Probiotic dosing of *Ruminococcus flavefaciens* affects rumen microbiome structure and function in reindeer

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Abstract  Highly cellulolytic bacterial species such as Ruminococcus flavefaciens are regarded essential for the microbial breakdown of cellulose in the rumen. We have investigated the effect of ruminal dosing of R. flavefaciens strain 8/94-32 during realimentation of starved reindeer (males, n=3). Microbiome function measured as in situ digestion of cellulose and food pellets (% DMD; dry matter disappearance) decreased after probiotic dosing. Microbial community analyses (>100,000 16S rDNA gene sequences for 27 samples) demonstrated that ruminal dosing influenced the microbiome structure; reflected by increased phylogenetic distances from background samples (unweighted UniFrac analysis) and reduced species diversity and evenness. Despite the inability to detect strain 8/94-32 post-dosing, the relative abundance of its affiliate family Ruminococcaceae remained consistent throughout the trial whilst a dominant peak in the genus Prevotella and decline in uncharacterized Bacteroidetes (uBacNR) was observed in treatment samples. No clear relationships were observed between the relative abundance of Ruminococcaceae, Prevotella and uBacNR with cellulose DMD, however Prevotella (negative) and uBacNR (positive) exhibited relationships with pellet DMD. These unexpected effects of ruminal dosing of a cellulolytic bacterium on digestibility, are relevant for other studies on rumen manipulation.

Keywords: metagenomics, Ruminococcus flavefaciens; reindeer; 454-pyrosequencing
Introduction

Reindeer (*Rangifer tarandus tarandus*) in northern Norway are ruminants herded in a pastoralistic system, that mainly graze on natural pastures from which they select a large variety of graminoids, woody plants, lichens and mosses [21]. They experience large seasonal variation in feed quality and abundance, particularly during winter when snow conditions occasionally result in years with poor grazing condition thus restricting natural plants availability [13]. To reduce starvation, Saami pastoral herders provide supplementary feed such as baled grass silage and hay as well as commercially produced pellet concentrate which has become increasingly common in Saami reindeer husbandry [31]. Supplementary feeding of reindeer reduces starvation, but digestion problems still occurs and is putatively inferred to the condition of the animals and their ability to digest and utilise the food provided [14, 22].

In the reindeer rumen functions the resident microbiome contributes to the deconstruction of fibrous feed. Starvation has been shown to effect the rumen microbiome structure [22], leading to our hypothesis that changes in the rumen microbiome following starvation are linked to a reduced rumen fibrolytic capacity. Strategies that combine feeding with the administration of fibrolytic “key species” during realimentation may improve hydrolysis and fermentation of plant polysaccharides and overall health and well-being of the host animal. The rumen contains multiple metabolic niches, and despite the diversity of rumen microbes a limited number of bacterial species is believed to carry out the key function of cellulose deconstruction [32]. Probiotic bacteria are used in the bovine rumen to support productivity in the host organism [16] and combat diarrheal syndromes [1]. In this current pilot study we have investigated whether dosing of cellulolytic bacteria might be used to positively affect the microbial community and the overall microbial fibrolytic activity within the reindeer rumen microbiome during realimentation. We have focused on a proficient cellulose-degrading isolate originating from the reindeer rumen as a candidate probiotic, namely *Ruminococcus flavefaciens* isolate 8/94-32 (Family *Ruminococcaceae*) [30]. Ruminal dosing of fibrolytic *Ruminococcus* to enhance fibre digestion has previously been tested in domestic sheep (*R albus* and *R. flavefaciens*; [17]) and cattle (*R. flavefaciens*; [7]) with varying success. To gain further insight into the impact of bacterial dosing on rumen biomass converting capacity and microbiome structure, and to evaluate the
potential to improve feeding regimes for reindeer in particular, we have conducted a starvation and
realimentation trial with reindeer. The effect of probiotic manipulations on the structure dynamics of
the reindeer rumen microbiome were characterized using 454-pyrosequencing of bacterial 16S rRNA
gene amplicons (V1-V3 region), and changes in the fibrolytic capacity of the rumen simultaneously
monitored using functional assays.

Materials and methods

Animals and diets

Three castrated male reindeer (aged 2 years, initial body mass 63.5-73.5kg) with rumen cannula
(U1039 Cannula Sheep 62mm, Macam Rubber, NSW, Australia) were maintained at the animal
housing facilities at the University of Tromsø, Norway. The animals had been accustomed to handling,
and before the study started they were placed in indoor boxes, where they were kept at temperature
8°C -17°C at simulated natural light-darkness cycles (70°N) during the trials. Animals were treated
against parasites with fenbendazol (Panacur vet., Intervet International B.V., Boxmeer, Netherland),
and the level of parasites was monitored by faecal sample analyses to avoid untreated parasite
infections during trials. The animals were offered commercially produced reindeer feed pellets (grass
meal 35.0%, ground oats 26.0%, ground barley 17.5%, sea weed meal 13.0%, molasses 5.0%,
vegetable fat 2.0%, silage protein concentrate 1.5%; FK Reinfør, Felleskjøpet Trondheim, Norway) all
days, except days of food deprivation (see “Animal experimental procedures”). Fresh water was
available ad libitum at all times.

Animal experimental procedures

All animals received identical treatment throughout the experiment, and samples from the three
animals were processed at the same time. Before the experiments started, measurements were carried
out to monitor the background levels of rumen pH, fibre digestion in the rumen (dry matter
disappearance (DMD); see below) and rumen bacterial composition (Background, before 4 days of
food deprivation and dosing). The experiment was divided into two parts, hereby referred to as Part A
(Control / C) and Part B (Treatment / T), which were conducted consecutively in time only separated
by a four days break during which the animals received water and food ad libitum. Part A of the
experiment included dosing the animals with non-inoculated bacterial medium (placebo), while Part B
included dosing the animals with bacterial suspensions (prepared and administered as described in the
following section). Rumen sampling for 454-pyrosequencing and fibre digestion measurements was
carried out at 09:00 and those for pH-measurements were at 09:00 and 14:00 on each of the time
points indicated in Fig. 1, as described in the following section. Rumenal dosing was carried out
immediately after sampling (Fig.1). The animal experiment was carried out in accordance with
regulations in the Norwegian Animal Welfare Act, and approved by the National Animal Research
Authority of Norway (FOTS ID 375).

Bacterial cultures and dosing
A viable inoculum of R. flavefaciens 8/94-32 was prepared growing the bacteria in pure culture on
liquid M8P medium [29] in an anaerobic chamber (Coy Laboratory Products, Ann Arbor, MI, USA)
with an atmosphere of N₂ (90%), CO₂ (5%), and H₂ (5%). The M8P culture medium contained
pelleted reindeer feed (1g / 100ml) commercially available from FK Reinfør Felleskjøpet, Norway as
substrate for the microbial growth. This was the same feed provided for the experimental animals
allowing the bacteria to adapt to the substrate prior to dosing. The pelleted feed provided in the culture
medium was sterilized by ionizing radiation from a radioactive cobalt source (60Co, dose up to 15.000
Gy/h) at the Institute for Energy Technology (Kjeller, Norway) to avoid contamination. After 72hrs of
growth the bacterial culture was examined by microscope to check for purity and density, centrifuged
(~2500rpm for 10min), and approximately two-thirds of the supernatant was removed to obtain as high
a concentration of the viable inoculum of R. flavefaciens as possible. Each animal was dosed with
250ml of the concentrated bacterial suspension through the rumen cannula immediately after rumen
sampling on the days indicated in Fig. 1.

Rumen sampling and pH measurements
Samples of rumen content were collected through the rumen cannula by suction using a rigid plastic
tube (diameter 15mm) attached to a manual suction device. The plastic tube was inserted several times
at different angles and depths, and the collected rumen content transferred to a beaker in which the
rumen pH was measured using a portable pH-meter (PHM201 with PHC2005 electrode, MeterLab,
Radiometer Analytical, France) immediately after sampling. Rumen samples were kept on ice until
they were transferred into an anaerobic chamber (within 40min after sampling) where subsamples
(approx. 20g) were transferred to vials stored at -80°C until DNA extraction.

Rumen fibrolytic activity

Ruminal capacity to digest fibre was determined by analysing dry matter disappearance (DMD) of the
commercially produced feed pellets and ground cellulose filter paper (Whatman no.1) based on the in
situ nylon bag technique described by Ørskov et al [38]. The filter paper was ground to pass through a
2mm sieve (Cyclotec 1093 Sample mill, Foss tecator, Denmark). Substrates (approx. 2g pellets or 1g
filter paper) were placed in nitrogen-free polyester bags (ANKOM Rumen sampling bags, pore size
50(+/−15)μm, 5 x 10cm; BarDiamond, ID, USA) and dried overnight at 45°C, before bags were
weighed, labelled and sealed (Super-Seal Bag Sealing Machine, Jencons Scientific, PA, USA). DMD-
measurements were carried out in triplicates, with three bags containing ground Whatman filter paper
(cellulose DMD) and three bags containing pellets (pellet DMD) incubated in the rumen of each
animal at each time point. The bags were attached to a thin nylon tube at intervals of approx. 8cm
distance (one string of each substrate), and incubated for 24 hours. After incubation, the bags were
washed in a washing machine (without detergent) at 40°C (“wool program”, without centrifugation)
and dried at 45°C for 48 hours, before re-weighing. Negative controls were generated by placing bags
in dH₂O for 24 hours, after which the bags were treated identically to the incubated bags. The DMD is
the difference in dry weight before and after ruminal incubation, corrected for the change in DM
observed in the negative controls. At days 3, 5, 12 and 16, one to three in situ bags were ripped and
hence excluded from the calculations (see Footnote in Fig.2).

Cell harvesting and DNA extraction

Cells from the liquid phase and particle fraction of whole rumen content samples were harvested from
sample aliquots (approx. 0.75-0.85g) in a protocol that included washing with DDS buffer (35) and
spinning, based on a procedure outlined in Kang et al [15]. In brief, whole rumen contents were
resuspended in DDS buffer and subjected to low speed centrifugation. From the supernatant, the
dissociated cells from the particle fraction along with cells in the liquid fraction were harvested and
concentrated by high-speed centrifugation. The dissociation and harvesting steps were repeated four
times for each sample to increase the amount of cells harvested. Harvested cells were then washed
with 10mM Tris-HCl, 1M NaCl, before extraction of DNA as described by Rosewarne et al [25] with
only minor alterations. Lysis of cells was carried out by incubation in lysis buffer (RBB+C lysis buffer
[37] at 70°C for 20min with gentle mixing and no beating, followed by precipitation with CTAB
(cetyltrimethyl ammonium bromide) buffer. The mixture was then treated with chloroform, followed
by treatment with phenol / chloroform / isoamyl alcohol, before DNA was recovered using
isopropanol precipitation. DNA was stored at -20°C until further analysis.

454-pyrosequencing
The variable region V1-V3 of the 16S rRNA gene of bacteria was amplified using the forward primer
(5'- CCT ATC CCC TGT GTG CCT TGG CAG TCT CAG CAA CAG CTA GAG TTT GAT CCT
GG -3'), which contains the 454 Life Sciences primer B sequence and the broadly conserved bacterial
primer 27F, and the reverse primer (5'-CCA TCT CAT CCC TGC GTG TCT CCG ACT CAG NNN
NNN NNT TAC CGC GGC TGC T -3'), which contains the 454 Life Sciences primer A sequence, the
broadly-conserved bacterial primer 515R and a unique 8-nt multiplex identifier used to tag each
amplicon [13] (Table S1). PCR amplifications were carried out in 50µl reaction volumes containing
25µl iProof High-Fidelity DNA Polymerase (Bio-Rad, Hercules, CA, USA), 1µl of each primer, 1µl
of DNA, and 22µl sterile water. The PCR conditions were as follows: initial denaturation at 98°C for
1min, followed by 25 cycles of 30sec at 98°C (denaturing), 45sec at 55°C (annealing), 45sec at 72°C
(extension), and a final extension of 7min at 72°C. Amplicons were checked for size and purity by
electrophoresis on a 1% agarose gel, and concentrations were measured using Quant-iT dsDNA BR
Assay Kit (Invitrogen, Carlsbad, CA, USA). All PCR products were mixed in equal molar amounts,
and the pooled sample was run on a 1% agarose gel, from which the pooled PCR product band was
excised and purified using the NucleoSpin Extract II kit (Macherey-Nagel, Düren, Germany). The
454/Roche GS FLX sequencing was carried out using the LIB-A chemistry at the Norwegian High-
Throughput Sequencing Centre (Oslo, Norway). Sequences have been deposited in the NCBI
Sequence Read Archive (SRA) under the accession number SRA051253 and BioProject 89735.

16S rRNA gene sequence analysis

The 16S rRNA gene sequences were processed using the QIIME software package [5] and removed
from the analysis if they were <350 or >550nt in length, contained ambiguous bases, had a mean
quality score <25, contained a homopolymer run exceeding 6 nt, or did not contain a primer or
barcode sequence. Error-correction, chimera removal and operational taxonomic unit (OTUs)
clustering was performed using USEARCH quality filtering with QIIME, which incorporates
UCHIME [10] and a 97% sequence identity threshold. Prior to comparison of 16S rRNA gene
sequences within different sampling points, each dataset was randomly “subsampled” using QIIME to
normalize each dataset and remove sample heterogeneity (1075 sequences were used for each animal
at each time-point). Evenness was measured using the Simpson index in QIIME. The most abundant
sequence in each OTU was chosen as the representative sequence. Representative sequences were
length of 150 and a minimum identity of 75%. Taxonomy was assigned to each OTU using the
Ribosomal Database Project (RDP) classifier [8] with a minimum support threshold of 80% and the
RDP taxonomic nomenclature. The alignment was filtered to remove gaps and hypervariable regions
using a Lane mask, and a maximum-likelihood tree was constructed from the filtered alignment using
FastTree [24]. Phylogenetic trees were used to measure Phylogenetic Distance (PD), and construct
unweighted UniFrac distance matrices [19] which were visualised using principal coordinates analysis.
Network maps were used to visualize correlations (Pearson’s correlation) between genera with more
than 0.5% relative abundance and environmental variables (pH, cellulose and pellet DMD). This
previous filtering step removed poorly represented genera and reduced network complexity. Linear
regressions were used to test the relationships between alpha diversity metrics and pH with pellet
DMD.
Results and Discussion

Dosing impacts changes in rumen microflora function and pH

Dosing of R. flavefaciens did not increase the digestibility during the bacterial suspension led to a decrease in the cellulolytic DMD (0.116% DMD, p = 0.03, 95% CI [7.6, 0.3]) and pellet DMD (0.05 %, p = 0.005, 95% CI [1.1, 1.2]) after the last ruminal dosing (Day 10) of each part of the trial (Fig. 2a and b). In a study with repeated ruminal dosing of R. flavefaciens to cows by Cique et al. [7] reports that the effect of dosing on digestibility is dependent on diet, with no change in digestibility observed for animals on a high-concentrate diet, and an increase in digestibility of animals on a high-concentrate diet (a level similar to that of high-forage-fed cows). Thus, although previous studies report absent or limited improvement in digestibility.

To determine whether animal manipulations in the form of feed-deprivation and ruminal dosing

affected rumen pH levels, measurements were taken at all sample points throughout the study (Fig. 1). Ruminococcus species to adult sheep fed low-quality Rhodes grass. Kruse et al. [7] reports the effect of dosing on digestibility is dependent on diet, with no change in digestibility observed for animals on a high-concentrate diet, and an increase in digestibility of animals on a high-concentrate diet (a level similar to that of high-forage-fed cows). Thus, although previous studies report absent or limited improvement in digestibility.

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resulted in an additional moderate drop in pH of approx. 0.6 from control levels, and the lowest
recording was five days after previous findings which measured lowest pH levels 1-2 days after
realimentation commences (Fig. 2c). Ruminal pH differed between the two food-deprivation periods
(Dep-C/T) being ~ 0.4 units lower during this period in the control trial (Fig. 2c). The observed
differences in pH suggest that the rumen microbial community may have been different at the
beginning of the placebo dosing and the bacterial dosing. Hence, changes in the microbial
communities may consequently not only have been caused by the dosing itself but partly also by
temporal fluctuations in the microbial community as reflected in the pH (Fig. 2c).

Rumen microbiome structure influenced by dosing

Community analysis was carried out based on a total of 108250 non-chimeric 454-generated reads.
The decrease of species evenness in Dos-T samples was presumably caused by a pronounced increase
in the genus *Prevotella* that approximately doubled in relative abundance during the period (>50% of
the total community: Fig. 2d). Comparison of alpha and beta diversity metrics illustrated that rumen
manipulation in the form of bacterial dosing influenced rumen microbiome structure. Measurements of
Phylogenetic Distance (PD) (the sum of all branch lengths in a 16S rRNA gene phylogenetic tree:
[12]) indicated that highest sample diversity was calculated for the background sample, whilst PD
decreased to its lowest levels during probiotic treatment and to a lesser extent during control dosing
periods (Fig. 2e). Post-dosing PD measurements showed that the diversity returned to levels similar to
those observed prior to dosing, in both the control and the treatment experiment. Species evenness (the
relative abundance of different species in the community) measured via the Simpson’s index was also
observed to be lowest during dosing with bacteria (Fig. 2f), after which it recovered to pre-dosing
levels.

The factors driving phylogenetic variation in microbial communities between different samples was
also determined via principle coordinate analysis (PCoA) of unweighted UniFrac matrices [18] (Fig.
3). Analysis of the total microbiome community showed that samples taken during probiotic dosing
(Dos-T) exhibited the largest distance from background samples and thus greatest change in
phylogenetic structure (Fig. 3a). Interestingly, control dosing (Dos-C) caused similar phylogenetic
variance in two of the three animals, suggesting the process of dosing administration regardless of the presence of the probiotic is the predominant source of variation in the data. Fig. 3a also inferred clustering by individual animal (e.g. Animal_09), highlighting that distinct lineages are present in each animal and are a source of variation. Inter-animal variation in microbiome structure, has previously been observed in other young [18] and adult ruminants [3, 33], even when fed the same diet and receiving the same treatment. Unweighted UniFrac matrices were additionally calculated with animals collectively considered at each time-point to accommodate for inter-animal variation and make the effects of control vs. treatment more evident (Fig. 3b). Analysis based on pooled microbiota confirms that distinct microbiome disturbances are caused by the act of dosing administration, in addition to revealing a clear response of the microbiome in the subsequent periods after dosing (pDos1-2). The microbiome community in control samples changed directionally until it returned to a structure more-similar to the background and feed-deprived states. Similar directional patterns were also observed in treatment samples albeit with an overall greater distance from background samples, further highlighting the greater variation caused by probiotic dosing. Interestingly both pDos-C-2 and pDos-T-2 clustered in close proximity, suggesting the recovery of microbiome community structure in response to dosing with either a placebo or probiotic was similar.

Comparison of OTUs against the Ribosomal Database Project demonstrated that the reindeer rumen microbiota was dominated by the phyla Bacteroidetes (54.5-77.6%) and Firmicutes (18.5-35.7%) (Fig. 4), which exhibits compositional consistency with previous metagenomic analysis described from the Svalbard reindeer rumen microbiome [23]. Family level analysis showed that Prevotellaceae (24.2-67.5%), and Ruminococcaceae (3.7-19.4%) dominated each sample, as did uncharacterised groups affiliated to the phylum Bacteroidetes and the orders Bacteroidales and Clostridiales (Fig. 4). There was a strong influence of ruminal dosing on the composition of different microbial lineages (Dos-T in Fig. 4). The most evident changes in relative abundance were an increase in Prevotellaceae (predominantly the genus Prevotella; Fig. 2d) and decrease of uncharacterised Bacteroidetes phytotypes (uBacNR). Interestingly the relative abundance of the Ruminococcaceae, the affiliate family of the dosed R. flavefaciens strain, remained at a similar level throughout the experiment (Fig. 2d). However all R. flavefaciens strain 8/94-32 –affiliated OTUs were below detection levels post-
dosing (Table S2), suggesting that the introduced bacterium did not persist in the rumen 72 or 96 hours after dosing. Absolute numbers for OTUs affiliated to *R. flavefaciens*, *R. albus* and *F. succinogenes* indicate dosing did not stimulate other closely related cellulolytic strains (Table S2).

Relating microbiome structure to function and pH

To gain insight into how community structure relates to function, we investigated for relationships between bacterial diversity and composition towards cellulose and pellet DMD. No clear correlation between diversity (PD) or evenness and cellulose DMD was observed (R: 0.30, p: 0.1 and R: 0.31, p: 0.1 respectively). However communities with higher diversity and evenness functioned more efficiently with respect to pellet DMD (Fig. 5a-b). Observations linking higher phylogenetic variation and evenness to improved community efficiency have been previously recognized in anaerobic biogas reactors [34, 36]. Both metrics are believed to play an important role in preserving the functional stability of microbial ecosystems via a higher capacity to use redundant functional pathways that enable more efficient responses to environmental changes [34]. A similar effect may be applicable to the reindeer rumen microbiome, an anaerobic ecosystem that exhibits similar metabolic properties to a biogas reactor (i.e. fibre hydrolysis and gas production) and which experiences environmental changes that affects microbial composition and functions.

Correlation analysis deduced that no relationships were observed with dominant *Bacteroidetes* genera and cellulose DMD, however a positive correlation was observed between uncharacterized members of the *Veillonellaceae* (Fig. 5d), a *Firmicutes*-affiliated family that has not been previously associated with known cellulolytic bacteria. The genera *Ruminococcus* and *Fibrobacter* had no observed relative abundance relationships with cellulose DMD. This result was not entirely unexpected given that the relative abundance of all OTUs exhibiting similarity (>97%) to known cellulolytic species *Fibrobacter succinogenes*, *R. albus* and *R flavefaciens* were all on the lower limit of detection and collectively amounted to less than 0.4% (Table S2).

Based on pellet components it is expected that high levels of protein, starch, soluble sugars and plant polysaccharides including cellulose and hemicellulosic substrates such as beta-glucans, arabinoxylans were readily available to the resident microbiome. The relative abundance of *Bacteroidetes*-affiliated
lineages displayed both negative (Prevotella) and positive (uBacNR) relationships with pellet DMD (Fig. 5d). The Bacteroidetes are specialized in the breakdown of complex plant polysaccharides and uncharacterised lineages have previously been described in the rumen of Svalbard reindeer that harbour enzymatic capabilities towards plant polysaccharides such as beta-glucans and arabinoxylans [20]. Negative associations between Prevotella and pellet DMD align with earlier ruminant studies that detect high levels of Prevotella simultaneous with negative correlations towards DMD [6]. Both findings are somewhat surprising since rumen Prevotella spp. have a renowned metabolic versatility, capable of utilizing a wide variety of proteins, peptides and monosaccharides as well as plant polysaccharides including starch, hemicellulose, cellulodextrins and pectin [2]. Firmicutes-affiliated genera Butyribrio and Pseudobutyribrio both demonstrated positive relationships with pellet DMD. Representatives for both Butyribrio and Pseudobutyribrio have been isolated from the rumen of Norwegian reindeer and demonstrate activity against xylan and carboxymethyl-cellulose [30].

Surprisingly no significant relationships were observed between Prevotella and pH (R: 0.356, p: 0.067) which was at its highest relative abundance levels when pH was lowest (Fig. 2c), although the relative abundance of its affiliate family Prevotellaceae did correlate positively to pH (R: 0.528, p<0.01). An important phenotype of ruminant Prevotella spp. is their remarkable capacity to grow efficiently at relatively low pH values [27]. Overall, negative correlations were observed between pH and pellet DMD (Fig. 5c) which is in agreement with in vitro and in vivo studies that indicate that fibre digestion can be severely inhibited by even modest declines in ruminal pH [26]. Cellulolytic rumen bacteria including R. flavefaciens, F. succinogenes and R. albus as well as the efficient saccharolytic bacterium Butyribrio fribrisolvens are highly sensitive to drops in pH [26]. In this study, all genera that correlated positively to pellet DMD had negative relationships to pH including the dominant uBacNR group and Butyribrio (Fig. 5d). Although only moderate, more acidic ruminal conditions were associated with dosing and could explain the lower digestibility of cellulose and dry matter in the pellet feed. Moreover the interconnections between dosing, pH and high Prevotella remain to be elucidated i.e. does treatment dosing cause a drop in pH thus providing a niche for
Prevotella to dominate, or does dosing result in a community shift towards Prevotella dominance whose fermentative metabolism cause pH levels to decline?

Probiotic dosing does not stimulate R. flavefaciens levels or DMD in the reindeer rumen

A probiotic effect of dosed fibrolytic bacteria through increased fibre digestion is dependent on the dosed bacteria being able to establish in the rumen, even if only for a short time during realimentation.

Previous attempts of Ruminococcus spp. dosing to adult ruminants has repeatedly demonstrated that the introduced bacteria rapidly declines post-dosing or does not persist. In the current study the animals were food deprived prior to dosing administration, which is an important difference in conditions from previous dosing studies. Viable cell counts of rumen contents from reindeer have previously shown that starvation for four days reduces the total viable populations of anaerobic bacteria in the rumen fluid by as much as 99.7% and bacteria adhering to the rumen solids by 95.9% [39]. A severe reduction in rumen microbes could result in “open ecological niches” to colonize, analogous to the immature rumen where rumen probiotic studies have experienced success [7, 16].

However, community analysis shows that the starvation period did not result in an unstable microbial community with only minimal changes in phylogenetic structure observed (Fig. 3). Functional studies confirmed a limited DMD effect during starvation periods (Fig. 2) and rumen function did not seem severely depressed. The latter is supported by the observation that the animals did not show any signs of diarrhoea during realimentation. Since the reindeer in this study were managing surprisingly well with the food deprivation, it is possible the opportunities for introduced bacteria to establish in the rumen were limited.

In conclusion, probiotic bacteria and their influences in combating digestive disorders in humans and animals have been previously demonstrated, albeit with inconsistent measures of success in ruminants. Although this current probiotic effort to enhance feed fermentation in reindeer during realimentation did not succeed to expectations, the community-wide analysis enabled an opportunity to measure microbiome structural responses to rumen manipulation via dosing. 16S rRNA gene analysis illustrates that dosing of a fibrolytic bacterium drives phylogenetic variance within reindeer rumen
microbiome structure, which ultimately results in reduced rumen biomass saccharolytic capacity.

Interestingly the introduction of a gram-positive \textit{Firmicutes}-affiliated bacterium largely impacted on

dominant gram-negative \textit{Bacteroidetes}-affiliated bacteria including uBacNR and \textit{Prevotella}.

Uncharacterised uBacNR was positively correlated to pellet DMD, which reinforces previous

metagenomic analysis that infer the major involvement of deeply branched \textit{Bacteroidetes} in fibre

hydrolysis in the reindeer rumen. Given the dominance of \textit{Prevotella} in instances of lower pellet DMD

we speculate \textit{Prevotella} metabolism to be directed towards utilizing soluble sugars and proteins in

instances of rumen disturbance (i.e. dosing). Finally, UniFrac data suggests that ruminal manipulations

via cannula entry cause disruptions in the microbiome structure irrespective of placebo or treatment
dosing. Our results, therefore, suggest that future rumen studies that incorporate cannula-based

experiments need to consider, during the design and analysis stages, the potential for this process to

impact the structure and function of the rumen microbiome.

\textbf{Acknowledgments} The authors thank Prof. Arnoldus Schytte Blix for help with the rumen fistulation of the

animals, Hans Edwin Lian for all help with animals, Alexandra Heuer for help with sampling during animal

experiments, and Prof. Michael Greenacre for help with DMD statistics. This study is linked to the framework of

the International Polar Year (IPY) as part of the consortium IPY \# 399 EALAT: \textit{Climate change and reindeer

husbandry}. Funding was provided by the Reindeer Husbandry Research Fund, University of Tromsø, Roald

Amundsen Centre for Arctic Research (University of Tromsø) and Centre for Sami Studies (University of

Tromsø). PBP is supported by The Research Council of Norway’s FRIPRO program (214042) and the European

Commission Marie Curie International Incoming Fellowship (PIIF-GA-2010-274303).

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**Titles and legends to figures**

**Figure 1** Animal experimental protocol. All animals underwent identical conditions and sampling procedures at all time. The experiment was divided into two periods, “Part A: Control” (C) and “Part B: Treatment” (T), that included four days of food deprivation, eight days of realimentation and placebo (= medium) or bacterial dosing, respectively, followed by 12 days of feeding post-dosing. Part B was carried out four days after Part A was finished after a four-day break period. Except for dosing content, un-inoculated media in the control period (Dos-C) and a suspension of *R. flavifaciens* strain 8/94-32 in the treatment period (Dos-T), experimental treatments, sampling and measurements were identical in both periods.

**Figure 2** Rumen functionality parameters and microbiota community characteristics during the feeding trial. (a,b) Dry matter disappearance (DMD) (n=3) of (a) cellulose (grinded Whatman filter paper) and (b) feed pellets measured using the *in situ* nylon bag technique. (c) Rumen pH (n=3). (d) Relative abundance of *Prevotella* and *Ruminococcaees* assigned OTUs in the rumen microbiota (n=3). (e) Phylogenetic Distance (PD) (n=3). (f) Evenness (Simpsons index) (n=3). Values are given as means with error bars representing SD. a mean of 8 measurements; b mean of 7 measurements; c mean of 6 measurements.

**Figure 3** Phylogenetic distances between samples depicted by principal coordinate analysis (PCoA) of unweighted UniFrac similarities. Samples from reindeer rumen were obtained from animals (n=3) during different feeding regimes and dosing treatment. (a) PCoA including all OTUs. (b) PCoA including OTUs pooled for all animals (n=3) at the various time points. Back., background; Dep-C, food deprived in control; Dos-C, placebo dosing in control; pDos1-C, first post-dosing in control; pDos2-C, second post-dosing in control; Dep-T, food deprived in treatment; Dos-T, dosing of bacteria; pDos1-T, first post-dosing in treatment; pDos2-T, second post-dosing in treatment.

**Figure 4** OTU-based community composition of rumen microbiota of reindeer (n=3) based on 454-pyrosequencing sequences assigned to OTUs at a 97% identity level. Bubble size denotes the relative
abundance (percentage) of the most abundant bacterial families at each time point. Phyla affiliation is indicated in parenthesis. B: *Bacteroidetes*, F: *Firmicutes*, P: *Proteobacteria*. Back., background; Dep-C, food deprived in control; Dos-C, placebo dosing in control; pDos1-C, first post-dosing in control; pDos2-C, second post-dosing in control; Dep-T, food deprived in treatment; Dos-T, dosing of bacteria; pDos1-T, first post-dosing in treatment; pDos2-T, second post-dosing in treatment.

**Figure 5** The relationships between diversity metrics, metadata and the relative abundances of dominant bacterial genera. (a-c) Linear regressions were used to test the relationships between alpha diversity metrics, pH and pellet DMD. Panels depict the relationships. PD: Phylogenetic Diversity (d) Correlation network showing the relationships between different bacterial genera and DMD (cellulose and pellet) and pH within the reindeer rumen. Nodes with a significant Pearson’s correlation ($p<0.05$) are connected by an edge with values indicated: positive correlations are displayed in blue, negative correlations in red. Node sizes of the different phylogenetic groups reflect their relative abundance. uBacNR: unclassified *Bacteroidetes* group.

**Supplementary Information**

**Table S1** 454-pyrosequencing statistics.

**Table S2** Absolute counts for reindeer rumen OTUs affiliated to known cellulolytic strains.
| N days | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 |
|-------|---|---|---|---|---|---|---|---|---|----|----|----|---|---|---|---|---|---|---|---|----|----|----|
| Food deprived (Dep-C) | | | | | | | | | | | | | | | | | | | | | | | | |
| Placebo dosing (Dos-C) | | | | | | | | | | | | | | | | | | | | | | | | |
| Post-dosing (pDos-C and pDos2-C) | | | | | | | | | | | | | | | | | | | | | | | | |
| Break | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 |
| Food deprived (Dep-T) | | | | | | | | | | | | | | | | | | | | | | | | |
| Bacterial dosing (Dos-T) | | | | | | | | | | | | | | | | | | | | | | | | |
| Post-dosing (pDos-T and pDos2-T) | | | | | | | | | | | | | | | | | | | | | | | | |
| Dosing of bacteria | X | X | X | | | | | | | | | | | | | | | | | | | | | |
| Placebo dosing | | | | | | | | | | | | | | | | | | | | | | | | |
| Rumen pH | X | X | X | X | | | | | | | | | | | | | | | | | | | |
| DMD measurements | X | X | X | X | | | | | | | | | | | | | | | | | | | |
| 454-pyrosequencing | X | X | X | | | | | | | | | | | | | | | | | | | | | |

Figure 1
Figure 5

(a) Relationship between P and Pelt DMD: $R = 0.50$, $p = 0.008$

(b) Relationship between S and Pelt DMD: $R = 0.45$, $p = 0.019$

(c) Relationship between T and Pelt DMD: $R = 0.69$, $p < 0.001$

(d) Network analysis of bacterial taxa and environmental parameters:
- **Firmicutes**
- **Bacteroidetes**
- **Spirochaetes**
- **Unclassified**

Environmental parameters:
- Positive correlation ($p<0.05$)
- Negative correlation ($p<0.05$)

Percentage levels:
- 0.5-1%
- 10-15%
- >30%

Bacterial taxa:
- Prevotella
- uBacNR
- Butyryrivio
- Treponema
- Pseudobutyryrivio
- Anaerovax
- Unclassified Veillonellaceae
Supplementary Material S2
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