the bronchi of asthmatic children and adult patients compared to controls (Bisgaard *et al*, 2007, Hilty *et al*, 2010). However, it needs to be clarified if the enrichment of *Haemophilus* is the cause or the consequence of the asthmatic stage or changed lung microbial structure. Nevertheless, this observation initiates the speculation that manipulation of the lung microbiome could help to change the development of asthma. One could imagine altering the lung microbiota in the early stages of microbial colonization in infants using probiotics as seen with the NEC children. But also a hypothetical inhaled lung microbial transplantation with a healthy microbiota, pre- or probiotics could also be feasible.

![Figure 3](image_url)

Even more controversial are the effects of the microbiota-brain-gut axis. Recent studies hypothesized based on germ-free mice (GF) models that the microbial composition influences cognitive responses. For example the lack of gut bacteria in GF mice reduced the plasma level of tryptophan – the precursor of serotonin – with the consequence of a reduced anxiety (Clarke et al., 2012). The level of anxiety was normalized after post colonization. Furthermore gut isolated *Bifidobacterium* and *Lactobacillus* produced *in vitro* the neurotransmitter Gamma-aminobutyric acid (Barrett et al., 2012). Additionally, experimental results indicate that the microbiota is connected with the brain by the level of cytokines (depended on immune responses) and the vagus nerve (controls gut motility). The authors suggest that manipulation of the gut microbiota could be an alternative strategy to treat neural-disorders such as anxiety and depressions (Foxx-Orenstein & William, 2012).

4. **Current strategies to manipulate the microbiota and the lack of the understanding complexity**

The idea of manipulating the microbiota is not new and in fact we are influencing the composition and the dynamics of the gut microbial community on a daily basis by diet. There are several other specific and non-specific approaches to alter the microbial composition, such as antibiotic treatment, vaccination, phage-therapy and food additives (pre-, pro- and synbiotics) and as mentioned before microbial transplantation. Antibiotics for example are rarely specific and never affects only one microbial species, but rather target by broad-spectrum the majority of the sensitive microbes. For this reason antimicrobials are becoming controversial not only due to continues increase in resistant pathogenic strains, but also since commensals are eliminated too (Foxx-Orenstein & William, 2012). This is especially problematic and we are only beginning to understand the consequences of the loss of the “good” bacteria and a healthy community structure. For example, the previous study of Jakobsson et al. revealed that 4 years after macrolide treatment, the gut microbial composition of the human patients was not completely regained and the antibiotic resistance genes were still more prevalent in the community compared to the control group (Jakobsson et al., 2010). Furthermore Theriot et al. (2014), reported that antimicrobial treatment of *Clostridium difficile* infections - an increasing world wide problem - changed the metabolic activities, as it increased bile salt concentration which in turn facilitates the germination of *C. difficile* from dormant into the virulent vegetative type, supporting the re-infection with *C. difficile* (Sorg & Sonenschein, 2008). The authors suggested that antibiotic treatment generally increases the risk of infections with *C. difficile*. This indicates that short-time application of antibiotics might promote long-time disorders of the host gut system with significant physiological disadvantages. Knowledge in how antibiotics would affect the total microbial community needs to be described to enumerate the damage by treatment.

Phage therapy is an alternative strategy to antibiotic treatment in which bacteria are attacked by prokaryote specific virus. This method has from a medical view the advantage that even if the pathogens develop resistance, phages evolve and overcome the resistance (Hyman & Abedon, 2010). Phage therapy has been experimentally shown to reduce the number of *Campylobacter jejuni*
in broilers (Wagenaar et al., 2005). But phages can vary in host-range, limiting the application of the method because both pathogenic and commensal strains are potentially affected by the treatment. There are currently no reports of phage therapy to reduce a single pathogenic strain and the response of the complex microbial community.

Vaccination is compared to antibiotics and phage-therapy a target-specific manipulation and should in theory not affect other microbes. Besides treating diseases, this approach has also been applied to change specifically metabolic pathways as for example the methane production in ruminants. One of the major CH₄ producing organisms is the archaeal genus of Methanobrevibacter (Leahy et al., 2010). A previous investigation targeted Methanobrevibacter to prevent methanogenesis. But the experiment finished with moderate success due to complexity of the community - When one type of methanogen was removed - related methanogens took over and removed the effect of methane depletion (Williams et al., 2009). This is another example of manipulating the microbiota without taking the complex community into account.

Diet and supplements are the easiest way to manipulate the microbiota. The nutrient composition can easily be adjusted to the needs of the individual animal, can be fast applied and affect in theory the entire gut microbial community. Food constantly shapes the gut microbial community and can significantly alternate the composition within a day (David et al., 2013). There are several strategies to target specific metabolic pathways. For example the addition of given metabolites (prebiotics) to the diet to enhance or inhibit the growth of certain microbes. One of the most famous cases is the food additive fructooligosaccharides, added to commercial probiotics, to stimulate the growth of commensal bacteria like Bifidobacterium in order to treat colon associated disorders such as acute colitis (Zheng et al., 2014).

Diet manipulation of livestock animals has a long history. For instance, organic acids (e.g. fumarate), secondary plant metabolites (Tannins, saponins), as well as fats and oil are used to reduce the methane emission or to improve the animal productivity (e.g. Kumar et al., 2014). The mechanisms behind the methane depletion are often barely or not described because the impact on the microbial community is not evaluated (Martin et al., 2010).

The application of living beneficial microorganisms (probiotic) is a strategy frequently applied to alter the gut microbiota. Most famous examples are the addition of Bifidobacterium and Lactobacillus to reduce bowl disease in humans (O’Mahony et al., 2005). In ruminants acetogens have been in focus as potential probiotic strains hence the prokaryotes utilize hydrogen and incorporate electrons of hydrogen into acetate - an alternative pathway to the methanogenesis (Martin et al., 2010). However the addition of acetogens was without success most likely due to the competition of methanogens that display higher affinity to hydrogen (Kumar et al., 2014).

5. Previous methodological limitations and the “big bang” in microbiological science

The problem with all manipulation approaches mentioned above is that in the past most strategies were evaluated by investigating single bacterial strains or cultivatable communities. It has been estimated that approximately 1% of the prokaryotes on earth can be cultivated. Thus 99% of the microbiota was neglected. Consequently, the lack of understanding complex microbial communities
becomes evident when looking at the example of antimicrobial treatments. As mentioned before, the effects of antibiotics on commensals are often not described. The “big-bang” of the microbiological research started when sequencing became commercially affordable. This journey began in 2004 with 454 Roche generating 200000 sequences (100-150bp) in a single run (Emrich et al, 2007). Currently microbial studies are designed to characterize the 16S rRNA gene and sequence metagenomic or metatranscriptomic DNA. This study focused on the phylogenetic ribosomal marker of the 16S rRNA gene that holds taxonomic information from phyla to species level (Janda & Abbot, 2007). The gene is circa 1592bp long and consists of 9 variable regions (V1-V9) encoding different degrees of the taxonomic resolution (Cai et al, 2013, Mizrahi-Man et al, 2013).

![Figure 4. Frequency of residues along the different base position of the 16s RNA gene, reveals the phylogenetic conserved and variable region (V1-V9) (Figure adapted from Ashelford et al [6]). The 16S rRNA gene position 341-806 covering the region of V3 and V4 is the primary phylogenetic marker region of this thesis.]

To characterize the complex microbial community, universal primers are designed based on cultivated and annotated strains in public databases to target most microbes. The major problem here is the small sequence length generated by the sequencing technology, currently 454 FLX generates reads with up to 1000bp. In reality the average sequence length is approx. 400-500bases. The latest technology of the Illumina sequencer, that generates more sequences than 454 FLX, currently reaches a read length of 150 bases for single end or 300 bases for paired-end sequencing. In both cases the read length limits the taxonomic resolution of the sequence hence not the full 16S rRNA gene is observed. Nevertheless previous mock culture sequencing and in silico testing revealed that sequencing of certain variable region of the 16S rRNA gene holds enough taxonomic information to receive genus information (Nelson et al, 2014). In this study we amplified mostly a region covering V3-V4 (Figure 4) with the fragment size of 465 bases for all studies using 454 FLX sequencing (Human Microbiome Project Consortium, 2012, Gulino et al, 2013). When several samples are sequenced at the same time (which is commonly the case for 16S amplicon libraries), the individual amplicon library needs to be barcoded in order to be recognized by the sequencing software. This can be done directly with barcoded primers in one single PCR run, but due to
potential miss priming with metagenomic DNA, barcodes instead are attached to the amplicon in a second PCR (Berry et al., 2013). After the sequences are generated the data are de-multiplexed; cleaned of primers, adaptors, chimeras, low quality reads are removed; and denoised. To generate operational taxonomic units (OTUs), similar to bacterial species level, the sequences are clustered at 97% sequence identity (Stackebrandt & Goebel, 1994). Finally they are assigned to public databases such as Greengenes that is dedicated to ribosomal RNA sequences.

6. Identify the driving forces shaping the microbial community (alpha and beta diversity)

To characterize the complex microbial structure and estimate the consequence of potential manipulations, common ecology parameters are applied of which some of the most common ones: Alpha diversity, Chao 1 richness, etc. are mentioned below.

Alpha diversity is such an ecological tool that enumerates the species distribution in a single sample based on species richness, evenness and diversity. The most direct alpha diversity measure is the total OTU count number, the primary “species” estimator at a given sequence depth. However it does not distinguish between re-occurring OTUs (Zaura et al., 2009). Therefore the Chao1 richness index estimator can be used as an alternative since it takes into account, the total number of observed species, and how many species were found only once and twice (Chao A, 1984). Evenness evaluates the distribution of individuals across microbial species and can be determined by for example the Pielous evenness index (Pielou, 1975).

Moreover the Shannon index combines species number and abundance but is biased towards rare species (Shannon & Weaver, 1949). The diversity measure based on the phylogenetic tree (PD) is more sensitive towards rare OTUs (Faith DP, 1992). The PD diversity measures the sum of the branch length of a phylogenetic tree of a given set of OTUs in a sample, however species abundances are neglected. As a general advice, microbial communities should be evaluated by multiple parameters, due to mentioned limitation in diversity estimation.

In order to estimate the compositional variations between treatments the beta diversity that compares differences between samples needs to be evaluated. Often the methods used are the Bray-Curtis dissimilarity, the unweighted and weighted Unifrac distance matrices. Bray-Curtis dissimilarity estimates how many species are shared between samples sites compared to the total species number observed (Bray & Curtis, 1957). This tool is very useful when comparing the abundances of bacterial species between samples. The unweighted Unifrac estimates distance between samples on the summed branch length of the phylogenetic tree of shared and non-shared OTUs (Lozupone & Knight, 2005). The weighted Unifrac metric includes the abundance of OTUs while calculating the branch length.

There are several tools to visualize dissimilarity between samples and to identify driving forces shaping the microbial community such as ordination. One of the most common methods is the principal coordinate analysis (PCoA). The PCoA transforms the distance metric (Bray-Curtis, Unifrac etc.) into a graphical configuration of a low multi-dimensional Euclidean space explaining the largest variances between the samples in a low dimensional space (usually the first three dimension) (Ramette A, 2007). The community clustering can further be characterized for instance
by the analysis of similarity (Anosim) that estimates the degree of separation of two or more groups (Clarke KR, 1993). Anosim measures the average rank distance within replicates compared to the mean rank distance between groups. The test generates an R-value of which 1 indicates highly dissimilarity and 0 no difference.

After observing if the manipulation changed the overall microbial community structure the difference in composition needs do be identified. Variations in relative frequency can be estimated by classical statistical methods, depending on the nature of the data set. For example if the data are parametric, variations in abundance between groups are evaluated by the Students t-test (if variables are paired) and the Welch’s t-test (if variables are not paired) when two populations are compared. If more than two populations are investigated a standard Anova test can be applied. In case of a non-parametric data set the Wilcoxon Rank Sum test is applicable for two populations comparison, for more than 2 populations the best test is Kruskal-Wallis Ranks Sum test. Finally, here to mention is that there is tremendous amount multivariate analysis methods to compare microbial communities and new statistical methods are developed. I focused on the listed methods since they have been applied in this thesis.

7. Microbes reticulated – Identifying interactions in complex networks

Difference in relative abundance hold little information of the interaction between the microbial communities within groups of diet or treatments and often the community is so complex that it is difficult to estimate the importance of single variations. However microbial interactions can display for example putative relationships of synergistic or antagonistic nature with importance for the microbial flora and the host eventually. Recent studies hypothesized that microbial interactions can be described by co-occurrence pattern based on the 16S rRNA gene amplicon observation and the enumeration of pairwise relationships between species (Faust & Raes, 2012).

Other ways to describe microbial interaction are rule-based models (Faust & Raes, 2012). Rule based approaches predict interaction on pre-formulated assumption of the presence and absence of certain OTUs in the presence of other OTUs. This model is difficult to apply in complex microbial communities when no advanced pre-knowledge is given.

Pairwise interactions can be applied to the complex microbial community based on abundance table using graph theory. Theoretically, if two OTUs or species (nodes) co-occur or co-exclude in a linear manner, the OTUs will be connected by a graph called “edge” that indicates positive or negative co-correlation. Every OTU can have multiple connections of which the sum is called the degree. These graph type interaction can be generated for example by Cytoscape (Saito et al, 2012) or Igraph R (Csardi & Nepusz, 2006). However there are several steps to be considered before generating a correlation network. First, false differences in abundance introduced by DNA extraction methods, variation in sequencing effort etc. needs to be removed from the OTU table by for example normalization or down sampling. Secondly, to prevent arbitrary correlation of highly abundant with low abundant OTUs, the abundance table should be log-transformed. As Faust & Raes indicated (2012), with large numbers of zeros often observed in OTU tables are not necessarily due to negative correlation but rather due to sequencing effort. This issue can be addressed by removing
rare species with the risk of missing potentially important bacteria. In the fourth step, a proper correlation measure needs to be chosen. Two of them are the parametric Pearson and non-parametric Spearman correlation. However, most problematic for co-occurrence networks is the choice of a biological relevant correlation cutoff value. Which degree of correlation predicts true interactions? (Figure 5) A Spearman Rho cutoff of 0.9 relates OTUs highly significant, however biological correlation not always follow laboratory conditions (Barberán et al, 2011). We used in this study the spearman cutoff of 0.6, as it explained the majority of the OTUs of the abundance table (Manuscript 4 and 5) with the least clustering effect. Furthermore, the chances of random correlation increases by the number of compared pairs; therefore multiple testing should be applied by for example applying the Bonferroni-corrected permutation p-value (p<0.05).

Figure 5. Correlation network of the black vulture facial microbes. Network A and B are based on the same OTU table of 26 black vultures, but A displays stronger co-correlations compared to B. Interactions were filtered for minimal sample count of 13. The networks indicate the problem of pairwise co-occurrence networks. Network A is divided into several components without interaction between the components and it lacks significantly negative co-correlations compared to B. But are all interactions of network B biological meaning full?
After the network is generated, the analyst is exposed to large quantity of correlations. Which connections are essential to sustain the functional microbiome? Which interactions are affected by the dynamics of the system?

Keystone species are taxa with a more important role than others in a given environment; whereas other organisms are functional redundant and their loss will not substantially harm the function of a system (Ley et al., 2006). Robert T. Paine first introduced the keystone concept in 1965 observing that the removal of the low abundant gastropod Charonia tritonis elevated the level of the starfish Acanthaster planci with introducing significant disturbances in the complex coral reef community. Thus, although the C tritonis belongs to a minority species, its occurrence is essential to the functional community in the reef. A few reports in host-microbial alliances have indicated bacteria with potential keystone character such as the enterotype investigation of the human gut that separates the European human population into the three types with Bacteroides, Prevotella, or Ruminococcus as central genus (Arumugam et al., 2011). Putative microbial keystone species have also been indicated using co-correlation networks in oral biofilms and soil (Duran-Pinedo et al., 2011, Lupatini et al., 2014).

Jordán F discussed in 2009 the usage of network analysis to determine keystone species. He highlighted the three measures of Degree, Betweenness centrality and Closeness centrality, to characterize potential important species. The degree is the average number of neighboring nodes (Figure 6). It estimates the impact of a species to the number of connections. Although several OTUs can have a low degree they still connect clusters and interact with many OTUs indirectly. Those OTUs will be neglected by average number of neighbouring nodes. This type of keystone node can be characterized by the Betweenness centrality, the proportion of the shortest path between all nodes and all nodes passing a given nodes. Thirdly there is the closeness centrality, which measures the average distance of a node from all others in a graph. Independent of which measure is used to identify keystone species, the methodology is new and the experimental confirmation is still needed.
Figure 6. Keystone estimators in microbial networks. Examples adapted from the Cytoscape plugin NetworkAnalyzer (Doncheva et al [7])

8. Discussion and future perspective

8.1. Pitfalls of methodology

The main method of microbial characterization of this study is the sequencing based 16S rRNA gene amplicon library. The method clearly has the advantage that it is culture independent and most importantly characterizes the complex microbial communities and not only focuses on few prevalent and isolated species when comparing treatments or manipulation as previously done by classical microbiological techniques. However the technique has several disadvantages that need to be highlighted in this section.

The PCR based method comes along with amplification based biases. For example chimera is one of the major errors that is generated during PCR, when strand extension was not finalized during an amplification cycle and similar biological sequences that should not joined are joined (Smyth et al, 2010). Also, minor variation in DNA concentration during library preparation can have significant effects in sequencing effort. To compensate this inconsistence, samples were randomly down sampled with the result that large parts of the reads were ignored. Rarefaction has the potential to introduce errors in the data interpretation as recently indicated and needs to be addressed in future
work (McMurdie & Holmes, 2014). Also to consider, despite the usage of universal primers, the oligonucleotides are designed based on previous isolated and characterized microbes. This is a significant problem since this thesis evaluated environments not described before and as previously mentioned, the major microbes on earth have not been investigated. Therefore there is the high risk that some bacteria will not be described by the 16S amplicon library approach. Additionally taxonomic results are affected by the choice of 16S rRNA gene variable region sequenced and the chosen primers (e.g. Mizrahi-Man et al., 2013). Thus comparison to previous published data especially on higher taxonomic levels such as genus needs to be taken with care if different primers and different variable regions are sequenced. Furthermore, the sequencing technology of 454 FLX has many sequencing errors such as homopolymers (amplicons with sequences of identical bases as for example AAAAAA) that make in average 1% of the 454 sequences (Gilles et al., 2011). Chimera and homopolymer artificially increase the diversity. To remove both error types, algorithms such as Uchime (chimera) and AmpliconNoise (for homopolymer detection, Quince et al., 2011) have been developed. AmpliconNoise is very computing intense. Homopolymers can be avoided by using the Illumina sequencer, however that reduces currently the taxonomic resolution due to shorter sequence length of this machine.

After the sequences are cleaned and trimmed of errors and OTUs are picked de novo, the sequences are subsampled to receive relative abundances for statistical evaluation. However the results cannot directly be associated to the “real” abundance of the microbes, because not every bacterial cell carries only 1 ribosomal 16S gene (Klappenbach et al., 2000; Klappenbach et al., 2001). The number varies largely between and within phyla and consequently hindering the estimation of the total abundance of biological entities. Copy number adjustments at higher taxonomic resolution are problematic making the successful assignment to drop from genus to phylum level due to the limited resolution of the short amplicon fragment or the lack of references in the database. This is this is not a problem when we enumerate treatment effects between biological samples. The limited taxonomic resolution of the amplified 16S rRNA gene fragments (usually assigned to only genus level) is a major drawback because we cannot associate biological relevant questions to species or subspecies.

Finally, this thesis investigates microbial communities at single time points. Crucial information about the dynamics and stabilities of systems are entirely neglected. This is partially due to the experimental setup but also because of sequencing costs.

8.2. Future perspectives

As sequencing prices drop significantly, microbiome profiling will eventually move to shot-gun metagenome sequencing instead of 16S rRNA analysis. The metagenome analysis has in theory the advantage that the total DNA of a given sample is characterized and therefore combinations of different genes can be used to assign taxonomy to the sequences. The metagenome also allows more accurate species number enumerations with single copy universal phylogenetic marker genes as recently reported (Sunagawa et al., 2013). Furthermore, not only bacteria and archaea, but also
fungi, protozoa, virus and metabolic genes are analyzed simultaneously, substantially increasing the resolution of the reticulated microbial world.

However the workflow of the metagenome analysis is currently less standardized as the 16S rRNA genes studies and new tools are rapidly developed to for example speed up the assembly of reads into contigs. This complicates comparisons with already published data sets. Nevertheless, standardization techniques will most likely be developed or improved in the near future.

One of the main goals of this thesis was to apply pairwise microbial networks to predict potential interactions and characterize OTUs of larger importance compared to the other OTUs of the microbial system. The use of co-correlation interactions in microbial surveys is still new and the strength of the methodology to predict real interactions depends on the type of data and is an open field of discussion. Future studies need to evaluate experimentally microbial networks and characterize the importance of the keystone species. Are single microbes more important for the host environment than others? And how does the microbial community change if those putative keystone taxa are removed?

In theory the importance of a given OTU host system could be estimated by removing it from the community by antibodies or keystone specifically designed phages. The consequences of the loss of this central microbe on the total microbial community and the host could then be evaluated.

Finally, this study claims to characterize the complex microbial community. And yet, the applied method, as mentioned before, characterizes only prokaryotes (with the exception for manuscript 5). However eukaryotes, such as fungi, protozoa, naturally occurring phages and animal viruses have substantial impact on the host and the microbial community. The first inter domain correlations studies have just recently been reported (Hoppe et al, 2014) and should be included in future studies.

9. References


**Picture References**


The murine lung microbiome in relation to the intestinal and vaginal bacterial communities
The murine lung microbiome in relation to the intestinal and vaginal bacterial communities

Kenneth Klingenberg Barfod1,2*, Michael Roggenbuck3†, Lars Hestbjerg Hansen3, Susanne Schjørring1, Søren Thor Larsen4, Søren Johannes Sørensen4 and Karen Angeliki Krogfelt1

Abstract

Background: This work provides the first description of the bacterial population of the lung microbiota in mice. The aim of this study was to examine the lung microbiome in mice, the most used animal model for inflammatory lung diseases such as COPD, cystic fibrosis and asthma. Bacterial communities from broncho-alveolar lavage fluids and lung tissue were compared to samples taken from fecal matter (caecum) and vaginal lavage fluid from female BALB/cJ mice.

Results: Using a customized 16S rRNA sequencing protocol amplifying the V3-V4 region our study shows that the mice have a lung microbiome that cluster separately from mouse intestinal microbiome (caecum). The mouse lung microbiome is dominated by Proteobacteria, Firmicutes, Actinobacteria, Bacteroidetes and Cyanobacteria overlapping the vaginal microbiome. We also show that removal of host tissue or cells from lung fluid during the DNA extraction step has an impact on the resulting bacterial community profile. Sample preparation needs to be considered when choosing an extraction method and interpreting data.

Conclusions: We have consistently amplified bacterial DNA from mouse lungs that is distinct from the intestinal microbiome in these mice. The gut microbiome has been extensively studied for its links to development of disease. Here we suggest that also the lung microbiome could be important in relation to inflammatory lung diseases. Further research is needed to understand the contribution of the lung microbiome and the gut-lung axis to the development of lung diseases such as COPD and asthma.

Background

Studies of the lung microbiome by culture independent techniques and its impact on lung immunity is a relatively new field and may contribute to new advances in understanding respiratory diseases [1]. Healthy human lungs have up until recently been considered to be sterile by culture-based techniques, but new evidence have identified microbial communities both in healthy humans and in those with disease [2-4]. The human microbiome project [5] did not originally include the lungs, but recently the Lung HIV Microbiome Project has published the first results in this field [6,7]. Investigations into lung microbiology and lung immunity in humans is limited largely because of technical, ethical considerations and small samples sizes, whereas the use of animal models can provide novel information useful in investigations into the importance of lung microbiome in the development of lung immunology. Effective utilization and development of animal models have recently been identified as one of the most important challenges in future lung microbiome research by the NIH [8]. Whereas many studies have focused on the gut microbiome and its impact on among others lung immunity and asthma, little work has been performed to examine the contribution of the lung microbiome on the pathogenesis of pulmonary diseases. Especially in inflammatory lung diseases such as asthma and COPD, the local microbiome may play an important role in the pathogenesis. The technical challenges related to the novel culture-dependent techniques include consistent extraction of useful DNA, the development of PCR methods and sampling methods for the less abundant bacterial load of the lungs.
We hypothesized that the problems with getting bacterial DNA from lungs was due to the presence of host DNA in the extractions. In this study, we have investigated the bacterial community from lungs of 20 mice using rDNA amplicon 454 pyrosequencing. We also performed a conventional cultivation study of 10 mouse bronchoalveolar lavage (BAL) fluids on different agar plates. Sampling methods and DNA extraction protocols were investigated systematically: one BAL sample still containing mouse cells (BAL-plus) and one BAL sample, where the mouse cells were removed (BAL-minus) by cytopsin. The bacterial communities in BAL samples were compared using DNA extractions from washed lung tissue, caecum samples and vaginal flushing. We chose to include vaginal samples for two major reasons. The vaginal microbiome of BALB/c has not previously been described and could have influence on microbial "priming" and transfer from mother to pup. In this study, it also serves a reference sample from a different mucoid epithelium than lung. The bacteria were classified by their sequence into Operational Taxonomic Units (OTU). An OTU is an approximation to taxonomy with a given cutoff for sequence similarity.

In this study, it also serves a reference sample from a different mucoid epithelium than lung. The bacteria were classified by their sequence into Operational Taxonomic Units (OTU). An OTU is an approximation to taxonomy with a given cutoff for sequence similarity.

Bacterial identification by culturing

Mouse BAL fluids, 200 μL per mouse, were cultivated on general growth media blood agar 5% (SSI, Denmark) and Chocolate Agar (SSI, Denmark) for fastidious bacteria and incubated at 37°C for 24 hours. Another set of plates with selective media was incubated under microaerophilic conditions (5%CO₂, 3%H₂, 5%O₂ and 87%N₂) at 37°C for 48 hours [11]. The bacterial colonies were subjected to routine identification by the Vitek2 system (Bio Mérieux, France).

DNA extraction and PCR

Isolation of bacterial DNA from frozen BAL or vaginal samples was done using Qiagen spin protocol (Qiagen, DNA mini kit Denmark) for body fluids with the following modifications: Tubes were thawed and centrifuged at 16,000 g for 5 min to spin down all the bacteria. The supernatant was discarded and the bacterial pellet was resuspended with 450 μL lysis buffer. Forty-five μL proteinase K and add 0.3 mL 0.1 mm zirconium/silica beads (Techum, Sweden) were added. Proceed with bead beating step using TissueLyser (Qiagen, Denmark) for 6 min at 30 Hz. [12]. Lysis was performed by incubating in heat block at 56°C for 10 min. and then at 95°C for 7 min. Proceed with protocol for body fluids from step 5. At the elution step, the AE buffer is preheated to 65°C and DNA elution is performed with 100 μL with 3 minutes incubation at room temp before final spin. Isolation of bacterial DNA from frozen caecal or tissue was done using Qiagen spin protocol for detection of pathogens from stool (Qiagen, DNA mini stool kit Denmark) with the following modifications: Add 1.4 mL of the ASL buffer and perform bead beating, lysing and eluding as described above for body fluids. For tissues samples, chlorine [10] and heat sterilized 3 mm steel bead (Qiagen, Denmark) was added to the samples along with the zirconium/silica beads for extra tissue disruption.

16S sequencing

Amplicon libraries of the 16S rRNA gene of caecum, BAL and vaginal samples were prepared with two PCR reactions.
In the first PCR, a 466 bp long fragment covering the variable region V3 and V4 of the 16S rRNA gene, was amplified with AccuPrime™ Pfx DNA Polymerase and the bacteria and archaea specific primers 341 F and 806R (Table 1). The reaction started with an initialization at 94°C for 2 min, followed by 44 cycles of denaturation at 94°C for 20 sec, annealing at 56°C for 30 sec. and elongation at 68°C for 40 sec. The reaction was completed with a final elongation at 68°C for 5 min. Due to the low DNA (<0.5 ng × μL⁻¹) concentration in the samples we needed to increase the cycle number above the standard of 30–35. This adjustment highly increased the risk of amplifying contamination from extraction buffer and other experimental used liquids. To minimize this possibility we chose the lowest cycle number with a clear amplification band in the agarose gel and no signals of negative controls from BAL procedure for DNA purification.

In the second PCR the adaptors were attached to the amplicon library elongating the fragment towards 526 bp with the primer TitA_341F and TitB_806R. The same reaction conditions of PCR I were applied in PCR II with a reduced cycle number of 15.

Initially we tried to apply the same procedure for the lung tissue samples but unspecific bands after gel-electrophoresis made it impossible to select the correct fragment size. To overcome this problem we chose the primer 27 F and 1492R amplifying the entire 16S rRNA gene which appeared to be more specific. The PCR I conditions were the same as mentioned above except that the annealing temperature was reduced to 55°C and the cycle number to 40. In this perspective the Tag-PCR reaction with TitA_341F and TitB_806R provided the selection for V3 and V4 as well as attaching the adaptors to the amplicons.

### Statistical analysis and bioinformatics

The 16S rRNA gene sequences obtained from one half a plate of a 454 - Roche - Titanium pyrosequencing run were quality filtered, trimmed and split into the corresponding animal samples with the Qiime pipeline version 1.6.0 using the default settings [17]. We considered only sequences with a minimum length of 250 bp. Chimeras were removed by UCHIME [18]. The operational taxonomic units (OTU) were picked de novo and clustered at 97% sequence similarity. The taxonomy was assigned using RDP classifier (bootstrap threshold 0.8) greengenes as reference database [19].

For statistical analysis, raw data were transferred into the open source statistical program “R” [20]. The non-parametric Wilcoxon test (W) evaluated variations of alpha diversity between two variables. We used the non-parametric Kruskal-Wallis-test when comparing more than 2 variables (KW). Dissimilarities in OTUs abundance between the samples were explained by KW and the sample clustering of the OTU count based Bray-Curtis distance metric were examined by the analysis of similarity (anosim).

### Results

To determine the airway bacterial microbiota of the BALB/cJ mouse model based on 16S rDNA gene sequencing, we have compared sequences found in the lungs with three different approaches, to sequences found in corresponding vaginal and caecal samples.

Over all sequence quality and results from all sample types

We generated a total of 908256 sequences. After quality filtering and chimera check, 27% of sequences were removed and 660319 sequences were further processed for OTU picking (sequences ranged between 3530 up to 31638 per animal sample). The de novo OTU clustering revealed 6487 OTUs. The OTU table was randomly subsampled to avoid differences based on sequencing effort leaving 3318 OTUs for further analysis (Rarefaction curve are shown in Additional file 1: Figure S5).

We found a total of 19 bacterial phyla in the samples analysed. The most dominant (>0.5% abundance) phyla observed were Acidobacteria, Actinobacteria, Bacteroidetes, Firmicutes, Proteobacteria and TM7. The difference in bacterial composition at the phylum level between sampling sites is shown in Figure 1A.

### Table 1 Primers

<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequence</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>27 F</td>
<td>5′-AGAAGTTTGTATCTGAGCCTAG-3′</td>
<td>[13]</td>
</tr>
<tr>
<td>341 F</td>
<td>5′-CTTAGGGGGRBCGAC-3′</td>
<td>[14, 15]</td>
</tr>
<tr>
<td>806 R</td>
<td>5′-GGACTAANNGGTATCTAAT-3′</td>
<td>[14, 15]</td>
</tr>
<tr>
<td>TitA_341F</td>
<td>5′-CGTATCGCTTCCTGAGCCTAG-3′</td>
<td>[16]</td>
</tr>
<tr>
<td>TitB_806R</td>
<td>5′-CTATGCGCCCTGACGAGCCTAG-3′</td>
<td>[16]</td>
</tr>
<tr>
<td>1492R</td>
<td>5′-GGITACACTTGTAGACTT-3′</td>
<td>[13]</td>
</tr>
</tbody>
</table>
In Additional file 2: Table S2 we have listed all the bacteria that were found, which were unique for the lung samples and which were shared between sampling sites.

The bacterial sequences of the lung samples

If we only look at the lung samples, the most dominant lung phyla found were Proteobacteria, Firmicutes, Actinobacteria, Bacteroidetes and Cyanobacteria. Additionally we observed Fusobacteria and Cyanobacteria in the lung and vaginal samples.

In order to highlight phyla variations in the lung community compared to vaginal and caecal communities, we first we took the three lung sample types: bronchoalveolar lavage fluids (BAL-plus), and BAL-minus, where the mouse cells have been removed by a spin protocol and finally lung tissue from the distal tip of the lung and considered them as one ecological community. In this lung community profile, Actinobacteria and Proteobacteria were clearly more abundant than in the caecum community (KW, p < 0.0001).

Then, looking at the differences between the three lung sample types, Firmicutes appeared (KW, p < 0.05) more abundant in lung tissue (57%) than in BAL samples (20%). The SR1 bacteria were found only in BAL-minus and Lung tissue samples, but Tenericutes was observed in all samples, except in the vaginal samples. Other phyla observed below 0.5% abundance were Chloroflexi, Deinococcus-Thermus, Fibrobacteres, Gemmatimonadetes, OD1, OP10, Planctomycetes, Verrucomicrobia, and WS3.

Comparing lung sampling methods we also found a significant variation for Actinobacteria and Proteobacteria, which were largely abundant in both type of BAL communities relative to the lung tissue samples (KW, p < 0.05). At phylum level, the composition of the lung tissue

Figure 1 Community composition. (A) Distribution of Phyla between sample types. LF-plus bronchoalveolar lavage (BAL) fluids and LF-minus is BAL where the mouse cells have been removed. LT is lung tissue and VF is vaginal flushing. (B) Venn diagram of identified shared and unique genera from each sampling site. All the lung type samples are considered here as one. (complete list shown in Additional file 3: Table S4). (C) The PcoA plot is generated of the Bray-Curtis dissimilarity metric based on OTU counts and explains the largest variance between all samples (PCoA plot 1 vs 3 and PCoA plot 2 vs. 3 are attached in Additional file 4: Figure S4). (D) Heat map of even subsampled OTU table. The dendrogram is two sited hierarchal clustered by abundance dissimilarity and the data are log transformed. Shown are only taxa, which counted for at least 0.05% of the generated sequences. The x-axis clusters the animal samples and the y-axis the taxonomical information. * marks Vaginal subcluster S1 and ** subcluster S2.
samples appeared to be very similar to the vaginal samples except for a larger abundance of Cyanobacteria in vaginal samples (KW, p < 0.05).

Bacterial sequences of the caecum
Looking at the caecum samples, they contained more Firmicutes and Bacteroidetes KW, p < 0.0001 than the lung samples and Acidobacteria and Cyanobacteria were absent. The phylum Bacteroidetes (29%) appeared to be the second most abundant after the Firmicutes (59%). The vaginal and the caecal communities only had Rumicococcus in common, a genus that was not observed in the lung microbiota. Three genera were found in caecal samples alone; Robinsoniella, Parasutterella and Ramlibacter. The low numbers of genera detected in the caecal samples is due to the depth of taxonomic information obtained for these particular OTU sequences towards the consensus lineage of the database.

Overlapping genera
For an overview comparison between the different sample types, we have merged the results found in the different lung communities and displayed the overlapping genera with caecum and vagina in a venn diagram. This diagram reflects 255 identified genera (summarized in Additional file 3: Table S4), that covers 76% of the sequences from BAL-plus, 68% from BAL-minus, 66% of vaginal and lung tissue community and 27% of sequences assigned to the caecum community (Figure 1B).

Lung samples, vaginal and caecum samples shared the 12 core genera Bacteroides, Barnesiella, Odoribacter, Alistipes, Mucispirillum, Lactobacillus, Streptococcus, Peptostreptococcus, Roseburia, Anaerotruncus, Oscillibacter, Pseudomonas. We observed Parabacteroides, Eubacterium, Marvinhoxydans, Butyrivibrio, Papillibacter, Bosa, Anaeroplasma, lung and caecum. The pulmonic and vaginal community shared 103 genera (Additional file 3: Table S4). Additionally Akkermansia was also found in the lung but only in one caecum sample in the raw data set.

Variability in community composition between samples obtained from the same sampling site (Beta_diversity)
To make a sample to sample comparison and illustrate the variation between our mice we have performed a principle coordinate analysis (PCoA) based on the Bray-Curtis dissimilarity between OTU count metric PCoA plot (Figure 1C), which explains the largest variance between all samples (Additional PCoA 2 and 3 are found in Additional file 4: Figure S4).

The caecal samples cluster together at a significant distance from lung and vaginal communities, confirmed by the analysis of similarity, anosim (R = 0.673, p = 0.001) The dissimilarity between the three lung communities was found to be little due to strong cluster overlap (anosim, R = 0.09, p = 0.05) when comparing only the lung distances.

We found large variation within the vaginal samples resulting in a division into subcluster 1 (S1), containing animal vaginal sample 8, 5 and 2, and subcluster 2 (S2), vaginal sample 3,4,6,9 and 10 (anosim, R = 0.72, p = 0.001). The separation is clearly shown in PCoA1 (Figure 1C) and PCoA3 (Additional file 4: Figure S4). Those samples that grouped into S1 were found to be less similar to caecum and lung communities, whereas samples grouping into S2 appeared more closely related to the lung microbiota.

A more detailed description of the taxa responsible for distinguishing bacterial communities in the lung, caecum and vagina is demonstrated using a heatmap dendrogram (Figure 1D).

We removed from the subsampled OTU table all observations accounting for less than 0.5% of the generated sequences to visualize the taxa with main impact on the community profile. This method provides maximal taxonomic resolution of each individual animal sample and directly reflects the PCoA plots since both analyses are based on OTU count dissimilarities.

For the caecum samples, 27% could be assigned to a taxonomic genus as mentioned before and the sequences belonged to Alistipes (16%) Anaeroplasmata (15.3%) and Porphyromonadaceae (7.9%).

Vaginal samples within S1 contained between 56-97% of Streptococcus, while vaginal samples within S2 only had 0.2 – 10% of the gram-positive bacterium, explaining why here appears to be such a distinction between the S1 and S2 groups. In addition to Streptococcus, notable contributions from Acinetobacter (6.2%), Sphingomonas (3.3%), Enterococcus (3.1%), and Polaromonas (1.8%) were also observed in the vaginal community.

All lung samples had representative sequences from genera including Staphylococcus (8.3%) Massilia (2.6%), Corynebacterium (2.2%), Pseudomonas (2.5%), Streptococcus (2.3%) and Sphingomonas (1.7%) without significant variation (KW, p > 0.05).

Even though the beta diversity measure indicated that there were minimal differences between the lung communities sampled using different methods, six major genera varied significantly (KW, p < 0.05). Acinetobacter, Pelomonas, and Schlegella were more abundant in the BAL-plus samples in comparison to the BAL-minus or the lung tissue samples. Arcobacter, and Polaromonas were highly associated with BAL-minus, whereas Brochothrix was only found in the lung tissue samples.
Richness and diversity of sample type (Alpha diversity)

To compare the OTU diversity between sample approaches and sampling sites, we have calculated the alpha diversity index. There were two key points we were interested in. First, we wanted to know if the alpha diversity of the BAL samples was higher or lower than the diversity of the lung tissue samples. A larger or comparable alpha diversity index would indicate that the BAL samples communities provide a representative snapshot picture of the microbial composition of the lung. However a lower alpha-diversity of the BAL samples would make functional assumption based on the BAL sampling difficult since a significant amount of taxa will not be described. Secondly, we expected that host cell removal from the BAL-minus material would reduce the diversity index because some bacteria could be stronger attached to the pulmonic cell surface than others and could be removed from the sample by centrifugation.

The bacterial community of the BAL-minus were in 50% of the cases (indicated by the median) richer than the BAL-plus (Figure 2A). We found this difference to be significant (W, p < 0.05).

There was no significant variation between the BAL-minus and lung tissue samples. The mouse caecum community is generally richer than all other tested communities, except of the upper quartile of the tissue samples. The vaginal microbiota appeared to be as rich as the lung tissue community.

In more than half of the BAL-minus samples, more unique OTUs were observed than in the lung tissue material (Figure 2B). The BAL-plus samples contained significantly less OTUs than the BAL-minus samples.

Figure 2 Alpha diversity plots. A: Chao1 richness estimator between sample types and individual samples (circles), LF-plus is bronchoalveolar lavage (BAL) fluids and LF-minus is BAL where the mouse cells have been removed. LT is lung tissue and VF is vaginal flushing. B: Observed unique OTUs and C: Shannon diversity estimator between sample types (s above) and individual samples (circles). The sequences (3350) were randomly even subsampled before calculating the alpha diversity. The boxplots show median, quartile, smallest and largest observations as well as outliers (circles). Significant variation is indicated by * (KW, p < 0.05).
(W, p < 0.001). The variation of Chao1 and observed OTUs comparing all pulmonic samples were significant (KW, p < 0.01). We observed the highest number of unique OTUs in the caecum samples, compared to vaginal and lung tissue microbiota (W, p > 0.05).

A slightly different picture was observed for the diversity index (Figure 2C). In most cases the alpha diversity of BAL-minus samples appeared to be larger than the BAL-plus and lung tissue samples. However, the variation of diversity between all pulmonic samples was not significant (KW, p > 0.05). The Shannon index varied significantly when comparing both BAL-plus and BAL-minus communities only (W, p < 0.05) and reflect the observation of Chao1 and unique OTU sequences.

In summary, the mouse cell-free BAL samples yielded a richer microbial community, had a larger alpha-diversity and contained more unique OTU in comparison to the samples with mouse cells. In addition, at least 50% of the alpha-diversity observations the BAL-minus show larger diversity indexes than the lung tissue samples. The upper quartile of the lung tissue samples varied largely for all three diversity indicators approaching larger diversity (Shannon), richness (Chao1) and observed OTUs as found for the caecum samples. This could be the result of non-proper flushing or contamination during the experimental process. However, the low diversity, richness and fewer OTUs in the lung tissue samples correspond to higher diversity, richness and more OTUs in the matching BAL samples. There is also a large overlap in beta-diversity based on OTU abundance of lung tissue samples with the BAL samples, suggesting that, a biased flushing is more likely to be the reason, than contamination.

Bacteria found via traditional culturing of BAL
To establish any possibly cultivable part of the lung microbiota and possible viable contaminations, we performed a conventional cultivation study of BAL fluids from 10 additional mice. Of the 40 different agar plates under various conditions with 200 μL BAL per plate from each of the 10 mice, we only found a few bacterial colonies on 5 plates originating from only 4 different mice. These bacterial colonies were all identified to be Micrococcus luteus with 99% probability by the Vitek2 system (Bio Mérieux, France).

Discussion
Methodology
In this work we have sequenced the lung bacterial 16S rRNA gene variable region V3/V4 with different methods and compared the results to gut and vaginal bacterial microbiome. We chose the V3/V4 region since Claesson et. Al [21] reported that it taxonomically characterizes microbial communities best without sequencing the entire 16S rRNA gene. Furthermore the same approach has been applied in multiple studies to study bacterial interaction with lakes, plants, humans and most important with mice [22-25].

In contrast to the general assumption, our results suggest that the lower airways in mice are not sterile and have a distinct bacterial microbiome that could probably influence airway diseases. A classic obstacle in the investigation of the microbiota of the lungs is the likelihood of contamination with bacteria from the upper respiratory tract (URT). This is especially true for the study of the human respiratory microbiome, because the procedure used has a high risk of contamination with oral microbiota [7]. In our study, this is bypassed by the invasive entry via the throat into trachea. We have extracted bacterial DNA from lung tissue, BAL with and without mouse cells and vaginal flushings. Our results show that it is possible to consistently obtain comparable sequences from the BAL fluid to use for community studies related the development of inflammatory disease in our mouse model.

The use of BAL as the sample for investigations has several advantages. The BAL sampling resembles the procedures used in humans, except that the work in animals bypasses both URT and oral microorganisms and samples the entire lung instead of just a local lung compartment. The microbial community has been shown to vary with the site of sampling in excised lung from a COPD lung transplant [26]. The removal of mouse cells in the BAL has the added advantage that we can use the lung cells and determine the lung inflammatory status of the mouse by staining and differentiating the immune cells [27]. A limitation of our study is the lack of comparison of our sequences with that of the upper respiratory flora. This could possibly be obtained by performing 16S rDNA sequencing on a matching nasal lavage sample for each mouse. This should be done in the future.

Our lung tissue samples showed some clustering that could indicate a sampling problem. In our study we sampled the distal tip of the left lung lobe after the BAL procedure was performed. The clustering could be a result of this BAL procedure not being equally effective between samples in the very low airways, sometimes leaving the distal tip un-flushed. This would predict a clustering showing one community equal to the one found in the BAL and one more rich and diverse representing the less rinsed tissue. If we were especially interested in the tissue associated microbiota, BAL should not be performed before sampling and mouse cells should not be removed from the BAL fluid before extraction.

Our results show that there are fewer OTUs in the BAL-plus samples with mouse cells and that the lung tissues samples have a large variation. This suggests that the removal of tissues and host cells is a viable approach,
when extracting DNA for the examination of the lung microbiome.

Another challenge when working with low bacterial loads is the risk of contamination from the environment or sampling procedures. Some contamination must be expected and taken into account when interpreting data. We believe that we have taken large precautions to insure sterility during procedures and we have used excess controls to check that our sampling procedure or experimental chemicals did not produce any sequences on their own in the PCRs. Culturing of the BAL used for DNA extraction did not yield many bacteria either. Furthermore, our sequences were very consistent between mice. This would suggest that any contamination was either negligible or at least distributed evenly between mice.

We did find large variation within the vaginal samples resulting in subclustering into groups we designated S1 and S2 (Figure 1C and D). S1 (vaginal samples 2, 5 and 8) was found to be much more distantly related to caecum and lung communities than the S2 group, which more closely resembled the lung microbiota. We believe this could be the result of a possible infection in the S1 vaginas, as these 3 samples contained 56-97% Streptococcus. In the present study, we did not monitor the stage of the estrous cycle at the time of sampling, which has been shown to change the bacterial profile of the vagina in animals and humans [28,29]. Mice have a daily fluctuation in estrous cycle, which in part could explain the subclustering of the vaginal microbiota. This should be taken into account in futures studies.

There was a concern that the vaginal sampling procedure would yield a large overlap in OTUs with the gut microbiota due to cross contamination during sampling. This has been shown not to be the case, as we show here there is a very little overlap between caecum and vaginal microbiota. To our best knowledge this is the first time that the BALB/c mouse vaginal bacterial community has been investigated with 454 Pyrosequencing for a full community study. This promises to be useful in futures studies of the "inheritance" of bacterial microbiome from mother to pup or vaginal microbiome related diseases such as vaginosis [28,30].

We faced two main obstacles: The low DNA concentration in all samples, except for the caecal material and unspecific primer binding in the tissue samples.

To overcome the low DNA concentration we increased the PCR cycle number. The large cycle number essentially could amplify any kind of contamination or primer bias such as chimeras, but we adjusted the rounds of cycles to the crucial experimental negative controls as described in the material and methods. Our results are confirmed by the observed community profile of previous human lung observations (discussed in detail below) and the low abundance of chimeras (<3% of quality trimmed sequences) [31,32].

The second obstacle was the non-specific binding of the primers in the lung tissue sample caused by the low amounts of bacterial DNA and large amounts of eukaryotic nucleic acids. Since the risk of contamination barely left space for adjustments, we chose to do a nested PCR and amplified a ~ 1500 bp long fragment of the 16S rRNA gene prior amplifying the hyper variable region V3/V4. Although both primer sets are universal and theoretically target all bacteria and archaea, the tendency to favor certain taxonomic groups cannot be excluded, thus one primer set should be preferred to compare the different samples.

Therefore we were expecting a significantly different clustering in beta-diversity of the lung tissue community in comparison to the BAL fluids. However the differences were small supporting our methodology.

The lung has a distinct bacterial community

It is not known from where we obtain our putative bacterial lung microbiota however it is most likely to be in a flux state with the environment. There is support for this notion in the hygiene hypothesis of the development of asthma and allergies [33]. We hypothesize that mice obtain the bacteria from their local environment and littermates influenced by handling by human, feed and water. But it is also a possibility that the core lung microbiome is established in utero, during and after birth in the very early life, as it is suggested with gut microbiota from human and animal studies [34-36].

The lung microbiota found via the culture independent methods described in our study of BALB/c mice, have been shown not to be just a subpopulation of the bacteria found in caecum samples and did not vary significantly between mice suggesting that a distinct lung microbiome exists. An example of this would be the sequence for Pelomonas 4818 (OTU ID), which was found in all our lung samples but not in any caecum samples. We did find 6 major genera that varied significantly between our different sampling methods for the lung bacterial community (KW, p < 0.05) (Additional file 5: Figure S3). Acinetobacter, Pelomonas were most abundant in the BAL-plus, where both Acinetobacter and Pelomonas have been associated with the human lung microbiota [4]. Arcobacter mostly found in BAL-minus has likewise been found to also be correlated with protection from skin allergy and protection from OVA allergy in mouse models [37-39] and found in human lungs [40]. Polaromonas, Schlegella and Brochothrix have not previously been found in BAL fluids from humans or mice and are considered environmental bacteria. We have found Prevotella and Veillonella spp. only in the lung and vaginal samples. These species have been suggested to be a distinct part of lung microbiome and mucus epithelia.
in humans and the absence of *Bacteroides* associated with asthma [3,41].

We have also compared the genera variation of vaginal cluster S1 and S2 against all lung samples. S1 varied significantly in 4 taxa (Figure 1C and D). Genera observed <50 sequences sum counts were not considered. This cut off value was chosen as an additional denoising criterion necessary for sequences with high PCR cycle number. *Staphylococcus* was more abundant in the pulmonary samples (KW, p < 0.05) than in S1. Also, *Anaerococcus* and *Massilia* were not observed in the S1 samples. The large abundance of *Streptococcus* in S1 (KW, p < 0.05) varied clearly from the lung samples. The vaginal cluster S2 with high similarity in beta diversity towards the lung samples varied in S2 genera, but all taxa added up to less than our chosen detection minimum of 50 sequences.

List of bacteria with possible influence on lung immunity

We wanted to identify the microbiota that possibly could influence lung immunity in our animal model. We created a list of interesting bacteria (prior to sequencing) at the genus, family or species level, based on other previous studies of both, human lung and animal models of disease. This list is found in Additional file 2: Table S2 and Additional file 6: Table S3. From our results we found bacteria associated with asthma and COPD in the mouse lung microbiome such as *Lachnospiraceae* and *Akkermansia muciniphila* [42] and *Shewanella, Comamonadaceae* [43], *Haemophilus, Streptococcus, Fusobacteria* [3]. No indications were observed for *Bartonellaceae, Globicatella, Ralstonia* nor *Nitrosonomonadaceae* from our premade list. No OTU sequence blasted could be assigned to *Clostridium difficile, Pseudomonas aeruginosa, Lactobacillus OTU 1865, Bacteroidales OTU 991 or Micrococcus luteus* from our list either. Sequences from the genera *Micrococcus* we isolated did not contain enough taxonomic information to differentiate between *Micrococcus luteus* found on our agar plates and other *Micrococcus* species.

The putative *Akkermansia muciniphila* was found in lung and in one caecum sample and is especially interesting as it is a mucin degrading bacterium and has been shown to influence gut mucus layer thickness [44]. Recently, it was reported that *Akkermansia muciniphila* is present in BALB/c caecum but not in fecal samples. The overall BALB/c caecal microbiome found in our study is also confirmed with the dominant phyla being *Firmicutes* (69.99%) and *Bacteroidetes* (22.07%) [45]. The presence of *Akkermansia muciniphila* in the lung mucus layer could be of importance in asthma characterized by thickening of the epithelium and increased mucus production [46]. Most of the lung-associated bacteria that we identified in Additional file 2: Table S2 could only be found in the mouse lung and vagina samples but not in the caecum. *Bifidobacterium animalis subsp. lactis,* and *Lactobacillus acidophilus NCFM* were added to the list of interesting species because of their use as probiotic bacteria in various mouse models and humans, and it would be interesting to know whether or not these bacteria are present in an unchallenged model. We found OTUs matching *Bifidobacterium animalis subsp. lactis, Bifidobacterium longum subsp. longum* and *Lactobacillus reuteri* the latter two not being on our list, in lung samples, but not in any caecum samples. *Bifidobacterium longum subsp. longum* have been found in human (meconium) and is regarded as one of the first colonisers of the gut originating from the mother [36]. Several strains of *Lactobacillus* have been shown to modulate allergic pulmonary inflammation, whereas *Lactobacillus reuteri* has been shown to reduce inflammation in BALB/c mice [47,48].

Impact on animal models of inflammatory lung disease

The influence of gut microbiota on lung immunity has been vastly explored and several studies have linked changes in the gut microbiome with changes in lung immunity in mice [42,49-51]. As it is becoming clear that the microbiome of the animal used in a particular model influences that animal’s immune status and ultimately affects the outcome of experiments, it is important to take precautions in the model design. Things known to influence gut microbiome composition in laboratory mice include probiotics, antibiotics, stress, handling, vendor/site of breeding and animal lineages [52-55] and it is possible that these factors will affect the lung microbiota as well. Most studies done on gut microbiota and lung immunity do not take lung residing bacteria into account when the data are interpreted. It is possible that the local lung effects seen could be the results of changes in the lung as well as in the gut. In our studies we always use age matched female mice from the same site of breeding (lot number) and distribution of the mice equally between groups as to avoid any littermate bias. It should possibly also be noted whether or not the mice have mothers that are sisters or not as this also effects gut and possibly lung microbiome in the offspring [52]. As an added layer of complexity we should remember that the total mouse microbiota do not only consists of bacteria but also fungi and viruses. In particular bacteriophages could influence gut or lung microbiology and indirectly have adverse effects on health [56].

Future studies into the lung microbiota of mice should include a comparison between nasal lavages and BAL to distinguish between upper and lower respiratory tract microbiota and possibly longitudinal studies with culture independent techniques.
Conclusions

BALB/cj mice were shown to have a lung microbiome that was distinct from their caecal but overlapping with their vaginal bacterial community. We have consistently amplified bacterial DNA from mouse BAL fluid and have shown that host DNA present in the DNA extraction step influences the community profiles obtained and that this needs to be taken into account when choosing methods, performing the analyses and prior to biological interpretation. Mouse models provide the means to obtain mechanistic insights into the lung microbiome. We believe that the lung microbiota should be considered when working with these mouse models of human disease and further research is needed to reveal the contribution of the lung microbiota to the pathogenesis of diseases such as respiratory disease common in infants (i.e. RSV), cystic fibrosis, COPD and asthma.

Availability of supporting data

All supporting data are included as additional files and all sequences used in this study are available in the NCBI Sequence Read Archive under study accession number SRP033710 (http://www.ncbi.nlm.nih.gov/sra).

Additional files

Additional file 1: Figure S5. Rarefaction curves. (A) Observed species – raw data. (B) Observed species after random even subsampling. The data shown in (A) are counted for all sequences generated. The graph.euven out after approx. 2000 sequences observed and revealed that the random even subsampled OTU table (B) at a sequencing depth of 3330 will be efficient to include also the rare OTUs. The subsampled OTU table (B) was used for the statistical analysis of this study and is the basis of the figures 1 and 2.

Additional file 2: Table S2. List of interesting taxa. This list shows the distribution of lung associated taxa between sampling methods and sites. Most of the lung-associated bacteria could only be found in the lung and vagina samples but not in the caecum. LF-plus is bronchoalveolar lavage (BAL) fluids and LF-minus is BAL where the mouse cells have been removed. LT is lung tissue. VF is vaginal flushing and caecum from the gut microbiota.

Additional file 3: Table S4. Distribution of genera between samples. The observations are based on the summarized subsampled OTU table (3318 OTUs) after singletons and doubletons were removed. We discriminated shared and unique genera of lung, vaginal and caecal environment.

Additional file 4: Figure S4. Additional PCoA 2 and 3. The axis of PCoA plots 2 and 3 explain the 62.9%/24% and 10.4%/2.28% of the variances respectively. Both plots show the large overlap of bronchoalveolar lavage (BAL) fluids BAL-plus with mouse cells in BLUE, BAL-minus (without mouse cells) in RED and lung tissue in ORANGE and support plot 1. Only in plot 3 the caecal GREEN community overlaps with the lung and vaginal community confirming its large distance from the other sample sites.

Additional file 5: Figure S3. Variation in lung genus composition. The genera shown counted up to at least 50 or more sequences in relative abundance and vary significantly among the lung communities. ANOVA, p < 0.05. LF-plus is bronchoalveolar lavage (BAL) fluids and LF-minus is BAL where the mouse cells have been removed. LT is lung tissue, VF is vaginal flushing and caecum from the gut microbiota.

Additional file 6: Table S3. Blast search – putative species identity. For further identification the representative sequence of each OTU of the Qiime pipeline output were picked and blasted. OTUs were only considered when the highest score, maximum identity and 100% query cover uniquely matched one species. Additional subspecies information corresponds to the best hit. It is also noted from how many different animals and from which sampling site the OTUs were found. LF-plus is bronchoalveolar lavage (BAL) fluids and LF-minus is BAL where the mouse cells have been removed. LT is lung tissue. VF is vaginal flushing and caecum from the gut microbiota.

Competing interests

The authors declare that they have no competing interests.

Authors’ contributions

KKB conceived and designed the study, carried out the animal work and DNA extractions, and drafted the manuscript. HRD did the 16S data generation, analysis and participated in the design of the study and manuscript. SSC performed the cultivation and bacterial identification. KAK, LHH, STL and SJS participated in design and coordination and helped to draft the manuscript. All authors read and approved the final manuscript.

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Author details

1Statens Serum Institut, Antillevv 9, 2300 Copenhagen S, Denmark. 2National Research Centre for the Working Environment, Lernø Parkalle 105, 2100 Copenhagen O, Denmark. 3Department of Biology, Microbiology, University of Copenhagen, Universitetsparken 15, 2100 Copenhagen O, Denmark.

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*Vitamin D impact on naïve and OVA exposed murine lung microbiota*
Vitamin D impact of the lung microbiota and OVA allergy in mice.

Michael Roggenbuck¹, Kenneth Barfod², Søren Johannes Sørensen¹, Shelley Gorman³*

¹Section of Microbiology, Department of Biology, University of Copenhagen, Denmark
²Det Nationale Forskningscenter for Arbejdsmiljø, Denmark
³Telethon Kids Institute, University of Western Australia, Australia

*Corresponding author
Shelley.Gorman@telethonkids.org.au

Abstract

In the past the lower respiratory tract of the lung was thought to be sterile. However, recent high throughput DNA sequencing methods have identified by testing the bronchoalveolar lavage of humans and mice that the lung carries a unique array of microbial flora, which may be dysregulated in asthmatics. We investigated the possible connection of changes to the lung microbiota with asthma development by using a murine model of allergic airway disease, induced by sensitization with ovalbumin (OVA) and Aluminium hydroxide (Alum). We also investigated the impact of sex and vitamin D deficiency; other factors that may modify the lung microbiome. The bacterial composition of the lungs of naïve BALB/c mice were compared with OVA-sensitized mice, with vitamin D deficiency induced by dietary restriction throughout life (from conception onwards). OVA sensitization changed the microbial composition significantly, by increasing levels of Acinetobacter, Haemophilus and Rothia bacterial species. Species of Acinetobacter, and Flavobacterium were elevated in the absence of dietary vitamin D, while decreased levels of Pseudomonas species were observed in naïve male mice that were vitamin D deficient. There was only a minor impact of dietary vitamin D in naïve female mice with increased levels measured of an unknown bacterial species. Interestingly, dietary vitamin D modified the lung microbial composition in different ways in OVA-exposed and naïve males. However, vitamin D increased the levels of the same bacterial species in the lungs of female mice whether they were naïve or sensitised with OVA treated mice.

Introduction

Novel culture independent techniques for microbial identification have within a decade changed the way we view the “non-pathogenic” bacteria or the microbiome that inhabits every crevasse of our bodies. Most microbiome research has focused on the gut microbiome and its relation to disease. Our airways used to be deemed sterile in the absence of infection, but recent studies suggest that complex communities of bacteria exist in both healthy and diseased lungs (Hilty et al., 2010; Huang et al., 2010; Beck et al., 2012; Morris et al., 2013). The putative role of this lung microbiome and the
metagenomics in the development of lung diseases, although in its infancy, is a rapidly growing field of research (Huang, 2013) (Huang et al, 2013).

Vitamin D deficiency has been recognized for its role in bone health, but in recent years there have been a growing focus on vitamin D in chronic diseases like allergic asthma (Foong & Zosky, 2013). However, a casual link between vitamin D status and the development of allergic asthma has not been established. It is known that the lack of vitamin D may affect lung development in utero (Zosky et al, 2011) and may be required to regulate the differentiation of both T-regulatory and dendritic cells for control of tissue inflammation (Nguyen et al, 2004; Taher et al, 2008; XYstrakis et al, 2006; Vasiliou et al, 2014). There are ongoing clinical trials in Denmark and the US that will determine the effect of supplementation of vitamin D to pregnant women and asthma in their offspring (Weiss, 2011).

Recently there has been focus on the potential for vitamin D to co-regulate both commensal bacteria and immune development to suppress asthma severity and/or development (Foong and Zosky, 2013) (Weiss, 2011). We recently showed that lifelong (from conception to adulthood) vitamin D deficiency enhanced elements of OVA-induced allergic airway disease in male but not in female mice (Gorman et al, 2013). Increased lung inflammation (Eosinophils and neutrophils) in male mice was correlated with a significant (4-5 fold) increased bacterial burden in the lungs (Gorman et al, 2013). Dietary vitamin D supplementation of initially-vitamin D-deficient male mice reversed these effects on increased lung inflammation and bacteria levels (Gorman et al, 2013). Gender bias in autoimmune diseases is well established and has been shown to be correlated to changes in gut microbiota in NOD mice (Yurkovetskiy et al, 2013). Using a customized 16S rRNA sequencing protocol to amplify the V3-V4 region, we have recently described the lung microbiome in female BALB/c mice in relation to the gut and vaginal microbiota (Barfod et al, 2013). Here, we use these methods to characterize how allergic sensitization, sex and vitamin D deficiency affect the lung microbiome. Using these methods, the purpose of this study was to examine how vitamin D status affects the lung microbiome in both sexes with or without induced OVA airway allergy.

Material and methods

Animal treatment. All experiments were performed according to the ethical guidelines of the National Health and Medical Research Council of Australia and with approval from the Telethon Kids Institute Animal Ethics Committee. Animals were held in the Bioresources Centre of the Telethon Kids Institute. Breeding animals were purchased from the Animal Resources Centre (Murdoch, Western Australia). As previously described (Gorman et al, 2013), female and male B6C3F1 mice were born to mothers fed a vitamin D3-containing (2280 IU vitamin D3/kg, SF05-34, Specialty Feeds, Perth, Western Australia: treatment known as VitD+0) or -deficient (0 IU vitamin D3/kg, SF05-33, Specialty Feeds: treatment known as VitD-0) diet and were continued to be fed the same diet until the end of experiment. An additional group was included to characterize the effects of short-term treatment with vitamin D by supplementing the diet of previously vitamin D-deficient mice with the vitamin D-containing diet for 4 weeks prior to sampling (Treatment
known as VitD-1). Mice were housed as previously described (Gorman et al, 2013). A detailed description of immune cells counts, cytokines and defensin levels measures in the bronchoalveolar lavage fluid (BALF) of these naïve mice are part of another unpublished manuscript. To characterize the lung microbiome, the right lung lobe was collected from these naïve 12-week old mice and snap-frozen in liquid nitrogen (n=10 mice per treatment).

For the induction of allergic airways disease, 12 week-old mice (From VitD+0, VitD-0 and VitD-1 treatments) were sensitized and boosted with ovalbumin (OVA, 1 µg: Sigma Chemical Company, St Louis, MO, USA) and Aluminium hydroxide (Alum, 200 µg: Serva, Heidelberg, Germany) as previously described (Gorman et al, 2013). There was a 2-week period between the sensitization and boost, and then 7 days after the boost, the respiratory tract was then challenged by inhalation of nebulized (UltraNebs, DeVilbiss, Somerset, PA, USA) OVA-in-saline (1 mg.ml⁻¹) aerosols for 30 minutes. All characterized physiological parameters including lung inflammation, airway hyper-reactivity and serum IgE levels have been previously published (Gorman et al, 2013). To characterize the lung microbiome, the right lung lobe was collected from these OVA-sensitized 15-week old mice and snap-frozen in liquid nitrogen (n=10-12 mice per treatment).

For both naïve and OVA-sensitized mice, serum levels of 25-hydroxyvitamin D were significantly elevated (>50 nmol.L⁻¹) in mice fed a vitamin D-containing diet and reduced (<20 nmol.L⁻¹) in mice fed the diet not supplemented with vitamin D (Gorman et al, 2013). OVA-sensitization did not affect circulating levels of 25-hydroxyvitamin D.

**DNA extraction and amplicon library construction.** DNA was extracted from a 2 mm³ sample of the left lungs using the DNeasy Blood and Tissue DNA extraction kit according to manufacturer’s protocol (Qiagen). To profile the microbial community of the lungs, we amplified the variable region V3-V4 of the 16S rRNA gene as previously reported (Barfod et al, 2013) with modifications using the primer pair of 341F and 806R (Hansen et al, 2012). The PCR reaction mix was composed of 5µl 5x Phusion buffer HF (7.5 mM MgCl₂, Finnzymes, Finland), 0.5µl 10mM dNTPs, 1.25µl 10µM of each primer, 0.25µl DNA polymerase (Hotstart Phusion 540L, 1 unit/µl Finnzymes) and 3.5 µl template. The PCR amplification protocol was adjusted to reduce the high cycle number of 45 rounds, down to 35. The PCR program started with 98°C 2 min initialization, followed by 35 cycles of denaturation at 98°C for 5 sec, annealing at 55 °C for 30 seconds and strand elongation at 60°C for 1 minute. The PCR was finalized by a single elongation step at 72°C for 5 minute and than cooled down to 4 °C. The size of the PCR product was evaluated using gel electrophoresis. The fragment was then excised and purified using the Montage Gel Extraction Kit (Merck Millipore). Negative controls were also excised at the position of the expected fragment size to investigate possible contamination in the following gel purification. Adaptors were added to the amplicons in a second PCR run under the same conditions as PCR I with a reduced cycle number of 15. Due to the low DNA concentration and the potential high risk of contamination we also sequenced the negative controls.

**Sequencing and data treatment.** The sequences were generated with 454 FLX Titanium (Roche Corp.). The reads were trimmed for low quality (minimal quality score = 25) using the Qiime
pipeline version 1.5.0 (Caporaso et al, 2010). Only sequences with a minimal length of 200bp were considered. Chimeras were removed using the Uchime algorithm (Edgar et al, 2011). Operational taxonomic units (OTUs) were picked de novo from quality checked reads and clustered at 97% sequence similarity using Uclust. Taxonomy was assigned using the RDP classifier (Version 2.2) method and Greengenes as reference database (Liu et al, 2008). All OTUs observed in the sequenced negative control were subtracted from the OTU table.

Statistical validation. Treatment-associated microbial profiling was evaluated with the Bray-Curtis dissimilarity and the Unifrac distance metric. The clustering was visualized using multidimensional scaling (Principal coordinate analysis = PcoA) and further characterized with the analysis of similarity (Anosim). Anosim measures the ranked distance between the treatments compared to the distance of samples within the same treatment group (Clarke KR, 1993) and is normalized to 1. Thus, the closer R to 1 the more different the treatments whereas R=0 indicates no difference. Abundance based variation between treatments were evaluated for normal distribution using the Shapiro-Wilk test. Due the non-parametric nature of the data we used the Wilcoxon Rank Sum test when comparing two treatments (P<0.05), whereas the Kruskal-Wallis test (P<0.05) was applied when more than 2 variables were compared.

Results

This study compares the 12 treatment groups shown in Table 1. First, we examined the impact of allergic sensitization on the lung microbiome as simulated by ovalbumin (OVA) exposure compared to naïve mice. Secondly, we evaluated the impact of dietary vitamin D on naïve mice, separately of the OVA sensitized mice. We then investigated the effect of vitamin D supplementation of initially vitamin D-deficient mice and finally we determined sex-related differences in response to OVA exposure and dietary vitamin D.
Table 1. Treatment groups. 1Mice were born to mothers that were fed a diet that did (+) or did not (-) contain vitamin and were maintained on these diets throughout the experiment unless otherwise stated. 2For some treatments, mice initially fed a vitamin D-deficient diet were then supplemented for 4 weeks with a diet containing vitamin D.

<table>
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<th>Treatment ID</th>
<th>OVA/Naïve</th>
<th>Vitamin D(_3) in diet(^1)</th>
<th>Vitamin D(_3) Supplementation(^2)</th>
<th>Sex</th>
<th>Biological replicates</th>
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<td>3. F-1 Naïve</td>
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</table>

OVA treatment significantly changed the murine lung microbial composition.

We evaluated the 16S rRNA gene composition of the lungs to determine the abundance of de novo picked operational taxonomic units (OTUs). After quality trimming and further data treatment 663961 sequences were identified with large variation in sequencing depth ranging from 95 to 26562. However, the Shannon diversity (Figure 1) indicates with approximately 750 reads/sample had enough saturation to investigate the most abundant microbes in the lung. We chose 1200 reads/sample for subsampling the OTU table at even depth and further statistical analysis, although at this depth, rare OTUs will be neglected as the Chao1 richness has not reached saturation (Figure 1).
Figure 1. Rarefied Chao1 richness and Shannon diversity to estimate subsampling depth.
As shown in the PcoA plot (Figure 2), the lung samples cluster according to the ‘OVA’ and ‘naïve’ treatments. The clustering, confirmed with analysis of similarity (Anosim, R=0.439), was significant (Permutation p-value=0.001). However, OVA-sensitization had no effect on the lung diversity. The naïve lung contained in average 138 OTUs (Similar to species) compared to the 128 OTUs in the OVA lung samples without significant variation (Wilcoxon, p=0.165). Also, microbial richness (Chao1, naïve=406.6, OVA=370.7) and diversity (Shannon index, naïve=4.37 versus 4.30 in OVA) did not explain the difference between the treatments (Wilcoxon, p>0.05).

To estimate which microbes generated the difference in clustering we assigned taxonomy to the OTUs. First we compared the microbial phyla distribution between the OVA-sensitized and naïve mice (Table 2). The lungs of naïve mice contained significantly larger relative proportions of Deinococcus-Thermus, OD1, TM7 and unknown bacteria. In contrast, the lungs of OVA-sensitized mice contained relatively more Cyanobacteria, Deferribacteres, Planctomycetes, Verrucomicrobia and unclassified sequences.

Table 2. Relative phyla frequencies of bacteria detected in the lungs of in naïve and OVA-sensitized mice. Differences between treatments were estimated with the Wilcoxon test with a significant p-value (P<0.05) highlighted with an asterisk (*).
To identify differences in OTU composition (taxonomic species level) of naïve and OVA-sensitized mice, we tested for significant variations using the Wilcoxon test (P<0.05). Of the most abundant OTUs (Observations based on more than 100 relative reads), 44 varied significantly in the lungs of naïve and OVA-sensitized mice. Lung samples from naïve mice contained 5 OTUs significantly more abundant compared to the OVA-sensitized mice that accounted for 10.5% of the naïve relative sequences. However, those OTUs were only classified as Bacteria (with Greengenes), OTUs Alphaproteobacteria, Cyanobacteria, OD2 and Proteobacteria. Furthermore few OTUs assigned to the family of Microbacteriaceae, as well as the genera of Deinococcus, OD1_genera_incertae_sedis, Methylobacterium and Paludibacter were significantly associated to the naïve samples. The lungs of OVA-sensitized mice carried increased proportions of the three bacterial OTUs not annotated to any known phyla. Furthermore 3 OTUs assigned to the family of Comonadaceae, Cyrptomonadaceae, Sphinomonadaceae, and 2 observations of Verrucomicrobiaceae were elevated in OVA exposed animals. Finally 17 of elevated OTUs in the lungs of OVA-sensitized mice were fully annotated to genus: Acidovorax, Acinetobacter, Aquabacterium, Blastococcus, Dechloromonas, Escherichia/Shigella, Haemophilus, Helio bacter, Janthinobacterium, Mucispirillum, Phyllobacterium, Rothia, Simonsiella, and Sphingomonas.

<table>
<thead>
<tr>
<th>Taxa</th>
<th>Naïve $\bar{x}$ in %</th>
<th>Ova $\bar{x}$ in %</th>
<th>Wilcoxon p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Acidobacteria</strong></td>
<td>0.5</td>
<td>0.5</td>
<td>0.332</td>
</tr>
<tr>
<td><strong>Actinobacteria</strong></td>
<td>13.4</td>
<td>14.9</td>
<td>0.274</td>
</tr>
<tr>
<td><strong>Bacteroidetes</strong></td>
<td>4.5</td>
<td>4</td>
<td>0.218</td>
</tr>
<tr>
<td><strong>Chloroflexi</strong></td>
<td>0</td>
<td>0.2</td>
<td>0.082</td>
</tr>
<tr>
<td><strong>Cyanobacteria</strong></td>
<td>6.3</td>
<td>11.5</td>
<td>&lt;0.001*</td>
</tr>
<tr>
<td><strong>Deferribacteres</strong></td>
<td>0</td>
<td>0.2</td>
<td>0.020*</td>
</tr>
<tr>
<td><strong>Deinococcus-Thermus</strong></td>
<td>1.5</td>
<td>0</td>
<td>0.007*</td>
</tr>
<tr>
<td><strong>Firmicutes</strong></td>
<td>5.7</td>
<td>8</td>
<td>0.548</td>
</tr>
<tr>
<td><strong>Fusobacteria</strong></td>
<td>0.1</td>
<td>0.1</td>
<td>0.002*</td>
</tr>
<tr>
<td><strong>Gemmatismonadetes</strong></td>
<td>0.1</td>
<td>0</td>
<td>0.082</td>
</tr>
<tr>
<td><strong>Nitrospira</strong></td>
<td>0</td>
<td>0.1</td>
<td>0.326</td>
</tr>
<tr>
<td><strong>OD1</strong></td>
<td>3.3</td>
<td>1.4</td>
<td>0.119</td>
</tr>
<tr>
<td><strong>Bacteria, unknown</strong></td>
<td>24.4</td>
<td>9</td>
<td>&lt;0.001*</td>
</tr>
<tr>
<td><strong>Planctomycetes</strong></td>
<td>0.1</td>
<td>0.6</td>
<td>0.024*</td>
</tr>
<tr>
<td><strong>Proteobacteria</strong></td>
<td>35.7</td>
<td>38.9</td>
<td>0.441</td>
</tr>
<tr>
<td><strong>SR1</strong></td>
<td>0.2</td>
<td>0</td>
<td>0.080</td>
</tr>
<tr>
<td><strong>TM7</strong></td>
<td>2.1</td>
<td>0.2</td>
<td>0.029*</td>
</tr>
<tr>
<td><strong>Verrucomicrobia</strong></td>
<td>1.9</td>
<td>10.1</td>
<td>&lt;0.001*</td>
</tr>
<tr>
<td>Unclassified sequences</td>
<td>0.1</td>
<td>0.3</td>
<td>&lt;0.001*</td>
</tr>
</tbody>
</table>
We next characterized separately the effect of dietary vitamin D on lung microbiomes of the naïve or OVA-sensitized mice. Again we used the previously described cluster analysis (Figure 1) to decide to use 1200 reads/sample for our analyses. Based on the Bray-Curtis dissimilarity we evaluated the compositional variations between the first 6 different naïve treatment groups listed in Table 1 and evaluated results with the Anosim R-value and the permutation p-value (P<0.05). As shown in Table 3, there were no significant differences between the treatment groups of female and male mice, except for a small difference between vitamin D-replete female and male mice (F+0 naïve vs M+0 naive).

Table 3. Bray-Curtis dissimilarities between treatments evaluated with Anosim R-value and permutation p-value, with a significant p-value (P<0.05) highlighted with an asterisk (*).

<table>
<thead>
<tr>
<th>Treatment comparisons</th>
<th>Anosim R-value</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>M+0 naive vs M-0 naive</td>
<td>-0.050</td>
<td>0.872</td>
</tr>
<tr>
<td>M-0 naive vs M-1 naive</td>
<td>0.099</td>
<td>0.073</td>
</tr>
<tr>
<td>M+0 naïve vs M-1 naïve</td>
<td>0.100</td>
<td>0.057</td>
</tr>
<tr>
<td>F+0 naïve vs F-0 naïve</td>
<td>0.010</td>
<td>0.38</td>
</tr>
<tr>
<td>F-0 naïve vs F-1 naïve</td>
<td>-0.040</td>
<td>0.712</td>
</tr>
<tr>
<td>F+0 naïve vs F-1 naïve</td>
<td>0.030</td>
<td>0.283</td>
</tr>
<tr>
<td>M+0 OVA vs M-0 OVA</td>
<td>-0.110</td>
<td>0.791</td>
</tr>
<tr>
<td>M-0 OVA vs M-1 OVA</td>
<td>-0.020</td>
<td>0.495</td>
</tr>
<tr>
<td>M+0 OVA vs M-1 OVA</td>
<td>0.120</td>
<td>0.119</td>
</tr>
<tr>
<td>F+0 OVA vs F-0 OVA</td>
<td>0.020</td>
<td>0.378</td>
</tr>
<tr>
<td>F-0 OVA vs F-1 OVA</td>
<td>0.100</td>
<td>0.196</td>
</tr>
<tr>
<td>F+0 OVA vs F-1 OVA</td>
<td>0.050</td>
<td>0.452</td>
</tr>
<tr>
<td>M-0 Naive vs F-0 Naive</td>
<td>-0.023</td>
<td>0.639</td>
</tr>
<tr>
<td>M-1 Naive vs F-1 Naive</td>
<td>-0.050</td>
<td>0.759</td>
</tr>
<tr>
<td>M+0 Naive vs F+0 Naive</td>
<td>0.120</td>
<td>0.035*</td>
</tr>
<tr>
<td>M-0 OVA vs F-0 OVA</td>
<td>0.068</td>
<td>0.293</td>
</tr>
<tr>
<td>M-1 OVA vs F-1 OVA</td>
<td>-0.082</td>
<td>0.639</td>
</tr>
<tr>
<td>M+0 OVA vs F+0 OVA</td>
<td>0.412</td>
<td>0.013*</td>
</tr>
</tbody>
</table>

We repeated the analysis with the unweighted Unifrac distance (Table 4) metrics that enumerates differences between groups by phylogenetic relation instead of the abundance based Bray-Curtis.
Here we only observed a minor but significant difference between the male mice fed a vitamin D deficient diet and initially vitamin D-deficient male mice then fed a vitamin D supplemented diet (M-0 naïve vs M-1 Naïve). However, the previously noted effect between the naïve male and female lung microbiomes observed by Bray-Curtis distance was gone. Because the Unifrac analysis was dependent on phylogeny and thus sequencing depth, the analysis was repeated with a higher subsampling level of 4000 reads/samples. However, we observed again no difference between the treatment groups, and additionally the difference of M-0 naïve and M-0 naïve at 1200 reads per sample (Table 4) was removed (data not shown).

Table 4. Unifrac dissimilarities between treatments evaluated with Anosim and permutation p-value (P<0.05)

<table>
<thead>
<tr>
<th>Treatment comparisons</th>
<th>Anosim R-value</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>M+0 naïve vs M-0 naïve</td>
<td>0.030</td>
<td>0.270</td>
</tr>
<tr>
<td>M-0 naïve vs M-1 naïve</td>
<td>0.120</td>
<td>0.031*</td>
</tr>
<tr>
<td>M+0 naïve vs M-1 naïve</td>
<td>0.060</td>
<td>0.147</td>
</tr>
<tr>
<td>F+0 naïve vs F-0 naïve</td>
<td>0.010</td>
<td>0.427</td>
</tr>
<tr>
<td>F-0 naïve vs F-1 naïve</td>
<td>-0.010</td>
<td>0.506</td>
</tr>
<tr>
<td>F+0 naïve vs F-1 naïve</td>
<td>0</td>
<td>0.462</td>
</tr>
<tr>
<td>M+0 OVA vs M-0 OVA</td>
<td>0.720</td>
<td>0.010*</td>
</tr>
<tr>
<td>M-0 OVA vs M-1 OVA</td>
<td>0.330</td>
<td>0.022</td>
</tr>
<tr>
<td>M+0 OVA vs M-1 OVA</td>
<td>0.640</td>
<td>0.008*</td>
</tr>
<tr>
<td>F+0 OVA vs F-0 OVA</td>
<td>0.210</td>
<td>0.192</td>
</tr>
<tr>
<td>F-0 OVA vs F-1 OVA</td>
<td>0.040</td>
<td>0.484</td>
</tr>
<tr>
<td>F+0 OVA vs F-1 OVA</td>
<td>0.320</td>
<td>0.105</td>
</tr>
<tr>
<td>M-0 Naive vs F-0 Naive</td>
<td>0.001</td>
<td>0.446</td>
</tr>
<tr>
<td>M-1 Naive vs F-1 Naive</td>
<td>0.030</td>
<td>0.299</td>
</tr>
<tr>
<td>M+0 Naive vs F+0 Naive</td>
<td>0.020</td>
<td>0.343</td>
</tr>
<tr>
<td>M-0 OVA vs F-0 OVA</td>
<td>0.380</td>
<td>0.043*</td>
</tr>
<tr>
<td>M-1 OVA vs F-1 OVA</td>
<td>0.005</td>
<td>0.513</td>
</tr>
<tr>
<td>M+0 OVA vs F+0 OVA</td>
<td>0.636</td>
<td>0.006*</td>
</tr>
</tbody>
</table>

The lack of difference between the treatments could have been masked by our sequencing method. A sequencing depth at 4000 reads/sample halved the number of biological replicates; however, at 1200 reads/sample rare OTUs are neglected due to the lack of saturated richness (Figure 1). In addition, in preliminary studies, we have detected a 4-fold increase in 16S rRNA sequences as well as significant increases in the number of neutrophils and macrophages detected in the lungs of male mice fed a vitamin D-deficient diet (Gorman et al, manuscript in preparation), suggesting that there may indeed be differences in the lung microbiome induced by a lack of dietary vitamin D in naïve
male mice. To examine this possibility, we evaluated the clustering effect directly on the relative of OTU counts by using the Kruskal-Wallis test (1200 reads/sample).

*Vitamin D depletion increased OTUs assigned to Acinetobacter and Flavobacterium but decreased OTUs assigned to Pseudomonas in naïve male mice.*

First we compared the naïve male mice and detected 10 OTUs that accounted for 5.5% of the relative sequences, with significant difference between the lungs of naïve M+0, M-0 and M-1 (n=10; =9; =10). Most prominent was an OTU of *Pseudomonas* (Figure 3), found largely in the lungs of M+0 samples followed by M-0 and then M-1. Species assigned to *Acinetobacter* were not found in the lungs of M+0, but observed in naïve M-0 and most samples of M-1. OTUs of *Flavobacterium* were more frequent in the lungs of mice fed a diet that lacked vitamin D diet (M-0), with little observed in the lungs of the mice from the M-1 and M+0 treatments.

*Figure 3. The most frequent OTUs with significant variation between the naïve male mice fed the vitamin D-containing diet (Kruskal-Wallis test=KW, p<0.05). The x-axis represents the biological replicates whereas the y-axis displays the OTUs (OTU counts are log-transformed).*

*There was only a limited effect of dietary vitamin D on the lung microbiome in naïve female mice.*

When examining the naïve female mice, only 5 OTUs were affected by dietary vitamin D with a minor proportion of sequences (0.4% of the female naïve sequences). A single OTU associated to *Enterobacteriaceae* was only observed in the F-0 naïve mice, and was not found in the lungs of F+0 naïve and F-1 naïve. The other four OTUs, which included *Pseudomonadaceae* were only observed in the lungs of F-1 naïve mice. No OTUs were observed in the lungs of the F+0 group.

*The effect of dietary vitamin D on the lung microbiome of OVA-sensitised mice.*

Next we evaluated the impact of dietary vitamin D on the lung microbiome of OVA-sensitised mice. The Bray-Curtis and Unifrac dissimilarity tests, evaluated by Anosim (At 1200reads/sample), came to different conclusions (Tables 3&4). According to the Bray-Curtis metric, there were no differences between in the lung microbiomes induced by dietary vitamin D in either male or female mice. However when comparing genders, by treatment, there was significant clustering of fully vitamin D repleted male and female mice (M+0 OVA and F+0 OVA) as observed for the naïve
animals. Both the Bray-Curtis and Unifrac tests suggested that dietary vitamin D did not change the composition of the lung microbiomes of OVA-sensitized female mice microbial composition and vitamin D treatments with the observations. However, there were significant variations in microbial composition identified in the lungs of OVA-sensitized male mice depending upon vitamin D treatment. The re-analysis at the higher sequencing depth of 4000 reads/sample was problematic as the Anosim permutation requires at least 4 biological replicates and the subsampling reduced the number of animal samples below 4. As for the naïve mice, we then using Kruskal-Wallis rank sum test (P<0.05) to determine the effects of dietary vitamin D on the OTU compositions of the lungs of OVA-sensitized mice.

The microbial profile is modified differently by dietary vitamin D in OVA-sensitized mice as compared to naïve animals.

In the lungs of male mice sensitized with OVA, 10 OTUs were identified that made up 12.3% of the total relative sequences, and these varied according to the vitamin D treatment. However, the variations were mostly due to OTUs taxonomically classified to microbial family and not genus. The most frequent OTU belonged to the family of Verrucomicrobiaceae, most often observed in M-1 OVA, followed by M+0 OVA and least observed in in M-0 OVA mice. Other OTUs detected including those belonging to Comamonadaceae and Janthinobacterium.

There were significant sex differences in the composition of the lung microbiomes of OVA-sensitized mice.

Twenty OTUs accounting for 2.2% of the total relative sequences were observed in the lungs of OVA-sensitized mice. Most prominent was one OTU highly prevalent in the lungs of female OVA-sensitized and barely detected in the male mice. This OTU was the same unclassified bacterial species identified in the lungs of naïve female mice. Furthermore, Escherichia/Shigella, Borea sp. and Rhodococcus were disproportionally represented in the male lung samples, whereas one OTU of Propionibacterium was only observed in the female lung.

Which bacterial species commonly colonize the lower respiratory tracts of mice?

To address this question, we compared the samples of this study with our previously published murine lung microbiome in short MLM (Barfod et al, 2013). The animal experiments of the present investigation were performed in in the Bioreosources Centre of the Telethon Kids Institute in Perth, Australia whereas the published data were conducted in the “Det Nationale Forskningscenter for Arbejdsmiljø“ in Denmark. To compare the lung microbial communities of BALB/c mice from these two locations, we clustered the sequences of naïve and OVA-sensitized mice together with the MLM mice using the Bray-Curtis analysis (Figure 4A). As shown in the first three PcoA1 axis the
lung samples of the lung microbiomes of the ‘Denmark’ BALB/c mice overlap with the bacterial communities of the ‘Australian’ BALB/c mice, although there is an obvious separation. The separation was confirmed with the Anosim. The samples of MLM were significantly different to the naïve (R=0.430, p=0.001) and OVA (R 0.434, p=0.01) samples. The Venn diagram of Figure 4B reveals that the naïve and OVA-sensitized Australian BALB/c mice share approximately 10-fold more OTUs with each other than with the MLM mice, respectively. However, 259 core OTUs accounted for 40, 51 and 59% of the relative sequences of the naïve and OVA-sensitised Australian BALB/c mice and Denmark BALB/c mice respectively, indicating that the most frequent OTUs were present in the lungs of all mice.

Figure 4. Core lung microbiome. A. Bray-Curtis dissimilarity between Australian (Naive & OVA-sensitized) and Danish mice (MLM) displayed with PcoA axis 1-3. B. OTU-based Venn diagram summarized at even depth.

Discussion

In this study, we identified that the lungs of naïve and OVA-sensitized mice are composed of significantly different microbial populations. Overall, We observed phyla previously reported in the
human and mice lung such as *Actinobacteria*, *Bacteroidetes*, *Firmicutes* and *Proteobacteria* (Charlsen *et al.*, 2012, Barfod *et al.*, 2013).

The composition of the lung microbiome of OVA-sensitized mice were similar to those observed in patients with asthma and chronic obstructive pulmonary disease (COPD). The lungs of OVA-sensitised mice were enriched in OTUs for the Cyanobacteria, bacteria previously associated with asthma in humans (Goleva, 2013). We observed that OVA sensitization enriched for OTUs that included *Acinetobacter* and *Haemophilus* species found in patients suffering from COPD (Erb-Downward *et al.*, 2011; Pragman *et al.*, 2011). OVA-sensitized mice also had more OTUs for *Rothia* species found in cystic fibrosis patients (Lim *et al.*, 2013) and isolated from lower respiratory tract of lymphoma patients (Cho *et al.*, 2013). In contrast, *Deinococcus-Thermus*, the COPD-associated phyla was reduced in the lung microbiome of OVA–sensitized mice (Erb-Downward *et al.*, 2011). However, all-in-all the OVA sensitization process appears to modify the lung microbiomes of mice in ways that are representative of respiratory pathologies like asthma and COPD.

The analysis of the effects of dietary vitamin D was problematic due to the large variation in sequencing depth achieved and therefore mixed results by the Anosim analysis of the dissimilarities between treatments. We acknowledge that each metric has pitfalls; the Bray-Curtis neglects rare OTUs, while the phylogenetic metric of Unifrac (Unweight) weighs strongly rare OTUs. Previously in our experience this has not been a problem as long as we can sample to a sequencing depth were species richness (Chao1) and OTUs rarefaction curves reach the plateau of saturation. However, the large variation of sequencing efforts between the samples reduced number of biological replicates at higher subsampling levels and removed all variation between the treatments. Therefore our analysis focused on direct comparison of OTU abundances between the treatments at the highest sequencing depth with the largest possible number of biological replicates and the saturated Shannon diversity.

There were several bacterial species affected by dietary vitamin D in the lungs of male naïve mice. Lifelong feeding with vitamin D reduced the prevalence of OTUs of *Acinetobacter* and *Flavobacterium*, whereas the proportion of *Pseudomonas* species increased. In female mice, only minor variations were observed, with the most common species assigned *Enterobacteriaceae*. These results suggest that vitamin D has only a minor effect on the composition of the lung microbial community in female mice compared to the males. The direct comparison of female with the male lung microbiome explained that the major difference was assigned to an unknown bacterial species. Furthermore the *Flavobacterium* species that reduced in male mice with vitamin D was more abundant in male compared to female lung samples. *Acinetobacter*, also negatively affected by vitamin D in male mice, were more abundant in female mice. These results are indications that the lung microbiome of female and male naïve mice respond differently to dietary vitamin D.

In the lungs of OVA-sensitized mice, more OTUs were affected by dietary vitamin D than observed in naïve mice. In addition, different bacterial species were affected by vitamin D in the OVA-sensitized mice than in the naïve animals.
References


Manuscript 3

Development of Methane emission from growing lamb fed milk replacer and cream for a prolonged period
Development of methane emission from lambs fed milk replacer and cream for a prolonged period

M. N. Haque1*, M. Roggenbuck2, P. Khanal3, M. O. Nielsen3, J. Madsen1*

1Department of Large Animal Sciences, Faculty of Health and Medical Sciences, University of Copenhagen, Groennegaardsvej 2, DK-1870 Frederiksberg C, Denmark

2Department of Biology, Microbiology, Faculty of Science, University of Copenhagen, Universitetsparken 15, 2100 København Ø, Denmark

3Department of Veterinary Clinical and Animal Sciences, Faculty of Health and Medical Sciences, University of Copenhagen, Groennegaardsvej 3, DK-1870 Frederiksberg C, Denmark

1*Corresponding authors. Tel: +45 35 33 30 92; fax: +45 35 33 30 55; E-mail address: jom@sund.ku.dk (J. Madsen)
Tel: +45 35 33 28 68; E-mail address: naha@sund.ku.dk (M. N. Haque)

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ABSTRACT

Methane (CH$_4$) emission was investigated in artificially reared growing lambs fed milk replacer and cream. This study was part of a larger study with 70 lambs, of which 18 lambs with an average body weight of 21±3.6 kg (mean±SD) were used. The lambs were housed in individual pens (1.5×1.5 m). From 3 until 180 days of age, they were fed either a restricted grass hay diet or a “Cream” diet (50% milk replacer and 50% cream) ad libitum until a daily maximum allocation of 2.5 L/d. In addition, rolled maize was fed ad libitum (maximum allowance 1 kg/d). After 180 days, two groups were placed together and supplied a hay diet. The CH$_4$ and carbon dioxide (CO$_2$) were measured in periods 1 to 4 (approx. 90, 150, 185 and 235 days of age, respectively). During periods 1 and 2, the measurements were performed on each of the 18 lambs individually for 8 hours, equally distributed in 3 periods over a 24-hours day. During periods 3 and 4, the measurements were performed on each of the 18 lambs consecutively for 30 minutes. Twenty-six lambs (out of 70), of which four lambs from the CH$_4$ measurement group, were slaughtered at the age of 180 days to collect rumen samples for microbiological study. The dry matter intake (DMI, g/d) was significantly lower (P<0.001) in the cream-fed group. The CH$_4$ production (g/d) was 84 and 87% lower in the cream group compared to the hay group during periods 1 and 2, respectively. The same group had a lower CH$_4$ emission per unit of DMI and DEI (P<0.001). The CH$_4$:CO$_2$ ratios were 0.0022 and 0.0036 in the cream group during periods 1 and 2, respectively. Within 4 days after changing the diet (period 3), the CH$_4$:CO$_2$ ratio of the ex-cream-fed lambs was 0.035, much higher compared to the CH$_4$:CO$_2$ ratio during period 1 (P<0.001). A significantly lower CH$_4$:CO$_2$ ratio (P<0.001) was observed in the cream group compared to the hay group during periods 3 and 4, respectively. The abundance of rumen methanogens was lower in the fluid portion of the cream group. In conclusion, the artificial rearing of lambs with milk replacer and cream nearly prevented CH$_4$ release. Switching from milk replacer and cream to a fibrous diet dramatically changed the CH$_4$:CO$_2$ ratio in the cream group within 4 days. The CH$_4$:CO$_2$ ratio remained lower for 50 days after the diet alteration.

Keywords: Abundance, Artificial rearing, Breath sample, Carbon dioxide, Fibrous diet, Rumen development

Abbreviations: ADFom, acid detergent fibre expressed exclusive of residual ash; aNDFom, neutral detergent fibre assessed with heat stable amylase and expressed exclusive of residual ash; BW, body weight; CO$_2$, carbon dioxide; CH$_4$, methane; CH$_4$:CO$_2$, ratio between methane and carbon dioxide; DE, digestible energy; DEI, digestible energy intake; DM, dry matter; DMI, dry matter intake; DNA, deoxyribonucleic acid; FA, fatty acid; HP, heat production; HPU, heat production unit; Lignin(sa), lignin determined by solubilisation of cellulose with sulphuric acid; LSM, least square mean; rRNA, ribosomal RNA; PCR, polymerase chain reaction; WG, weight gain.
1. Introduction

Enteric fermentation is the second largest source of greenhouse gas emissions, contributing approximately 40% to total emissions, of which the contribution of small ruminants is 10% (Gerber et al., 2013). Methane (CH\textsubscript{4}) production from ruminants is a result of the microbial fermentation of feeds in the rumen. Moreover, enteric CH\textsubscript{4} production leads to a loss of productive energy ranging from 2 to 12% of the gross energy intake in ruminants, depending on the level of feed intake and diet composition (Johnson and Johnson, 1995). Therefore, due to nutritional and environmental considerations, efforts to mitigate CH\textsubscript{4} emissions from ruminants, especially through dietary manipulation, are receiving great attention. The addition of lipids or fats was recently proven to mitigate CH\textsubscript{4} emission while increasing the dietary energy content (Machmuller and Kreuzer, 1999; Machmuller et al., 2000). The CH\textsubscript{4}-suppressing mechanism of fats is believed to be induced by the reduction of organic matter fermentation, fibre digestibility and, consequently, the methanogenic pathway and more importantly by the direct inhibition of methanogens in the rumen through the hydrogenation of unsaturated fatty acids (Johnson and Johnson, 1995). A strong effect of lipid supplementation on CH\textsubscript{4} reduction (up to 73%) has been reported in sheep (Machmuller and Kreuzer, 1999). Most studies have investigated a short-term CH\textsubscript{4} mitigation strategy, and their persistency is still in question. Therefore, it is crucial to determine a long-term mitigation strategy through dietary manipulation. Such an approach could be the artificial rearing of young ruminants through liquid feeding to avoid microbial fermentation in the rumen. In newborn ruminants, CH\textsubscript{4} production and energy loss through CH\textsubscript{4} are absent due to a non-functional rumen and lack of microbial fermentation (Eadie, 1962). The establishment of rumen microorganisms and fermentation in neonatal lamb begins at the age of 3-4 weeks (Wardrop and Coombe, 1960), during which the type of diet is the primary factor that affects the relative growth of the digestive organs (Wardrop, 1960). Moreover, the metabolic and physical development of the rumen during the growing phase depends on solid feed consumption (Baldwin et al., 2004). Increased supply of milk to dairy calves produced a higher daily weight gain and slower rumen development (Khan et al., 2007). Similarly, Smith (1959) reported that rumen development will not occur in milk-fed calves for an abnormally prolonged time (up to 32 weeks). The initiation of fibrous feed consumption and fermentation processes is required to inoculate and establish the anaerobic rumen microbial ecosystem and develop proper rumen function in young ruminants (Baldwin et al., 2004). We hypothesised that feeding milk replacer for a prolonged period would affect the rumen microbial populations, fermentation rate and gas production, especially CH\textsubscript{4} production in lambs. We further hypothesized that the prolonged feeding of a liquid fat (dairy cream) would have a sustained suppressive effect on the enteric CH\textsubscript{4} production. Therefore, the objective of this study was to investigate the rumen fermentation and CH\textsubscript{4} emissions of lambs that were reared on a diet of milk and cream compared to lambs that were reared on a conventional grass-hay diet.
2. Material and methods

2.1. Animals, housing and feeding

This experiment complied with the guidelines of the Danish Ministry of Justice with respect to animal experimentation and care of animals under study. This study was part of a larger study with 70 lambs born to twin-pregnant ewes. Only 18 lambs with an average body weight 21±3.6 kg (mean±SD) were selected in this study to describe the effects of feeding milk replacer and cream on CH4 emissions. Within each twin pair, one lamb was assigned to each of the two treatments, which were termed the “Hay” and “Cream” groups. The lambs suckled their dams until 3 days of age, after which the ewes were separated from their lambs. Initially, the lambs were housed in a large barn that was maintained with proper ventilation and at a temperature of approx. 18-22°C. The lambs were fed individually in smaller pens (1.5 × 0.75 m) with sawdust as a bedding material. When the lambs reached 60 days of age, they were transferred to larger pens (1.5 × 1.5 m). From 3 days until 56 days of age, the “Hay” group was fed milk replacer (180 g of milk powder g/L; Elitemilk Lamb, Vifoarm; DLA Group, Galten, Denmark) from a suckling bucket and received grass hay from 14 days of age. From 57 days of age, the hay group was fed only hay without any milk replacer (Fig. 1). The daily allowances of milk replacer and hay for the hay group were adjusted on a weekly basis to achieve moderate daily live weight gains of approx. 225 g/d.

Lambs in the second group, which was termed the “Cream” group, received 50% milk replacer and 50% dairy cream (Osted Ost og Mejeri ApS, Lejre, Denmark) ad libitum (until the daily predefined maximum of 2.5 L/d up to 180 days of age). Rolled maize (Maize flakes; R2 Feed Partner A/S, Hedensted, Denmark) was fed ad libitum (until the predefined maximum allowance of 1 kg/d). The milk replacer-dairy cream mix was fed from a suckling bucket. From 3 to 7 days of age, the lambs in both of the groups were fed four times a day and twice daily thereafter at approx. 07:00 h and 16:00 h, respectively. A small amount of barley straw (approx. 10 g/d) was fed to the both groups, and for cream lambs, this feeding was to prevent disorders of rumen function. The amount of DM from the ingested barley straw was considered insignificant. Therefore, this was not added to the total DMI for both of the groups. Water was available ad libitum at all times, and a vitamin-mineral mix was provided based on requirements (NRC, 2007). The daily amount that was fed to the lambs was recorded daily during periods 1 and 2. The feed residues and weight of the lambs were recorded once per week. The daily ingested amount was calculated considering the weekly supply and refusal. The weight gain was calculated considering the differences in the weekly body weight changes during periods 1 and 2. The feed intake and body weight were not recorded during periods 3 and 4. At 180 days of age, a total of 26 lambs (13 from each treatment) out of 70 were slaughtered for the collection and analysis of the rumen contents for rumen microbial diversity, of which 4 lambs (2 from each treatment) were from the CH4 measurement groups. The remaining lambs were thereafter managed together and were fed the hay diet. The CH4 measurements were performed in 4 periods at approximately 90, 150, 185 and 235 days of age. Periods 3 and 4 were held at 4 and 50 days after the transfer of the cream lambs to a normal hay diet, respectively. In order to make a comparable CH4 estimation during periods 3 and 4, two lambs were added to each group to make similar the number of animals as in periods 1 and 2. The added
lambs with closer average BW in the respective groups were selected from the flock that was reared with a same feeding regime as in the cream and hay groups, respectively.

2.2. Sampling and analysis of feed samples

The chemical composition, nutritive value and energy content of the feed that was used during periods 1 and 2 are shown in Table 1. Feed samples were taken from both of the groups during each of the measurement periods. Immediately after collection, the samples were stored in a freezer. Before laboratory analysis, the samples were mixed together to create a composite sample. The samples were dried at 103°C to determine the dry matter percentage. The crude ash content was determined according to EU (2009). The aNDFom, ADFom and lignin(sa) were determined according to Van Soest et al. (1991). Both aNDfom and ADFom were expressed exclusive of residual ash. Lignin(sa) was determined by solubilisation of cellulose with sulphuric acid. The crude protein content was determined according to Licita et al. (1996). The crude fat content was determined following ISO-11085 (2008). The digestibility of the feed ingredients of the hay diet were calculated according to NRC (2001). The digestibility of the feed ingredients in the diet of the cream group were determined according to Moller et al. (2000). The digestible energy content was based on the chemical composition of the individual ingredients, and was calculated according to NRC (2001).

2.3. Sampling and microbial analysis

Both solid and liquid samples were collected from the rumen. Immediately after the collection of the total contents, the solid part was separated from the liquid by filtering with double-folded cheese cloth. The samples were immediately stored at -40°C after collection. During the analysis phase, the DNA was extracted from 0.5 g of ruminal fluid and solid content using the Genomic Mini AX Soil Spin Kit (A&A Biotechnology). To study the archaeal composition in the ruminal samples, the variable regions V3 and V4 of the 16S rRNA gene phylogenetic marker were amplified using the primer 341F (5’-CCTAYGGGRBGCASCAG-3’) and 806R (5’-GGACTACNNNGGGATCTAAT-3’) by the Phusion Hot Start Polymerase 540L (Neefs et al., 1991; Yu et al., 2005). The polymerase reaction (PCR) mixture contained 5 µl of 5× Phusion HF buffer (7.5 mM MgCl2, Finnzymes, Finland), 0.5 µl of 10 mM dNTP mixture, 0.25 µl of Phusion Hot Start DNA Polymerase (1 unit/µl, Finnzymes), 1.25 µl of each primer (10 µM), and 2 µl of template. The PCR reaction started at 98°C for 30 seconds, followed by 30 cycles of 98°C for 5 seconds, 56°C for 20 seconds and 72°C for 20 seconds. The reaction was finalised with 72°C for 5 minutes. The amplicon fragment of 466 bp was elongated to 526 bp by adding sequencing adaptors and barcodes (Roche FLX) to the PCR product following the same conditions as for the first PCR with a shortened cycle number of 15. The sequences were generated using 454 GS FLX Titanium. For methanogen quantification, the sequences were cleaned of low quality reads and split into individual animal samples using the default settings of Qiime (Caporaso et al., 2010). Chimera were removed by USEARCH UCHIME (Edgar et al., 2011). Operational taxonomic units (OTUs) were de novo picked by UCLUST, and the taxonomy was assigned to the OTUs with the RDP classifier method and Greengenes reference database (Liu et al., 2008).
2.4. Installation and gas measurement

The gas measurements were performed every 15 seconds in the 4 experimental periods. During periods 1 and 2, each of the 18 lambs was measured individually for 8 hours equally distributed in 3 periods over a 24-hour day. During periods 3 and 4, the measurements were taken on each of the 18 lambs consecutively for 30 minutes during the day without considering the potential diurnal variation. Prior to measuring the breath sample, the pens of the lambs were covered by Plexiglas to restrict the air movement as much as possible. However, the pens were not completely airtight. The glass was transparent to avoid blocking the views of the lambs from each other. The breath concentrations of CH\textsubscript{4} and CO\textsubscript{2} were measured using a continuous gas analyser Gasmet DX-4030 (Gasmet™, 2010) based on Fourier Transformed Infrared Radiation. The inlet filter of the Gasmet was fitted inside the pen to collect concentrated breath samples. After being received in the inlet, the breath sample passes through the filter and thereafter through the Gasmet analyser to determine the concentrations of CH\textsubscript{4} and CO\textsubscript{2}. Before each measurement, the equipment was calibrated with known standard gases to verify the accuracy of the measurement. On each experimental day, the background concentrations of CH\textsubscript{4} and CO\textsubscript{2} were measured. The measurements were remotely monitored via the internet using TeamViewer (TeamViewer©, 2013).

2.5. Calculation

The CH\textsubscript{4} and CO\textsubscript{2} emissions from the lambs were calculated according to the CO\textsubscript{2} method (Madsen et al., 2010). The barn concentrations of CO\textsubscript{2} (590, ppm) and CH\textsubscript{4} (6.9, ppm) were subtracted from the exhaled concentrations of the lambs to obtain the actual breath concentration. A ratio between CH\textsubscript{4} and CO\textsubscript{2} (CH\textsubscript{4}:CO\textsubscript{2}) was determined. The heat production (watt) of the lambs was calculated following Equation (1) as described by CIGR (2002). The excretion of CO\textsubscript{2} (L/d) was calculated according to Pedersen et al. (2008) and mentioned in Equation (2). The amount of CH\textsubscript{4} (g/d) was calculated as described by Madsen et al. (2010) in Equation (3).

\[ HP = 6.4 \times BW^{0.75} + 145Y \]  
\[ CO_2 = HPU \times 180 \times 24 \]  
\[ CH_4 = CO_2 \times \frac{CH_4}{CO_2} \times 0.714 \]

Where:

HP=heat production of the animals

BW\textsuperscript{0.75}=metabolic body weight of the animals

Y=daily weight gain of the animals

HPU=heat-producing unit \( \frac{HP}{1000} \)

180=L of CO\textsubscript{2}/HPU/hour
2.6. Statistical analyses

The raw data were processed to obtain the average emission per lamb per day. The day average data were then fitted with a linear model using the statistical software R version 3.0.0 (R Development Core Team, 2013). The primary model for periods 1 and 2 was fitted with all of the possible influential variables [body weight, weight gain (WG), groups, periods and dry matter intake]. The final model (Equation 4) was selected for periods 1 and 2 by the stepwise elimination of the non-significant variables. The model (Equation 5) for periods 3 and 4 was fitted with groups and periods. The model was validated using an analysis of variance (ANOVA) on the Akaike Information Criterion. The model residuals were checked for normality and homoscedasticity by visual inspection, producing qqplots.

\[ y_{ij} = \mu + \alpha_i + \beta_j + Xy_{ij} + \varepsilon_{ij} \]  

(4)

\[ y_{ij} = \mu + \alpha_i + \beta_j + \varepsilon_{ij} \]  

(5)

Where \( y_{ij} \) is the response variable, \( y = [\text{CH}_4 \text{ g/d, CH}_4 \text{ g/kg WG, CH}_4 \text{ g/kg DMI, CH}_4 \text{ g/MJ DEI and CH}_4:CO_2 \text{ ratio for periods 1 and 2; and only the CH}_4:CO_2 \text{ ratio for periods 3 and 4} \] \) of group \( i \), and period \( j \), \( \mu \) = overall mean, \( \alpha_i \) = group (cream and hay), \( \beta_j \) = measurement period [1 and 2 (Equation 4), and 3 and 4 (Equation 5)], \( Xy_{ij} \) = total dry matter intake of group \( i \), and period \( j \), and \( \varepsilon_{ij} \) is the model residuals. Although the period as a fixed variable was not significant, this was included in the model to determine the trend of CH\textsubscript{4} production over the time. The least square means (LSM) were extracted from the model using the package lsmeans as described by Russell (2013). A multiple comparison was performed using Tukey’s pairwise comparisons using the function glht from the multcomp package (Hothorn et al., 2008). For microbiological analysis, the rarified relative sequence counts of the methanogens were checked for normal distribution using the Shapiro Wilk test. Due to the non-normal distribution of the dataset (Shapiro Wilk; \( W=0.703 \), \( P<0.001 \)), the non-parametric two-sample Wilcoxon Rank Sum test (cut-off \( p \)-value, \( P<0.05 \)) was used to evaluate the diet-induced differences in methanogen abundance between the variables.
3. Results

3.1. Body weight and dry matter intake

The body weight, daily weight gain and feed intake of the experimental lambs are shown in Table 2. The body weight (BW, kg) was not different in the two groups within the periods. During period 2, a significant increase in the BW was observed in both of the groups (P<0.001) compared to that in period 1. The average daily weight gain was 30% and 22% higher (P=0.02) in the cream compared to the hay group during periods 1 and 2, respectively. The total dry matter intake (DMI, g/d) was significantly lower in the cream compared to the hay group (P<0.001). However, there was a period effect on the DMI for both of the groups, resulting in a greater intake (P<0.001) during period 2. The neutral detergent fibre (NDF, g/d) intake was extremely low, and the total dietary fat intake was much higher (P<0.001) in the cream compared to the hay group. The digestible energy intake (DEI, MJ/d) during period 1 was different between the groups (P=0.016). However, no difference (P>0.05) was found in DEI (MJ/d) between the groups during period 2, although there was a tremendous increase compared to the amount that was observed during period 1 (Table 2).

3.2. Methane production

The CH₄ output (g/d) was significantly (P<0.001) lower in the cream compared to the hay group (Table 3). Feeding milk replacer and cream resulted in 84% less CH₄ output compared to the hay group (P<0.001) during period 1. The reduction in the same group was greater (87%) during the 2nd period (P<0.001). However, the CH₄ (g/d) yield in each group was not different between periods 1 and 2.

The CH₄ release (g/kg WG) was significantly lower in the cream group (P<0.001) compared to that of the hay group between the periods. The same results were observed (P<0.001) for CH₄ expressed in terms of dry matter intake (DMI, g/d) and digestible energy intake (DEI, MJ/d) (Table 3). A regression analysis of CH₄ output (g/d) according to the body weight (kg) explained this difference more clearly (Fig. 2). This figure illustrates an increasing trend of CH₄ (g/d) in the hay group with increased BW over time. However, in the cream group, the CH₄ (g/d) yield was almost steady across time, with increased body weight.

The CH₄ output (g/d) was positively correlated (r=0.74 and 0.71) with the digestible energy intake (DEI, MJ/d) in the hay groups during periods 1 and 2 (Fig. 3a). In case of cream group the correlation between CH₄ release (g/d) and DEI (MJ/d) was negative (r=-0.12 and -0.32, respectively) during periods 1 and 2. The CH₄ emission (g/MJ DEI) was very different in the two groups (P<0.001), even though a similar CH₄ (g/MJ DEI) output was observed in the cream group during periods 1 and 2. The DMI (g/d) was strongly correlated (r=0.73 and 0.72) with the CH₄ (g/d) in the hay group during periods 1 and 2 (Fig. 3b). However, a negative correlation (r=-0.25 and -0.58) was observed between CH₄ (g/d) and DMI (g/d) in the cream group during the same periods.
This result indicates that the CH₄ (g/d) emission was steady in the cream group, although the DMI increased over time.

3.3. CH₄ and CO₂ ratio (CH₄:CO₂) after changing the diet

An extremely quick response was observed in the CH₄ emission change in the 180-day-old cream-fed lambs after the lambs were transferred to a normal hay diet. Within four days of changing the diet (period 3), the CH₄:CO₂ ratio of the ex-cream-fed lambs was 0.035±0.0011 (mean±SE), which was much higher than 0.0022±0.00036 (mean±SE) in the same lambs during period 1 at 90 days of age (P<0.001). Fifty days after the diet alteration (period 4), the ratio increased (P<0.001) further to 0.039±0.0015 (mean±SE) compared to the ratio that was recorded during period 1 (Fig. 4). However, no difference in the CH₄:CO₂ ratio was found in the cream group between periods 1 and 2 (P>0.05). Similar results of the CH₄:CO₂ ratios were also observed in the same group after the diet alteration during periods 3 and 4.

The CH₄:CO₂ ratios in the hay group were 0.069±0.0013, 0.067±0.0008, 0.070±0.0014 and 0.046±0.0013 (mean±SE) in periods 1, 2, 3 and 4, respectively (Fig. 4). A multiple comparison of the CH₄:CO₂ ratio from the 4 measurement periods within the hay group showed no significant difference (P>0.05) between the ratios during periods 1, 2 and 3. However, the CH₄:CO₂ ratio in the same group was significantly lower (P<0.001) during period 4 compared to that during period 1. When comparing the CH₄:CO₂ ratio between the groups during periods 3 and 4, the cream group had a significantly lower CH₄:CO₂ ratio (P<0.001) compared to that of the hay group (Fig. 4).

3.4. Methanogens and morphology of rumen wall

The 16S rRNA gene was amplified with universal primers to study the archaeal community (further details in section 2.3). The amplicon libraries were further processed using high-throughput sequencing and were quality trimmed according to the recommendations of the default settings of Qiime (Caporaso et al., 2010). After the basic data treatment, de novo OTU picking and taxonomy assignment, 4680 archaeal sequences (75 OTUs) were received for downstream analysis. The full data describing the bacteria will be presented in a separate publication. The majority of the generated sequences (3704) belong to the Methanobacteriaceae family, which includes most of the known methanogenic prokaryotes. In hay-fed lambs, the methanogens were composed of Methanobrevibacter (93.85%), Methanosphaera (1.5%), Methanocorpusculum (4%) and unassigned Methanobacteriaceae (0.15%) sequences. The cream-fed group was composed of only Methanobrevibacter. To compare the relative abundances between both of the diet groups, the sequences were randomly and evenly subsampled, and the sequencing-induced differences were eliminated.

A small but significant increase in the methanogens count was observed in the solid phase of the rumen digesta in the cream compared to that in the hay-fed lambs (Wilcoxon-Rank-Sum test, P=0.036) (Fig. 5). In contrast, higher counts were found in the hay-fed lambs when looking at the ruminal fluid phase. The cream-fed lambs barely carried any methanogenic sequences in the rumen fluid (Wilcoxon Rank-Sum test P=0.013).
A visual inspection of rumen wall clearly demonstrate a poor development of rumen mucosa, with little papillary growth and light yellow coloration that were considered to be associated with a lack of microbial fermentation in the cream group (Fig. 6a). In contrast, in the hay group, excellent mucosal development and distinctive papillary growth were observed (Fig. 6b).
4. Discussion

4.1. Feed intake and daily weight gain

The diet of the cream group contained 50% dairy cream along with milk replacer and produced a very high total fat content. In addition, rolled maize is rich in starch and is a source of readily fermentable carbohydrate. These two components produced a diet with a high energy concentration and reduced the DMI (g/d) in cream group. Haddad and Younis (2004) reported a reduced DMI in lambs with 5% fat added to a high concentrate fattening diet. Similar results have been declared in lambs from 15 to 180 days by supplementing 25 to 75 g (per kg of concentrate) of coconut oil (Bhatt et al., 2011). Moreover, Machmuller and Kreuzer (1999) described that the DMI was not affected by feeding a ration with 30 to 35 g/kg coconut oil, whereas feeding 70 g/kg coconut oil reduced the DMI in adult sheep. The diet in this study was completely different from those that were mentioned earlier, but the markedly lower DMI in the cream group was due to a higher energy content in the diet. Although the DMI was lower in the cream group, the total digestible energy intake (MJ/d) was very similar in the cream and hay group, indicating that the reduction in the DMI was due to the higher digestible energy content in the milk replacer and cream diet. This result confirms that ruminants can be reared on a diet of milk and other liquids for a longer period without an adverse effect on the daily DE intake and the occurrence of digestive disorders. It should be noted that a negligible amount of barley straw was provided to the lambs that were fed the liquid diet to avoid digestive disturbances. The lambs in the cream group were fed the milk replacer and cream diet from birth. Therefore, these lambs were considered well adapted to the diet. The rate of daily weight gain (g/d) was higher in the cream group presumably reflecting better energy utilisation compared to that of the hay group. This difference in the daily weight gain between two groups might have caused by the moderate growth rate in the hay at a restricted feed intake. However, the gross body weight was not different between the groups presumably because of the low protein content, which restricted their lean growth to some extent.

4.2. Breath sampling and methane estimation

The estimation of CH₄ by the CO₂-method makes it possible to conduct measurements while keeping the animals in their natural environment (Madsen et al., 2010). However, in this study, we slightly modified the measurement technique. The individual pens were covered with transparent Plexiglas to restrict air movement, but the normal behaviour and movements of the lambs were confirmed by periodic inspection throughout the entire duration of the experiment. Therefore, the breath samples are assumed to be from the normal metabolism of the lambs. Another important aspect should be considered is the length of the measurement because the concentrations of the breaths are not always constant. During the development phase of the CO₂ method, Madsen et al. (2010) found that approx. 2-3% of the breath sample is enough to estimate CH₄ with the CO₂ method. Another study of dairy cows demonstrated that 2-3 days of measurement in the automatic milking system (AMS) is enough for CH₄ estimation (Lassen et al., 2012). In this study, we used 8 hours of measurement during the first two periods and 30 minutes during periods 3 and 4. The length of the measurements is longer than in the previous study of Haque et al. (2014), who measured dairy cows for 5 days during milking in AMS with an average of 6 minutes, and the number of visits to the AMS was 2.6 per day, producing a measurement time of approx. 15.6
minutes per day per animal, which is less than the 30 minutes in this study during periods 3 and 4. Moreover, the variation in the concentration of CH₄ and CO₂ as measured in the pens is much less compared to those in the AMS (Madsen and Bertelsen, 2012). Therefore, the shorter measurements during the last two periods are considered reasonable.

4.3. Long-term dietary impact on methane emission

Highly significant differences in CH₄ production (g/d) were observed between the two experimental groups. It was expected that the CH₄ emission from the cream lambs would be markedly lower than that of the hay fed lambs because of the extremely high level of total fat and almost no fibre in the cream diet. Moreover, it can be assumed that the continuous feeding of milk and cream in liquid form may have influenced the significantly lower CH₄ release in cream group. The very different feeding in the cream group up to 180 days of age probably retarded the normal development of the rumen, and very negligible microbial fermentation occurred in lambs as evidenced by the extremely low CH₄ (g/d) emission. Yurteven and Ozturk (2009) found that feeding a corn-based diet to adult sheep greatly increased the proportion of propionate, whereas the proportion of acetate decreased. Feeding milk replacer and cream along with rolled maize in this study may have enhanced the proportion of propionic acid production by reducing the proportion of acetate, thereby reducing the levels of CH₄ output. Several studies of adult sheep and lambs have reported remarkable reductions of up to 73% in the CH₄ emissions by supplementing coconut oil (Machmuller and Kreuzer, 1999; Machmuller et al., 2000). In this study, the CH₄ release was 84-87% lower in the cream group, which is fairly consistent with the results of (Machmuller and Kreuzer, 1999), who reported a 73% reduction by the addition of 3.5 to 7 % coconut oil to the diet. The same author in another study reported a 43-57% CH₄ reduction by supplementing 3 and 6% coconut oil (Machmuller, 2006). Although a different fat source was used in this study, cream is also characterised by high levels of medium-chain fatty acids, similar to coconut oil products. In fact, coconut oil is one of the only other fat sources except for milk fat from ruminants that contains very large amounts of medium-chain (C₁₂:C₁₆) fatty acids. Furthermore, Czerkawski et al. (1966) found that a momentarily high concentration of fatty acid in the rumen fluid might be more important to suppress CH₄ release. In this study, the remarkable CH₄ suppression in the cream group was probably linked to an accumulation of medium-chain saturated fatty acids in the rumen fluid, which reduced the supply of organic matter and had inhibitory effects against the methanogens. This result is also supported by the result of the microbial analysis in this study, in which almost no methanogens were found in the fluid portion in the cream-fed group (Fig. 5). Moreover, very little solid contents were found in the cream group compared to the hay group, indicating that the total abundance of methanogens in the cream group was lower.

4.4. Rumen morphology and ruminal microbial biodiversity

The rumen wall was collected at slaughter. No histomorphological measurement was performed. Photographs of the rumen wall (Fig. 6) clearly demonstrate the visible differences in the morphological structure. It is possible that the limited roughage diet and supply of liquid milk in the cream group for a prolonged period suppressed the morphological development of the rumen wall, the growth of rumen papillae and, more importantly, the growth of the rumen microbial
population. The same argument was mentioned by Jasper and Weary (2002) in dairy calves. Similarly, Baldwin et al. (2004) found that fibrous feed consumption and fermentation are crucial for rumen development and for the establishment of rumen microbes. In this study, although the cream group received rolled maize and a small amount of barley straw, the ingested amount was too low to initiate rumen development. In the hay group, the proper development of the rumen was obvious because of the presence of a sufficient amount fibre in the diet. Similarly, Khan et al. (2011) reported that the provision of hay to calves promotes rumen development.

Methanogenic archaea do not directly assimilate cellulose and are dependent of the fibrolytic bacteria, which provide carbon dioxide and hydrogen during fermentation (Liu and Whitman, 2008). Previous studies have demonstrated that fatty acids supplied to the diet are toxic for fibrolytic bacteria and protozoa and consequently resulted in CH₄ depletion (Machmuller et al., 2003; Hook et al., 2010). Thus, it was expected that the relative numbers of methanogens would significantly be reduced in the cream-fed group. The results suggest that the extreme cream diet feeding did not eliminate the archaea but rather reduced the diversity of the methanogens to the genus Methanobrevibacter. The methanogens appeared to be adapted to the solid substrate in the cream group. A significant shift of the methanogens from the ruminal fluid to the solid material was observed in the cream group compared to the hay group, indicating an adaptation of the microbial community to the dietary conditions. The rolled maize that was added to sustain crucial microbial metabolic activities in the rumen system of the cream group certainly enhanced the possibility of survival of the methanogenic archaea in the solid phase of the rumen contents. However, the level of CH₄ release decreased probably due to the increased production of propionate as described by Yurtseven and Ozturk (2009), suggesting possible interactions of the methanogens with other microorganisms in the solid rumen material and their adaptation to the diet, which needs to be confirmed by future studies.

4.5. Response of lambs to the fibrous feed

The intense response of the CH₄:CO₂ ratio in the cream group within 4 days of changing the diet indicates that feeding a fibrous diet would quickly initiate the rumen fermentation, which appears to be independent of the feeding regime earlier in life. The significantly lower CH₄:CO₂ ratio in the cream group 50 days after the diet alteration attributes an important assumption, i.e., although the cream group responded very quickly to the fibrous diet, we assume that artificially reared ruminants will take longer to emit an equal amount gas (CH₄ and CO₂) as from the ruminants reared under normal feeding regime. The effect of artificial feeding would last for an extended period, presumably because of the possibility of residual effects. Therefore, the result in the ex-cream-fed group does not necessarily imply that the CH₄ suppressing effect of an extreme diet is transitory. Further studies are required to confirm this hypothesis. It should be acknowledged that the CH₄:CO₂ ratio in the hay group during period 4 was slightly lower, which might not be a representative gas emission from normal metabolism, presumably because the lambs were free in the barn, and some handling was involved to get the lambs into the measurement pens. In addition, a 30-minute measurement time was followed during this period. The situation again demonstrates
that to obtain a normal breath sample for accurate CH₄ estimation, it is important to maintain the animals in their normal movement.

5. Conclusions

The artificial rearing of lambs with milk replacer and cream nearly prevented CH₄ release. The abundance of rumen methanogens was lower in the fluid portion of the cream group, and the rumen archaea were mostly adapted in the solid phase of the rumen content. Switching from milk replacer and cream to a fibrous diet dramatically changed the fermentation pattern and, consequently, the CH₄:CO₂ ratio in the cream group within 4 days. The CH₄:CO₂ ratio remained lower for 50 days after the diet alteration. Feeding milk replacer and cream to the lambs up to 180 days reduced the CH₄ emissions for an extended period. However, it cannot be excluded that the effect is long-lasting.

Conflict of interest

None

Acknowledgements

The authors sincerely acknowledge all of the members of the project “Impact of pre- and postnatal dietary interactions on postnatal metabolic and endocrine function” for their support to conduct this study.
References


Gerber, P.J., Steinfeld, H., Henderson, B., Mottet, A., Opio, C., Dijkman, J., Falcucci, A., Tempio, G., 2013. Tackling climate change through livestock – A global assessment of emissions and
mitigation opportunities. Food and Agriculture Organization of the United Nations (FAO), Rome, Italy.


Table 1

Chemical composition, digestible nutrients and energy content of the feeds.

<table>
<thead>
<tr>
<th>Items</th>
<th>Grass hay</th>
<th>Cream</th>
<th>Milk powder</th>
<th>Rolled maize</th>
</tr>
</thead>
<tbody>
<tr>
<td>'DM (g/kg)</td>
<td>931.0</td>
<td>429.2</td>
<td>956.1</td>
<td>895.0</td>
</tr>
<tr>
<td>Ash(g/kg DM)</td>
<td>68.2</td>
<td>8.3</td>
<td>71.0</td>
<td>6.2</td>
</tr>
<tr>
<td>aNDFom(g/kg DM)</td>
<td>504.0</td>
<td></td>
<td>41.1</td>
<td></td>
</tr>
<tr>
<td>ADFom(g/kg DM)</td>
<td>323.4</td>
<td></td>
<td>40.0</td>
<td></td>
</tr>
<tr>
<td>Lignin(sa) (g/kg DM)</td>
<td>35.0</td>
<td></td>
<td>9.0</td>
<td></td>
</tr>
<tr>
<td>CP (g/kg DM)</td>
<td>208.0</td>
<td>43.3</td>
<td>225.1</td>
<td>85.0</td>
</tr>
<tr>
<td>cFat (g/kg DM)</td>
<td>37.1</td>
<td>380.0</td>
<td>236.1</td>
<td>19.3</td>
</tr>
<tr>
<td>DE (MJ/kg DM)</td>
<td>12.7</td>
<td>35.8</td>
<td>20.4</td>
<td>15.9</td>
</tr>
</tbody>
</table>

1Calculated as g/kg of fresh material;

DM: dry matter;
aNDFom, neutral detergent fibre assessed with heat stable amylase and expressed exclusive of residual ash;

ADFom, acid detergent fibre expressed exclusive of residual ash;

Lignin(sa), lignin determined by solubilisation of cellulose with sulphuric acid;

CP: crude protein;

cFat: crude fat;

DE: digestible energy.

Table 2

Least square means (LSM) of the body weight, weight gain and nutrient intake of the lambs (n=18) of hay and cream group during periods 1 and 2.
Parameters | Hay | Cream | RSE | $R^2$ | Significance
--- | --- | --- | --- | --- | ---
| **Period 1** | **Period 2** | **Period 1** | **Period 2** |  
| BW (kg) | 20.9<sup>a</sup> | 33.7<sup>b</sup> | 21.8<sup>a</sup> | 34.7<sup>b</sup> | 5.33 | 0.59 | <0.001 |
| WG (g/d) | 151.2<sup>b</sup> | 204.1<sup>b</sup> | 197.3<sup>b</sup> | 250.1<sup>c</sup> | 58.53 | 0.24 | 0.004 |
| 1DMI (g/d) | 650.9<sup>b</sup> | 1096.5<sup>d</sup> | 237.1<sup>a</sup> | 682.7<sup>c</sup> | 176.60 | 0.75 | <0.001 |
| NDFI (g/d) | 147.7<sup>c</sup> | 341.6<sup>d</sup> | 0.9<sup>a</sup> | 2.8<sup>b</sup> | 56.81 | 0.84 | <0.001 |
| FL (g/d) | 12.7<sup>a</sup> | 36.2<sup>a</sup> | 197.7<sup>b</sup> | 221.2<sup>b</sup> | 60.47 | 0.70 | <0.001 |
| DEI (MJ/d) | 8.0<sup>a</sup> | 14.2<sup>c</sup> | 8.8<sup>b</sup> | 15.1<sup>c</sup> | 2.87 | 0.56 | <0.001 |

1 DMI: dry matter intake excluding the amount from barley straw;  
NDFI: neutral detergent fibre intake;  
FI: fat intake;  
RSE: residual standard error;  
$R^2$: goodness of fit of the linear model;  
Significance indicates the model P value;  
<sup>abcd</sup> superscripts indicate differences (P<0.05) tested by Tukey’s pairwise comparison within group and between the same period across the groups.

Table 3

Least square means (LSM) of the CH₄ production from the lambs (n=18) fed a hay- or cream-based diet at two time points during periods 1 and 2.

Parameters | Hay | Cream | RSE | $R^2$ | Significance
--- | --- | --- | --- | --- | ---
| **Period 1** | **Period 2** | **Period 1** | **Period 2** |  
| CH₄ (g/d) | 19.9<sup>bc</sup> | 19.1<sup>c</sup> | 3.2<sup>bc</sup> | 2.4<sup>c</sup> | 2.39 | 0.96 | <0.001 |
| CH₄ (g/kg WG) | 116.3<sup>c</sup> | 113.9<sup>c</sup> | 11.5<sup>ab</sup> | 9.1<sup>a</sup> | 14.05 | 0.95 | <0.001 |
| CH₄ (g/kg DMI) | 31.1<sup>c</sup> | 34.3<sup>cd</sup> | 4.3<sup>ab</sup> | 1.1<sup>a</sup> | 4.02 | 0.93 | <0.001 |
| CH₄ (MJ/DEI) | 2.5<sup>c</sup> | 2.6<sup>cd</sup> | 0.4<sup>ab</sup> | 0.2<sup>a</sup> | 0.29 | 0.94 | <0.001 |

WG: weight gain;  
DMI: dry matter intake;  
MJ: megajoule;
DEI: digestible energy intake;
RSE: residual standard error;
$R^2$: goodness of fit of the linear model;
Significance indicates the model P value;

$\text{abcd}$ superscripts indicate differences (P<0.05) tested by Tukey’s pairwise comparison within group and between the same period across the groups.
Figure captions

Fig. 1. Experimental feeding period of the lambs (n=18) in the cream and hay group.

Fig. 2. Daily CH₄ production of individual lambs (n=18) as affected by the body weight (kg) in the cream and hay group during periods 1 and 2.

Fig. 3. Methane production (g/d) of individual lambs (n=18) in relation to the digestible energy intake (MJ/d) and DMI (g/d) in two groups during periods 1 and 2.

Fig. 4. The CH₄:CO₂ ratio of the lambs (n=18) in two groups during periods 1, 2, 3 and 4 at the age of 90, 150, 185 and 235 days, respectively. The error bar indicates the standard error of the mean (SE). The values above the bars indicate the average per period.

Fig. 5. Relative sequence counts of methanogens of the lambs (n=26) in cream and hay groups. The ruminal solid and fluid phases of the diet groups are distinguished.

Fig. 6a. Photograph of the rumen wall from a lamb from the cream group at 180 days of age showing a poor development of rumen mucosa, with little papillary growth and light yellow coloration associated with a lack of microbial fermentation in the rumen.

Fig. 6b. Photograph of the rumen wall from a lamb from the hay group at 180 days of age showing excellent mucosal development with dark coloration due to microbial fermentation in the rumen.

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The microbial profile of the rudimentary rumen
The microbial profile of the rudimentary rumen

Michael Roggenbuck¹, Md Najmul Haque², Jørgen Madsen²*, Søren Johannes Sørensen¹*

¹Department of Biology, Microbiology, Faculty of Science, University of Copenhagen, Universitetsparken 15, 2100 København Ø, Denmark

²Department of Large Animal Sciences, Faculty of Health and Medical Sciences, University of Copenhagen, Groennegaardsvej 2, DK-1870 Frederiksberg C, Denmark

*Corresponding authors

Email address:
jom@sund.ku.dk
sjs@bio.ku.dk

Abstract

In our previous study we artificially prevented the development of the rumen in lambs by feeding a colostrum ”mimicking” diet composed of saturated fat, milk replacer, maize flakes from day 0 to day 150 and reduced the methane emission of 87%, without apparent health effects of the animal. Here we described the microbial community associated with the undeveloped rumen, compared to the hay fed control animals at the age of 180 days. We found that both ruminal fluid and solid fractions are very low in diversity. Few bacterial species closely related to known isolated and characterized fore- or hindgut bacteria dominated the samples. The inhibited rumen microbiota was mostly composed of easy fermentable carbohydrate degraders such as Bifidobacterium and the mucin degrader Akkermansia. The ruminal fluid – that carried barely any methanogens, contained large levels of the propionate producing Megasphaera and acetogenic Blautia – both volatile fatty acids are alternative electron sinks for hydrogen. Furthermore we observed by microbial network analysis direct and indirect co-correlation and exclusions of Methanobrevibacter. Our observations revealed that the bacterial species assigned to Bifidobacterium and Akkermansia had indirect negative exclusion affects with Methanobrevibacter. Our results give insights into the microbial community of an artificially underdeveloped rumen

Introduction
The global livestock sector contributes a significant share to anthropogenic greenhouse gas (GHG) emission. Enteric fermentation of ruminants is one of the major sources of anthropogenic greenhouse gas and contributes about 40% (Gerber et al., 2013) with methane as the one of the key gases associated to global climate change. During the complex ruminal fermentation, fiber rich plants are converted into volatile fatty acids (VFAs) (e.g. acetic acid, propionic acid and butyric acid) while CO2 and hydrogen are produced. The hydrogen accumulation in the rumen is prevented by the three known ruminal metabolic reactions, of acetate-, propionate- and methano-genesis (McAllister & Newbold, 2008). In case of the first two pathways, the hydrogen and CO2 are converted to VFAs, useable by the host as energy source. However during methanogenesis the electrons of hydrogen are transferred to CO2 and CH4 and water is formed. Thus, enteric CH4 is not only an environmental hazard in terms of global warming (Gerber et al., 2013) but also it is associated with 2-12% of feed energy loss from the ruminants (Johnson & Johnson, 1995). Therefore, it is crucial to take immediate steps for mitigation of CH4 emission from livestock. There are several strategies under investigation to decrease the enteric CH4 emission as for instance vaccination against (Williams et al., 2009) against methanogenic archaea. However most common approaches apply alternating feeding strategies (e.g. Martin et al., 2010). Most ideas tested so far have had moderate success partly due to complexity of the rumen microbiota (Martin et al., 2010). For example when the targeted methanogen is eliminated by vaccination from the rumen, other methanogenic archaea take over and removed the depletion effect (Williams et al., 2009). However in the young ruminants there is little fermentation during first three months of life due to the undeveloped foregut. The rumen development follows three levels. (I) From day of birth up to 3 weeks, lambs are considered not ruminants, (II) while up to 8 weeks the juveniles are in the transition phase and older (III) are considered adult and fully ruminant (Wardrop & Coombe, 1961). The diet of newborn lambs, the colostrum, is composed of large quantities of proteins, fat, milk, carbohydrates and water. To mimic these diet conditions we designed a cream based diet compositional similar to the standard milk replacer for the young ruminants. In our previously published study, we artificially depressed the development of the rumen (Haque et al., 2014) by feeding lambs from day 0 up to 150 days with milk replacer, cream (32% saturated fat) and maize flakes and compared the animal development with lambs fed with hay (Hay group). We showed substantial methane depletion up to 87 % in the cream fed group compared to the hay group, without apparent health effects of the host. We observed close to no no methanogens in the ruminal fluid fraction, but larger relative levels of a single Methanobrevibacter sp. in solid fraction compared to the control (hay) group. Although no health effects were observed, the rumen wall of the cream group was barely developed and its size was rudimentary compared to the hay group.

In this study we investigate the complex microbial community of the cream and hay fed animals at the age of 150 days. We aim to characterize the microbial flora associated to
the undeveloped rumen and focused on the co-occurrences and co-exclusions of *Methanobrevibacter* sp. Furthermore we characterize differences associated with effects on the ruminal fraction (fluid, solid). We hypothesize that the rumen of the cream fed group gives new insights into the role of *Methanobrevibacter, the microbiome* and potentially highlight antagonistic strains.

**Material and methods**

*The animal study.* The full animal study has been published here (Haque et al., 2014). In short, the experimental lambs suckled their dams until 3 days of age. The lambs were housed in the individual pens in a large stable. One group of lamb labeled as "cream" were fed 50% milk replacer and 50% dairy cream *ad libitum* until a daily maximum allocation of 2.5 L/d. In addition, rolled maize was fed *ad libitum* (Maximum allowance 1 kg/d). The control group labeled as "hay" were fed milk replacer and cream from 3 days up to the weaning (8 weeks) of age and there after only hay up to the 6 months of age. Methane and CO$_2$ were measured in periods 1 to 2 (Approx. 90, 150, respectively). The methane emission was calculated according to Madsen et al (2010). The emission rate observed counted up to 87% reduction of CH$_4$ in the cream group in compared to the hay fed lambs. A total of 22 animals from two groups were slaughtered at the age of 6 months. The animals were euthanized by intravenous injection of propofol (5–6 mg i.m. kg$^{-1}$ body weight; B. Braun, Melsungen, Germany) followed by decapitation. The rumen contents were removed and filtered with double fold cheesecloth to separate solid portions from the liquid. Thereafter, The rumen content was directly filled into the sterile falcon tubes. And frozen immediately at -20°C and shortly after transferred into the -80°C freezer.

**DNA extraction and amplification of 16S rRNA gene.** To each 0.5g of rumen fluid or solid sample, 1ml extraction buffer (50mM Tris-HCl, 5mM EDTA, 3% SDS) and 0.5 g sterile glass beads was added. Cells were mechanically disrupted by FastPrep (MP Biomedicals) 5.5m/sec for 30 seconds. After removal of cell debris and other rumen solid material by centrifugation (13000 rpm for 1 min), the DNA was further cleaned and extracted using the Genomic Mini AX soil Spin A&A Biotechnology. We amplified 466bp of the 16S rRNA gene covering the hypervariable regions of V3 and V4 by using the primer 341F (CCTAYGGGRBGCASCAG) and 806R (GGACTACNNGGGTATCTAAT) (Yu et al., 2005, Neefs et al., 1991). The polymerase chain reaction (PCR) mix contained 7.5mg MgCl2 (Phusion HF Buffer, Finnzymes, Finland), 1 units/µl Phusion HOT Start DNA polymerase, equal amounts of both primers (10µM), and 2µl of template. PCR reaction started with an initial activation at 98°C for 30 sec, followed by 30cycles of denaturation 98°C for 5s, annealing for 20s at 56°C, and
the elongation for 20s at 72°C. The reaction was finished with a final extension for 5 minutes at 72°C. PCR products were ladder sized in agarose gels (1%), cut and purified with QIAEX II Extraction KIT (Qiagen). Adaptors and tags for multiplexing the sequencing run were added to the PCR product of every sample by using the TAG-primers supplied by Roche and repeating the first PCR with 15 cycles. The second PCR product was purified as described before and submitted to the sequencing center. The sequences were generated with 454 GS FLX Titanium Roche.

**Data treatment.** The received sequences were cleaned of low quality reads and split up into the different sample tags using the Qiime (version 1.5.0) default settings (Caporaso et al, 2010). Chimeras were removed using the Usearch algorithm (Edgar et al, 2011). The operational taxonomic units (OTUs) were de novo picked using the uclust algorithm at 97% sequence identity. Taxonomy was assigned with the RDP classifier (version 2.2) and Greengenes as reference database (Liu et al., 2008).

After quality trimming we received 539883 sequences with a mean count of 12000 sequences per animal. Of the cream solid samples, we generated in average 10485 reads per animal (n=11, min: 5823; max: 28911), of the cream fluid fraction 8298 (n=10, min: 2213; Max: 14522), of the hay solid fraction 3860 reads (n=12, min: 16534, max: 38903), and the hay fluid fraction 5381 (n=11, min: 13012, max: 32349).

**Statistics and network analysis.** The OTU table was randomly even subsampled at 3327 reads per sample, to remove bias by different sequencing efforts. All statistical tests were done in the open source statistical software R (Hornik, 2012). We verified the grouping of the microbial community composition according to the diets and ruminal fractions using the non-parametric analysis of similarity (Anosim) in which an R value close to 1 indicates strong grouping, and 0 reveals no difference between the treatments. A permutation test (999 iterations) was applied to estimate the significance of the anosim R value (Clarke KR, 1993). Furthermore, differences in diversity between the groups were evaluated by the Shannon index and tested for significance with the non-parametric Wilcoxon- Rank-Sum test (W). Finally, microbial taxa (on phylum, genus and OTU level) with significantly varying abundance between the diet groups were identified using the Wilcoxon-Rank-Sum test (W).

Before generating the OTU based microbial network, the OTU table was filtered for all OTUs found less than 50 counts (<0.01% of the total sequences) throughout all samples to reduce complexity and remove singletons. The pairwise-relationships were generated by computing the Spearman-rank coefficient (Cut off ± 0.6) and further tested for significance by permutations (999 iterations) followed by multiple test correction (Bonferroni) p<0.05 (Barberán et al, 2011). Co-occurrences often suffer from large number of zeros (Faust & Raes, 2012), to address this problem we considered only co-occurrences based >8 (sample counts) observations larger than zero.
Furthermore we aimed to characterize potentially more important bacterial or archaeal OTUs in the “rudimentary” rumen with focus on the interaction of *Methanobrevibacter*. Therefor we applied a concept commonly used to characterize hub nodes in social networks that have been recently discussed in biological food webs such as betweenness centrality (Jordán F, 2009). The betweenness centrality (bw) of an OTU (v) is the sum of shortest paths between OTU (x) and OTU (y) on which OTU (v) lies on, divided by the total number of shortest path between OTU (x) and OTU (y). The bw is normalized to 1, thus the closer bw of given OTUs is to 1, the larger the influence of that bacterial species on the total community. The bw is used to evaluate the network conditions, however its true biological relevance is an area of active research.

**Results**

We analyzed the microbial composition of the ruminal fluid and solid fraction of 11 cream fed animals and 12 lambs fed with hay in the age of 180 days. In both feeding groups, two samples of the fluid fraction were not successfully amplified reducing the number of biological replicates to 10 and 11 of the cream and hay group respectively. The cleaned data set contained in total 34335 *de novo* picked operational taxonomic units (OTUs) at 97% sequence identity. However due to the large variation in read depth between the samples, the OTU table needed to be subsampled in order to remove the sequencing effort. However there were two variables to be considered, the sample number (crucial for post analysis) and the diversity (the aim of the study was to characterize the total community of the cream group samples). Therefor the Chao1 richness was estimated to choose the level of subsampling. As figure 1 shows, all samples of the cream group fluid are observed at 3000 reads per sample. Additionally the Chao1 richness reached the plateau of saturation for most of the cream group solid fraction and hay group fluid fraction samples, thus covering the majority of the microbiota. However for the hay solid samples, rare OTUs will potentially be underrepresented in the analysis. Nevertheless, since this analysis focuses on the interaction of the cream group community we choose the subsampling level of 3327 reads/sample in order to keep the full sample number of the fluid and solid fractions.
Figure 1 Rarefaction of evenly subsampled sequences. The Chao1 richness saturation was reached for all cream group samples of both ruminal fractions. Of the hay diet samples, the majority of the samples did not reach the plateau at 3000 sequence counts. Therefore rare OTUs will potentially be underrepresented in the hay group.
Cream group microbiota is significantly less diverse compared to hay fed animals

At even sequencing depth we observed 13273 OTUs. Generally, the ruminal fluid contains less microbial OTU counts than the solid phase samples independent of feeding groups (Figure 1). Furthermore the OTU distribution between the ruminal fractions shown in table 1 reveals that the ruminal cream fluid contains significantly fewer phylotypes than the cream solid and the two hay group fractions. This observation was confirmed by the Shannon diversity revealing a significant lower diversity of cream fed animals in fluid (W=0, p<0.001) or solid W= 13, p=0.001) phase when compared to hay fed animals (Figure 2).

As shown in figure 3 (Venn-Diagram), 61 OTUs (0.46%) were observed in all four ruminal fractions, whereas 11614 (87.5%) were associated to the individual fractions and 1598 (12.07%) were shared partly between the fractions.

When looking into the individual OTU distribution (microbial species only found in one animal) the solid content of hay fed animals carried more unique OTUs (\(\bar{x}:38.0\%\), min: 13.7\%, max: 65.6\%) per animal not shared with other individuals of the same feeding group compared to the cream fed animals (\(\bar{x}:31.6\%\), min: 12.1\%, max: 45.3\%) (Wilcoxon, p<0.05). A similar picture was found in the hay fluid phase that contained more OTUs (\(\bar{x}:29.7\%\), min:13.9\%, max:49.9\%) in the hay group compared to the cream fed animals (\(\bar{x}:17.7\%\), min:7.5\%; max:43.6\%) (Wilcoxon, p<0.05).

![Shannon diversity comparison between feeding groups and ruminal fraction.](image)

Figure 2. Shannon diversity comparison between feeding groups and ruminal fraction.
Table 1. OTU observations in the ruminal fractions at even read depth.

<table>
<thead>
<tr>
<th>Ruminal fraction</th>
<th>Mean</th>
<th>Min</th>
<th>Max</th>
<th>Wilcoxon-Rank-Sum test</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cream fluid</td>
<td>202</td>
<td>129</td>
<td>262</td>
<td>Cream fluid vs hay fluid; W=0; p&lt;0.001</td>
</tr>
<tr>
<td>Cream solid</td>
<td>448</td>
<td>275</td>
<td>699</td>
<td>Cream solid vs hay solid; W=11; p&lt;0.001</td>
</tr>
<tr>
<td>Hay fluid</td>
<td>747</td>
<td>456</td>
<td>963</td>
<td>Hay fluid vs hay solid; W=28; p&lt;0.01</td>
</tr>
<tr>
<td>Hay solid</td>
<td>1020</td>
<td>233</td>
<td>1434</td>
<td>Cream fluid vs cream solid; W=0; p&lt;0.001</td>
</tr>
</tbody>
</table>

Figure 3. Venn-diagram of relative OTU distribution between feeding groups and ruminal fractions.

Microbial composition between ruminal fluid and solid varies strongly independent of diet

However to determine how much the cream group microbial composition varied from the hay group we generated the Bray-Curtis dissimilarity (Figure 4). We observed differences between ruminal fluid and solid when ignoring feeding types confirmed by the Anosim of R=0.413 (P<0.001). However when including diet differences we observed four clusters without overlap evaluated by the Anosim R-value 0.832 (P<0.001) explaining the
strongest dissimilarity between the four groups shown by the principle coordinate analysis (PcoA) of figure 4.

![Figure 4. Microbial cluster analysis with principal coordinate analysis (PcoA). Shown are PC1 and PC2, explaining the largest variance of the Bray-Curtis dissimilarity between the samples based on the subsampled (3000 reads/sample) OTU table.](image)

To hypothesis about the physiological properties of the microbial communities we assigned taxonomy to the observed OTUs. Most sequences of the cream (Fluid:98.8%; solid:96.2%) and hay group (Fluid:98.8%; solid:96.2%) were assigned to phylum level. There were no unclassified sequences. We found *Firmicutes* to be the most prominent phyla in rumen liquid and solid independent of the diet with a similar distribution throughout all samples (Table 2). The second most prevalent phyla *Bacteroidetes* varied only in the ruminal fluid fraction with twice as many relative sequences in the hay group compared to the cream samples. Furthermore only few of *Fibrobacteres* were observed in the cream fed animals. *Euryarchaeota*, including the group of methanogens (in this study all OTUs assigned to the family of *Methanobacteriaceae*) were barely observed in the ruminal cream fluid but accounted for up to 1.2% in cream solid samples. The rumen solid of the cream contained
larger levels of *Actinobacteria*, *Proteobacteria* and *Verrucomicrobia* present only a minority in animals fed with hay.

Table 2. Average phyla distribution in % between feeding groups and ruminal fractions. (Variations were enumerated with the Wilcoxon test, *p*<0.05). Significance was highlighted with an asterisk (*).

<table>
<thead>
<tr>
<th>Taxon</th>
<th>Cream solid ¯x in%</th>
<th>Hay solid ¯x in%</th>
<th>P-value</th>
<th>Cream fluid ¯x in%</th>
<th>Hay fluid ¯x in%</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Euryarchaeota</td>
<td>1.0</td>
<td>0.4</td>
<td>0.037*</td>
<td>0.1</td>
<td>1.3</td>
<td>&lt;0.001*</td>
</tr>
<tr>
<td>Actinobacteria</td>
<td>12.3</td>
<td>2.5</td>
<td>0.002*</td>
<td>11.7</td>
<td>1.0</td>
<td>&lt;0.001*</td>
</tr>
<tr>
<td>Bacteroidetes</td>
<td>9.3</td>
<td>10.0</td>
<td>0.834</td>
<td>14.9</td>
<td>29.8</td>
<td>0.004*</td>
</tr>
<tr>
<td>Firmicutes</td>
<td>57.3</td>
<td>73.7</td>
<td>0.007*</td>
<td>52.9</td>
<td>48.4</td>
<td>0.331</td>
</tr>
<tr>
<td>Spirochaetes</td>
<td>0.0</td>
<td>0.1</td>
<td>0.001*</td>
<td>0.2</td>
<td>0.5</td>
<td>0.005*</td>
</tr>
<tr>
<td>Synergistetes</td>
<td>0.3</td>
<td>0.0</td>
<td>&lt;0.001*</td>
<td>0.3</td>
<td>0.5</td>
<td>0.021*</td>
</tr>
<tr>
<td>Tenericutes</td>
<td>0.0</td>
<td>0.1</td>
<td>&lt;0.001*</td>
<td>0.0</td>
<td>0.1</td>
<td>&lt;0.001*</td>
</tr>
<tr>
<td>TM7</td>
<td>0.0</td>
<td>0.8</td>
<td>&lt;0.001*</td>
<td>1.3</td>
<td>0.8</td>
<td>0.001*</td>
</tr>
<tr>
<td>Verrucomicrobia</td>
<td>12.3</td>
<td>0.6</td>
<td>&lt;0.001*</td>
<td>1.7</td>
<td>0.9</td>
<td>0.562</td>
</tr>
</tbody>
</table>

We further characterized the microbial community by assigning the sequences to genus level and observed 149 genera. In the cream samples 14 genera enumerated more than 1% relative abundance and accounted for 77% of the subsampled sequences assigned to the fluid fraction. In the cream solid samples only 12 genera were observed larger than 1%, however these genera reached only 48.8% of the sequences. In the hay solid and fluid samples, 7 and 6 genera respectively reached at least 1% or larger levels but accounted for only 15.6 and 28.2% of the generated sequences. Therefor the majority of the genera above 1% were found in the cream fed animals. Thus it is not surprising that the significant differences of genera distribution between both feeding groups were associated to the dominance of few taxa in the cream fed animals as listed in table 3. For example the cream fed animals contained larger levels of *Megasphaera*, *Bifidobacterium*, *Succinivibrio*, *Roseburia*, *Acidaminococcus*, *Akkermansia*, *Bacteroides*, *Olsenella* in the fluid phase compared to the hay group. The hay fed animals carried significantly more of *Fibrobacter*, *Ruminococcus*, as well *Selenomonas*. The fluids of both feeding groups shared evenly the genera *Succinlasticium*, *Prevotella*, *Ruminobacter*, *Anaerovibrio*, *Phascolarctobacterium* and *Oscillibacter*. 
Table 3. Variations of the most abundant genera between cream fluid and solid fraction. Differences in relative abundances (in %) were characterized with the Wilcoxon test (P<0.05). Significance was highlighted with an asterisk (*)

<table>
<thead>
<tr>
<th>Genera</th>
<th>Cream fluid $\bar{x}$ in %</th>
<th>Cream solid $\bar{x}$ in %</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Acetivibrio</td>
<td>0</td>
<td>0.5</td>
<td>&lt;0.001*</td>
</tr>
<tr>
<td>Acidaminococcus</td>
<td>1.7</td>
<td>0.1</td>
<td>&lt;0.001*</td>
</tr>
<tr>
<td>Acinetobacter</td>
<td>0</td>
<td>0.6</td>
<td>&lt;0.001*</td>
</tr>
<tr>
<td>Actinomyces</td>
<td>0.2</td>
<td>0.2</td>
<td>0.974</td>
</tr>
<tr>
<td>Akkermansia</td>
<td>1.7</td>
<td>12.3</td>
<td>0.008</td>
</tr>
<tr>
<td>Alistipes</td>
<td>0</td>
<td>3</td>
<td>&lt;0.001*</td>
</tr>
<tr>
<td>Anaerovibrio</td>
<td>1.8</td>
<td>0</td>
<td>0.002*</td>
</tr>
<tr>
<td>Bacteroides</td>
<td>1.5</td>
<td>0.9</td>
<td>0.748</td>
</tr>
<tr>
<td>Barnesiella</td>
<td>0</td>
<td>0.5</td>
<td>&lt;0.001*</td>
</tr>
<tr>
<td>Bifidobacterium</td>
<td>12.4</td>
<td>9.8</td>
<td>0.847</td>
</tr>
<tr>
<td>Blautia</td>
<td>0.8</td>
<td>2</td>
<td>0.088</td>
</tr>
<tr>
<td>Clostridium</td>
<td>0.1</td>
<td>0.8</td>
<td>0.035*</td>
</tr>
<tr>
<td>Coprococcus</td>
<td>0.1</td>
<td>1.4</td>
<td>&lt;0.001*</td>
</tr>
<tr>
<td>Desulfovibrio</td>
<td>0</td>
<td>0.8</td>
<td>&lt;0.001*</td>
</tr>
<tr>
<td>Escherichia/Shigella</td>
<td>0</td>
<td>1.6</td>
<td>&lt;0.001*</td>
</tr>
<tr>
<td>Faecalibacterium</td>
<td>0.8</td>
<td>0.3</td>
<td>0.016*</td>
</tr>
<tr>
<td>Lactobacillus</td>
<td>1</td>
<td>0.3</td>
<td>0.674</td>
</tr>
<tr>
<td>Megasphaera</td>
<td>16</td>
<td>2.3</td>
<td>0.002*</td>
</tr>
<tr>
<td>Methanobrevibacter</td>
<td>0</td>
<td>1</td>
<td>&lt;0.001*</td>
</tr>
<tr>
<td>Mitsuokella</td>
<td>3.4</td>
<td>0.1</td>
<td>0.062</td>
</tr>
<tr>
<td>Olsenella</td>
<td>1.4</td>
<td>2</td>
<td>0.797</td>
</tr>
<tr>
<td>Oscillibacter</td>
<td>0.3</td>
<td>0.6</td>
<td>0.148</td>
</tr>
<tr>
<td>Parabacteroides</td>
<td>0</td>
<td>0.3</td>
<td>&lt;0.001*</td>
</tr>
<tr>
<td>Phascolarctobacterium</td>
<td>1</td>
<td>0.3</td>
<td>0.689</td>
</tr>
<tr>
<td>Prevotella</td>
<td>8.6</td>
<td>0.2</td>
<td>0.001*</td>
</tr>
<tr>
<td>Roseburia</td>
<td>8.7</td>
<td>0.8</td>
<td>0.028*</td>
</tr>
<tr>
<td>Ruminobacter</td>
<td>5.6</td>
<td>0</td>
<td>0.079</td>
</tr>
<tr>
<td>Ruminococcus</td>
<td>0.2</td>
<td>2.5</td>
<td>&lt;0.001*</td>
</tr>
</tbody>
</table>
As in the solid phase the majority of the variation in genera abundance was associated with the significant prevalence of *Akkermansia, Bifidobacterium, Succiniclasticum, Alistipes, Blautia, Escherichia/Shigella* and *Lactobacillus* in cream fed animals in comparison to hay solids. The cream diet negatively affected the occurrence of *Roseburia, Prevotella, Oscillibacter* and *Fibrobacter*.

The variation of genera between the cream fluid and cream solid fraction are of great interest due to the fact that *Methanobrevibacter* was barely found in the fluid but relatively elevated in the solid samples (compared to hay group). Dissimilarities between fluid and solid can potentially indicate the excluding activities in the fluid fraction and promoting factors of the solid fractions for *Methanobrevibacter*. For example *Anaerovibrio, Megasphaera, Prevotella* and *Succinivibrio* occurred in significant larger levels of the cream fluid whereas *Akkermansia, Alistipes* and *Ruminococcus* were prevalent in the solid fraction. No differences were observed for *Bifidobacterium, Blautia* and *Succinicularis* (Full list in table 3). *Blautia* is of special interest since this genus utilizes the hydrogen and CO₂, with acetate as final end product and thus competes with the methanogenesis for the electron donor.

### Enrichment of easy fermentable carbohydrate utilizers in rudimentary rumen

Furthermore the OTU cluster analysis at figure 5 revealed that the major differences between the cream group microbial communities are shown in figure 4 (PCoA) is due to the elevation of few bacterial species (OTUs). We approximated known metabolic function by comparing the observed OTUs with previously cultivated and characterized microbes. Therefor, the representative sequences of the *de novo* picked OTUs were assigned to species level using the fully annotated 16s RNA gene library of Genebank (NCBI). Annotations were only considered when the best hit was uniquely assigned to the Greengenes predicted genera.

The best Genebank hits in the cream group samples are listed together with known metabolic function (based scientific literature) in Table 4. The major OTU (>100 relative reads) annotation based on the short 16S rRNA gene observed are associated to putative

<table>
<thead>
<tr>
<th>Schwerlitzia</th>
<th>0.7</th>
<th>0</th>
<th>0.078</th>
</tr>
</thead>
<tbody>
<tr>
<td>Streptococcus</td>
<td>0</td>
<td>0.6</td>
<td>0.270</td>
</tr>
<tr>
<td>Succiniclasticum</td>
<td>9.2</td>
<td>9.6</td>
<td>0.847</td>
</tr>
<tr>
<td>Succinivirio</td>
<td>5.4</td>
<td>0</td>
<td>&lt;0.001*</td>
</tr>
<tr>
<td>Synergistis</td>
<td>0</td>
<td>0.2</td>
<td>0.002*</td>
</tr>
<tr>
<td>Turicibacter</td>
<td>0</td>
<td>0.6</td>
<td>&lt;0.001*</td>
</tr>
<tr>
<td>Veillonella</td>
<td>0.6</td>
<td>0</td>
<td>0.634</td>
</tr>
</tbody>
</table>
fat, carbohydrates and VFA degraders as well as acetogenic \textit{(Blautia)} and propionic hydrogen utilizers (e.g. \textit{Megasphaera elsdonii}, and \textit{Succiniclasticum} sp.) previously isolated from rumen or hindgut and characterized by cultivation. We have shown the variations in abundance of phyla and genera composition of cream lambs versus hay fed lambs. However it is difficult to speculate about interaction of \textit{Methanobrevibacter} by simple abundance comparison between samples.

Table 4. Genebank species identity of OTUs observed in cream group samples and their metabolic function in rumen and gut environment. The representative sequences of the most prevalent OTUs (with more than 100 relative reads) were picked and blasted. Metabolic substrates and products of the species were associated to the Genebank hits if the species have been previously isolated from a rumen or a gut system.

<table>
<thead>
<tr>
<th>Closest relative species</th>
<th>Sequences length</th>
<th>Query cover in %</th>
<th>E-value</th>
<th>Identity in %</th>
<th>Substrates</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>\textit{Akkermansia muciniphila}</td>
<td>410</td>
<td>100</td>
<td>0</td>
<td>99</td>
<td>Mucin</td>
<td>Derrien \textit{et al.}, 2004; Lukovac \textit{et al.}, 2014</td>
</tr>
<tr>
<td>\textit{Alistipes onderdonkii}/\textit{A. finegoldii}/\textit{A. sahii}</td>
<td>424</td>
<td>100</td>
<td>0</td>
<td>100</td>
<td>Mono and oligosugars</td>
<td>Song \textit{et al.}, 2006</td>
</tr>
<tr>
<td>\textit{Anaerovibrio lipolyticus}</td>
<td>428</td>
<td>100</td>
<td>0</td>
<td>99</td>
<td>Fat</td>
<td>Henderson C, 1971</td>
</tr>
<tr>
<td>\textit{Bacteroides dorei}/\textit{B. uniformis}</td>
<td>424</td>
<td>100</td>
<td>0</td>
<td>100</td>
<td>Mono and oligosugars</td>
<td>Bakir \textit{et al.}, 2006, Song \textit{et al.}, 2006</td>
</tr>
<tr>
<td>\textit{Barnesiella intestinihominis}</td>
<td>424</td>
<td>100</td>
<td>0</td>
<td>99</td>
<td>Mono and disaccharides</td>
<td>Morotomi \textit{et al.}, 2008</td>
</tr>
<tr>
<td>\textit{Bifidobacterium thermophilum}/\textit{Bifidobacterium thermacidophilum}</td>
<td>413</td>
<td>100</td>
<td>0</td>
<td>100</td>
<td>Carbohydrates</td>
<td>Mitsuoka &amp; Choji 1977</td>
</tr>
<tr>
<td>\textit{6)/Bifidobacterium longum}</td>
<td>409</td>
<td>100</td>
<td>0</td>
<td>100</td>
<td>Carbohydrates</td>
<td>Sela \textit{et al.}, 2008</td>
</tr>
<tr>
<td>\textit{Blautia glucerasea}</td>
<td>404</td>
<td>100</td>
<td>0</td>
<td>99</td>
<td>CO2 and hydrogen</td>
<td>Park \textit{et al.}, 2013</td>
</tr>
<tr>
<td>\textit{Blautia faecis}/\textit{Blautia schinkii}</td>
<td>404</td>
<td>100</td>
<td>0</td>
<td>98</td>
<td>CO2 and hydrogen</td>
<td>Park \textit{et al.}, 2013</td>
</tr>
<tr>
<td>\textit{Blautia}</td>
<td>404</td>
<td>99</td>
<td>0</td>
<td>97</td>
<td>CO2 and</td>
<td>Park \textit{et al.}, 2013</td>
</tr>
<tr>
<td>Organism</td>
<td>Sugar Type</td>
<td>Percentage</td>
<td>pH Value</td>
<td>Remarks</td>
<td></td>
<td></td>
</tr>
<tr>
<td>----------</td>
<td>------------</td>
<td>------------</td>
<td>----------</td>
<td>---------</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Clostridium disporicum</strong></td>
<td>Sugars</td>
<td>100</td>
<td>0</td>
<td>Horn N, 1987</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Coprococcus comes</strong></td>
<td>-</td>
<td>98</td>
<td>-</td>
<td>-</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Coprococcus eutactus</strong></td>
<td>-</td>
<td>95</td>
<td>-</td>
<td>-</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Desulfovibrio piger</strong></td>
<td>VFA, ethanol and hydrogen</td>
<td>99</td>
<td>-</td>
<td>Loubinoux et al, 2002</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Faecalibacterium prausnitzii</strong></td>
<td>Sugars</td>
<td>99</td>
<td>-</td>
<td>-</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Lactobacillus taiwanensis/Lactobacillus johnsonii</strong></td>
<td>Mono and oligo sugars</td>
<td>100</td>
<td>-</td>
<td>Wang et al, 2009</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Lactobacillus reuteri/Lactobacillus vaginalis</strong></td>
<td>-</td>
<td>100</td>
<td>-</td>
<td>-</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Megasphaera elsdenii</strong></td>
<td>Lactate</td>
<td>100</td>
<td>-</td>
<td>Kung &amp; Hession, 1995</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Methanobrevibacter sp. thaueri, oralis, archaean, gottschalki</strong></td>
<td>CO2 and hydrogen</td>
<td>99</td>
<td>-</td>
<td>Miller &amp; Lin, 2002</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Mitsuokella jalaludinii</strong></td>
<td>Glucose</td>
<td>100</td>
<td>-</td>
<td>Lan et al, 2002</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Olsenella profusa</strong></td>
<td>Mono and oligo sugars</td>
<td>96</td>
<td>-</td>
<td>Kraatz et al, 2011</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Olsenella umbonata</strong></td>
<td>Fructose, mucin</td>
<td>100</td>
<td>-</td>
<td>Kraatz et al, 2011</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Oscillibacter ruminantium</strong></td>
<td>Sugars and VFAs</td>
<td>94</td>
<td>-</td>
<td>Lee et al, 2013</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Parabacteroides merdae</strong></td>
<td>Sugars</td>
<td>100</td>
<td>-</td>
<td>Sakamoto et al, 2006</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Phascolarctobacterium succinatutens</strong></td>
<td>Succinate</td>
<td>97</td>
<td>-</td>
<td>Watanabe et al, 2013</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Prevotella ruminicola</strong></td>
<td>Arbutin, oligo sugars</td>
<td>100</td>
<td>-</td>
<td>Avgušt et al, 1997</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Butyrivibrio fibrisolvens/Rose buria hominis</strong></td>
<td>Starch</td>
<td>98</td>
<td>-</td>
<td>Klieve et al, 2003</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Ruminobacter amylophilus</strong></td>
<td>Starch</td>
<td>97</td>
<td>-</td>
<td>Anderson KL, 1995</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
The co-occurrence between OTUs can help to predict interactions or describe ecological niches of microbes, characterize their connectivity and identify putative key taxa with the larger impact on the community. Therefore we created the co-presence OTU network of solid fraction of the cream group samples since this fraction contained higher frequent levels of Methanobrevibacter compared to the solid fraction of the control (Hay group).

**Figure 5.** Individual community profile. The hierarchical cluster in the dendrograms of the heatmap was generated by the Euclidean distance to compare the animal samples (x-axis) with the most abundant OTUs (y-axis).
Cream group Methanobrevibacter displays negative and positive co-relation to other bacterial species

The microbial interactions of figure 6 are based on 58 OTUs (nodes) connected by 248 total edges (co-occurrences) of which 43.5% were negative and 56.5% were positive correlated. The OTUs of the generated network belonged to the 7 phyla of Actinobacteria, Bacteroidetes, Euryarchaeota, Firmicutes, Proteobacteria, Synergistetes and Verrucomicrobia. Interactions are displayed after applying the unweighted force-directed algorithm. The force directed layout has no effects on the conditions of the co-occurrences but minimizes graphically the overlap of edges. Three clusters (I-III) were found, of which each cluster is based mainly on positive correlation whereas the co-occurrences between clusters are of negative nature. Most interesting here are the hypothetical relations of Methanobrevibacter sp. found in cluster III, co-occurring with two OTUs assigned to Lachnospiraceae and Ruminococcaceae (III) but negatively correlated to the three OTUs of Bacteroides and Roseburia in cluster I (Figure 6).

Theoretically, Methanobrevibacter is indirectly also connected to the other 53 bacterial species observed in figure 6 with potential positive or negative effects on methanogenesis. To estimate which bacteria have the largest impact on the microbial network in the solid fractions we evaluate the impact of each microbial species (OTU) on the total interactions by generating the betweenness centrality. Each cluster
Figure 6 Pairwise co-occurrence network. Shown are the correlations found in the cream group solid fractions (n=11). Correlations are predicted on the log-transformed even OTU table using Spearman with the minimal cutoff ± 0.6 and Bonferroni corrected p-values (P<0.05). Co-occurrences were only considered when observed in at least 72% of the samples (n=8). The central network shows all interactions after applying the unweighted force-directed algorithm (Cytoscape). Three clusters were observed of which each contains a central OTU (identified with the highest betweenness centrality). Correlations of the OTUs assigned to the tentative acetogenic Blautia genus were found in cluster I and II whereas most exclusion were associated to OTUs of cluster III. Positive occurrences of the methanogenic Methanobrevibacter were only observed in cluster III and direct negative edges were revealed with OTUs of cluster I.

contained one central bacterial species associated to most negative connections of the counteracting cluster. The OTUs with the highest betweenness centrality of cluster I-III were taxonomically assigned to Bifidobacterium, Akkermansia (both indirectly co-exclusive with Methanobrevibacter) and Ruminococcaceae respectively (indirectly positive co-occurring with Methanobrevibacter). Microbial species assigned to the potential acetogenic genus of Blautia were found only in cluster I and II but not in III.
Diet is key driver of microbial composition of cream group – no correlation with pH and rumen weight

The observed interactions are most likely diet driven. In order to investigate the possible host factor impact on the microbial community composition we evaluated co-correlation of the rumen wall weight, as well as the pH of the rumen content against all genera of the solid and fluid fraction. We observed no correlations of Methanobrevibacter or other assigned genera in the cream group to the listed host factors (Table S1 & S2), indicating that diet is the key driver of the microbial community of the cream group animals. Hay group correlations were not listed since most OTUs were not assigned to genus level.

Discussion

In our previous report we found when lambs fed with cream (fat, milk, protein) from day of birth until 180 days, the animals emitted 84% less methane than the conventional diet group (hay). Despite that, the cream group animals were healthy and even gained faster weight than hay fed animals, the papillae of the rumen wall was little under developed. We observed that there were close to no methanogens (OTUs assigned to the family of Methanobacteriaceae) in the ruminal fluid fraction of the cream group animals, but larger level of a single species of Methanobrevibacter in the ruminal solid fraction compared to the hay group. The aim of this manuscript was to estimate potential interaction of Methanobrevibacter with the total microbial community and characterize the total microbial community.

The rumen content is composed of the two phases of fluid and solid, essential for the rumen development and full fermentation (VI-Baldwin et al, 2004). The solid phase is the major fermentation anchor, while fluid transports metabolites for major resorption further to omasum and abomasum. In adult ruminant the ingested solid plant material holds longer in the reticulorumen where the fermentation takes place and the rumen development is stimulated. The ruminal fluid is a mix of water digestive enzymes, metabolites and moves faster forward to the omasum and abomasum as the solid fraction. In this study we found that the ruminal fluid and solid fraction varied significantly in microbial composition in both, cream and hay fed animals. Both fractions of the hay group were almost equally diverse, whereas the ruminal fluid of the cream group was significantly less diverse (Shannon) than the solid phase.

We suggest that diet was the primary selection factor of the microbial community composition since there were no detected correlations of the rumen microbes towards pH or rumen weight in the cream group samples.
In adult sheep during ruminal fermentation, carbohydrates are fermented producing volatile fatty acids mainly acetate, propionate and butyrate and form hydrogen (Heinrichs, et al, 2005). Cellulose, hemicellulose and pectins are fermented by the fibrolytic bacteria. Due to the fat, milk and starch nature of the cream diet we only observed few potential fiber degraders such as Ruminococcus albus. The common cellulose degrader Fibrobacter however was only observed in the hay group samples. Most of the hay OTUs were not assigned to any known genera, whereas the cream diet was highly selective for close relatives of previously isolated and characterized species. The cream group fluid, with excluding conditions for methanogens was enriched for fat (Anaerovibrio sp.), starch (Succinivibrio sp.) and protein degraders (Prevotella sp.) as well as propionate producers (Megasphaera sp.) (Table 4).

Both cream fractions contained evenly large levels of the sugar fermenting Bifidobacterium sp. (Biavati & Mattarelli, 1991) compared to the hay group. Interestingly, the cream diet favored the abundance of the tentative propionate producing Succiniclasticum sp. and acetate enriching Blautia sp. The observation is supported by the large levels of Megasphaera in the cream fluid (The fraction were no methanogens were observed). Megasphaera is a known propionate producer and tested probiotic strain for methane reduction in ruminants (Aikman et al, 2011). Our result suggests alternative hydrogen utilization of cream group animals because both, propionogenesis and acetogenesis, are competitive pathways to the methanogenesis, (Ungerfeld EM, 2013, Carrillo-Reyes et al, 2014). The shift to propionate production has been previously reported in ruminants for changes in diet composition from fiber diet to starch feed (Martin, 2010). This hypothesis is additionally supported by fact that Blautia is significantly enriched in the cream group compared to the hay group in both ruminal fractions. Also, in the microbial co-occurrence network, Blautia positively correlates with several carbohydrate and organic acid degraders that produce hydrogen and putatively could be utilized by Blautia sp. (Figure 6. Cluster I and II).

However this does not explain the enriched relative levels of the single species of Methanobrevibacter in the cream group solid fraction, despite the large reduction in methanogenesis. The pairwise relationship of the cream solid fraction revealed OTUs in positive co-presence of Methanobrevibacter (Figure 6, Cluster III). Unfortunately these OTUs were not further taxonomically assigned than family level (Ruminococcaceae and Lachnospiraceae). Thus it is difficult to speculate about potential metabolic activities that
favored the enriched frequency of *Methanobrevibacter*. However the microbial network analysis revealed that, *Bacteroidetes* and *Roseburia* directly negatively correlated with *Methanobrevibacter*, *Akkermansia* and especially *Bifidobacterium* indirectly negatively affected the methanogen occurrence in favor of the tentative acetogenic *Blautia* sp (Figure 6, Cluster I and II).

Additionally, several previous observations found that the 16S rRNA gene abundance does not correlate to the methane emission (Kong *et al*, 2010, Zhou *et al*, 2009). Therefor single enriched *Methanobrevibacter* sp. could simply emit lesser methane, while the major methane producers are inhibited by the cream diet. This is in agreement with a previous report showing that diet reduced the diversity of *Methanobrevibacter* on species level, with a significant decline in methane emission (Danielsson *et al*, 2012).

Curiously despite the substantial reduction of methane we reported no apparent health effects of the host (Haque *et al*, 2014) in the cream group. One scenario could have been the development of subacute ruminal acidosis (SARA) (Jouany *et al*, 2006), the result of enrichment of VFAs such as lactate when easy fermentable carbohydrates are fed to the herbivore and the subsequent drop in pH. But we observed no variation in pH between the two feeding groups as reported in our previous study (Najmul *et al*, 2014). Interestingly is the prevalence of *Akkermansia*. The mucin-degrading bacterium *Akkermansia* has only recently been reported in rumen samples of cattle (Dowds *et al*, 2008). It has also been found that *Akkermansia municipals* growth is *in vitro* not supported by substrates such as maltose, rhamnose, cellbiose and the key rumen fermentation metabolites of acetate, fumarate, succinate and butyrate, this is supported by the few co-correlation or co-exclusion of *Akkermansia* with Cluster I and III. The large levels of *Akkermansia* could indicate enriched mucus production in the reticulo-rumen of the cream group. We can only speculate how this would affect the lamb health during the maturation over longer period. In an additional set of animals (rumen samples were not collected) we fed lambs as in the cream group animals, but changed the diet after 180 days to hay (Haque *et al*, 2014). The diet alternation increased the methane emission within a week to almost the same level as animals fed with hay during the entire experimental period. Therefore it appears that the rumen microbiota could be converted back to normal. In future studies it needs to be investigated in what timeframe and if microbial community will reach the same diversity when switched back from cream to hay feed.

Controversy. The cream-milk-maize replacer leads to a substantial reduction in methane emission without obvious health effects of the host for the period of experimentation. However, this treatment is commercial costly and thus unlikely applied in large scale. Nonetheless, our study highlights possible combination of synbiotics consortia, in which
the growth of, *Bifidobacterium*, *Megasphaera* and *Succiniclasticum* are stimulated. *Megasphaera* has already been tested as probiotic, with a decreased methane emission (Aikman et al, 2011, Klieve et al, 2003). The development of symbiotic consortia with living *Megasphaera*, *Succinivibrio* in combination with other microbes such as *Bifidobacterium* and *Succiniclasticum* and propionogenesis favoring diet conditions could potentially substantially lower the methane emission in livestock ruminants.

Summary and conclusion

We have shown here for the first time, the reticulated microbiota of an undeveloped rumen system without health effect of the lamb responsible for 87% methane depletion. Furthermore we have shown here that the ruminal fraction of cream fed animals differed largely from hay treated animals. Our observation revealed that diet was the major driving force selecting the microbial community composition with minor impact of the host conditions. We also found potential *Methanobrevibacter* excluding microbial species (for example *Bifidobacterium*) together with known anti-correlating (*Megasphaera*) microbes that need further experimental evaluation using co-occurrence networks.

References


**Supplementary tables**
Table S1. Co-correlation of most abundant genera of cream group fluid fraction with rumen weight and pH, evaluated with Spearman.

<table>
<thead>
<tr>
<th>Genera</th>
<th>Weight of reticulorumen Rho; p-value</th>
<th>pH Rho; p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Acidaminococcus</td>
<td>0.209; 0.54</td>
<td>-0.082; 0.82</td>
</tr>
<tr>
<td>Akkermansia</td>
<td>-0.009; 0.98</td>
<td>0.377; 0.25</td>
</tr>
<tr>
<td>Anaerovibrio</td>
<td>-0.474; 0.14</td>
<td>-0.169; 0.62</td>
</tr>
<tr>
<td>Bacteroides</td>
<td>0.255; 0.45</td>
<td>-0.155; 0.65</td>
</tr>
<tr>
<td>Bifidobacterium</td>
<td>0.573; 0.07</td>
<td>-0.373; 0.26</td>
</tr>
<tr>
<td>Megasphaera</td>
<td>0.591; 0.06</td>
<td>0.064; 0.86</td>
</tr>
<tr>
<td>Phascolarctobacterium</td>
<td>-0.067; 0.85</td>
<td>-0.505; 0.11</td>
</tr>
<tr>
<td>Prevotella</td>
<td>-0.418; 0.2</td>
<td>0.118; 0.73</td>
</tr>
<tr>
<td>Roseburia</td>
<td>0.336; 0.31</td>
<td>-0.255; 0.45</td>
</tr>
<tr>
<td>Ruminobacter</td>
<td>-0.362; 0.27</td>
<td>0.337; 0.31</td>
</tr>
<tr>
<td>Ruminococcus</td>
<td>-0.433; 0.18</td>
<td>0.358; 0.28</td>
</tr>
<tr>
<td>Succiniclasticum</td>
<td>-0.336; 0.31</td>
<td>0.445; 0.17</td>
</tr>
<tr>
<td>Succinivibrio</td>
<td>0.218; 0.52</td>
<td>-0.245; 0.47</td>
</tr>
</tbody>
</table>

Table 2. Co-correlation of most abundant genera of cream group solid fraction with rumen weight and pH, evaluated with Spearman.

<table>
<thead>
<tr>
<th>Genera</th>
<th>Weight of reticulorumen Rho; p-value</th>
<th>pH Rho; p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Akkermansia</td>
<td>-0.255; 0.45</td>
<td>-0.036; 0.92</td>
</tr>
<tr>
<td>Alistipes</td>
<td>-0.018; 0.97</td>
<td>-0.055; 0.88</td>
</tr>
<tr>
<td>Bifidobacterium</td>
<td>0.391; 0.24</td>
<td>-0.6; 0.06</td>
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<tr>
<td>Blautia</td>
<td>-0.564; 0.08</td>
<td>0.136; 0.69</td>
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<tr>
<td>Coprococcus</td>
<td>-0.4; 0.23</td>
<td>0.236; 0.49</td>
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<tr>
<td>Escherichia/Shigella</td>
<td>-0.073; 0.84</td>
<td>-0.045; 0.9</td>
</tr>
<tr>
<td>Lactobacillus</td>
<td>-0.029; 0.93</td>
<td>0.296; 0.38</td>
</tr>
<tr>
<td>Megasphaera</td>
<td>-0.46; 0.15</td>
<td>-0.173; 0.61</td>
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<tr>
<td>Methanobrevibacter</td>
<td>0.373; 0.26</td>
<td>-0.291; 0.39</td>
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<tr>
<td>Olsenella</td>
<td>-0.173; 0.61</td>
<td>-0.155; 0.65</td>
</tr>
<tr>
<td>Oscillibacter</td>
<td>0.2; 0.56</td>
<td>-0.018; 0.97</td>
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<tr>
<td>Prevotella</td>
<td>-0.101; 0.77</td>
<td>0.532; 0.09</td>
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<tr>
<td>Roseburia</td>
<td>0.091; 0.8</td>
<td>-0.109; 0.75</td>
</tr>
<tr>
<td>Ruminococcus</td>
<td>0.291; 0.39</td>
<td>-0.036; 0.92</td>
</tr>
<tr>
<td>Succiniclasticum</td>
<td>0.1; 0.78</td>
<td>-0.036; 0.92</td>
</tr>
</tbody>
</table>
Manuscript 5

The microbiome of carrionovore
The microbiome of a carrionovore

Michael Roggenbuck¹, Ida Bærholm Schnell Dresen²,³, Nikolaj Blom⁴,⁵, Jacob Bælum⁴, Mads Frost Bertelsen³, Thomas Sicheritz Pontén⁴, Søren Johannes Sørensen¹, M Thomas P Gilbert², Gary R. Graves⁶,⁷, Lars H. Hansen⁸*

¹ Department of Biology, Section of Microbiology, University of Copenhagen, 2200 Copenhagen, Denmark
² Center for GeoGenetics, Natural History Museum of Denmark, University of 2200 Copenhagen, Denmark
³ Center for Zoo and Wild Animal Health, Copenhagen Zoo, 2000 Frederiksberg, Denmark
⁴ Center Biological Sequence Analysis, Technical University of Denmark, 2800 Kgs. Lyngby, Denmark
⁵ Center for Biosustainability, Technical University of Denmark, 2970 Hørsholm, Denmark
⁶ Department of Vertebrate Zoology, National Museum of Natural History, Smithsonian Institution, 20013-7012 Washington, DC, USA
⁷ Center for Macroecology, Evolution and Climate, University of Copenhagen, 2200 Copenhagen, Denmark
⁸ Department of Environmental Science, Aarhus University, 4000 Roskilde, Denmark
*Corresponding Author: gravesg@si.edu; lhha@dmu.dk

Vultures are carrionvores that fill a key ecosystem niche in which they have evolved a remarkable tolerance to bacterial toxins in decaying meat. Here we report the first deep metagenomic analysis of the vulture microbiome. Through face and gut comparisons of 50 vultures representing two species, we demonstrate a remarkably conserved low diversity of gut microbial flora. The gut samples contained an average of 281 operational taxonomic units (OTUs) per specimen, compared to 1149 OTUs on the facial skin. Clostridia and Fusobacteria, widely pathogenic to other vertebrates, dominated the gut microbiota. We reveal a likely fecal-oral-gut route for their origin. DNA of prey species detectable on facial swabs was completely degraded in the gut samples from most vultures, suggesting that the gastro-intestinal tracts of vultures are extremely selective. Our findings show a strong adaption of vultures and their bacteria to their food source, exemplifying a specialized host-microbial alliance.

Introduction

The microbiota of vertebrates rapidly begin to decompose their hosts after death¹ During the subsequent breakdown of tissue, these microorganisms excrete toxic metabolites, rapidly rendering
the carcass a hazardous food source for most carnivorous and omnivorous animals. Some scavengers such as vultures, however, are able to consume carrion without suffering any apparent ill-effects. Indeed these carrionivores may have to wait for decay to occur before they are able to scavenge carcasses with tough skins, thus substantially increasing the risk of exposure to toxins. Furthermore, to obtain access to the inside of such carcasses, they often insert their heads directly in the body cavities of decaying prey, thereby exposing their head and neck to pathogenic bacteria. Although the majority of the ingested bacteria will not survive the acidic gastric passage prior to hindgut colonization, toxins such as botulinum survive the passage into the hindgut, possibly seriously compromising the health of the consumer. Furthermore the pioneering feeding experiments of Houston & Cooper revealed that spore forming pathogens such as Bacillus anthracis can be recovered from vulture feces.

To investigate how vultures are able to tolerate this challenging dietary niche, we characterized the functional microbiome of the facial skin and the hindgut (large intestine) of the two most widespread species of vultures in the New World (Coragyps atratus [black vulture] \( n = 26 \); Cathartes aura [turkey vulture] \( n = 24 \); both collected in Nashville US). Here we show that the acidic gastrointestinal tract of vultures is a strong filter of the microbiota ingested from decaying carcasses resulting in a significantly less diverse hindgut flora dominated by Clostridia and Fusobacteria which are pathogenic to most vertebrates.

**Results**

*Facial DNA reveals prey composition*

We initially investigated whether an individual’s diet, as assayed from facial swabs, influenced the bacterial composition of the vulture’s face and hindgut. This was done via PCR amplification and deep sequencing of a mammalian-generic mitochondrial 16S rRNA gene. Amplification of potentially contaminating human DNA was reduced by the addition of blocker oligos during PCR. Although these primers have been successfully used in other dietary DNA studies, we detected mammalian amplicons in only four (8 %) of the vulture hindgut samples (all turkey vultures). In contrast, mammalian DNA was readily amplified from most facial swabs (~90%), representing nine mammalian families (Bovidae, Canidae, Cervidae, Didelphidae, Equidae, Leporidae, Mephitidae, Procyonidae, Suidae). The majority of mammalian families were observed in both vulture species (Supplementary table 1 = ST1). Despite use of human-specific blocking primers, human DNA was recovered from one turkey vulture facial swab and hindgut sample, most likely a laboratory contaminant but possibly from ingested sewage. The difference in mammalian DNA on facial swabs and from the hindgut reflects the significant breakdown of dietary DNA in the vulture gastrointestinal tract pointing to extraordinarily harsh chemical conditions that may be an adaptation to consumption of toxic carrion. Therefore the microbial flora colonizing the vulture gastro-intestinal tract has adapted to survive these harsh conditions.

To investigate the carrionvore microbiota, we PCR amplified and deep sequenced a microbial ribosomal phylogenetic marker from each DNA extraction.
The microbial communities of both vulture species display similar patterns

A clear distinction was observed between the microbial communities (Figure 1.I) of the facial skin (black vulture, n=26, 5306-10427 reads/animal, $\bar{x}=7764 \pm 1414$; turkey vulture, n=24, 4244-10320 reads/animal, $\bar{x}=7229 \pm SD=1826$) and hindgut samples (black vulture, n=26, 6096-12700 reads/animal, $\bar{x}=9319 \pm 1745$; turkey vulture, n=23, 4518-13220 reads/animal, $\bar{x}=8405 \pm 2449$). The facial microbial community was significantly more diverse than that of the hindgut (Figure 1.II), inconsistent with previous observations from other vertebrates such as humans\(^9\). The skin and hindgut microbiota of turkey and black vultures largely overlapped, consistent with the observation that both species routinely feed on the same prey species and often at the same carcasses (Figure 1.I).
Figure 1. Facial skin and hindgut exhibit largely different microbial communities.

(I) Microbial clustering based on Bray-Curtis dissimilarity matrix (visualized by principal coordinate analysis). Facial skin and hindgut communities exhibited minor overlap (ANOSIM; $R=0.744$, $P=0.001$). Hindgut (ANOSIM, $R=0.333$, $P=0.001$) and skin (ANOSIM, $R=0.321$, $P=0.001$) communities showed minor clustering within vulture species.

(II) Variation in diversity (Shannon index) between facial skin and hindgut. The hindgut community was significantly less diverse than the facial community (Wilcoxon Rank Sum test, $P<0.001$). The facial communities of black vultures were more diverse than those of the turkey vulture whereas the hindgut samples displayed the opposite pattern (Wilcoxon-Rank-Sum test, $P<0.01$). There were no differences between male and female vultures in clustering (not shown) of hindgut (ANOSIM, $R=0.063$, $P>0.05$) or facial (ANOSIM, $R=0.025$, $P>0.05$) communities.

**Clostrida and Fusobacteria prevail in the vulture gut**

Given the challenge of penetrating the hide of large mammals, vultures often enter large carcasses through natural orifices, in particular the anus. This increases the likelihood of ingesting anaerobic fecal bacteria such as Clostridia and Fusobacteria. Indeed, both bacterial groups were detected at high frequencies in the gut of both vulture species. The percentage of Clostridia sequence counts relative to total counts within the guts of individuals was largely similar in black vultures (min=26% & max=85%, $\bar{x}=50\%\pm 18$) and turkey vulture (min=26% & max=84%, $\bar{x}=55\%\pm 17$; Wilcoxon $P>0.05$). In contrast, percentages of Fusobacteria were significantly lower in the guts of black vultures (min=0.2% & max=54%, $\bar{x}=21\%\pm 16$) than in turkey vultures (min=2% & max=69%, $\bar{x}=31\%\pm 16$; Wilcoxon, $P<0.05$).

Percentages of Clostridia on facial skin swabs was significantly lower in black vultures (min=7% & max=40%, $\bar{x}=24\%\pm 10$) than in turkey vultures (min=8% & max=68%, $\bar{x}=26\%\pm 16$; Wilcoxon, $P<0.001$). Percentages of Fusobacteria were similar on the facial skin of black vultures (0% & max=31%, $\bar{x}=6\%\pm 9$) and turkey vultures (min=0.2% & max=23%, $\bar{x}=3\%\pm 5$, Wilcoxon, $P>0.05$) as shown in Figure 2.I. Although no comparable studies of facial skin microbiomes are available for comparison, these observations are unusual with regards to available vertebrate facial microbiomes, such as those of human (predominantly Actinobacteria), frogs (predominately Actinobacteria and Betaproteobacteria), and salamander (predominately Betaproteobacteria). Fusobacteria have not been reported on human skin. Clostridia on the other hand have been found to colonize parts of the human skin with lowered oxygen availability, for example the buttocks, but not the face.
I. Carnivore
II. Herbivore

Omnivore
Carrionovore

0.05

Turkey Vulture (hindgut)
Turkey Vulture (skin)
Black Vulture (hindgut)
Black Vulture (skin)
Figure 2. Hindgut microbial flora of wild and zoo vultures is highly conserved.
(1) Mean bacterial class distribution found on facial skin and in the hindgut of black and turkey vultures (see details in supplementary note=SN1/SN2). (II) Comparison of microbial communities of zoo bird feces and vulture hindguts (UPGMA tree based on Unifrac metric). The hawk, owl and vultures in the zoo received fresh meat. *Clades collapsed.

Oral-fecal route of gut bacteria

The recovery of bacteria known to be linked to carcass degradation from vulture facial swabs renders it likely that at least part of the vulture facial microbiome, particularly Clostridia and Fusobacteria, are not necessarily skin colonizers, but originate from physical contact with food sources or soil clinging to the carcasses. Analysis of facial skin swabs revealed 22200 OTUs probably equivalent to bacterial species (black vulture, n= 26; 824-3179 OTUs/vulture; \( \bar{x} = 1464 \pm 553 \); turkey vulture, n= 24; 420-1537 OTUs/vulture; \( \bar{x} = 809 \pm 276 \)). Facial skin swabs contained significantly more microbes than hindgut samples (Welch’s \( t \)-test, \( P < 0.001 \)). We detected 3008 OTUs in hindgut samples (black vulture, n= 26; 164-281 OTUs/vulture; \( \bar{x} = 210 \pm 34 \); turkey vulture n= 23; 221-748 OTUs/vulture; \( \bar{x} = 362 \pm 113 \)). Given that > 85% of the microbial OTUs recovered at even sequencing depth (at 97% sequence similarity) were unique to the face, there appears to be strong selection during the passage from the mouth to the gut. About half of the OTUs found in the hindgut occurred exclusively in the hindgut. However, OTUs detected on facial skin and in the hindgut accounted for 98% of all gut sequences, including all bacterial specimen assigned to Clostridia and Fusobacteria. This provides strong evidence that the majority of hindgut microbes have the same origin as facial bacteria. Furthermore, the average number of hindgut species for each vulture that were unique to that individual was only 10% (black vulture, \( \bar{x} = 8.3\% \pm 6.3 \), 3.5-36.7%; turkey vulture, \( \bar{x} = 12.6\% \pm 7.4 \), 1.5-26.6%; Welch’s \( t \)-test \( P<0.001 \)) (ST3, re-clustered at 96% sequence similarity for comparison with previous study\(^ {14} \). Thus approximately 90% of the vulture gut bacteria are shared between individuals. In comparison, the average unique OTU composition of the mammalian gut has been shown to be 56%\(^ {14} \). This observation demonstrates a stronger selection for the observed microbial community of vultures compared to mammals.

Gut microbes are highly conserved between captive bred and wild vultures

To explore which components of the hindgut microbiome are unique to vultures, we generated additional avian metagenomic data using fecal samples obtained from several other species obtained from a single location (Copenhagen Zoo). Samples included two captive-bred turkey vultures, three predatory species (red tailed hawk [Buteo jamaicensis], African spotted owl [Bubo africanus], and red-legged seriema [Cariama cristata]), a non-predatory Amazon parrot (Amazona sp.), and the American flamingo (Phoenicopterus ruber). Recent studies of human gut dynamics
have demonstrated that the gut microbiome can rapidly adjust to dietary modifications\textsuperscript{15}. In addition, the gut microbiota of alligators has been observed to vary seasonally with diet \textsuperscript{16}. It is notable that despite having similar diets in the zoo, the fecal microbiome of the zoo-kept turkey vultures was considerably different from those of the hawk and owl (Figure 2.II, ST4 &ST5), but remarkably similar to the hindgut microbiome of the wild sampled vultures. This indicates that phylogenetic differences in the digestive physiology of vultures override the importance of diet in the assembly of the hindgut microbiota.
Discussion

Both, Clostridia and Fusobacteria are common soil bacteria. However Clostridia species have been documented as the cause of severe food poisoning in both humans, and chickens and are responsible for periodic die-off’s of wild birds such as waterfowl and shorebirds\(^{17-21}\). Although the flesh-degrading Fusobacteria have been reported to colonize the hindgut of living omnivorous and carnivorous animals, in humans they do so at negligible abundances and have recently been shown to promote colon cancer (<1%)\(^{14, 22, 23}\). Alligators, which all scavenge carrion, exhibit similar frequencies of Clostridia and Fusobacteria as those observed in vultures, but vary over season\(^{16}\). However hyenas, mammalian carrion feeders, did not contain notable levels of Fusobacteria\(^{14}\).

Thus, even though the vulture hindgut microbiota originated from the diet, we speculated that its composition is primarily shaped by the oral-gut passage and the hindgut properties. The frequency of Clostridia and Fusobacteria (Figure 3.I.) in the hindgut raises the question of whether these bacteria simply outcompete other bacterial groups or if their presence is actually promoted for the physiological benefit of the vultures. The former option suggests that vultures are passive hosts that tolerate the bacteria and their toxins without receiving benefit, whereas the latter posits that the relationship is more mutualistic in that bacteria receive a predictable flow of protein rich food in an anaerobic environment and the vultures obtain nutrients provided by bacterial degradation of carrion. Indeed we observed genes that encode tissue-degrading enzymes and toxins associated with *Clostridium perfringens* in the metagenome of the turkey vulture hindgut with shotgun sequencing (ST6).

Curiously, more than 90% of all microbial interactions in the hindguts of both vulture species (Figure 3.II&III) were of a positive nature. However, all negative co-occurrence interactions were assigned to either Clostridia and/or Fusobacteria, suggesting their competitive nature. Therefore, the most likely scenario is that Clostridia and Fusobacteria outcompete other bacterial groups in the anaerobic hindgut, and that vultures benefit from the bacterial breakdown of carrion while tolerating bacterial toxins. Some scavenging birds are known to harbor antibodies against toxins such as botulinum\(^{24}\) and we speculate that vultures are unusually tolerant of toxins.

In summary, our findings demonstrate that wild vultures host a similar but very unique gut microbiome that is dominated by two major groups of bacteria that likely originate from their food.
To determine essential functions of vulture homeostasis contributed by the gut microbiota, germ-free vultures will need to be experimentally exposed to the microbes observed in this study, using mammalian investigations as a model\textsuperscript{25}.

**Methods**

*The sampling of the wild vultures.* *Coragyps atratus* (Black vultures) were live-trapped at carcasses of road-killed deer at the same location over a period of several days. They were transported to a central facility within several hours of trapping and were then euthanized with CO\textsubscript{2} by USDA (United States Department of Agriculture) personnel under authority of the US Fish & Wildlife Service. Vultures were necropsied and sampled within 30-45 minutes of death. Investigators were gloved, masked, and gowned during the necropsies. *Cathartes aura* (Turkey vulture) were shot at roosts, bagged individually, and quickly chilled to 2-4 °C. Vultures were then transported to the processing facility where they were refrigerated from 2-6 hours before necropsy and sampling. Briefly, the hindgut (large intestine) samples were collected in a relatively sterile manner in a sheltered warehouse. Carcasses were opened ventrally from throat to vent to expose the entire gastrointestinal tract. We sampled sequentially from anterior to posterior. For all hindgut sampling, we isolated a 3-4 cm section of the large intestine about 2-3 cm above the cloaca with medical hemostats. A new sterile syringe was used for each sample. We injected 2-3 ml of sterile water through the wall of the hindgut with a sterile syringe (discarded after each aspiration), gently massaged the hemostat-blocked section of the hindgut while the needle was still inserted, and then aspirated the wash liquid with the syringe. The aspirant was injected directly into a cryotube containing RNAlater. Facial skin samples were taken with sterile polyester swabs and collected in RNAlater.

*Diet of captive bred birds.* The hawk and owl were fed with whole mice and day old chicks. The turkey vultures received whole mice, chicks, rats, rabbits, and guinea pigs, and horse and goat meat. Furthermore the flamingo diet was composed of commercial pellets containing maize, wheat, and fishmeal manufactured by Kasper Faunafood, the Netherlands and Nutrazu, UK respectively. The parrot was fed on seeds fruits and vegetables. Finally, the seriema received a mix of day-old chicks, mice, mealworms, commercial dog food, commercial dried insect-mix, boiled eggs and a mix of vegetables. The fecal samples were sterile sampled and stored at -80°C prior to DNA extraction.

*Mammalian DNA survey.* The DNA was extracted from facial swabs and hindgut aspirates with the FastDNA Spin kit for Soil (MP Biomedicals). 16S mammalian mtDNA was PCR amplified using mammalian primer-set 16S mam1 (CGGTTGGGTTGACCTCGGA) and 16S mam2 (GCTGTTATCCCTAGGGTAACT), and a blocking oligo 16S mam_blkhum2 (GCCACCTCGGAGCAAGACCC)\textsuperscript{26}. Both 16S mam1 and 16S mam2 were 5’-labelled with unique 8-nucleoc tags (with at least 2 differences between tags). All samples were amplified in duplicate with different tag-combinations. This primer set is generic to mammals, excluding humans and related primates, and was specifically chosen to limit the chance of recovering a human signal that
may derive from handling of the vultures during collection and DNA extraction. PCR was performed using the enzyme Amplitaq Gold (Applied Biosystems, Foster City, CA) in 25 µl volumes that contained 1 mM MgCl₂, 1x buffer, 0.4 µM each primer, 0.1 mM mixed dntp, 0.1 µl Taq Gold, and 1 µl purified DNA. In addition, 4.0 µM of a human blocking probe was added to each reaction to ensure as low an amplification of human DNA as possible. PC conditions were as follows: 95°C for 5 min enzyme activation followed by 40 cycles of 95°C for 12 s, 59°C for 30 s, and 70°C for 25, and 1 cycle for final extension of 7 min at 70°C. Post PCR, amplicons were visualized on 2% agarose gels stained with GelRed (Biotium, Hayward, CA). The PCR products were pooled in equimolar ratios and purified using Qiaquick PCR purification Kit (Qiagen, Valencia, CA) principally following the manufacturer’s guidelines, although including a 5 min incubation time at 37°C prior to elution of the DNA. The purified PCR products were then build into indexed Illumina MiSeq libraries using the NEBNext Quick DNA Sample Prep Master Mix Set 2 (NEB, Ipswich, MA, USA). MiSeq sequencing was performed following the manufacturer's protocol for paired-end reads, 150 bp settings. The Illumina reads were trimmed using default settings in AdapterRemoval, except using a minimal read length of 25 bp. After quality trimming, the paired sequences were merged using customized Perl scripts if the overlap was 100%. The trimmed and merged sequences were sorted in the sample-specific tag-combinations using Geneious (Version 6.1.6 created by Biomatters). Sequences with tag-sequence errors were removed from further analysis. From each tag-combination, OTUs with 97% similarity and a minimum size of 10 sequences were identified using Usearch. OTUs were blasted and assigned to family using Geneious (Version 6.1.6 created by Biomatters). Only families present in both unique tag-combinations from the same vulture sample were kept. 16S mammal sequences from facial swaps from two black vultures were excluded because only 1 tag-combination amplified properly. All mammalian families observed are listed in table ST1.

16S rRNA gene amplicon sequencing – preparation and data generation. We amplified the hypervariable region V3-V4 of the 16s rRNA gene using the 341F (5’-CCTAYGGGRBGCASCAG-3’) and 806R (5’-GGACTACNNGGGTATCTAAT-3’) primer and Phusion 540 L DNA Polymerase). The PCR reaction mix was composed of 5 µl 5x Phusion buffer HF (7.5 mM MgCl₂, Finnzymes, Finland), 0.5 µl 10mM dNTPs, 1.25 µl 10 µM of each primer, 0.25 µl DNA polymerase (Hotstart Phusion 540L, 1 unit/µl Finnzymes) and 1 µl template (same DNA extract used in Mammalian DNA survey). The reaction started with an initialization at 98°C for 30 seconds, followed by 30 cycles of denaturation at 98°C for 5 sec, annealing at 56°C for 20 sec. and elongation at 72°C for 10 sec. The reaction was completed with a final elongation at 72°C for 5 minutes. In the second PCR the adaptors were attached to the amplicon library following the conditions of PCR I with only 15 cycles.

The 16S rRNA gene amplicon sequences were generated by one full plate of 454 - Roche –FLX Titanium. The obtained observations were quality filtered, trimmed and split into the corresponding animal samples with the Qiime pipeline version 1.6.0 using the default settings. Chimeras were removed by Uchime. Based on 811016 trimmed sequences with the average length of 400bp (Min=223, max=523), operational taxonomic units (OTU) were picked de novo and clustered at 97% sequence similarity (Similar to species level). We generated 83,797 sequences of the 7 zoo bird samples in the range of 10,932-13,716 reads per sample that were used for de novo OTU
picking. The taxonomy was assigned using the Rd p classifier and Greengenes as reference database.

The rarefaction curves (Figure S1) show how many unique species (OTUs) were observed in a given count of sequences. Both skin and hindgut curves reach asymptotes with fewer than 1000 sequences. With an average of 4544 sequences per sample, enough sequences were generated to characterize the microbial community of both skin and hindgut. Therefore we removed the difference in sequencing effort by randomly subsampling the OTU table at even sequencing depth of 4544 observations. All further prokaryotic post analyses were based on the even OTU table.

For statistical analysis, the even OTU table was transferred into the open source statistical program “R”. The normality of the raw data was evaluated with the Shapiro-Wilk test ($\alpha = 0.05$). When variables were normally distributed variation between two groups was investigated with the Welch’s $t$-test ($\alpha = 0.05$), otherwise we used the nonparametric Wilcoxon Rank-Sum test ($\alpha = 0.05$) to evaluate differences in OTU alpha diversity between black and turkey vultures. Dissimilarities in OTU abundances between the samples were explained by the OTU count based Bray-Curtis distance metric and examined by the analysis of similarity (ANOSIM).

**Microbial networks.** Bacteria that colonize the same environment potentially co-occur, sharing the same niche, or exclude each other while competing for the same resources. This relationship has been characterized in multiple investigations by generating the Spearman co-occurrence network based on the relative OTU counts. Positive pairwise correlations hypothetically indicate interactions such as symbiosis, mutualism, or commensalism, whereas negative pairwise correlations potentially signal competition, mutual exclusion, or parasitism.

To reduce complexity, we omitted OTUs represented by fewer than 50 sequence observations. The network was generated using the CoNet plugin for Cytoscape based on the non-parametric Spearman correlation coefficients with a minimal cutoff threshold of $\rho = 0.6$ ($P<0.01$, Bonferroni corrected). We present correlation data for OTUs that were detected in at least 50% of the gut samples (n=13 for black vultures, n=11 for turkey vultures). The OTUs of the network of figure 3.II represent 84% of the relative sequences of the black vulture samples, whereas the OTUs of figure 3.III account for 73% of the relative sequences of the turkey vulture samples. The different interaction types are listed in table S7.

**Hindgut metagenomic survey.** DNA from the hindgut of one turkey vulture was shotgun sequenced using the same DNA extract used to characterize prey composition and the microbial community. DNA in the extract was fragmented to 200-300 base pairs using Biorupter and the Illumina library was build by applying the NEB Blunt end Kit E6070. The library was amplified twice using different indexes in a 50 μl volume PCR mix containing 2X Phusion High-Fidelity PCR Master Mix, 1 μM of Primer inPE1.0 (5’-AAT GAT ACG GCG ACC ACC GAG ATC TAC ACT CTT TCC CTA CAC GAC GCT CTT CCG ATC T), 20 nM of primer inPE2.0 (5’-GTG ACT GGA GTT CAG ACG TGT CTT CCG ATC T), 1 μM of an Illumina multiplex primer (5’-CAA GCA GAA GAC GGC ATA CGA GAT NNN NNN GTG ACT GGA GTT C, where the N stretch corresponds to a 6 nucleotides index tag), and 12 μl DNA extract.
The PCR run was initialized at 98°C for 30 seconds, and continued with 14 cycles of denaturation at 98°C for 10 seconds, annealing at 65°C for 30 seconds and elongation at 72°C for 60 seconds. The reaction was finished with final step at 72°C for 5 minutes. PCR products with the length of 325-425bp were gelcut and purified using QIAquick gel purification KIT and eluted in 22 µl EB buffer. The sequencing reaction followed the standard procedure of Illumina Inc. HiSeq2000 for paired-end reads.

Raw reads were trimmed using the Cutadapt software with settings of minimum length 30 bp, minimum quality 30, and removal of all overrepresented sequence. Cleaned raw reads were subject to an in-house chain-mapping using BWA-MEM as the mapping tool against bacterial genomes, vulture genome (in process of submission), human genome, and virus database in the given order. The bacterial database consists of the 2942 completely sequenced and draft genomes available in Genbank as of January 22th 2014, and the Virus database consists of the complete Virus reference database from NCBI. All trimmed raw reads were additionally subject to BWA-MEM mapping against virulence genes, and to the genomes of Meleagris gallopavo (UMD2), Gallus gallus (Galgal4), Anas platyrhynchos (BGI_duck_1.0), Equus caballus (EquCab2), Felis catus (Felis_catus_6.2), Bos taurus (UMD3.1), Sus scrofa (Sscrofa10.2), Rattus norvegicus (Rnor_5.0), Ictidomys tridecemlineatus (Spetri2), and Oryctolagus cuniculus (OryCun2.0) as representatives of prey animals. All mappings were performed with paired-end data and the criteria for a positive hit were that both reads should map with >80% of the read length in addition to the default settings in BWA-MEM.

The majority of the data generated were assigned to vulture DNA (43.5%), unmapped reads (49%), bacteria (6.1%). Reads mapped against viruses were below 0.01%. The sequences were submitted to the virulence factor database (VFDB). The best hits are listed in table S6.

References


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Author Contributions

Michael Roggenbuck and Lars H. Hansen analyzed the microbial survey and are the main authors of this manuscript. Michael Roggenbuck, Lars H. Hansen and M. Thomas P. Gilbert generated the microbial data. Ida Bærholm Schnell and M. Thomas P. Gilbert profiled the mammalian dietary composition. Nikolaj Blom, Jacob Bælum and Thomas Sicheritz Pontén did the metagenomic analysis. The sampling was performed by Gary Graves (wild vultures) and Mads F Bertelsen (zoo samples). Søren Johannes Sørensen helped interpret the data. This project was conceived and designed by Gary R. Graves, M. Thomas P. Gilbert and Lars H. Hansen. All authors have read and understood the manuscript.

Author Information

The microbiome sequence data from this study have been submitted to Sequence Read Archive (SRA) and are available accession no. SRP040754. The authors declare no competing financial interests. Reprints and permissions information is available at www.nature.com/reprints. Readers are welcome to comment on the online version of the paper. Correspondence and requests for materials should be addressed to G.R.G (gravesg@si.edu) and L.H.H (lhha@dmu.dk).

Supplementary Information
**Supplementary Note 1. Additions to the skin microbiota.** After taxonomy assignment the OTUs observed in the skin samples were divided into 59 microbial classes of the microbial skin community, dominated by Clostridia, Gammaproteobacteria, Actinobacteria, Bacilli, Betaproteobacteria, Fusobacteria, Flavobacteria, Bacteroidia, Alphaproteobacteria, Thermomicrobia, Cyanobacteria, and Epsilonproteobacteria (see list ST2). Black vulture carried more Actinobacteria, Epsilonproteobacteria, and Thermomicrobia whereas the turkey vulture face contained significantly more Betaproteobacteria and Clostridia (Wilcoxon-Rank-Sum test, \( P < 0.05 \)). Archaea accounted to only 0.3% of the relative skin sequences.

60.15% of black and 63.75% of turkey vulture of the facial skin sequences were assigned to genus level observing bacteria *Acinetobacter, Arthrobacter, Clostridium, Flavobacterium, Herbaspirillum, Ignatzschinera, Psychrobacter* and *Sphaerobacter* to be the most prominent (>1%) skin bacteria. The genera distributions between both bird species are listed in table ST8.

**Supplementary Note 2. Additions to the gut microbiota.**

15 different phyla were observed in the hindgut samples. However the majority of the hits belong to the three groups of Firmicutes (63.8%), Fusobacteria (26.5%), Proteobacteria (8.3%). The black vulture samples contained in average twice as many Proteobacteria as found in turkey vultures. The turkey vultures accommodated significantly more sequences assigned to Fusobacteria (Wilcoxon, \( P < 0.05 \)).

Furthermore, when looking at the phylogenetic microbial class level, Bacilli and Epsilonbacteria were more abundant in black vultures hindgut samples (Wilcoxon, \( P < 0.05 \)). Turkey vultures carried larger amounts of Actinobacteria and Fusobacteria (Full list is shown in table ST4). Clostridia levels were similar between both bird species.

The 7 taxa of figure 3.I explained 74% of the relative sequences at species level (97% sequences similarity) of black and turkey vulture together (listed in ST9). *Fusobacterium* was more prevalent in turkey vulture. There was no difference between both vulture specimens in the levels of the most observed *Clostridium* OTU that was found in all samples. As indicated in the Figure 3.I., *Catellicoccus* and *Campylobacter* were more prevalent in black vultures, whereas *Peptostreptococcus* was mainly associated to turkey vultures (Wilcoxon, \( P < 0.05 \)).

**Supplementary Note 3. Additional results of the Zoo bird fecal samples.**

The richest sample was the hawk feces followed by the owl whereas the parrot and the wild vultures contained the least diverse fecal microbial communities (Figure S1). The zoo vultures were richer than the wild vultures.

The Gammaproteobacteria was the most abundant class in all zoo bird feces with the exception for the flamingo and the two turkey vultures (Table ST5). Clostridia were only not observed in seriema feces, but accounted for up to 20% of the reads in the other birds samples, besides for the vultures and the flamingo (51 and 55% respectively). Fusobacteria were not found in the parrot, seriema, hawk and owl. Flamingo and the zoo turkey vultures fecal samples contained similar levels of
Clostridia and Fusobacteria. However, the major difference between the flamingo and vultures was described on genus level were the flamingo feces contained 18% of *Megamonas*, only observed below 0.1% in vultures (wild and zoo samples). Of the 12 microbial classes observed in the wild turkey vultures, only 10 were found in the zoo turkey vultures. The profile of the zoo vultures were highly similar to the wild animals with the exception of finding 16% Bacteroidia that was barely found in the wild animals. This observation is most likely due to the diet difference between wild life and captivity.

**Supplementary table 1.** Mammalian taxa detected on facial skin swabs and in the hindgut of *Cathartes aura* (Turkey vultures) and *Coragyps atratus* (Black vultures). Numbers indicate the percentage of vultures in which taxa were detected.

<table>
<thead>
<tr>
<th>Family</th>
<th>Black vulture skin n=23</th>
<th>Turkey vulture skin n=22</th>
<th>Black vulture hindgut n=24</th>
<th>Turkey vulture hindgut n=21</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bovidae</td>
<td>100</td>
<td>91</td>
<td>0</td>
<td>19</td>
</tr>
<tr>
<td>Canidae</td>
<td>30</td>
<td>27</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Cervidae</td>
<td>100</td>
<td>100</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Didelphidae</td>
<td>4</td>
<td>41</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Equidae</td>
<td>9</td>
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<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Felidae</td>
<td>0</td>
<td>5</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Homo sapiens</td>
<td>0</td>
<td>5</td>
<td>0</td>
<td>5</td>
</tr>
<tr>
<td>Leporidae</td>
<td>4</td>
<td>5</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Mephitidae</td>
<td>0</td>
<td>9</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Procyonidae</td>
<td>0</td>
<td>14</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Suidae</td>
<td>26</td>
<td>18</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>
**Supplementary table 2.** Microbial class (mean) distribution - Skin; relative abundance in %  
(Significance: Wilcoxon P<0.05)

<table>
<thead>
<tr>
<th>Class</th>
<th>Black vulture n=26</th>
<th>Turkey vulture n=24</th>
<th>W; P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>$\bar{x}$ ± SD [min; max] in %</td>
<td>$\bar{x}$ ± SD [min; max] in %</td>
<td></td>
</tr>
<tr>
<td>Actinobacteria</td>
<td>15.5 ± 8.6 [3.3; 33.9]</td>
<td>10.6 ± 10.5 [1.5; 40.6]</td>
<td>427; 9.75E-03</td>
</tr>
<tr>
<td>Alphaproteobacteria</td>
<td>2.3 ± 1.3 [0.8; 5.5]</td>
<td>3.7 ± 3.5 [0.2; 13.5]</td>
<td>253; 3.57E-01</td>
</tr>
<tr>
<td>Bacilli</td>
<td>9.6 ± 8.2 [1.1; 31.6]</td>
<td>13.1 ± 10.8 [0.9; 33]</td>
<td>245; 2.79E-01</td>
</tr>
<tr>
<td>Bacteroidia</td>
<td>5.9 ± 10 [0.1; 36.4]</td>
<td>2.3 ± 2.5 [0.1; 8.4]</td>
<td>300; 9.92E-01</td>
</tr>
<tr>
<td>Betaproteobacteria</td>
<td>5.4 ± 4.9 [0.7; 20.1]</td>
<td>11 ± 11 [0.6; 38.3]</td>
<td>191; 3.13E-02</td>
</tr>
<tr>
<td>Clostridia</td>
<td>24.4 ± 10.3 [7.5; 40.5]</td>
<td>26.2 ± 15.7 [7.9; 68.2]</td>
<td>351; 2.98E-08</td>
</tr>
<tr>
<td>Cyanobacteria</td>
<td>0.8 ± 1.3 [0; 6.3]</td>
<td>3.7 ± 8.2 [0; 36.6]</td>
<td>218; 1.07E-01</td>
</tr>
<tr>
<td>Epsilonproteobacteria</td>
<td>1.8 ± 3 [0; 12.8]</td>
<td>0.1 ± 0.2 [0; 0.8]</td>
<td>525; 6.26E-06</td>
</tr>
<tr>
<td>Flavobacteria</td>
<td>6.2 ± 5.3 [0.2; 20.2]</td>
<td>2.3 ± 2.2 [0; 9.1]</td>
<td>479; 3.23E-04</td>
</tr>
<tr>
<td>Fusobacteria</td>
<td>6.1 ± 9.0 [0; 31.5]</td>
<td>3.0 ± 5.3 [0.2; 23.6]</td>
<td>191; 4.9E-02</td>
</tr>
<tr>
<td>Gammaproteobacteria</td>
<td>13.1 ± 8.1 [3.4; 47]</td>
<td>16.5 ± 7.7 [4.7; 32.9]</td>
<td>209; 7.28E-02</td>
</tr>
<tr>
<td>Thermomicrobia</td>
<td>3.9 ± 5.1 [0; 19.3]</td>
<td>0.5 ± 1.1 [0; 4.8]</td>
<td>511; 2.32E-05</td>
</tr>
</tbody>
</table>
Supplementary table 3. Unique bacterial species (OTUs*) observation distinguished by environment and sex in %. *De novo picked OTUs at 96% sequences similarity. (Significance: Wilcoxon $P<0.05$)

<table>
<thead>
<tr>
<th>Sample site</th>
<th>$\bar{x} \pm SD$ [min; max] in %</th>
<th>W; $P$-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Black vulture; skin</td>
<td>20.6 ± 7.4 [11; 41.6]</td>
<td>257; 4.06E-01</td>
</tr>
<tr>
<td>Turkey vulture; skin</td>
<td>20.7 ± 7.8 [7.9; 37.9]</td>
<td></td>
</tr>
<tr>
<td>Black vulture; hindgut</td>
<td>6.4 ± 6.3 [1.5; 26.6]</td>
<td>422; 5.05E-03</td>
</tr>
<tr>
<td>Turkey vulture; hindgut</td>
<td>10.9 ± 7.4 [3.5; 36.8]</td>
<td></td>
</tr>
<tr>
<td>Black vulture; skin; female</td>
<td>23.4 ± 7.3 [11.8; 41.6]</td>
<td>36; 8.28E-02</td>
</tr>
<tr>
<td>Black vulture; skin; male</td>
<td>17 ± 6.7 [11; 31.2]</td>
<td></td>
</tr>
<tr>
<td>Turkey vulture skin; female</td>
<td>20.2 ± 6.6 [7.9; 28.9]</td>
<td>74; 5.16E-01</td>
</tr>
<tr>
<td>Turkey vulture; skin; male</td>
<td>23.9 ± 9.5 [10.8; 37.9]</td>
<td></td>
</tr>
<tr>
<td>Black vulture; hindgut; female</td>
<td>6.4 ± 6.1 [2.2; 26.6]</td>
<td>6.5; 9.53E-01</td>
</tr>
<tr>
<td>Black vulture; hindgut; male</td>
<td>8.3 ± 7.1 [1.5; 21.2]</td>
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<tr>
<td>Turkey vulture; hindgut; female</td>
<td>11 ± 9.2 [3.5; 36.8]</td>
<td>54; 7.93E-01</td>
</tr>
<tr>
<td>Turkey vulture; hindgut; male</td>
<td>10.8 ± 4.1 [6.6; 18]</td>
<td></td>
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</table>
Supplementary table 4. Microbial class (mean) composition – hindgut; relative abundance in %.
Only taxa >1% shown. (Significance: Wilcoxon, P<0.05)

<table>
<thead>
<tr>
<th>Class</th>
<th>Black vulture n=26</th>
<th>Turkey vulture n=23</th>
<th>W-value; P-value</th>
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<tbody>
<tr>
<td></td>
<td>$\bar{x} \pm SD$ [min; max]</td>
<td>$\bar{x} \pm SD$ [min; max]</td>
<td></td>
</tr>
<tr>
<td>Actinobacteria</td>
<td>0.2 ± 0.2 [0.0; 0.8]</td>
<td>1.2 ± 2.0 [0.0; 7.6]</td>
<td>417; 7.06E-03</td>
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<td>Alphaproteobacteria</td>
<td>0.3 ± 0.6 [0; 2.2]</td>
<td>0.1 ± 0.2 [0; 1]</td>
<td>370; 8.26E-02</td>
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<td>Bacilli</td>
<td>16.6 ± 15.0 [0.1; 58.9]</td>
<td>5.2 ± 7.3 [0.2; 26.6]</td>
<td>436; 1.98E-03</td>
</tr>
<tr>
<td>Betaproteobacteria</td>
<td>1.3 ± 2.4 [0; 9.7]</td>
<td>1.3 ± 1.5 [0.1; 5.5]</td>
<td>246; 4.08E-01</td>
</tr>
<tr>
<td>Clostridia</td>
<td>50.5 ± 18.1 [26.1; 84.8]</td>
<td>55.6 ± 17.4 [25.5; 83.6]</td>
<td>240; 3.50E-01</td>
</tr>
<tr>
<td>Epsilonproteobacteria</td>
<td>6.0 ± 5.5 [0.5; 17.1]</td>
<td>1.1 ± 2.4 [0.0; 10.7]</td>
<td>514; 2.61E-06</td>
</tr>
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<td>Fusobacteria</td>
<td>21.2 ± 15.9 [0.2; 54]</td>
<td>31.1 ± 16.3 [1.7; 68.5]</td>
<td>191; 4.99E-02</td>
</tr>
<tr>
<td>Gammaproteobacteria</td>
<td>3.6 ± 5.1 [0; 21.3]</td>
<td>2.9 ± 2.9 [0.1; 10.7]</td>
<td>279; 8.85E-01</td>
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</table>
**Supplementary table 5.** Microbial class observations - Zoo bird feces; relative abundance in %. Only taxa >1% shown.

<table>
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<tr>
<th>Class</th>
<th>Parrot n=1</th>
<th>Owl n=1</th>
<th>Hawk n=1</th>
<th>Seriema n=1</th>
<th>Zoo turkey vulture n=2</th>
<th>Flamingo n=1</th>
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<tbody>
<tr>
<td>Actinobacteria</td>
<td>0</td>
<td>13.6</td>
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<td>Alphaproteobacteria</td>
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<td>1.5</td>
<td>0.2</td>
<td>0</td>
<td>0.4</td>
</tr>
<tr>
<td>Bacilli</td>
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<td>1</td>
<td>10.8</td>
<td>0</td>
<td>0.7</td>
<td>0.6</td>
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<tr>
<td>Bacteria;Other</td>
<td>0</td>
<td>0.3</td>
<td>6.7</td>
<td>0.1</td>
<td>0.2</td>
<td>1.9</td>
</tr>
<tr>
<td>Bacteroidia</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>16.8</td>
<td>2.4</td>
</tr>
<tr>
<td>Betaproteobacteria</td>
<td>0</td>
<td>6.9</td>
<td>0.3</td>
<td>0</td>
<td>1.5</td>
<td>0.2</td>
</tr>
<tr>
<td>Clostridia</td>
<td>7.3</td>
<td>4.9</td>
<td>20.5</td>
<td>0</td>
<td>51.6</td>
<td>55</td>
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<td>Epsilonproteobacteria</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Erysipelotrichi</td>
<td>0</td>
<td>0</td>
<td>0.2</td>
<td>0</td>
<td>0.8</td>
<td>1.8</td>
</tr>
<tr>
<td>Flavobacteria</td>
<td>0</td>
<td>2.1</td>
<td>5.9</td>
<td>0</td>
<td>0.1</td>
<td>2.8</td>
</tr>
<tr>
<td>Fusobacteria</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>26.2</td>
<td>13.3</td>
</tr>
<tr>
<td>Gammaproteobacteria</td>
<td>91.8</td>
<td>47.7</td>
<td>28.2</td>
<td>98.7</td>
<td>0.6</td>
<td>15</td>
</tr>
<tr>
<td>Sphingobacteria</td>
<td>0</td>
<td>3.5</td>
<td>0.6</td>
<td>0</td>
<td>0</td>
<td>0.1</td>
</tr>
<tr>
<td>Thermomicrobia</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>2.2</td>
</tr>
<tr>
<td>TM7_genera_incertae_sedis</td>
<td>0</td>
<td>1.2</td>
<td>0.2</td>
<td>0</td>
<td>0</td>
<td>0.2</td>
</tr>
<tr>
<td>Verrucomicrobiae</td>
<td>0</td>
<td>6.1</td>
<td>1.4</td>
<td>0</td>
<td>0</td>
<td>0.6</td>
</tr>
</tbody>
</table>
Supplementary table 6. Virulence factors observed in a single turkey vulture shot-gun metagenome. Shown are only genes with full read length.

<table>
<thead>
<tr>
<th>Strain (VFDB ID)</th>
<th>Gene description</th>
<th>Coverage of the gene in %</th>
<th>Depth (cover of each position in the gene)</th>
<th>Length of the gene</th>
<th>Bases covered</th>
</tr>
</thead>
<tbody>
<tr>
<td>Clostridium perfringens (VF0378)</td>
<td>plc-phospholipase C</td>
<td>100</td>
<td>188</td>
<td>1197</td>
<td>1197</td>
</tr>
<tr>
<td>Clostridium perfringens (VF0388)</td>
<td>colA-collagenase</td>
<td>100</td>
<td>138</td>
<td>3315</td>
<td>3315</td>
</tr>
<tr>
<td>Clostridium perfringens (VF0382)</td>
<td>pfoA-perfringolysin O</td>
<td>100</td>
<td>134</td>
<td>1503</td>
<td>1503</td>
</tr>
<tr>
<td>Clostridium perfringens (VF0389)</td>
<td>nagH-hyaluronidase</td>
<td>100</td>
<td>133</td>
<td>4887</td>
<td>4887</td>
</tr>
<tr>
<td>Clostridium perfringens ATCC1312 (VF0391)</td>
<td>nanH-sialidase</td>
<td>100</td>
<td>106</td>
<td>1149</td>
<td>1149</td>
</tr>
<tr>
<td>Clostridium perfringens (VF0391)</td>
<td>nanJ-exo-alpha- sialidase</td>
<td>100</td>
<td>99</td>
<td>3522</td>
<td>3522</td>
</tr>
<tr>
<td>Clostridium perfringens (VF0390)</td>
<td>cloSI-alpha-clostripain</td>
<td>100</td>
<td>89</td>
<td>1575</td>
<td>1575</td>
</tr>
<tr>
<td>Clostridium perfringens (VF0389)</td>
<td>nagI-hyaluronidase</td>
<td>100</td>
<td>78</td>
<td>3894</td>
<td>3894</td>
</tr>
<tr>
<td>Clostridium perfringens (VF0389)</td>
<td>nagJ-hyaluronidase</td>
<td>100</td>
<td>76</td>
<td>3006</td>
<td>3006</td>
</tr>
<tr>
<td>Clostridium perfringens 13(VF0391)</td>
<td>nanI-exo-alpha- sialidase</td>
<td>100</td>
<td>75</td>
<td>2085</td>
<td>2085</td>
</tr>
<tr>
<td>Clostridium perfringens (VF0389)</td>
<td>nagK-hyaluronidase</td>
<td>100</td>
<td>74</td>
<td>3492</td>
<td>3492</td>
</tr>
</tbody>
</table>
**Supplementary table 7.** List of interactions between the bacteria within the vulture gut. The results are based on the spearman co-correlations (cutoff=0.6, permutation test, p<0.05) based on the relative and log-transformed OTU table.

<table>
<thead>
<tr>
<th>Interaction types</th>
<th>Black vulture in % (n=26)</th>
<th>Turkey vulture in % (n=23)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Number of total interactions</td>
<td>100 (133)</td>
<td>100 (167)</td>
</tr>
<tr>
<td>Negative co-correlation (total)</td>
<td>8 (10)</td>
<td>10 (17)</td>
</tr>
<tr>
<td>Positive co-correlations (total)</td>
<td>92 (123)</td>
<td>90 (150)</td>
</tr>
<tr>
<td>Negative co-correlations within the same microbial classes</td>
<td>2 (3)</td>
<td>5 (9)</td>
</tr>
<tr>
<td>Positive co-correlations within the same microbial classes</td>
<td>86 (114)</td>
<td>81 (135)</td>
</tr>
<tr>
<td>Negative co-correlations between microbial classes</td>
<td>5 (7)</td>
<td>5 (8)</td>
</tr>
<tr>
<td>Positive co-correlations between microbial classes</td>
<td>7 (9)</td>
<td>9 (15)</td>
</tr>
<tr>
<td>Negative co-correlations between Fusobacteria and Clostridia</td>
<td>3 (4)</td>
<td>2 (3)</td>
</tr>
<tr>
<td>Positive co-correlations between Fusobacteria and Clostridia and Clostridia</td>
<td>0 (0)</td>
<td>5 (9)</td>
</tr>
</tbody>
</table>
**Supplementary table 8.** Microbial genera (mean) composition – Skin; relative abundance in %. (Significance: Wilcoxon, \( P < 0.05 \)) Only taxa above 1% relative abundance (mean) of either bird species are listed.

<table>
<thead>
<tr>
<th>Class</th>
<th>Black vulture n=25 ( \bar{x} \pm SD \ [\text{min; max}] ) in %</th>
<th>Turkey vulture n=24 ( \bar{x} \pm SD \ [\text{min; max}] ) in %</th>
<th>W; ( P )-value</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Acinetobacter</em></td>
<td>1.6 ± 1.4 [0.4; 7.3]</td>
<td>3.7 ± 3.7 [0.1; 12.5]</td>
<td>199; 4.51E-02</td>
</tr>
<tr>
<td><em>Arthrobac</em>ter</td>
<td>6.1 ± 5 [1.1; 23.4]</td>
<td>4.7 ± 7.7 [0.2; 32]</td>
<td>429; 9.47E-03</td>
</tr>
<tr>
<td><em>Brucella</em></td>
<td>0.6 ± 0.6 [0; 2.1]</td>
<td>2 ± 2.7 [0; 10.3]</td>
<td>191; 3.12E-02</td>
</tr>
<tr>
<td><em>Catellicoccus</em></td>
<td>1.7 ± 3.3 [0.1; 16]</td>
<td>0.6 ± 2.4 [0; 11.5]</td>
<td>522; 6.45E-06</td>
</tr>
<tr>
<td><em>Clostridium</em></td>
<td>6.2 ± 4.9 [0.9; 20.1]</td>
<td>5.6 ± 4.8 [0.6; 20.0]</td>
<td>328; 5.75E-01</td>
</tr>
<tr>
<td><em>Flavobacterium</em></td>
<td>2.3 ± 2.9 [0; 11]</td>
<td>0.6 ± 1.3 [0; 6.5]</td>
<td>469; 7.06E-04</td>
</tr>
<tr>
<td><em>Herbaspirillum</em></td>
<td>3.9 ± 4.3 [0; 17]</td>
<td>9.1 ± 9.8 [0.1; 33.8]</td>
<td>192; 3.29E-02</td>
</tr>
<tr>
<td><em>Ignatzschineria</em></td>
<td>1.8 ± 4.6 [0; 23.6]</td>
<td>2.8 ± 4.2 [0; 16.7]</td>
<td>256; 3.94E-01</td>
</tr>
<tr>
<td><em>Peptostreptococcus</em></td>
<td>1.1 ± 2.4 [0; 12.6]</td>
<td>4.1 ± 5.5 [0; 22.1]</td>
<td>201; 4.96E-02</td>
</tr>
<tr>
<td><em>Prevotella</em></td>
<td>4.2 ± 7.8 [0; 28.2]</td>
<td>0.4 ± 0.4 [0; 1.6]</td>
<td>364; 1.96E-01</td>
</tr>
<tr>
<td><em>Pseudomonas</em></td>
<td>1.1 ± 1 [0; 3.6]</td>
<td>3.6 ± 2.8 [0; 9.8]</td>
<td>109; 141E-04</td>
</tr>
<tr>
<td><em>Psychrobacter</em></td>
<td>5 ± 3.7 [1; 11.8]</td>
<td>2.6 ± 3.6 [0.2; 17.4]</td>
<td>451; 2.48E-03</td>
</tr>
<tr>
<td><em>Sphaero</em>bac*ter</td>
<td>2.5 ± 3.2 [0; 12.1]</td>
<td>0.2 ± 0.4 [0; 1.3]</td>
<td>511; 2.22E-02</td>
</tr>
<tr>
<td><em>Tissierella</em></td>
<td>1.4 ± 2.6 [0.2; 13.3]</td>
<td>1 ± 1.3 [0; 4.1]</td>
<td>374; 1.35E-01</td>
</tr>
<tr>
<td><em>Vagococcus</em></td>
<td>0.4 ± 0.5 [0; 2.3]</td>
<td>1.9 ± 2.6 [0; 11.7]</td>
<td>141; 1.60E-03</td>
</tr>
</tbody>
</table>
**Supplementary Table 9.** Distribution of most frequent gut species (OTUs) highlighted in figure 3.1. (Significance: Wilcoxon, \(P<0.05\))

<table>
<thead>
<tr>
<th>OTUs</th>
<th>Black vulture n=25 ± SD [min; max] in %</th>
<th>Turkey vulture n=24 ± SD [min; max] in %</th>
<th>W; (P)-value</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Campylobacter</em>_1</td>
<td>4.6 ± 4.7 [0.2; 14.1]</td>
<td>0.2 ± 0.6 [0; 2.4]</td>
<td>31; 1.12E-07</td>
</tr>
<tr>
<td><em>Catellicoccus</em>_1</td>
<td>12.1 ± 11.5 [0; 34.8]</td>
<td>1.9 ± 4.6 [0; 16.4]</td>
<td>60; 2.82E-06</td>
</tr>
<tr>
<td>Unclassified Clostridiales_2</td>
<td>5.3 ± 4.7 [0; 14.2]</td>
<td>6.4 ± 6.2 [0; 23.7]</td>
<td>306; 6.89E-01</td>
</tr>
<tr>
<td><em>Clostridium</em>_5</td>
<td>16.9 ± 17.6 [0.2; 53.8]</td>
<td>14.1 ± 12.8 [0.5; 44.9]</td>
<td>305; 7.05E-01</td>
</tr>
<tr>
<td><em>Fusobacterium</em>_1</td>
<td>19.8 ± 14.4 [0.2; 49.4]</td>
<td>30.7 ± 15.6 [2.2; 63.6]</td>
<td>405; 1.30E-02</td>
</tr>
<tr>
<td>Unclassified Peptostreptococcaceae_1</td>
<td>17.1 ± 15.8 [0.1; 49.6]</td>
<td>7.1 ± 8.7 [0.3; 38.4]</td>
<td>144; 2.82E-03</td>
</tr>
<tr>
<td><em>Peptostreptococcus</em>_1</td>
<td>2 ± 4.1 [0; 16.7]</td>
<td>7.4 ± 10.8 [0; 43.9]</td>
<td>452; 5.86E-04</td>
</tr>
</tbody>
</table>
The giraffe (Giraffa camelopardalis) rumen microbiome
The giraffe (Giraffa camelopardalis) rumen microbiome

Michael Roggenbuck¹, Cathrine Sauer²,³, Morten Poulsen², Mads F. Bertelsen³, & Søren J. Sørensen¹

¹Department of Biology, Microbiology, University of Copenhagen, Copenhagen O, Denmark; ²Department of Animal Science, Aarhus University, Tjele, Denmark; and ³Copenhagen Zoo, Centre for Zoo and Wild Animal Health, Frederiksberg, Denmark

Correspondence: Søren J. Sørensen, Department of Biology, Microbiology, University of Copenhagen, Universitetsparken 15, DK-2100 Copenhagen O, Denmark. Tel.: +45 5182 7007; fax:+4535322040; e-mail: SJS@bio.ku.dk

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Keywords
rumen; microbiome; giraffe; wild life; diet.

Abstract
Recent studies have shown that wild ruminants are sources of previously undescribed microorganisms, knowledge of which can improve our understanding of the complex microbial interactions in the foregut. Here, we investigated the microbial community of seven wild-caught giraffes (Giraffa camelopardalis), three of which were fed natural browse and four were fed Boskos pellets, leafy alfalfa hay, and cut savanna browse, by characterizing the 16S rRNA gene diversity using 454 FLX high-throughput sequencing. The microbial community composition varied according to diet, but differed little between the ruminal fluid and solid fraction. The giraffe rumen contained large levels of the phyla of Firmicutes and Bacteroidetes independent of diet, while Prevotella, Succinclasticium, and Methanobrevibacter accounted for the largest abundant taxonomic assigned genera. However, up to 21% of the generated sequences could not been assigned to any known bacterial phyla, and c. 70% not to genus, revealing that the giraffe rumen hosts a variety of previously undescribed bacteria.

Introduction
It has been estimated that there are 75.3 million wild and 3.57 billion domestic ruminants on earth belonging to six ruminant families of Antilocapridae, Cervidae, Bovidae, Giraffidae, Moschidae, and Tragulidae (Hackmann & Spain, 2010). The majority of research on the microbial community of ruminants has focused on livestock members of the Bovidae, while little is known about the microbiome of wild ruminants.

With a height of 5–6 m and a body weight of up to 1400 kg, the Giraffa camelopardalis (family Giraffidae) is one of the largest existing ruminants (Hall-Martin, 1977). The daily dry matter intake The word “circa” doesn’t fit here. It needs to be as in the original submitted text “approximates” 1.6% and 2.1% of body weight, for males and females, respectively (Pellew, 1984).

In the wild, giraffes are browsers – ruminants that feed on leaves, shoots, fruits, flowers, and even twigs of many different species of trees and shrubs (Leuthold & Leuthold, 1972). Browse generally has higher crude protein and lignin contents than grasses and may also contain other secondary plant metabolites such as tannins (Shipley et al., 1998). In contrast, grasses are usually richer in fibers.

Zoo diets are often designed to mimic the natural diet of an animal. As the wild diet items vary greatly in availability, palatability, and nutrient content throughout the year, it is, however, very difficult to replicate the natural diet in captivity. In addition, the nutrient requirements of most captive wildlife species are unknown, making it even more difficult to design an appropriate diet, and diet related problems have been reported for captive giraffes (Potter & Clauss, 2005; Clauss et al., 2007). The impact of neither natural nor captive diets on the giraffe rumen microbiota has not been investigated.

Like other herbivores, the giraffe depend on symbiosis with microorganisms in the digestive system to utilize cellulose and hemicellulose. In the rumen, the central organ of foregut fermentation of large herbivores, microorganisms ferment fibers and produce along with several other metabolites volatile fatty acids – the major energy source of the host (Saengkerdsub & Ricke, 2013). This has driven coevolution between the animal host and the microorganisms, where microorganisms have specialized in utilizing specific organic compounds reflecting differences in composition of the diet ingested by the animal (Ley et al., 2008a, b).
In this pilot study, we investigated the ruminal microbiota of seven giraffes fed with Boskos pellets, leafy alfalfa hay, and cut savanna browse. However, due to the logistics of the project, only four animals (group I) were fed continuously as described above. The other three giraffes (group II) were first kept under the same conditions as group I, but 4–6 days prior to sampling fed natural browse from surrounding enclosure.

It has been demonstrated that diet composition alter the rumen microbiota of calves (Pitta et al., 2010) and as the giraffes had eaten different diets for a longer period than the mean particle retention time of 40 h reported for giraffes (Clauss et al., 1998), we hypothesized that the rumen microbiota would be distinct between feeding group I and II.

To investigate this, we characterized simultaneously the bacterial and archaeal communities of the solid and fluid fraction of rumen content of the giraffe via a culture independent approach using the 16S rRNA gene phylogenetic marker and 454 FLX Titanium high-throughput sequencing amplifying the hypervariable region of V3–V4. Our study gives first insights into the giraffe rumen microbiome.

**Material and methods**

**Sample collection**

Samples of solid and fluid reticulo-ruminal content were collected from seven juvenile giraffes (G. camelopardalis), six males and one female, weighing 491 ± 92 kg.

The giraffes originated from private game parks in the Republic of South Africa and were brought to a central facility as part of a physiological research project. Here, the animals received a diet of Boskos pellets [WES Enterprises (Pty) Ltd, South Africa], leafy alfalfa hay, and various species of fresh cut savannah browse for 1–2 months. Four animals (A, B, C, D) continued the diet described above (diet group I), whereas three giraffes (E, F, G) were fed on natural browse only in a central facility as part of a physiological research project. Following anesthesia for physiological research, animals were euthanized and immediately underwent a thorough postmortem examination. Contents of the reticulo-rumen were sampled by collecting a handful of material from six different locations (see Fig. 1). Samples from all locations were mixed in a filter bag with 0.5-mm pore size (Grade Blender Bags, VWR, Denmark) to separate the fluid and the solid fractions. Immediately after sampling, the filter bag was stored at c. 5 °C for c. 2 h until the fluid and the solid part were separated. Thirty milliliter of each fraction was sampled and kept at –18 °C for up to 15 days until shipment on dry ice. After shipment, samples were stored at –80 °C until analysis.

**DNA preparation, sequencing and data treatment**

Subsamples of 0.5 g of each sample were transferred to a tube together with 1 mL extraction buffer (50 mM Tris-HCl, 5 mM EDTA, 3% SDS) and sterile glass beads. Cells were mechanically disrupted by FastPrep (MP Biomedicals) 5.5 m s⁻¹ for 30 s. Cell debris was removed by centrifugation, and the supernatant was incubated in 465 μL ammonium acetate (5 M) for 5 min at 4 °C. The reaction was stopped by adding 2x volume of reaction mix of Guanidine–HCl (7 M) to the tube. The genomic DNA was purified using the Genomic Mini AX SOIL Spin Kit (A&A Biotechnology).

The hypervariable region V3–V4 of bacterial and archaeal 16S rRNA genes was simultaneously amplified using the primers 341F (5'-CCTAYGGGRBGCASCAG-3') and 806R (5'-GGACCTACNGGTTATCTAAT-3') and the AccuPrime™ Pfx DNA Polymerase (Neefs et al., 1991; Yu et al., 2005). The PCR mix contained 2.5 μL 10x AccuPrime™ Buffer (dNTPs included), 1.25 μL of each primer (10 μM), 1 μL template and 0.15 μL AccuPrime™ Pfx DNA Polymerase plus sterile water with a final reaction volume of 25 μL. The reaction started with an initialization at 94 °C for 2 min, following 30 cycles of denaturation at 94 °C for 20 s, annealing at 56 °C for 30 s, and elongation at 68 °C for 40 s. The constructions of the amplon libraries were divided into two steps to avoid biased amplification due to unspecific adaptor binding to the template (Berry et al., 2011). The size of the PCR product was evaluated (fragment length; 466 bp) using gel electrophoresis. The fragment was then excised and purified using the Montage Gel Extraction Kit (Merck Millipore). In a second PCR of 15 cycles, adaptors were added to the amplicons using the same reaction conditions as mentioned above. After additional fragment size (526 bp) evaluation and excision, the amplicon concentration was measured using Qubit dsDNA HS Assay Kit (Invitrogen). The concentration was adjusted to 1.0x E + 80 copies μL⁻¹ and sent for sequencing. The sequences were generated by the 454 FLX – Titanium at the ‘National Danish High-Throughput DNA Sequencing Center’ in Copenhagen, Denmark. The reads were trimmed for low quality (minimal quality score = 25) and denoised using the Quimera pipeline version 1.3.0 (Caporaso et al., 2010; Quince et al., 2011). Only sequences with a minimal length of 200 bp were considered. Chimeras were removed using the UCHIME algorithm (Edgar et al., 2011). Operational taxonomic units (OTUs) were picked de novo from quality checked reads and clustered at 97% sequence similarity.
using UCLUST. Taxonomy was assigned using the RDP classifier (version 2.2) method with a bootstrap cutoff value of 0.8 and GREENGENES as reference database (Liu et al., 2008). Variations between samples (diet group I vs. II and fluid vs. solid sample) were checked for normal distribution using the Shapiro–Wilk test (significance value $P < 0.05$). When normally distributed, we evaluated the variables with the Welch’s $t$-test, if nonparametric, the Wilcoxon rank-sum test with the significance value of $P < 0.05$ was preferred. The microbial community composition was evaluated with the Bray–Curtis (BC) and the Euclidean matrix. The clustering was confirmed by the $R$ value ($0 =$ highly similar; $1 =$ highly dissimilar) of the analysis of similarity Insert here in brackets (Anosim) and significance permutation $P < 0.05$.

**Results**

After quality check (material and methods), we received 30,400 sequences with an average length of 376 bp (min 204, max 400, Fig. 2 – length distribution). The additional denoising and chimera check removed 6077 sequences as primer bias indicated sequences, leaving 24,323 sequences for downstream analysis. The number of sequences of each animal varied from 448 to 4339.

We used rarefaction to randomly subsample the sequences and evaluate diversity, as rarefaction (Gotelli & Colwell, 2001) is a method widely used for characterizing microbial diversity (Koenig et al., 2011; Koren et al., 2013; Charlop-Powers et al., 2014; Davenport et al., 2014; Ding & Schloss, 2014).

We characterized the richness estimator (Chao1) and the diversity (Shannon) index to evaluate the depth of the generated sequences (Fig. 3) (Colwell & Coddington, 1994; Hill et al., 2003). The richness and diversity curve did not reach the plateau of saturation for all samples at 500 sequences per sample. Thus, the most abundant taxa will be described but more rare species are potentially underrepresented. To remove bias by sequencing effort, we used a subset of 548 reads per sample. The
The subsampled OTU table contained 22% of the total generated sequences but more than 50% of the total OTUs. Therefore, two samples (Table 1) were disregarded as the sequences generated accounted for < 10 sequences. These samples were not part of the downstream analysis.

We observed in total 1692 OTUs spread throughout all samples. After subsampling, the OTUs table contained 878 OTUs. Of the subset OTUs, 45.9% were uniquely associated to diet group I, 22.7% were shared between both diets and 31.7% were only observed in the samples of group II. Rumen solid and fluid shared 27.7% of all OTUs identified, whereas 29.5% were unique to solid and 42.8% unique to fluid. Additionally, every animal carried on average 28% (min 11%, max 54%) of unique OTUs (Table 1) not shared with other giraffes at 97% sequence identity and without dissimilarity between fluid or solid rumen content (Welch’s t-test, \( P = 0.905 \)) or the diet groups (Welch’s t-test, \( P = 0.688 \)).

**Diet shapes microbial communities**

To investigate variations in microbial distribution between diet groups and ruminal fractions of the giraffe rumen, the diversity between the sampling sites was compared. No significant difference in richness (Chao1) or diversity (Shannon) indexes was found between neither feeding types nor ruminal fractions (Table 2).

Additionally, we observed if the samples were more similar in microbial composition within group I and II as well as in fluid or solid fraction, by generating an OTU relative abundance-based BC dissimilarity matrix. The principal coordinate analysis (PCoA; Fig. 4) revealed no distinct differences in the microbial distribution of solid or fluid fraction. Only the samples from animals A and B clustered according to sample fraction. However, giraffe E, F, and G, fed only on browse, grouped together in distance of animal A, B, C, and D, which had received Boskos pellets, alfalfa hay, and cut savanna browse.

**Firmicutes and Bacteroidetes most prevalent phyla**

A total of 21 phyla were observed for all samples combined (Fig. 5). The majority of the relative sequence counts were assigned to Firmicutes (50%) and Bacteroidetes (30%). Furthermore, both ruminal fractions were composed of Proteobacteria (4%), Cyanobacteria (1%), Actinobacteria (1%), Euryarchaeota (2%), and the candidate division TM7 (1%) without significant variation.
However, despite the difference in clustering between the diet groups, there were no significant variations on phyla level (Wilcoxon, \( P > 0.05 \)). The phyla of Acidobacteria, Chloroflexi, Crenarchaeota, Fibrobacteres, Fusobacteria, Gemmatimonadetes, Nitrospira, Planctomycetes, Spirochetes, Synergistetes, Tenericutes, Verrucomicrobia, candidate phylum WS3 and candidate division SR1 were detected to each constitute below 1% relative abundance. c. 7% of the relative sequences were not assigned to any known bacterial phyla. There was no variation in read length of the unassigned representative sequences compared with the ones identified (Wilcoxon, \( W = 82069, P = 0.101 \)). This indicates that the length of the reads is sufficient to receive at least phylum taxonomic information. Furthermore, there was no difference in sequence length between the diet groups or the ruminal fractions (Wilcoxon, \( P > 0.05 \)).

Majority of the sequences were not assigned to genus

On the phylogenetic family level only 53% of the sequences were assigned to known taxa of Ruminococcaceae (21%), Lachnospiraceae (11%), Prevotellaceae (10%), Veillonellaceae (7%), Methanobacteriaceae (2%), Porphyromonadaceae (1%), and Streptophyta (1%). Furthermore, only 28% of all sequences were assigned to genus level. Of 78 genera, 24 were observed only in the solid and 23 only in the fluid content, whereas 31 genera were found in both fractions. The most abundant genera (identified), independent of ruminal fraction, were Prevotella (6%), Succinivibrionaceae (5%), Oscillobacter (4%), Methanobrevibacter (2%), Ruminococcus (1%), Barnesiella (1%), and Pseudobutyrivibrio (1%). Besides the absence of Coprococcus and Ruminococcus in feeding group II, there was no further diet-dependent

<p>| Table 2. Diversity comparison of ruminal fluid vs. solid fraction and diet group I vs. II |</p>
<table>
<thead>
<tr>
<th>Diversity index</th>
<th>Fluid mean [min; max]</th>
<th>Solid mean [min; max]</th>
<th>Ruminal fraction comparison ( W; P )</th>
<th>Diet group I mean [min; max]</th>
<th>Diet group II mean [min; max]</th>
<th>Diet comparison ( W; P )</th>
</tr>
</thead>
<tbody>
<tr>
<td>Chao1 richness estimator</td>
<td>396; [320; 462]</td>
<td>338; [215; 578]</td>
<td>25; 0.2677</td>
<td>360 [215; 578]</td>
<td>368 [219; 538]</td>
<td>20; 0.570</td>
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<tr>
<td>Shannon diversity estimator</td>
<td>6.5; [6.0; 6.9]</td>
<td>6.0; [4.7; 7.3]</td>
<td>24; 0.3434</td>
<td>6.1 [4.7; 6.9]</td>
<td>6.4 [5.5; 7.3]</td>
<td>16; 1</td>
</tr>
</tbody>
</table>

Listed are the nonparametric Chao1 richness (compares OTUs only once observed against OTUs found exactly twice) and the Shannon diversity index (relates OTU counts and their respective relative occurrences in the complex community). Differences were investigated with Wilcoxon rank-sum test \( (W) P < 0.05 \).

The table shows impact of quality trimming and chimera check on the sequences generated and the OTU survey. Distinguished are the seven giraffes (Sample ID) A–G together with the corresponding ruminal fluid (f) and solid fraction (s). Giraffe A–D is diet group I, whereas giraffe E–G belong to diet group II. Total OTUs are all observations found in the individual animal. Unique OTUs in % is the fraction of bacteria or archaea found in the specific giraffe and not shared with other individuals.
variation on genus level between the feeding groups. Furthermore, a comparison of the ruminal fractions revealed higher levels of *Oscillibacter* in the fluid phase.

All archaeal sequences (1060 sequences, constituting c. 2% of all sequences in the study) belonged to the family *Methanobacteriaceae*, specifically to the genera *Methanobrevibacter* (98% of the sequences) and *Methanosphera*. Almost all methanogenic sequences (97%) were observed in the ruminal solid phase of two giraffes (A and B) of diet group I. However, there was no overall difference of *Methanobrevibacter* relative abundance between the diet groups or the ruminal fractions (Table 2). This observation is independent of sequencing depth due to the fact that the two giraffes had higher levels of *Methanobrevibacter* compared with other giraffes (for example the solid phase of animal C, or the fluid phase of animal D) with similar or larger read counts per sample (Table 1).

To determine which OTUs caused the similarity and dissimilarity among the animals on the two diets, we compared the individual community profiles by generating two-sided dendrograms (Fig. 6). The giraffes did not cluster according to fluid and solid fraction as expected from the PCoA plot (Fig. 4). We observed three groups with similarities in microbial composition (Fig. 6). In cluster 1, giraffe B (only fluid fraction), C, and D (fluid and solid) grouped together by sharing c. 50% of the displayed OTUs. In cluster 2, the solid content of giraffe E, F, and both fraction sites of giraffe G shared only c. 26% of OTUs. Cluster 3 was composed of giraffe A (fluid and solid) and B (solid) appeared to be most different from the other giraffes. Both animals carried large amounts of *Methanobrevibacter* (12%), and *Succiniclasticum* (30%), in relative abundance. Most importantly, the giraffes of cluster 1 and 3 received *Boskos* pellets, cut browse, and alfalfa hay (diet group I), whereas cluster 2 represents the giraffes browsing naturally in the period of 4–6 days before sampling (diet group II). We observed several OTUs with significantly different levels between both feeding groups explaining the diet based clustering in Fig. 4, however, the majority of these OTUs were assigned to different taxonomic levels hindering taxonomic level based comparison between the diets. There were no significant variations between the ruminal fractions of the major OTUs shown in Fig. 6 (Wilcoxon, *P* > 0.05).
Discussion

Herbivores have adapted to live in symbiosis with microorganisms with the ability to metabolize plant material, thereby being able to retrieve energy from otherwise indigestible organic material. One of the most developed symbiotic systems is the reticulo-rumen of ruminants (Hume & Warner, 1980; Hackmann & Spain, 2010). This organ is a fermentation vat where the host provides buffered anaerobic conditions necessary for the microorganisms to utilize cellulose-rich plant material. The rumen content can be divided into two phases of rumen fluid (mix of host fluids, microorganisms, and metabolites) and solid material (feed particles and fiber-adhered microorganisms) (Chen et al., 2008). It has previously been reported that members of the complex microbial community have different substrate specificities creating differences between the two ruminal phases in the community composition in cattle (Cho et al., 2006).

In this study, no significant variation in the microbial distribution was found between the fluid and solid fractions (Fig. 4, Table 2). Instead, variation in microbial composition was higher among animals than between the solid and fluid fraction of the rumen content of each individual animal (Figs 4 and 6).

Although we observed 24 genera only found in the fluid fractions and 23, including the often fiber-associated genus Fibrobacter, only in the solid phase, these genera...
accounted for < 1% and were not found in all fluid or solid fractions (Kobayashi et al., 2008). The minor variation in the abundance of Oscillobacter between the ruminal fractions can either be the result of a small sample size and/or be the result of differences in diet composition between giraffes.

Diet was previously demonstrated to affect the composition of the rumen microbiome in cattle (Fernando et al., 2010) with impact on the microbial phyla distribution.

The phyla of Bacteroidetes (together with Firmicutes) contain some of the primary fiber-degrading bacteria in the rumen, and the abundance of these taxa therefore have a great effect on the ability of the host animal to utilize fiber-rich diet items (Brulc et al., 2008), thus it is not surprising that the diet composition impacts on the microbial community.

In giraffes, a ratio of Firmicutes to Bacteroidetes of 50:30 was observed across ruminal fractions and diets. This is similar to the ratios reported for wild goats (fluid 56:38; solid 40:39) (Cunha et al., 2011), ruminants of intermediate feeding type (i.e. eating both browse and grass) and forage-mix fed cattle (43:33, whole rumen content) (Petri et al., 2013). Henderson et al. (2013), however, found that the occurrence of Firmicutes and Bacteroidetes in cattle and sheep are dependent of the DNA extraction method. The abundance of Firmicutes, for example, was favored by bead beating in contrast to a phenol–chloroform DNA extraction without bead beating. Thus, direct comparison between rumen studies needs to be taken with caution. Interestingly, the same study revealed larger levels of Bacteroidetes compared with Firmicutes in sheep (independent of DNA extraction method) concluding that Firmicutes is not the dominant phyla in all ruminants.

Furthermore, no difference between the two diet groups on phylum level, diversity and richness was found, although a diet-dependent clustering in microbial composition was observed. Giraffes E, F, and G (diet group II), grouped closer together than giraffes A, B, C, D (diet group I), fed pellets with alfalfa hay and browse. Giraffes E, F, and G initially received the same diet as A, B, C, and D, but were given the opportunity to browse naturally for 4–6 days before sampling.

Recent investigations show that the human gut microbiota significantly diverges within 1 day of change of diet (David et al., 2013), however, we can only speculate how fast the microbial community of the reticulo-rumen alternates after a shift in diet composition. Considering that the mean retention time of particles in giraffes has been reported to be c. 40 h (Clauss et al., 1998), it appears likely that the variation of cluster 2 vs. 1 and 3 resulted from giraffes E, F, and G feeding only on browse.

An average 28% of the OTUs were unique to the individual animal but the majority of the OTUs observed were shared between the giraffes despite the large difference in abundance. It has previously been shown that the microbial gut communities in vertebrates cluster highly according to the preferred diet (Ley et al., 2008a, b). Thus, it seems likely that the variations in microbial composition between giraffes were feed driven and that the biome of cluster 2 most closely reflects those of free ranging giraffes.

Approximately 70% of all generated sequences from the giraffe rumen microbiome could not be assigned to genus level, and as much as 21% of all sequences could not be assigned even at phylum level. Although Coprococcus and Ruminococcus levels varied significantly between the feeding groups (Table 3), the major differ-

<table>
<thead>
<tr>
<th>Genus</th>
<th>Fluid mean abundance [min; max] in %</th>
<th>Solid mean abundance [min; max] in %</th>
<th>Variation by ruminal fraction W, P-value</th>
<th>Diet group I [min; max] in %</th>
<th>Diet group II [min; max] in %</th>
<th>Variation by diet W, P-value</th>
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<tr>
<td>Ruminococcus</td>
<td>1.1 [0; 4.9]</td>
<td>1.1 [0; 5.7]</td>
<td>16; 0.868</td>
<td>1.5 [0; 5.7]</td>
<td>0.6 [0; 1.82]</td>
<td>17; 0.931</td>
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<td>Butyribrio</td>
<td>0.8 [0.2; 1.3]</td>
<td>1.8 [0.4; 4.6]</td>
<td>25.5; 0.221</td>
<td>0.9 [0; 2.0]</td>
<td>2.3 [1.1; 4.6]</td>
<td>5.5; 0.088</td>
</tr>
<tr>
<td>Coprococcus</td>
<td>0.8 [0; 1.8]</td>
<td>0.5 [0; 1.6]</td>
<td>11.5; 0.362</td>
<td>0.9 [0.18; 1.8]</td>
<td>0 [0; 0]</td>
<td>32; 0.007</td>
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<td>Methanobrevibacter</td>
<td>0.1 [0; 0.2]</td>
<td>3.1 [0; 12.4]</td>
<td>15; 0.715</td>
<td>2.8 [0; 12.4]</td>
<td>0 [0; 0]</td>
<td>21; 0.340</td>
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<td>Molydella</td>
<td>0.5 [0.2; 2.4]</td>
<td>0.2 [0; 0.7]</td>
<td>20; 0.728</td>
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<td>0.7 [0; 2.4]</td>
<td>6; 0.084</td>
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<td>Oscillobacter</td>
<td>6.8 [1.8; 11.7]</td>
<td>1.9 [0; 4.1]</td>
<td>4; 0.034</td>
<td>4.76 [0.4; 11.7]</td>
<td>2.3 [0; 4.9]</td>
<td>21; 0.441</td>
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<td>Paraprevotella</td>
<td>1.1 [0; 2.7]</td>
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<td>12.5; 0.458</td>
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<td>0.4 [0; 0.9]</td>
<td>17; 0.931</td>
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<td>Prevotella</td>
<td>5.8 [2; 13]</td>
<td>5.9 [0.5; 26.8]</td>
<td>10.5; 0.289</td>
<td>3.8 [0.5; 12.9]</td>
<td>10.3 [2.4; 26.8]</td>
<td>7; 0.147</td>
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<td>Pseudobutyribrio</td>
<td>0.7 [0; 2.1]</td>
<td>1.4 [0; 2.7]</td>
<td>26; 0.192</td>
<td>0.9 [0; 2.7]</td>
<td>1.3 [0; 1.6]</td>
<td>9.5; 0.306</td>
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<td>Pyramatobacter</td>
<td>0.2 [0.07]</td>
<td>0.2 [0.1]</td>
<td>14; 0.602</td>
<td>0.2 [0.1]</td>
<td>0.2 [0; 0.54]</td>
<td>16; 1</td>
</tr>
<tr>
<td>Ruminobacter</td>
<td>0.7 [0; 2.2]</td>
<td>0.5 [0; 2.7]</td>
<td>12; 0.396</td>
<td>0.8 [0; 2.7]</td>
<td>0 [0; 0]</td>
<td>30; 0.017</td>
</tr>
<tr>
<td>Ruminococcus</td>
<td>1.8 [0.4; 4.4]</td>
<td>1.8 [0; 6.9]</td>
<td>15; 0.743</td>
<td>2.5 [0; 6.9]</td>
<td>0 [0; 0.5; 1.3]</td>
<td>7; 0.147</td>
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</tbody>
</table>

Differences were investigated with Wilcoxon rank-sum test (W) P < 0.05.
ences were observed for OTUs assigned to different taxonomic levels (Fig. 6). As metabolic specialization of microorganisms may be very diverse even at the taxonomic level of order, family or genus, our data reveal only little functional information about the microbial communities in the giraffe. Hence, studying the microbial communities of wild ruminants at a deeper taxonomic level is crucial to compare microbial functionality of wild and domestic ruminants. Hopefully, this will be possible as more data from these environments become available from future studies.

Conclusion
This is to the best of our knowledge, the first study of the rumen microbiome of the giraffe. Our results indicate that diet of the giraffe might be a key driving force in shaping the microbial diversity of the rumen. Future studies are needed to investigate differences in composition of microbial communities of browsers and grazers by examining animals receiving carefully controlled diets. Furthermore, our samples contained large amounts of novel bacteria. We believe that the giraffe is a reservoir for novel bacteria. We believe that the giraffe is a reservoir for never described microbial communities deserving further characterization. Additionally, fungi and protozoa of the giraffe reticulo-rumen were not investigated here and need future attention.

Acknowledgements
The authors would like to thank the Danish Cardiovascular Giraffe Research project (DaGIR) for the opportunity to collect the samples and Michael Petersen from Copenhagen Zoo for designing the giraffe graphic of Fig. 1. The study was funded by Det Strategiske Forskningsråd (DSF) from the Novenja project. The research of M.P. was granted by the Danish Agency for Science, Technology and Innovation through an individual Postdoc stipend and C.S. by a PhD scholarship from Aarhus University. The authors declare that they have no competing interests.

Ethical approval
Permission to euthanize the animals after experimentation was granted by the Gauteng Province of South Africa.

Authors’ contribution
M.R., C.S., M.F.B., and S.J.S. designed the study. C.S. and M.P planned the sampling. C.S. carried out the animal sampling. M.R. extracted DNA from samples and generated 16S RNA gene data, and initial data analysis. All authors participated in data interpretation and drafting the manuscript.

References


Co-authorship statement
3A. Co-authorship statement

All papers/manuscripts with multiple authors which is part of a PhD thesis should contain a co-author statement, stating the PhD student’s contribution to the paper.

1. General information

PhD student

<table>
<thead>
<tr>
<th>Name</th>
<th>Michael Roggenbuck</th>
</tr>
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<tbody>
<tr>
<td>Civ.reg.no.</td>
<td>180185-3427</td>
</tr>
<tr>
<td>(If not applicable, then birth date)</td>
<td></td>
</tr>
<tr>
<td>E-mail</td>
<td><a href="mailto:MichaelRoggenbuck@bio.ku.dk">MichaelRoggenbuck@bio.ku.dk</a></td>
</tr>
<tr>
<td>Department</td>
<td>Section of microbiology, Department of Biology</td>
</tr>
<tr>
<td>Principal supervisor</td>
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<th>Name</th>
<th>Søren Johannes Sørensen</th>
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<tbody>
<tr>
<td>E-mail</td>
<td><a href="mailto:SJS@bio.ku.dk">SJS@bio.ku.dk</a></td>
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</table>

2. Title of PhD thesis

Characterization and manipulation of the reticulated microbiome in vertebrates

3. This co-authorship declaration applies to the following paper/manuscript:

Title: The murine lung microbiome in relation to the intestinal and vaginal bacterial communities

Author(s): Barfod KK*, Roggenbuck M*, Hansen LH, Schjørring S, Larsen ST, Sørensen SJ & Krogfelt KA

Journal: BMC Microbiology;

Vol/13

DOI: 10.1186/1471-2180-13-303

4. Contributions to the paper/manuscript made by the PhD student

What was the role of the PhD student in designing the study?

The PhD student is shared first author

KKB conceived and designed the study, carried out the animal work and DNA extractions, and drafted the manuscript. MR did the 16S data generation, analysis and participated in the design of the study and manuscript. SSC performed the cultivation and bacterial identification. KAK, LHH, STL and SJS participated in design and coordination and helped to draft the manuscript. All authors read and approved the final manuscript.
### The PhD School of Science

#### 4. Contributions to the paper/manuscript made by the PhD student

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<td>How did the PhD student participate in data collection and/or development of theory?</td>
<td>MR did the library generation, data generation, analysis, analysis</td>
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<tr>
<td>Which part of the manuscript did the PhD student write or contribute to?</td>
<td>Participated in data analysis and drafting the manuscript.</td>
</tr>
<tr>
<td>Did the PhD student read and comment on the final manuscript? Yes</td>
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#### 5. Material in the paper from another degree / thesis:

Articles/work published in connection with another degree/thesis must not form part of the PhD thesis. Data collected and preliminary work carried out as part of another degree/thesis may be part of the PhD thesis if further research, analysis and writing are carried out as part of the PhD study.

<table>
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<th>Does the paper contain data material, which has also formed part of a previous degree / thesis (e.g. your master's degree)?</th>
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Please indicate which degree / thesis: ______________________________________________________________________________________

Please indicate which specific part(s) of the paper that has been produced as part of the PhD study:

The characterization of the lung microbiome is the focus of this publication and one of the central sample sites of this PhD thesis.

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The co-author statement should always be signed by the first author, the corresponding/senior author and the PhD student. If there are two or three authors the statement must always be signed by them all.

#### 6. Signatures of co-authors:

<table>
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<tr>
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<td>Kenneth Klingenberg Barfod</td>
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<td>12/09-2014</td>
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<td>[Signature]</td>
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<td>250814</td>
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<td>Karen Angeliki Kroghfelt</td>
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<td>11.09.14</td>
<td>Michael Roggenbuck</td>
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When completed, please send the form to the PhD secretary at the department.

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3A. Co-authorship statement

All papers/manuscripts with multiple authors which is part of a PhD thesis should contain a co-author statement, stating the PhD student’s contribution to the paper.

1. General information

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2. Title of PhD thesis

Characterization and manipulation of the reticulated microbiome in vertebrates

3. This co-authorship declaration applies to the following paper/manuscript:

Title: Vitamin D impact of the lung microbiota and OVA allergy in mice

Author(s): Michael Roggenbuck, Kenneth Barfod, Søren Johannes Sørensen, Shelley Gorman

Manuscript in draft: In preparation

4. Contributions to the paper/manuscript made by the PhD student

What was the role of the PhD student in designing the study?

The PhD student performed the majority of the data collection, analysis, and paper writing in this study. Michael Roggenbuck, Shelley Gorman and Kenneth Barfod designed this study together. Shelley Gorman conducted the animal experiments and is the corresponding author of the manuscript. Søren Johannes Sørensen participated in the data interpretation and helped drafting the manuscript.

All co-authors discussed the results of this manuscript and provided help in writing the manuscript.

How did the PhD student participate in data collection and/or development of theory?

MR did the, DNA extraction, 16S library generation, data generation, bioinformatic and multivariate
4. Contributions to the paper/manuscript made by the PhD student

<table>
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| Did the PhD student read and comment on the final manuscript? | Yes | 

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5. Material in the paper from another degree / thesis:

*Articles/work published in connection with another degree/thesis must not form part of the PhD thesis.

*Data collected and preliminary work carried out as part of another degree/thesis may be part of the PhD thesis if further research, analysis and writing are carried out as part of the PhD study.*

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<td>15.09.2014</td>
<td>Søren Johannes Sørensen</td>
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<tr>
<td>11.09.2014</td>
<td>Shelly Gorman</td>
<td>Confirmed by email</td>
</tr>
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When completed, please send the form to the PhD secretary at the department.

[www.science.ku.dk/english/research/phd/student/contact/departments/]
3A. Co-authorship statement

All papers/manuscripts with multiple authors which is part of a PhD thesis should contain a co-author statement, stating the PhD student's contribution to the paper.

1. General information

<table>
<thead>
<tr>
<th>PhD student</th>
<th>Principal supervisor</th>
</tr>
</thead>
<tbody>
<tr>
<td>Name: Michael Roggenbuck</td>
<td>Name: Søren Johannes Sørensen</td>
</tr>
<tr>
<td>Civ.reg.no. (If not applicable, then birth date) 180185-3427</td>
<td></td>
</tr>
<tr>
<td>E-mail: <a href="mailto:Michael.roggenbuck@bio.ku.dk">Michael.roggenbuck@bio.ku.dk</a></td>
<td>E-mail: <a href="mailto:SJS@bio.ku.dk">SJS@bio.ku.dk</a></td>
</tr>
<tr>
<td>Department: Section of microbiology, Department of Biology</td>
<td></td>
</tr>
</tbody>
</table>

2. Title of PhD thesis

Characterization and manipulation of the reticulated microbiome in vertebrates

3. This co-authorship declaration applies to the following paper/manuscript:

Title: Development of Methane emission from growing lamb fed milk replacer and cream for a prolonged period

Author(s): Haque MN, Roggenbuck M, Kanal P, Nielsen MO & Madsen J

Journal: Journal of Animal Feed Science and Technology (In print)

4. Contributions to the paper/manuscript made by the PhD student

What was the role of the PhD student in designing the study?

Analysis of methanogen distribution between the different feeding groups

How did the PhD student participate in data collection and/or development of theory?

MR did the, DNA extraction, 16S library generation, data generation, bioinformatic analysis and methanogen related statistics.
4. Contributions to the paper/manuscript made by the PhD student

Which part of the manuscript did the PhD student write or contribute to?

Contributed to the methanogen analysis in the material and methods, results and discussion section

Did the PhD student read and comment on the final manuscript? Yes

5. Material in the paper from another degree/thesis:

Articles/work published in connection with another degree/thesis must not form part of the PhD thesis. Data collected and preliminary work carried out as part of another degree/thesis may be part of the PhD thesis if further research, analysis and writing are carried out as part of the PhD study.

Does the paper contain data material, which has also formed part of a previous degree/thesis (e.g. your master's degree)?

Please indicate which degree/thesis:

Yes: No:

Please indicate which specific part(s) of the paper that has been produced as part of the PhD study:

The characterization of the methanogens is the essential part of the rumen microbiome as one of the major investigations of this PhD thesis.

The co-author statement should always be signed by the first author, the corresponding/senior author and the PhD student. If there are two or three authors the statement must always be signed by them all.

6. Signatures of co-authors:

<table>
<thead>
<tr>
<th>Date</th>
<th>Name</th>
<th>Signature</th>
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<tbody>
<tr>
<td>11/03/2014</td>
<td>M. Najmul Haque</td>
<td></td>
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<tr>
<td>11/8/2014</td>
<td>Jørgen Madsen</td>
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<tr>
<td>11/09/2014</td>
<td>Mette Olaf Nielsen</td>
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<tr>
<td>11/9/2014</td>
<td>P. Kanal</td>
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<tr>
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Revised 14 May 2014
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2. Title of PhD thesis

Characterization and manipulation of the reticulated microbiome in vertebrates

3. This co-authorship declaration applies to the following paper/manuscript:

Title: The microbial profile of the rudimentary rumen

Author(s): Roggenbuck M, Haque NMD, Madsen J & Sørensen SJ

Journal: In draft.

4. Contributions to the paper/manuscript made by the PhD student

What was the role of the PhD student in designing the study?

Characterization of diet and host physiology impact of the complex microbial community

How did the PhD student participate in data collection and/or development of theory?

The PhD student did the, DNA extraction, 16S library generation, data generation, bioinformatics analysis, microbial network analysis and multivariate statistics.

Which part of the manuscript did the PhD student write or contribute to?
4. Contributions to the paper/manuscript made by the PhD student

Main author of the manuscript.

Did the PhD student read and comment on the final manuscript? Yes

5. Material in the paper from another degree / thesis:

*Articles/work published in connection with another degree/thesis must not form part of the PhD thesis.*

*Data collected and preliminary work carried out as part of another degree/thesis may be part of the PhD thesis if further research, analysis and writing are carried out as part of the PhD study.*

Does the paper contain data material, which has also formed part of a previous degree / thesis (e.g. your master’s degree)  

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Please indicate which degree / thesis: ________________________

Please indicate which specific part(-s) of the paper that has been produced as part of the PhD study:

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2. Title of PhD thesis

Characterization and manipulation of the reticulated microbiome in vertebrates

3. This co-authorship declaration applies to the following paper/manuscript:

Title: The microbiome of a carrionovore

Author(s): Michael Roggenbuck, Ida Bærholm Schnell Dresen, Nikolaj Blom, Jacob Bælum, Mads Frost Bertelsen, Thomas Sicheritz Pontén, Søren Johannes Sørensen, M Thomas P Gilbert, Gary R. Graves, Lars H. Hansen

Journal: Nature Communications (In revision)

4. Contributions to the paper/manuscript made by the PhD student

What was the role of the PhD student in designing the study?

Michael Roggenbuck and Lars H. Hansen analyzed the microbial survey and are the main authors of this manuscript. Michael Roggenbuck, Lars H. Hansen and M. Thomas P. Gilbert generated the microbial data. Ida Bærholm Schnell and M. Thomas P. Gilbert profiled the mammalian dietary composition. Nikolaj Blom, Jacob Bælum and Thomas Sicheritz Pontén did the metagenomic analysis.
4. Contributions to the paper/manuscript made by the PhD student

The sampling was performed by Gary Graves (wild vultures) and Mads F Bertelsen (zoo samples). Søren Johannes Sørensen helped interpret the data. This project was conceived and designed by Gary R. Graves, M. Thomas P. Gilbert and Lars H. Hansen. All authors have read and understood the manuscript.

How did the PhD student participate in data collection and/or development of theory?

The PhD student analyzed the microbial survey and is the shared main authors of this manuscript.

Which part of the manuscript did the PhD student write or contribute to?

Data generation, interpretation and drafting the manuscript

Did the PhD student read and comment on the final manuscript? Yes

5. Material in the paper from another degree / thesis:

Articles/work published in connection with another degree/thesis must not form part of the PhD thesis. Data collected and preliminary work carried out as part of another degree/thesis may be part of the PhD thesis if further research, analysis and writing are carried out as part of the PhD study.

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The microbial survey.

The co-author statement should always be signed by the first author, the corresponding/senior author and the PhD student. If there are two or three authors the statement must always be signed by them all.

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<td>Michael Roggenbuck</td>
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<tr>
<td>12.9.14</td>
<td>Nikolaj Blom</td>
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<tr>
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<td>Jacob Bælum</td>
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<tr>
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<td>Søren J. Sørensen</td>
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<tr>
<td>12.9.14</td>
<td>Gary R. Graves</td>
<td>Not reachable due to expedition. However, statement is also included.</td>
</tr>
<tr>
<td>12/09-14</td>
<td>Lars H. Hansen</td>
<td></td>
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<tr>
<td>12.9.14</td>
<td>M Thomas P Gilbert</td>
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2. Title of PhD thesis

Characterization and manipulation of the reticulated microbiome in vertebrates

3. This co-authorship declaration applies to the following paper/manuscript:

Title: The giraffe (Giraffa camelopardalis) rumen microbiome

Author(s): Michael Roggenbuck, Cathrine Sauer, Morten Poulsen, Mads F. Bertelsen, Søren Johannes Sørensen
Journal: FEMS, Microbial Ecology
10.1111/1574-6941.12402

4. Contributions to the paper/manuscript made by the PhD student

What was the role of the PhD student in designing the study?

The PhD student designed the study together with the other co-authors.

How did the PhD student participate in data collection and/or development of theory?

MR did the DNA extraction, 16S library generation, data generation, bioinformatic and multivariate analysis.

Which part of the manuscript did the PhD student write or contribute to?

Data generation, interpretation and drafting the manuscript

Did the PhD student read and comment on the final manuscript? Yes

1/2

Revised 14 May 2014
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Yes: 
No: x

Does the paper contain data material, which has also formed part of a previous degree / thesis (e.g. your master’s degree)
Please indicate which degree / thesis: ____________________________
Please indicate which specific part(-s) of the paper that has been produced as part of the PhD study:
The giraffe microbial survey

The co-author statement should always be signed by the first author, the corresponding/senior author and the PhD student. If there are two or three authors the statement must always be signed by them all.

6. Signatures of co-authors:

Date | Name | Signature
-----|------|--------
1/9 - 2014 | Cathrine Sauer, | [Signature]
1/9 - 2014 | Morten Poulsen | [Signature]
1/9 - 2014 | Mads Frost Bertelsen | [Signature]
1/9 - 2014 | Michael Roggenbuck | [Signature]
1/9 - 2014 | Soren Johannes Sorensen | [Signature]

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www.science.ku.dk/english/research/phd/student/contact/departments/
The characterization and manipulation of the reticulated microbiome in vertebrates