*Supplementary Material*

# Metatranscriptomics reveals the active bacterial and eukaryotic fibrolytic communities in the rumen of dairy cow fed a mixed diet

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## Supplementary Methods

**Messenger RNA enrichment by rRNA capture**. Our in house-procedure is based on the widely used rRNA subtractive hybridization principle. In total , 18 capture probes targeting the large (LSU) and the small (ssu) subunit rRNA of all rumen microorganisms (bacteria, archaea, protozoa, fungi) identified at the genus level were designed (Table S5). Ribosomal RNA sequences of good quality were extracted from the SILVA database (RRID:SCR_006423) (Pruesse et al., 2007). The generated rRNA database comprised about 23,000 sequences belonging to 204 rumen genera (Table S6). For each set of sequences (16S, 18S, 23S, 28S), iterative multiple alignments of rRNA sequences were performed in order to highlight conserved regions. Sequences from the same genus were aligned through MEGA 6 (RRID:SCR_000667) (Tamura et al., 2013) using ClustalW (RRID:SCR_008620) (Larkin et al., 2007) and each alignment was manually curated. Alignments of genera were then aligned successively step by step, considering the phylogenetic position of each genus. In this way, 9 master alignments were obtained (one for each target except for bacterial 16S rDNA resulting in two master alignments). Probes were determined in conserved regions using the HiSpOD

software (RRID:SCR_014403) (Dugat-Bony et al., 2011). Default parameters were used, except for probe length, max similarity of the probe and the non-target sequences (% identity) which were fixed to 24 or 25 nt and 75%, respectively. Probes presenting least potential cross-hybridizations with any coding sequences (CDS) and none with CDS from rumen microorganisms were selected. Capture probes were designed in different regions of the rDNA in order to enhance enrichment effectiveness. Enrichment was performed with a starting material of 10 µg of total RNA. Only DEPC-treated solutions and RNase-free material were used. Solutions were those recommended for the Dynabeads Oligo(dT)25 kit (Invitrogen, Thermo Fisher Scientific, Waltham, MA, USA). In the first step, polyadenylated RNA (eukaryotic and some prokaryotic mRNA) were isolated by capture using Dynabeads Oligo(dT)25 following the manufacturer instructions and kept on ice until the end of the procedure. Capture probes polyadenylated at 5' position were added at different concentrations (Table S5) to polyA(-) RNA to allow probes to hybridize to their targets during 20 min at 37°C. Probe-rRNA complexes were captured with Dynabeads Oligo(dT)25 as for polyA(+) RNA. This step was repeated three times. Finally, polyA(+) RNA and prokaryotic mRNA-enriched RNA were pooled and ethanol precipitated. One aliquot of the total RNA sample was also used as a control for RNA degradation by following all the steps without rRNA capture (control RNA). Another aliquot of the same sample was treated using a commercial kit (MicrobExpress, Ambion, Thermo Fisher Scientific, Waltham, MA, USA) according to the recommendations of the manufacturer. Quality of the control RNA and effective removal of rRNA were controlled using the Agilent 2100 Bioanalyzer using RNA Nano Chip (Agilent Technologies, Santa Clara, CA, USA).

The efficiency of the enrichment method was assessed both qualitatively and quantitatively, and compared qualitatively to a commercial kit (MicrobExpress, Ambion, Life Technologies SAS, Saint Aubin, France) specifically designed for the removal of bacterial rRNAs (Figure S3). Our in-house capture method enabled the removal of more than 80% of bacterial and protozoal ssu rRNA and about 33 and 54 % of archaeal and fungal rRNA, respectively to be removed (Table S7). The method was less effective for archaeal and fungal rRNA, possibly due to the designed probes not covering all the diversity of these organisms in the rumen. Nonetheless, mRNA enrichment allowed a doubling of the number of available mRNA reads in the present RNA-seq data (Table S8), without introducing any bias (Pearson's correlation=0.96) (Figure S4). Therefore, the mRNA-enriched RNA sample was retained for functional analysis.

## Supplementary Figures

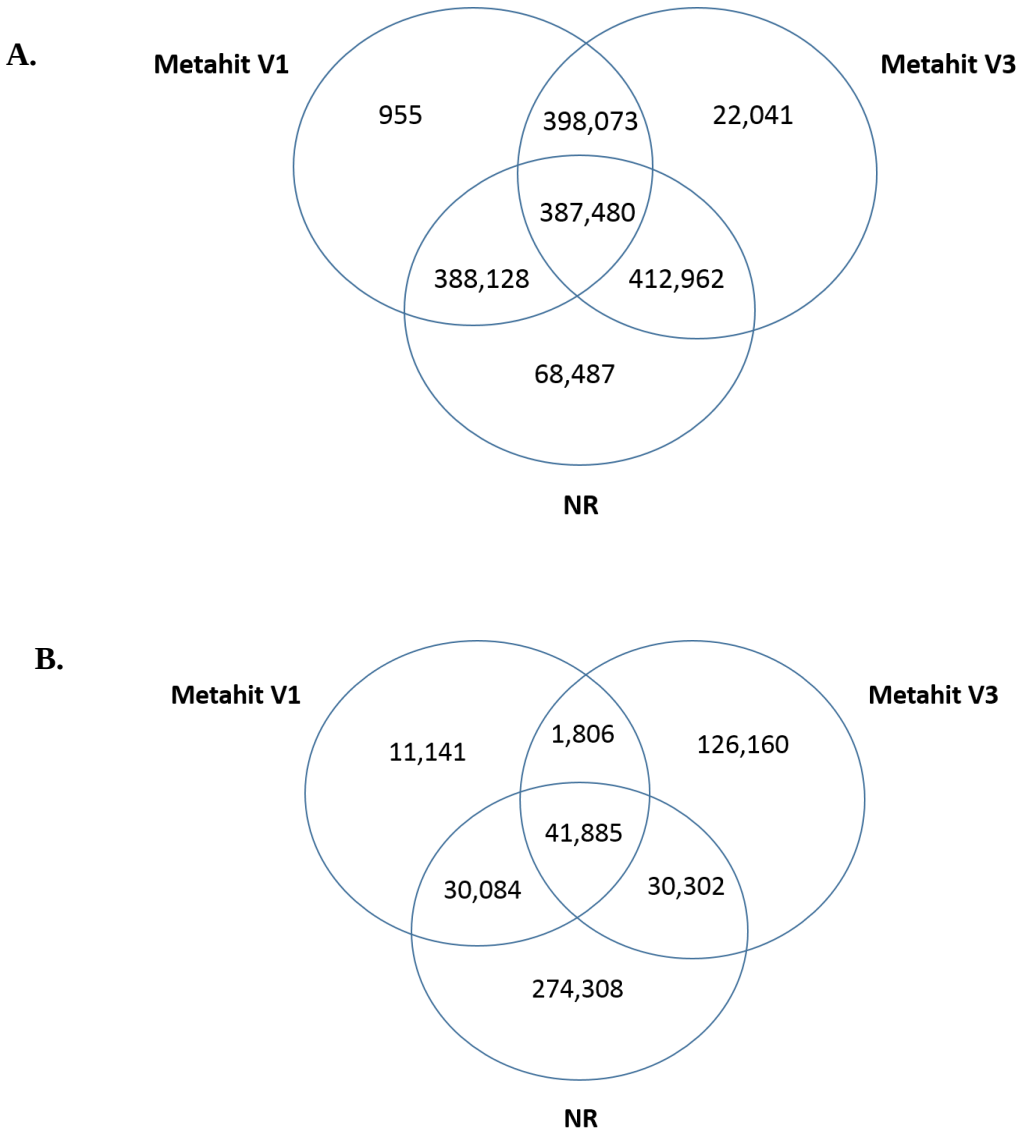
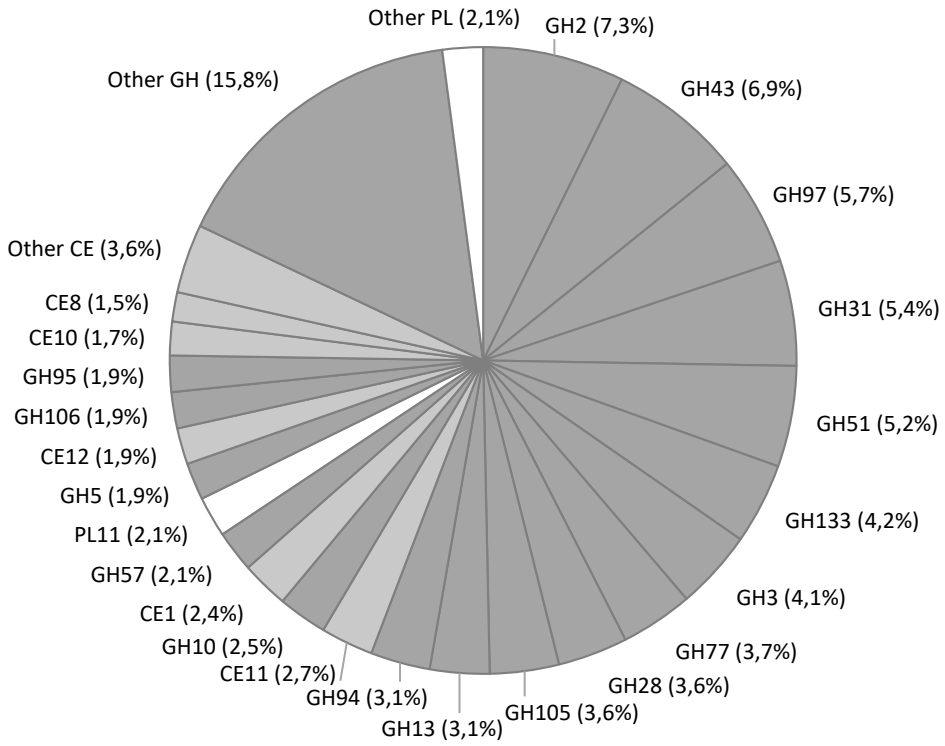



**Figure S1**. Evaluation of using three databases (NCBI non-redundant protein (NR), Metahit V1, Metahit V3) to annotate the present rumen metatranscriptome dataset.

A. Amount of reads giving a BLAST hit with each of the three databases. Overlaps between circles indicate the amount of reads that have led to a match with a sequence from 2 or 3 databases. Non-overlapping zones indicate the amount of reads matching with only one database.
B. Amount of reads giving the best bitscore within each database. Overlaps between circles indicate the amount of reads which have led to a match with a sequence from 2 or 3 databases.

***Prevotella***




***Ruminococcus***

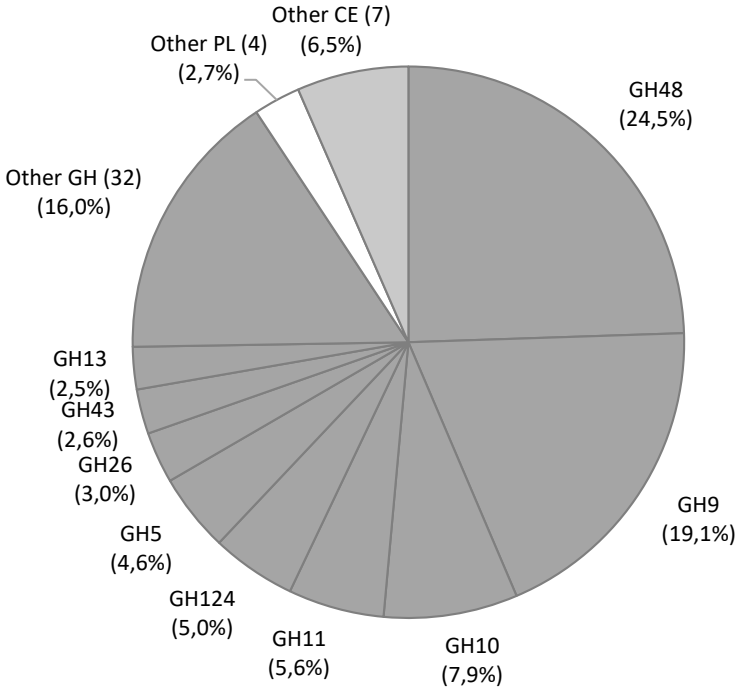



**Figure S2**. Distribution of glycoside hydrolase (GH), carbohydrate esterases (CE), and polysaccharide lyases (PL) transcripts assigned to the main bacterial genera identified (*Prevotella*, *Ruminococcus*, *Fibrobacter*) using the lowest common ancestor method (bitscore>90). The number of families gathered in "other" is indicated in parentheses.

***Fibrobacter***

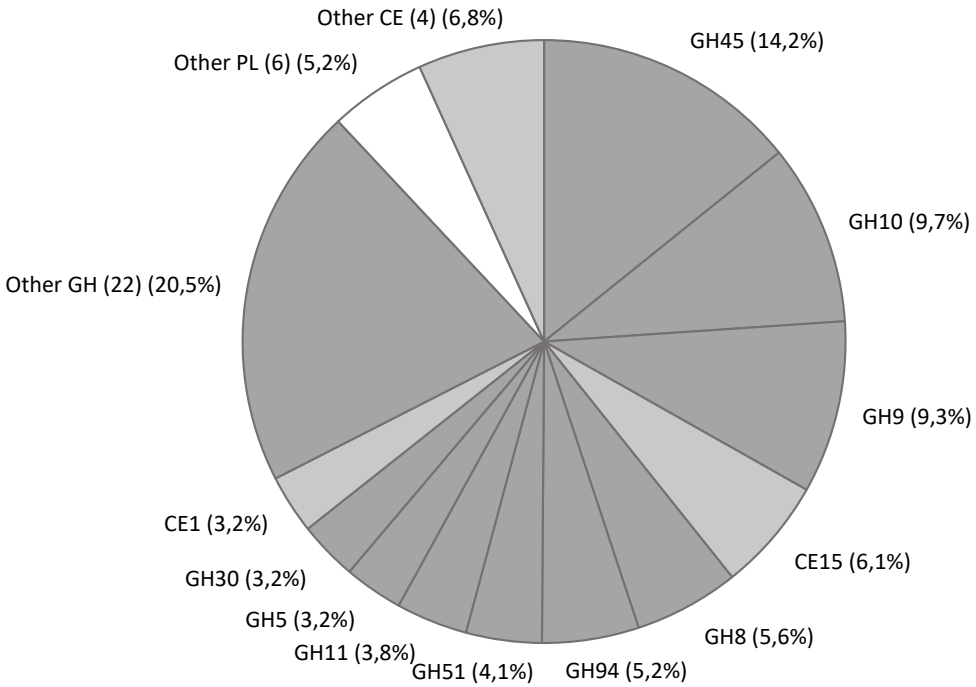



**Figure S2 (continued)**. Distribution of glycoside hydrolase (GH), carbohydrate esterases (CE), and polysaccharide lyases (PL) transcripts assigned to the main bacterial genera identified (*Prevotella*, *Ruminococcus*, *Fibrobacter*) using the lowest common ancestor method (bitscore>90). The number of families gathered in "other" is indicated in parentheses.

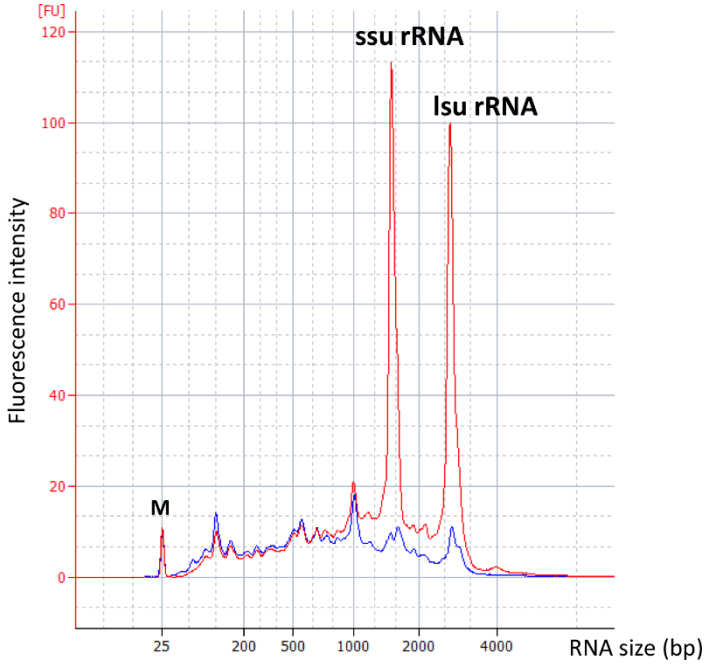



**Figure S3**. Efficiency of mRNA enrichment methods. Electrophoregrams of total RNA treated with the MicrobExpress kit (Ambion, Life Technologies SAS, Saint Aubin, France) (red) or using the in-house method (blue) that indicate improved performance of the later for removing rRNA peaks (ssu rRNA, lsu rRNA). M: internal marker.

Enrichment was performed with the starting material of 10 µg of total RNA for the 2 methods. Resulting mRNA-enriched fractions were resuspended in the same volume and one tenth was used for quality control.

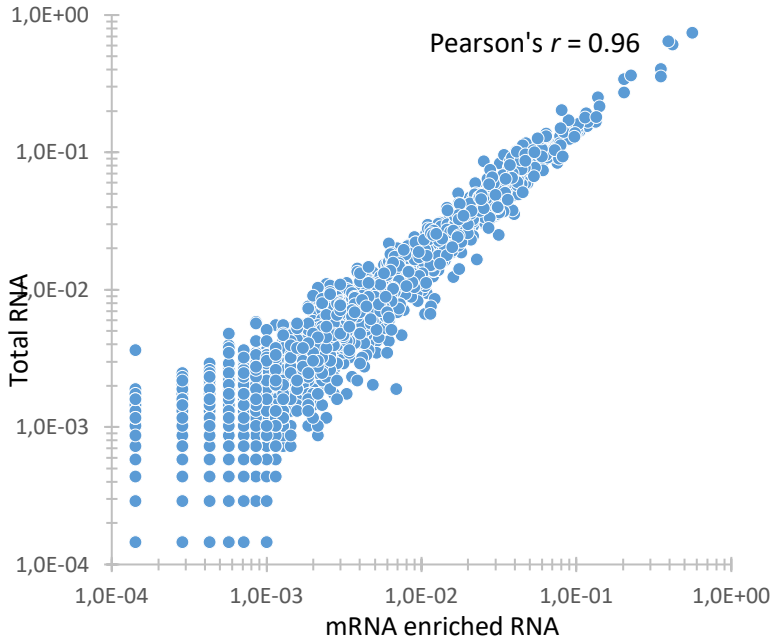



**Figure S4.** Correlation between relative abundances of KEGG Orthology groups observed with the total RNA and the in house mRNA-enriched method. Pearson's correlation r = 0.96.

# Supplementary Tables

**Table S1**. Relative abundances of rumen microorganisms at the family level based on ssu rRNA analysis.

Excel file Data sheet 2

**Table S2.** Glycoside hydrolase (GH), carbohydrate esterase (CE), polysaccharide lyase (PL), auxiliary activities (AA), and carbohydrate binding module (CBM) families identified in non-rRNA reads using the dbCAN software (e-value<1e-05). Substrate, known activities, number of reads corresponding to each family, and their relative abundance are indicated.

Excel file Data sheet 3

**Table S3.** Summary of BLASTX results obtained with the non-rRNA reads annotated as CAZyme by the dbCAN software and their taxonomic binning using the MEGAN software. BLASTX comparisons were performed against the CAZy database (no e-value threshold) and the non-redundant protein database (e-value<1e-05). Only best BLAST hits are reported.

Excel file Data sheet 4

**Table S4.** Improvement of BLAST results of reads detected as CAZymes by dbCAN, using draft genomes of switchgrass adherent bacteria from Hess *et al.* (2011)

| Genome bin | Phylogenetic order | Number of better hits compared with NCBI-NR | Average gain in identity percentage (%) | Minimum gain in identity percentage (%) | Maximum gain in identity percentage (%) |
|---|---|---|---|---|---|
| AC2a | Bacteroidales | 145 | 13.38 | 0.31 | 43.89 |
| AH | Bacteroidales | 101 | 12.41 | 0.6 | 46.57 |
| AGa | Bacteroidales | 87 | 18.00 | 1.00 | 54.47 |
| AJ | Bacteroidales | 65 | 6.23 | 0.03 | 32 |
| BOa | Clostridiales | 51 | 17.52 | 0.89 | 36.17 |
| AIa | Clostridiales | 35 | 10.23 | 0.67 | 39.69 |
| AQ | Bacteroidales | 35 | 16.04 | 0.85 | 42.73 |
| AFa | Spirochaetales | 31 | 7.55 | 0.04 | 37.74 |
| AMa | Spirochaetales | 13 | 7.21 | 1.66 | 25.58 |
| APb | Clostridiales | 13 | 13.90 | 1.85 | 47.06 |
| AN | Clostridiales | 12 | 5.97 | 0.95 | 17.34 |
| AWa | Clostridiales | 5 | 10.84 | 1.67 | 30 |
| AS1a | Clostridiales | 4 | 11.46 | 1.85 | 27.94 |
| ATa | Clostridiales | 2 | 11.14 | 2.22 | 20.05 |

**Table S5.** Statistics of the curated database dedicated to the ruminal ecosystem and the number of targeted genera. ssu : small subunit; lsu : large subunit.

| | | Number of rDNA sequences | | Number of genera |
|---|---|---|---|---|
| | | ssu | lsu | |
| Prokaryotes | Bacteria | 18000 | 4800 | 179 |
| | Archaea | 142 | 34 | 10 |
| Eukaryotes | Protozoa | 36 | 3 | 10 |
| | Fungi | 22 | 27 | 5 |

**Table S6**. Capture probes designed specifically for ribosomal RNA (rRNA) removal from rumen total RNA and the amount added for capture.

| Microorganisms | Target | Capture probe | 5'-3' sequence | Amount per reaction |
|---|---|---|---|---|
| Bacteria | 16S rRNA | 16Sbact514 | HCGTATTACCGCGGCTGCTGGCACG | 20 pmol |
| | | 16Sbact900 | GCTTGTGCGGGYCCCCGTCAATTCC | |
| | | 16Sbact780 | GCGTGGACTACCAGGGTATCTAATC | |
| | 23S rRNA | 23Sbact1050 | TGGCTGCTTCYAAGCCAACATCCT | 24 pmol |
| | | 23Sbact1920 | GACAAGGAATTTCGCTACCTTAGGA | |
| | | 23Sbact2240 | AGTTTGACTGGGGRGGTCGCCTCCT | |
| Archaea | 16S rRNA | 16Sarch830 | CCCCCGCCAATTCCTTTAAGTTTCA | 1 pmol |
| | | 16Sarch1004 | TCGCTCGTTGCCTGACTTAACAGGA | |
| | 23S rRNA | 23Sarch1907 | TACCTTAAGAGGGTYATAGTTACCC | 1.2 pmol |
| | | 23Sarch2572 | CACGACGGTCTAAACCCAGCTCACG | |
| Protozoa | 18S rRNA | 18Sprot522 | CTATTAGAGCTGGAATTACCGCGGC | 0.25 pmol |
| | | 18Sprot1146 | CCACCAACTAAGAACGGCCATGCAC | |
| | 28S rRNA | 28Sprot481 | CCAATCACACCCTAACCGGCTAAGC | 0.3 pmol |
| | | 28Sprot178 | CACACGCTATACGAGGCTTGCACTC | |
| Fungi | 18S rRNA | 18Sfung789 | CATTACTTCGGTCCTAGAAACCAAC | 0.25 pmol |
| | | 18Sfung1060 | AGCCTTGCGACCATACTCCCCCCGG | |
| | 28S rRNA | 28Sfung638 | CTGCAGACCTCAATCTCTCCGATTG | 0.3 pmol |
| | | 28sfung1941 | GGACCGTTAGGAACGCACCGGACAT | |

**Table S7.** Efficiency of the mRNA enrichment method as determined using qPCR and RNA-Seq data.

| | RT-qPCR data<br>rRNA copy numbers/μg RNA ± SD | | | RNA-Seq data<br>rRNA reads (% total initial reads) [(1)] | |
|---|---|---|---|---|---|
| | Before enrichment | After enrichment | rRNA removal (%) | Before enrichment | After enrichment |
| 16S bacteria | $2.83\times10^{10} \pm 6.60\times10^{9}$ | $3.23\times10^{9} \pm 1.51\times10^{9}$ | 88.78 | 92.9 | 85.4 |
| 16S archaea | $2.29\times10^{8} \pm 1.69\times10^{7}$ | $1.51\times10^{8} \pm 2.63\times10^{7}$ | 33.77 | | |
| 18S protozoa | $1.13\times10^{10} \pm 5.64\times10^{9}$ | $2.15\times10^{9} \pm 1.15\ 10^{9}$ | 80.94 | | |
| ITS fungi | $1.13\times10^{6} \pm 1.56\times10^{5}$ | $4.97\times10^{5} \pm 1.72\ 10^{5}$ | 54.79 | | |

qPCR was carried out in triplicate on two different RNA extracted from the rumen sample analysed in the present study. Each value is the mean ± standard deviation for n=6 determinations.[(1)] based on the identification of rRNA with SortMeRNA (Kopylova et al., 2012)

**Table S8.** Statistics of RNA sequencing and reads trimming, merging, and assembling.

| | Total RNA | mRNA enriched RNA |
|---|---|---|
| **Paired-end raw reads (n)** | 24,282,144 | 20,922,080 |
| Total size (nt) | 4,551,466,369 | 3,855,176,363 |
| No. of paired-end reads with Q>30 | 20,746,664 | 18,145,720 |
| **Merged reads (n)** | 9,863,588 | 8,719,248 |
| Average length (nt) | 169 | 168 |
| No. of rRNA reads | 9,163,861 | 7,450,287 |
| No. of non-rRNA reads | 699,727 (7.09%) | 1,268,961 (14.55%) |
| **Contigs (n)** | 12,240 | 23,150 |
| Average length (nt) | 354 | 357 |
| Range length | 201-4,442 | 201-4,616 |

**Table S9.** Oligonucleotide primers used for real-time quantitative PCR

| Microbial groups | Primer sets | Sequence (5'-3') | Fragment length (bp) | Reference |
|---|---|---|---|---|
| Total bacteria | 520F | AGCAGCCGCGGTAAT | 220 | Edwards et al. (2007, 2008) |
| | 799R2cor | CAGGGTATCTAATCCTGTT | | |
| Methanogenic archaea | MB1174f | GAGGAAGGAGTGGACGACGGTA | 233 | Ohene-Adjei et al. (2007) |
| | Arch1406-1389r | ACGGGCGGTGTGTGCAAG | | |
| Protozoa | P-SSU-316f | GCTTTCGWTGGTAGTGTATT | 223 | Sylvester et al. (2004) |
| | P-SSU-539r | CTTGCCCTCYAATCGTWCT | | |
| Neocallimastigomycota | Anaerobic_fungi-F | GAGGAAGTAAAAGTCGTAACAAGGTTTC | 120 | Denman and McSweeney (2006) |
| | Anaerobic_fungi-R | CAAATTCACAAAGGGTAGGATGATT | | |

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