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## Identifying active rumen epithelial associated bacteria and archaea in beef cattle divergent in feed efficiency using total RNA-seq

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### ABSTRACT

To date, the role of ruminal epithelial attached microbiota in cattle feed efficiency is undefined. In this study, we aimed to characterize transcriptionally active bacteria and archaea attached to the rumen epithelial wall and to determine whether they differ in cattle with varied feed efficiency. RNA-sequencing was performed to obtain the rumen epithelial transcriptomes from 9 of the most efficient (low RFI) and 9 of the most inefficient (high RFI) animals. The bacteria and archaea 16S rRNA transcripts were identified using an in-house developed pipeline, enriched from filtered reads that did not map to the bovine genome. Archaea from unclassified genera belonging to the *Euryarchaeota* phylum showed the most activity on the rumen epithelium of low RFI ( $81.3 \pm 1.9\%$ ) and high RFI ( $76.4 \pm 3.0\%$ ) steers. Bacteria from the *Succinivibrionaceae* family showed the greatest activity of bacteria on the low RFI ( $28.7 \pm 9.0\%$ ) and high RFI ( $33.9 \pm 8.8\%$ ) epithelium. Of the bacterial families, *Campylobacteraceae* and *Neisseriaceae* had significantly greater activity on the low RFI epithelium ( $p < 0.05$ ) and are known to play a role in oxygen scavenging. Greater activity of rumen epithelial attached oxygen scavenging bacteria may provide more optimal feed fermentation conditions, which contributes to high fermentation efficiency in the rumen.

### 1. Introduction

Ruminant animals, such as cattle, contain a large and diverse community of microorganisms within the rumen that consist of bacteria, archaea, protozoa, and fungi (Kamra, 2005). The rumen microbes are often classified into three populations based on their localization: the rumen fluid-associated microbes, the feed particle-associated microbes, and the rumen epithelial wall-associated microbes (McCowan et al., 1978; Olubobokun and Craig, 1990). Among these three fractions, the functions of the liquid-associated and feed particle-associated microbes have been extensively studied. The microbes of these two fractions play a major role in feed degradation and fermentation, which generates a large supply of substrates for the host to utilize (Bergman, 1990). The end products of fermentation, such as hydrogen and carbon dioxide, are converted to methane (CH<sub>4</sub>) by methanogenic archaea that belong to the phylum *Euryarchaeota* (Liu and Whitman, 2008; Morgavi et al., 2010).

Differences in the composition and activity of the rumen microorganisms may contribute to variation in host feed efficiency through their influence on feed digestion, fermentation, and CH<sub>4</sub> production. The feed

efficiency of an individual can be measured by residual feed intake (RFI), which is calculated as the difference between actual feed intake and expected feed requirements for growth and maintenance (Basarab et al., 2003). Animals with low RFI (L-RFI) are considered feed efficient while those with high RFI (H-RFI) are deemed feed inefficient. Feed efficient cattle produce approximately 25% less CH<sub>4</sub> than inefficient cattle (Hegarty et al., 2007) and consume 3.77 kg less feed per day for similar growth and body weight (Basarab et al., 2003). Studies of methanogens within the rumen fluid of cattle differing in feed efficiency, have found that the abundance of total methanogens were not different between RFI groups (Zhou et al., 2009; Zhou et al., 2010). However, the feed inefficient individuals had a more diverse methanogenic community and greater abundance of *Methanosphaera stadtmanae* and *Methanobrevibacter* sp. Strain AbM4 compared to efficient animals (Zhou et al., 2009). When the ruminal fluid bacterial community was assessed, it was discovered that the abundance of particular bacterial phylotypes were associated with feed efficiency (Hernandez-Sanabria et al., 2012). In a latter study comparing the active feed-associated microbiota between L- and H-RFI steers, it was found that both the bacterial

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community and the archaeal community was more active in H-RFI steers compared to that of L-RFI steers (Li et al., 2017). Such variation was proposed to be one of the driving forces contributing to the variation of host feed efficiency.

To date, the previous findings have been solely focused on rumen digesta associated microbiota; and there have been no studies examining the rumen epithelial attached microbes (epimural community) in relation to feed efficiency. It has been found that the predominant bacteria identified on the epithelium belong to the phylum *Firmicutes* (Li et al., 2012). Characterization of epimural bacteria have found they have specific roles in the rumen such as; mucosal protection, urea hydrolysis, epithelial recycling, and oxygen scavenging (Holovska et al., 2002; Cheng and Wallace, 1979; McCowan et al., 1978; Cheng et al., 1979). The hydrolysis of urea from the rumen wall by ureolytic bacteria provides ammonia for microbial protein synthesis, which is a major source of protein for the host. Oxygen scavenging by facultative anaerobes on the epithelium removes oxygen, thereby protecting obligate anaerobes and maintaining optimal fermentation conditions inside the rumen.

Compared to the knowledge of liquid and feed-associated microbiota, that of the rumen epimural microbiota is limited. Chen et al. (2011) reported that the epimural bacteria *Treponema* sp., *Ruminobacter* sp. and *Lachnospiraceae* sp. were associated with subacute ruminal acidosis (SARA) in Holstein dairy cows. Petri et al. (2013) found that in beef cattle, epimural bacteria *Atopobium*, cc142, *Lactobacillus*, *Olsenella*, RC39, *Sharpea*, *Solobacterium*, *Succiniclaticum* and *Syntrophococcus* were associated with rumen acidosis. These studies suggest that both the epimural microbiota and the gene expression within host cells play important roles in maintaining rumen conditions that are more favorable for fermentation. Based on the functions of these microbiota, we speculate that the rumen epithelial attached bacteria could also influence cattle feed efficiency. The objective of this study was to investigate the differences in activity of rumen epithelial attached bacteria and archaea between low and high feed efficient beef cattle. While the previous studies mainly examined DNA samples, the activities of the epimural microbiota have not been examined. Therefore, the RNA level analysis was performed to assess the active epimural microbiota, where the relative abundance of bacterial and archaeal 16S rRNA transcripts obtained from RNA-sequencing (RNA-seq) was used to measure the potential activity of the epimural microbial community.

## 2. Materials and methods

### 2.1. Animals and rumen tissue collection

All experimental procedures were approved by the University of Alberta Animal Care and Use Committee for Livestock (Moore-2006-55) and animals were raised in accordance with the Canadian Council of Animal Care guidelines (CCAC, 1993). At 10 months old, 175 Hereford x Aberdeen Angus hybrid steers at the University of Alberta Roy Berg Kinsella Research Station (Alberta, Canada) were placed under feedlot conditions as described by Kong et al. (2016). Briefly, the growing diet was fed for 90 days and steers had a one-week adaptation period before starting a 90-day period on a finishing diet. Feed intake data was collected throughout the feeding trial using the GrowSafe automated feeding system (GrowSafe Systems Ltd., Airdrie, Alberta, Canada) and the RFI after the finishing diet was used to classify steers as L-RFI (feed efficient;  $RFI < -0.5$ ), medium RFI (M-RFI;  $-0.5 \leq RFI \leq 0.5$ ), or H-RFI (feed inefficient;  $RFI > 0.5$ ) (Nkrumah et al., 2006). Steers were humanely euthanized after the feeding trial and rumen tissue (4-cm<sup>2</sup>) was collected from the central region of the ventral sac. The tissue samples were processed following Chen et al. (2011). Briefly, the tissue was rinsed with sterile phosphate-buffered saline (PBS, pH = 6.8) before being placed in RNAlater solution (Invitrogen, Carlsbad, CA) and stored at -80 °C until further processing. After thawing the rumen tissue, papillae (~80 mg) were obtained using sterile scissors and scalpels. RNA extraction and sequencing were performed on the rumen papillae from

nine extreme L-RFI ( $RFI = -1.40$  to  $-2.33$  kg/day) and nine extreme H-RFI ( $RFI = 1.32$  to  $3.23$  kg/day) steers.

### 2.2. RNA extraction and sequencing

The mirVana kit (Ambion, Austin, TX) was used to isolate total RNA from rumen papillae (~80 mg) according to the manufacturer's instructions. Then the RNA integrity, concentration, and purity of total RNA were measured using the Agilent 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA) and Nanodrop 2000 c spectrophotometer (Thermo Scientific, Wilmington, DE).

Rumen tissue samples of the top ten and bottom ten RFI animals were used for RNA extraction. And only 18 samples (N=9 for each RFI group) with an RNA integrity number greater than 7 were prepared for total RNA sequencing using the TruSeq RNA Sample Preparation v2 kit (Illumina, San Diego, CA) as previously reported (Kong et al., 2016). Briefly, the total RNA was directly subjected to cDNA synthesis without mRNA enrichment step, with the aim to identify host and microbial transcripts simultaneously. The resulting double-stranded cDNA was then subjected to end repair and 3'-end adenylation before ligation of index adapters. Polymerase chain reaction (PCR) (15 cycles) was used to enrich cDNA fragments that have an adapter attached and amplify the cDNA libraries. The cDNA libraries were then validated and quantified with the Agilent 2200 TapeStation (Agilent Technologies) and Qubit fluorometer (Invitrogen, Carlsbad, CA), respectively. Lastly, the libraries were sequenced on the Illumina HiSeq 2000 system at the McGill University and Génome Québec Innovation Centre (Québec, Canada) to obtain high quality paired-end reads (2 × 100 bp; Average quality score ≥ 33). The raw sequencing data have been deposited at publicly available NCBI's Gene Expression Omnibus Database with GEO Series accession number GSE76501 (<http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE76501>).

### 2.3. Identification and quantification of bacterial and archaeal 16S rRNA transcripts

There was a total of  $41,991,418 \pm 6,835,817$  (mean ± SD) high quality, paired reads (i.e. transcripts) generated per sample from RNA-seq that were first aligned to the bovine reference genome, UMD3.1 (Ensembl v83.31) using the software packages TopHat2 (v2.0.9; Kim et al., 2013) and Bowtie2 (v2.1.0; Langmead and Salzberg, 2012). The overall read alignment rate to the bovine reference genome was  $85 \pm 3.72$  % and the transcripts were analyzed by Kong et al. (2016). Forward and reverse reads that could not be aligned to the bovine genome ( $12,905,961 \pm 4,882,393$  (mean ± SD)) were combined to increase the length and then input to SortMeRNA (v1.99-beta; Kopylova et al., 2012) to obtain bacterial and archaeal 16S rRNA reads. The joined 16S rRNA sequences ( $2,687,989 \pm 1,350,507$  (mean ± SD)) were then input into the Mothur program (Schloss et al., 2009) and analyzed using an in-house developed pipeline (Li et al., 2016). As the transcripts could be mapped to different regions of 16S rRNA genes which lead to the over-estimating of the microbes, we used an in-house built regional enriched references for taxonomic classification. Briefly, archaeal 16S rRNA sequences were mapped to an in-house reference based on the V6-V8 regions-enriched rumen-specific archaea database (Janssen and Kirs, 2008) with addition of newly identified phylotypes, and bacterial 16S rRNA sequences were mapped the V1-V3 region-enriched GreenGenes database (version gg\_13\_5\_99; DeSantis et al., 2006). *De novo* chimera detection was conducted using UCHIME (Edgar, 2010) with default settings, and the taxonomic assignment of chimera-removed sequences was performed using the naive Bayesian method with a minimum confidence of 0.8. Bacteria were only classified down to the family level, while archaea were classified to the genus level because the sequences (96-200 bp) were too short to be reliably classified at the lower taxonomic levels. Three animals from each of the RFI groups were removed from further analyses due to their low number of transcripts

detected for bacteria and archaea. The proportion of 16S rRNA transcripts for every bacterial and archaeal phylotype was determined for every sample and the average proportion for each phylotype was calculated for each RFI group. Only phylotypes with an average abundance of 16S rRNA transcripts  $\geq 0.5\%$  in at least one RFI group are described in this study. After filtering low abundance phylotypes, Kruskal-Wallis Test was used to determine statistical significance (FDR adjusted  $p < 0.05$ ) between RFI groups in the relative abundance of 16S rRNA transcripts for each remaining phylotype.

### 3. Results & Discussion

#### 3.1. Identification of microbial transcriptional activity using RNA-seq method

The commonly used DNA-based methods for examining the epimural microbiota can only reveal the composition of the microbial community and not the microbial activity. Therefore, this study examined the epithelial attached microbes using RNA-seq, allowing for the measurement of the transcriptional activity of microbes in relation to feed efficiency. This adds more knowledge about the rumen microbiota in addition to previous studies mainly focused on the rumen liquid-associated and particle-associated microbiota.

Both the archaeal and bacterial community were identified through an RNA-seq approach. There were  $59,166 \pm 35,148$  (mean  $\pm$  SD) bacterial 16S rRNA transcripts (ranged from 17,293 to 96,379) and  $31,999 \pm 20,416$  (mean  $\pm$  SD) archaeal 16S rRNA transcripts (ranged from 10,245 to 84,639) identified per epithelial sample ( $n = 6$  per group). The percentage of the microbial rRNA transcripts of each sample (15–25%) was comparable with Mann et al. (2018) (2.33–7.74%), where a similar approach was also applied to reveal the active microbial community of

the rumen epithelium. The good's coverage of the obtained microbial transcripts of each sample was above 0.90, indicating the assigned transcripts were sufficient for downstream analyses. It should be noted that a very strict filtering criteria (good's coverage  $> 0.90$ ) was applied to ensure the obtained data can fully represent the microbial community, therefore six samples ( $10,058 \pm 4,681$  bacterial transcripts with range 3,683 to 14,120;  $2,460 \pm 1,088$  archaeal transcripts with range 1,255 to 4,334) were removed from downstream analyses due to their low good's coverage. As the samples with lower coverage may lead to biased taxonomy assignments and further influence downstream statistical analyses, they were therefore excluded.

#### 3.2. Identification and quantification of bacterial 16S rRNA transcripts

There were no significant differences in the relative abundance of transcripts between the RFI groups (adjusted  $p > 0.1$ ) for each phylum. Based on the previous studies using DNA-based methods, we hypothesized that the activity of rumen epithelial bacteria from the phylum *Firmicutes* would be the highest since *Firmicutes* was identified as the predominant bacterial phylum on the epithelium (Li et al., 2012). Surprisingly, the *Proteobacteria* phylum had the most abundant 16S rRNA transcripts in both RFI groups followed by *Firmicutes* and *Bacteroidetes* (Fig. 1). There was a higher relative abundance of transcripts from *Proteobacteria* on the L-RFI epithelium ( $54.5 \pm 9.0\%$  (mean  $\pm$  SEM)) than on the H-RFI epithelium ( $43.3 \pm 8.4\%$ ) (adjusted  $p < 0.1$ ). Other phyla detected were *Firmicutes*, *Bacteroidetes*, *Synergistetes*, *Actinobacteria*, and *Cyanobacteria* (Fig. 1). This suggests that although *Firmicutes* had the highest abundance on the epithelium, it is not necessary that they have the highest activity (at RNA level). In the study by Kang et al. (2013), it was also reported that the activity of *Proteobacteria* in the rumen was higher than *Firmicutes* even though *Proteobacteria* had lower

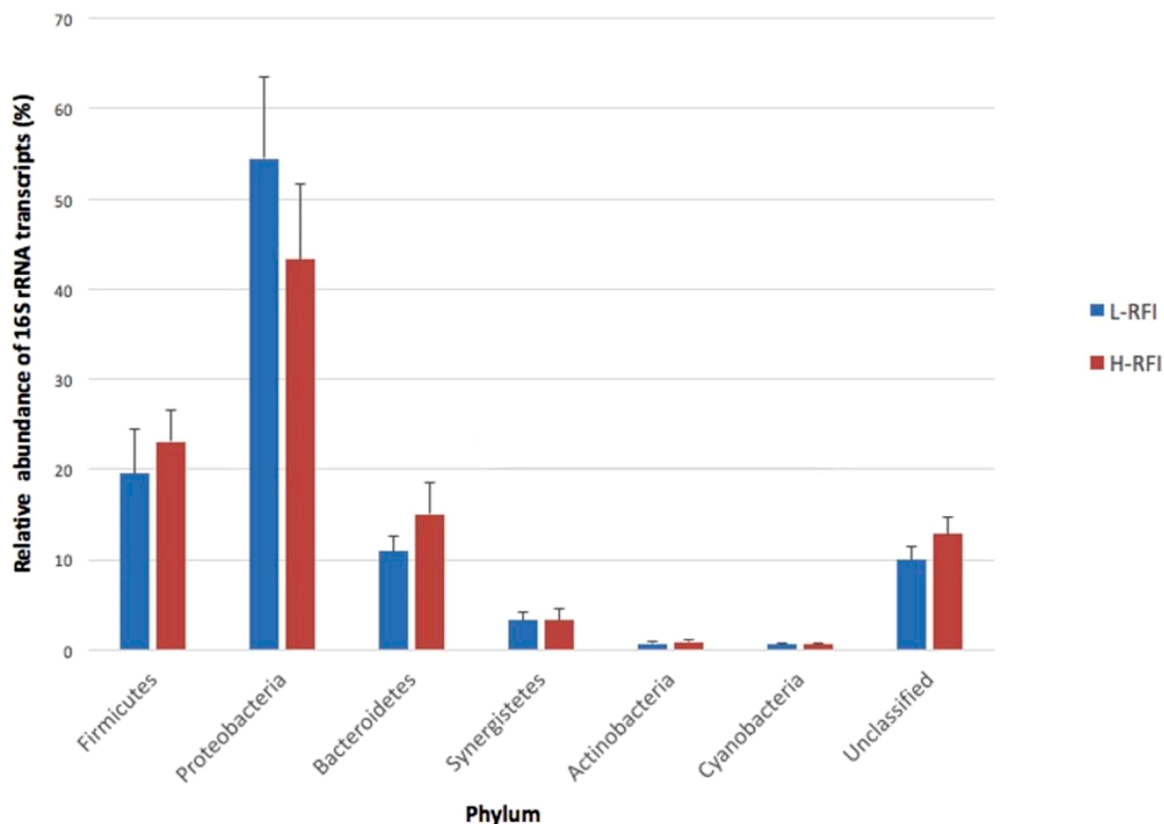


Fig. 1. The relative abundance of 16S rRNA transcripts belonging to each bacterial phylum on the rumen epithelium of L-RFI ( $n = 6$ ; blue) and H-RFI ( $n = 6$ ; red) beef steers. Relative abundance is given as a percentage and data are presented as mean  $\pm$  SEM (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.).

abundance. Although a harsh cell lysis technique was included in the current study, the predominance of *Proteobacteria* of the epimural tissue may be because they are more easily lysed compared to *Firmicutes* (Yuan et al., 2012). The currently available databases contain far more versions of proteobacteria reads compared to other bacterial phyla. As there were large numbers of unidentified reads, they may belong to *Firmicutes* or *Bacteroidetes* or other phyla, yet there is no supporting evidence due to the lack of more in-depth reference databases. However, in our previous study where three microbial profiling methods were compared for the same samples, RNA-seq and RNA amplicon-seq did reflected a *Proteobacteria* predominant community while DNA amplicon-seq showed a *Bacteroidetes* predominant community (Li et al., 2016), which may further support our results that although *Proteobacteria* may not be numerically predominant, they are the most active microbial group within the rumen epithelium. Petri et al. (2020) reported that the rumen epimural microbiota may be affected by the different feed additives offered in animal feed. A recent study by Anderson et al. (2021) claimed that the variation in rumen epimural microbiota can be attributed to multiple factors such as different host species, geographic region, diet, age, farm management practice, time of year, and hypervariable region sequenced, yet a core community was found across studies. Therefore, we speculated that the variation in epimural bacteria between the current study and the other studies may also be due to these multiple factors.

When the bacterial 16S rRNA transcripts were classified to the family level, the identified families included *Campylobacteraceae*, *Comamonadaceae*, *Coriobacteriaceae*, *Desulfobulbaceae*, *Dethiosulfovibrionaceae*, *Erysipelotrichaceae*, *Lachnospiraceae*, *Lactobacillaceae*, *Mogibacteriaceae*, *Neisseriaceae*, *Prevotellaceae*, *Ruminococcaceae*, *Succinivibrionaceae*, *Veillonellaceae*, *S24-7*, *p-2534-18B5*, unclassified *Bacteroidetes*, unclassified *Cyanobacteria*, unclassified *Firmicutes*, unclassified *Proteobacteria*, and families that could not be classified below kingdom bacteria (Fig. 2).

*Succinivibrionaceae*, which belongs to the *Proteobacteria* phylum, had the highest relative abundance of bacterial transcripts on both the L-RFI (28.7 ± 9.0%) and H-RFI (33.9 ± 8.8%) epithelium. Although *Succinivibrionaceae* transcripts were numerically higher in the H-RFI group, it was not statistically significant (adjusted  $p > 0.1$ ). The *Succinivibrionaceae* family contains the genera *Anerobiospirillum*, *Ruminobacter*, *Succinimonas*, and *Succinivibrio* (Stackebrandt and Hespell, 2006). It is known that *Succinivibrio dextrinosolvens* has urease and other nitrogen assimilation enzymes such as glutamine synthetase and glutamate (Patterson and Hespell, 1985). Although the *Succinivibrionaceae* family contains ureolytic species to convert urea to ammonia, the activity of this family does not significantly differ between RFI groups. However, analysis at the genus or species level is needed to examine whether there are differences in the activity of urease producers between L- and H- RFI cattle.

Of the bacterial families, *Campylobacteraceae*, *Neisseriaceae*, and unclassified *Proteobacteria* had significantly greater transcripts on the L-RFI epithelium (adjusted  $p < 0.05$ ) than on the H-RFI epithelium. The L-RFI group had 5.8 ± 1.3% of transcripts from *Campylobacteraceae*, while the H-RFI group had 2.4 ± 0.5%. *Neisseriaceae* contributed 10.8 ± 3.8% and 1.0 ± 0.4% of total transcripts on the L- and H- RFI epithelium, respectively. Unclassified *Proteobacteria* had 5.0 ± 0.8% transcript abundance in the L-RFI group and 2.6 ± 0.5% in the H-RFI group. Both *Campylobacteraceae* and *Neisseriaceae* are *Proteobacteria*. *Campylobacteraceae* contains the genera *Campylobacter* and *Arcobacter*, which are known to colonize mucosal surfaces (Lastovica et al., 2014). Most *Campylobacter* species have oxidase activity that catalyzes the reduction of oxygen to water (Lastovica et al., 2014). *Neisseriaceae*, which contains the genus *Neisseria*, are also known to colonize mucosal surfaces and have oxidase activity (Jurtshuk and Milligan, 1974). Because the *Campylobacteraceae* and *Neisseriaceae* families have oxygen scavenging functions and have significantly higher activity in L-RFI steers, our data

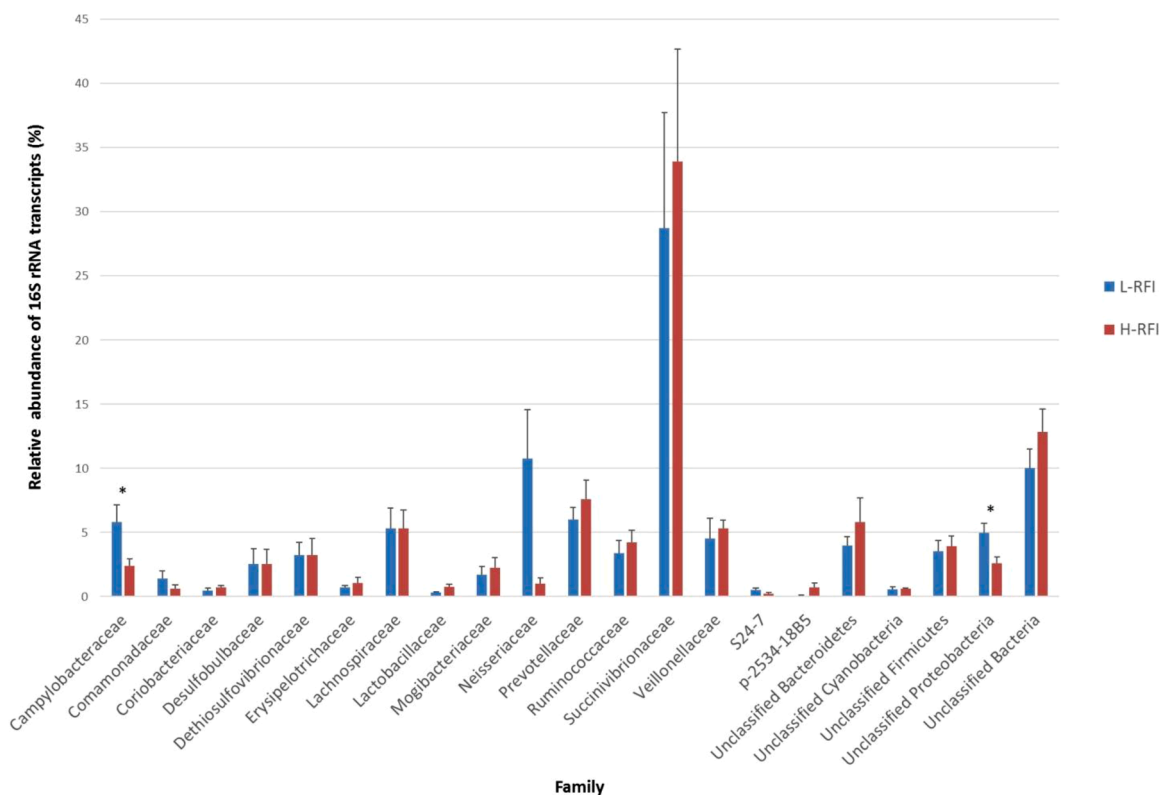


Fig. 2. The relative abundance of 16S rRNA transcripts belonging to each bacterial family on the rumen epithelium of L-RFI ( $n = 6$ ; blue) and H-RFI ( $n = 6$ ; red) beef steers. Relative abundance is given as a percentage and data are presented as mean ± SEM (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.).



suggests that L-RFI animals have better oxygen removal from the rumen and maintenance of the anaerobic condition for fermentation. Optimal fermentation conditions result in effective feed fermentation and may contribute to high feed efficiency by providing an increased supply of energetic substrates for the host to utilize as energy. It is known that the rumen digesta and liquid-associated microbes are obligate anaerobic organisms, any trace amount of oxygen may influence their fermentation capacity within the rumen. The higher activity of oxygen scavenging microbes in the rumen epithelium of L-RFI steers may therefore ensure the strict anaerobic condition in the rumen, thus the content and liquid-associated microbes can be more effective in digesting the feed.

Furthermore, there was significantly lower transcript abundance from *Lactobacillaceae* in the L-RFI group ( $0.3\% \pm 0.0\%$ ) compared to the H-RFI group ( $0.8 \pm 0.2\%$ ) (adjusted  $p < 0.05$ ). *Lactobacillaceae* belongs to the *Firmicutes* phylum, and it includes species that metabolize sugars to lactic acid, which lowers the pH of their environment. This suggests that the H-RFI epithelium may have a greater localized production of lactic acid, which may damage the rumen epithelium and have a negative effect on feed efficiency by decreasing absorptive capacity. In this study, however, we did not check the whole rumen epithelium for damage.

### 3.3. Identification and quantification of archaeal 16S rRNA transcripts

At the phylum level, *Euryarchaeota* and an unclassified phylum were detected from the transcripts (Fig. 3). *Euryarchaeota* contributed to  $89.2 \pm 0.6\%$  (mean  $\pm$  SEM) and  $88.4 \pm 1.0\%$  of the relative abundance of archaeal 16S rRNA transcripts from the epithelial tissue of the L- and H-RFI groups, respectively. The archaeal genera consisted of *Methanopyrus*, *Methanobrevibacter*, unclassified genera from the phylum *Euryarchaeota*, and genera that could not be classified at any taxonomic level below kingdom archaea (Fig. 4). The majority of the archaeal 16S rRNA transcripts were from genera that were unclassified *Euryarchaeota*. They contributed  $81.3 \pm 1.9\%$  and  $76.4\% \pm 3.0\%$  of total archaeal transcripts from the L- and H- RFI epitheliums, respectively. There was also a high relative abundance of transcripts from the archaeal genera that could not be classified below kingdom and from *Methanobrevibacter*. The

unclassified archaea had  $10.8 \pm 0.6\%$  relative transcript abundance on the L-RFI epithelium and  $11.6 \pm 1.0\%$  on the H-RFI epithelium. *Methanobrevibacter* relative transcript abundance was lower on the L-RFI epithelium ( $5.8 \pm 1.5\%$ ) compared to on the H-RFI epithelium ( $9.3 \pm 2.8\%$ ). However, there was no significant difference found between the RFI groups in the relative abundance of 16S rRNA transcripts for any of the archaeal phylotypes (adjusted  $p > 0.1$ ).

Based on the previous studies, Archaea belonging to *Methanobrevibacter* was the most predominant in the liquid and solid phase of rumen microbiota, we hypothesized that this genus of archaea is also the most active in the rumen epimural archaeal community.

It has been found that the most abundant rumen epimural methanogens, as characterized by 16S rRNA gene sequencing (DNA based method), are from the order *Methanobacteriales* and the genus *Methanobrevibacter* (Pei et al., 2010; Seedorf et al., 2015). The active archaeal community represented by the transcripts obtained in the current study differed significantly as compared to the previous studies. Such variation between DNA-level and RNA-level assessment suggests that while *Methanobrevibacter* are highly abundant at the DNA level, they are not the most transcriptionally active archaeal genus on the epithelium. Methanogens from unclassified genera of *Euryarchaeota* might be highly active on the epithelium, indicating that they may be responsible for a majority of the CH<sub>4</sub> production on the epithelium and that methanogenesis may be a major role of epimural archaea. However, their abundance is low compared to their activity. It has been reported that only 25.5% of epithelial associated methanogens are unidentified *Euryarchaeota* based on a clone library method (Pei et al., 2010). Other than methanogens, the unclassified genera belonging to *Euryarchaeota* may also possibly include halobacteria and thermophilic archaea (Gao and Gupta, 2007). The high proportion of unclassified reads in RNA-seq datasets signify the advanced capability of RNA-seq to detect the archaeal community on the epithelium. However, a rumen-specific archaea reference with the most updated data included is necessary for better taxonomically describe the unclassified reads. Further pure culture-based studies need to be conducted to identify the unclassified *Euryarchaeota* to determine which methanogenic genus is the most active on the epithelium and to study its functions. Additionally, we

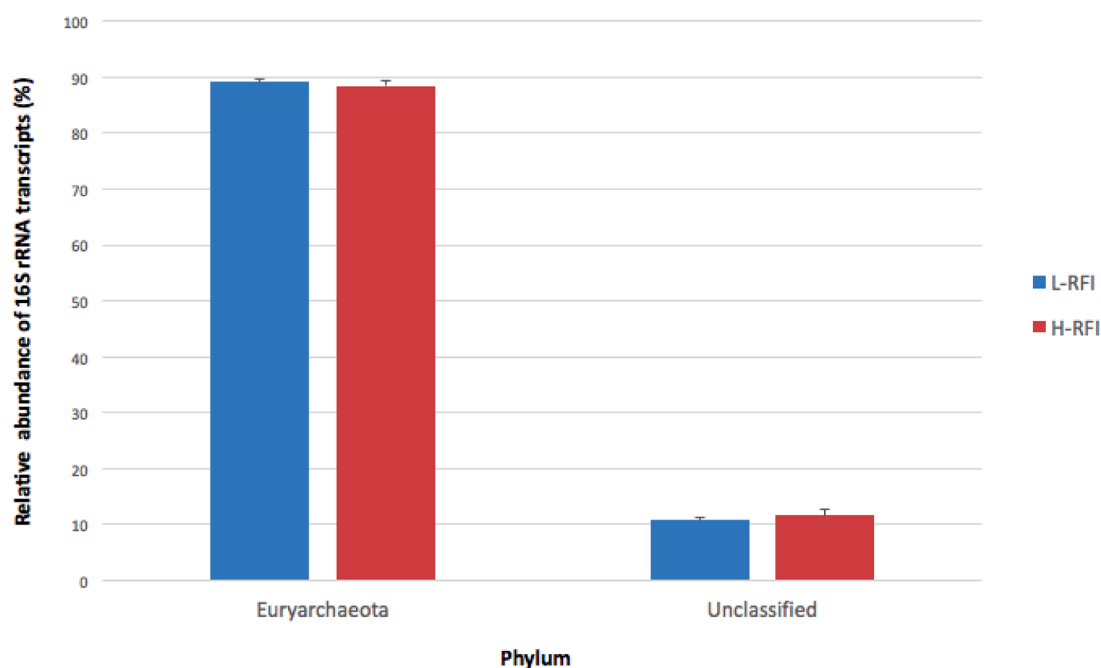
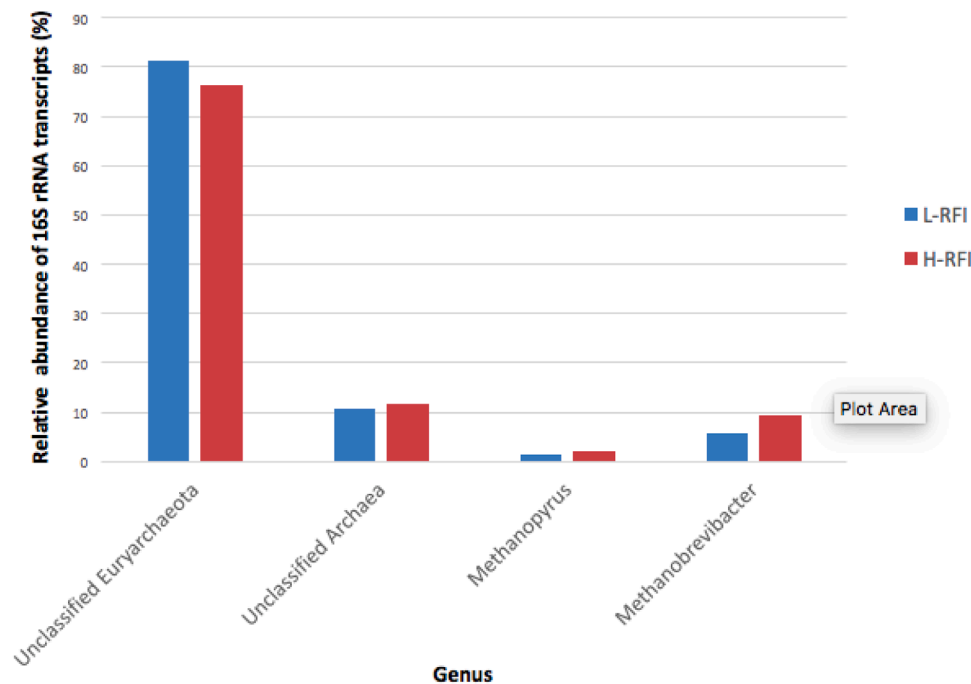


Fig. 3. The relative abundance of 16S rRNA transcripts belonging to each archaeal phylum on the rumen epithelium of L-RFI ( $n = 6$ ; blue) and H-RFI ( $n = 6$ ; red) beef steers. Relative abundance is given as a percentage and data are presented as mean  $\pm$  SEM (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.).



**Fig. 4.** The relative abundance of 16S rRNA transcripts belonging to each archaeal genus on the rumen epithelium of L-RFI ( $n = 6$ ; blue) and H-RFI ( $n = 6$ ; red) beef steers. Relative abundance is given as a percentage and data are presented as mean  $\pm$  SEM (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.).

found that archaea that could not be classified below kingdom level (unclassified archaea) also seem to be quite active on the epithelium (8.2% - 14.2% relative activity), which may have roles other than in methanogenesis. This is the first study detecting a phylum other than *Euryarchaeota* and *Crenarchaeota* in the rumen (Shin et al., 2004; Janssen and Kirs, 2008). One of the limitations of the current study is that owing to the lack of well-annotated rumen-specific archaeal databases, many of the archaeal reads were not able to be assigned to known taxa, making it difficult to clearly address how epimural archaeal community contribute to host RFI. Although the Rumen and Intestinal Methanogen-DB (RIM-DB) developed by Seedorf et al. (2014) may allow better taxa assignment, it has not been updated recently. Therefore, we have adopted the V6-V8 regions-enriched database published by Janssen and Kris (2008) and added newly identified phylotypes as the reference used in this study, aiming to allow a wider coverage of the reads. However, as shown by the data, such amendment is still far from sufficient to provide a clear overview of the archaeal community as our reads were very short and may not overlap or only with a few bp overlap between each other even if they were all from the same regions (V6-V8 regions for archaea). As such, it is necessary to include newly identified rumen methanogen phylotypes into this reference (e.g. an updated version of RIM-DB) so that to allow more precise taxa assignment. Using other marker genes (e.g. *mcrA* gene) for archaeal taxa assignment may be an alternate method to increase the resolution of the archaeal community. However, as archaea/bacteria ratio of 16S rRNA gene was proposed to be associated with methane emissions (Tapio et al., 2017), examining 16S rRNA gene allows further analyses with different purposes. Future work to improve the coverage of the rumen archaea reference database and to enhance sequencing depth to achieve higher archaeal reads may be helpful to identify the unclassified archaeal phylum to determine how they contribute to the functions of rumen epithelium.

Moreover, we had predicted there would be lower methanogen activity on the L-RFI epithelium since L-RFI steers produce less  $\text{CH}_4$  than H-RFI steers, however, we found no significant difference between the L- and H- RFI epithelium in the relative activity of any of the archaeal phylotypes. This suggests that the difference in  $\text{CH}_4$  production between

the RFI groups may be predominantly due to differences in the activity of the methanogens in the rumen content. Therefore, studying the activity of methanogens associated with rumen content may be a superior indicator of methanogenesis.

#### 3.4. Advantage of utilizing RNA-seq for microbial profiling

RNA-seq has been recently adopted for a wide range of studies, such as identifying the microbial community of the rumen epithelial microbiota (Mann et al., 2018), assessing the microbiota in human blood (Loohuis et al., 2018) and measuring the active microbes in soil (Bang-Andreasen et al., 2020). As shown in these studies, RNA-seq can be utilized as an alternative tool for microbial profiling. Since both the active microbiota and all other transcripts of the sample are assessed simultaneously, the obtained microbial data can be directly compared with transcripts of the host species. In the current study, although 100bp PE sequencing platform was used when the experiment was performed, and the reads were shorter compared to the recent sequencing platforms (250bp/300bp PE), all the obtained reads were assembled to contigs prior to processing. Therefore, the taxa assignment was based on the contigs which were long enough to cover multiple variable regions of the 16S rRNA gene of bacteria and archaea. The microbial data obtained in this study, together with our previous findings on host transcriptome of the same tissue samples (Kong et al. 2016) will allow us to perform downstream analyses identifying the host-microbial interactions in the rumen epithelium.

#### 4. Conclusions

Approximately 80% of the archaeal activity on the rumen epithelium was from unclassified genera from phylum *Euryarchaeota*, which emphasizes the need for further studies to classify unknown rumen methanogens. Our study showed that the relative activity of archaeal phylotypes on the epithelium was not significantly different between L- and H- RFI steers, which suggests that rumen epithelial attached methanogens may not contribute to differences in  $\text{CH}_4$  production and variations in feed efficiency. The most transcriptionally active epithelial

attached bacteria were from the phylum *Proteobacteria*, which was unexpected as *Proteobacteria* DNA abundance is not as high as *Firmicutes* on the epithelium (Li et al., 2012). There was no significant difference between RFI groups in the activity of ureolytic bacterial phylotypes; however, we found that the families *Campylobacteraceae* and *Neisseriaceae*, which contain oxygen scavenging bacteria were significantly more active on the L-RFI epithelium. Therefore, L-RFI steers may have more optimal rumen fermentation conditions for feed fermentation and generation of energetic substrates for energy production by the host.

### CRedit authorship contribution statement

**Rebecca S.G. Tan:** Data curation, Formal analysis, Writing – original draft, Writing – review & editing. **Mi Zhou:** Data curation, Writing – original draft, Methodology, Writing – review & editing. **Fuyong Li:** Formal analysis, Methodology, Writing – review & editing. **Le Luo Guan:** Conceptualization, Resources, Software, Supervision, Funding acquisition, Writing – review & editing.

### Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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