Diversity and Taxonomic Identity of Rumen Bacterial Community in Cattle Fed Different Diets

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Abstract
In this study, the identity of rumen bacterial community and their taxonomic classification in Zebu heifers (n=24) fed 4 different diets (range grasses, Bracharia MulatoII, Azolla and Cassava leaf meal) was surveyed using metagenomics sequencing of the 16SrRNA gene. Rumen liquor samples were collected from the heifers from which a total of 192 DNA samples were amplified and the resulting 16S rRNA sequences compared to the existing sequences at the National Centre for Biotechnology Information (NCBI) BLAST database through the MetagenAssist . Bioinformatics analyses indicated that 17 operational taxonomic units (OTUs) were present at the phylum level. Of these, 43.3% were affiliated to the phylum Firmicutes, 27.2% Bacteroidetes, 22.8% Proteobacteria and 1.7% Euryarchaeota. The remaining were Cyanobacteria (1.4%), Chloroflexi (1%) while Actinobacteria, Verrucomicrobia, Spirochaetes, Tenericutes, Planctomycetes, Elusimicrobia, Lentisphaerae, Armatimonadetes, Fibrobacteres, Synergistetes and Arthropoda were all below 1%. Both Time and Diet had significant effect on the abundance of microbes but did not affect their diversity. Different diets therefore can affect the abundance of rumen microbiome and eventually the performance of animals.

Introduction
The microorganisms in the rumen, have formed a complex ecosystem that is well suited to diverse diets (Clauss et al., 2010, Stevens & Hummes 1998). A symbiotic relationship has developed over time between ruminants and these ruminal microorganisms which enables the digestion of fibrous materials that the animal ingests (Dehority & Orpin1997). As stated by Rawls et al. (2004), the relationships between different microorganisms and the host animals affects their performance. In the rumen, the microorganisms receive substrates delivered through the ingested feeds. Through the process of fermentation, the microorganisms become valuable source of nutrients to the host animal (Mizrahi, 2013). Techniques based on the 16SrRNA have made it possible to analyse complex ecosystems thus allowing for the determination of how diverse and abundant are the communities of microorganisms (Amann et al., 1995). Metagenomics makes it possible to identify uncultured microorganisms, their evolution and functional relationships (Thomas et al., 2012, Sabree et al., 2009). The 16S rRNA gene is an excellent phylogenetic marker (Pace, 1997) as it comprises regions that are highly variable to highly conserved. The differences in sequences of these regions are used to differentiate the microorganisms and determine their phylogenetic relationships. Further, rRNA gene fragments can be obtained without previous cultivation of the microorganisms through the construction of 16SrDNA libraries. This is achieved by amplifying the 16SrRNA, obtained from samples by using polymerase chain reaction (PCR). In this way, a list 16S rRNA genes is then developed. The composition of the microbes can be determined through sequence analysis and comparing the analysis with appropriate reference sequences in databases to infer their phylogenetic affiliation (Illumina, 2013). According to Saro et al. (2012), feeding fibrous materials to ruminants can affect ruminal microbial community and increase ammonia nitrogen (Belanche et al., 2012). Ruminants convert the light energy captured by plants into edible compounds such as milk and meat (Nathani et al., 2015). The present study was carried out to identify and document composition of rumen bacteria of Zebu heifers feeding on a variety of feeds through metagenomics sequencing of 16S rRNA genes.

Methods and Study Site
The feeding trial was carried out at the KALRO Kiboko Research Centre in Makindu Sub County, Makueni County and the laboratory work was done at the International Livestock Research Institute, Nairobi The procedures used in the experiment were approved by the committee in charge of animal care and use at the Kenya Agricultural and Livestock Research Organization.

A completely randomised design of experiment was used in the trial that ran for 14 weeks, during which, twenty four (24) Small East African Short horned Zebu yearling heifers with mean live weight of 109.8 ± 18.4 kg were used. The heifers were randomly assigned into 12 pens with each pen housing two heifers. The 6 dietary treatments were then allocated to two pens each such that each treatment had four heifers as follows:

Treatment 1; Control consisting of a mixture of range grasses hay (composed mainly of *Eragrostis superba*), Treatment 2; Control plus sundried cassava leaves, treatment 3 Control plus sun dried Azolla. Treatment 4 to 6 were similar to treatment 1 to 3 but with the basal diet changed from range land grasses to Brachiaria Mulato II hay. The content of the Crude protein (CP) of the basal feeds was used in determining how much supplement to give to each of the animals according to their live weights, such that the diets provided 16% CP as recommended for growing animals (NRC, 1996).

Rumen fluid was collected from all the experimental animals on the 14th day of the experiment, first in the morning before feeding, and every other hour for 7 hours. The animals were restrained in a crash and a flexible stomach tube inserted through the mouth. An attached suction pump was used to withdraw the samples which were then put in 10 mL cryotubes and placed in liquid nitrogen until used for extraction of DNA. Extraction of DNA was done using the Zymo Soil Extraction Kit (ZYMO Research, USA) following the protocol of the manufacturer. Briefly, 150 uL of the rumen liquor was added to a ZR BashingBead™ Lysis Tube followed by 750 □L of solution for lysis. The Lysis Tube was then fixed in a bead beater which had tube holder assembly (Disruptor Genie™) and followed by processing for 5 min at maximum speed. Thereafter, The same ZR BashingBead™ Lysis Tube was then spinned for 1 min at a speed of 10,000 x g. 400□L of the supernatant was then placed in a Zymo-Spin™ IV Spin Filter and spinned for 1 min at a speed of 7,000 x g. 1,200 µL of buffer for binding was then mixed with the flow through. 800 µL of the mixture was placed in a Zymo-Spin™ IIC Column and spinned for 1 min at a speed of 10,000 x g. This step was repeated after discarding the flow through. Once all the steps had been followed, the eluted DNA quality was confirmed by electrophoresis on a 0.8 % agarose gel and visualized under ultraviolet light.

The primers used for PCR amplification and those for sequencing were similar to those used in a previous study by Caporaso et al. (2010a). According to that study, PCR primers were developed on the basis of the 16S rRNA's V4 region. A 12 – base error correcting Golay code was barcoded to the 806R reverse primer. This supports pooling of up to 2,167 different samples in each lane, and both PCR primers (515F and 806R) contained sequences of the Illumina flow cell adapter regions. The V4 region of the 16S rRNA was amplified using 515F universal primers (5′- AGAGTTTGATCMTGGCTCAG -3′), and 806R (5′- CGGTTACCTTGTTACGACTT-3′) according to Caporaso et al., 2010b. An amplification mixture was prepared consisting of: 10x Dream Taq buffer (2.5µlL, 10mM dNTPs (0.5µL), 10 nM of each primer, DNA template (1µL) and 0.2 µL of DreamTaq polymerase (Thermo Fisher Scientific USA). The amplification was carried out on a Thermo cycler (Applied Biosystems, USA) using the manufacturer's protocol. Amplified fragments were visualized on a 0.8 % agarose gel under ultraviolet light (Fig. 1). The PCR amplicons were purified on a QIAquick DNA Gel Extraction Kit (QIAGEN, CA).

Results

Table 1 shows the results of sequencing amplicons using the Illumina platform described above. In total 19,430,463 reads were generated before quality control. This was reduced to 11,838,743 after the removal of duplicates. When filtered by length and error, 3,149,693 reads remained. These gave rise to 27, 929 OTUs after filtering also by length and error. The bacterial community structure, (which comprises of composition, abundance and diversity) in the samples are shown in Figure 2. Bacterial community composition analysis showed seventeen bacterial Phyla with three; Firmicutes (43.3%), Bacteroidetes (27.2%), and Proteobacteria (22.9%) having a higher relative abundance than other phyla. The other 14 phyla, each represented <2% of all the bacterial sequences namely, Euryarchaeta, Chloroflexi, Cyanobacteria, Actinobacteria, Verrucomicrobia, Spirochaetes, Tenericutes, Plantomycetes, Elusimicrobia, Lentisphaerae, Armatimonadetes, Fibrobacteres, Synergistetes and Arthropoda. The abundance for Firmicutes in the control diet, Azolla, Bracharia, Bracharia mixed with cassava, Bracharia mixed with Azolla and cassava leaf meal alone was 49.8, 44.3, 39.1, 42, 44.7 and 41.5 (% of total sequences, SE = 0.02%), respectively. Proteobacteria was most abundant in cassava leaf meal treated heifers 26.5%, followed closely by Azolla treated heifers at 26%. For the other diets, the abundance of Proteobacteria was 23.9% and 19.2% respectively for Bracharia, and the control diet. The abundance of Bacteroidetes phylum was highest in the Bracharia and cassava leaf meal treated heifers at 30%. This was followed by Bracharia alone, Cassava leaf meal alone, and the control diet at 28.5%, 26.9% and

25.9% respectively. Among the different phyla, there were those whose abundance was significantly affected by the feed used, while others were not affected (Table 2). Proteobacteria, Actinobacteria, Spirochaetes, Bacteroidetes, Euryarchaeta and Armatimonadetes were significantly affected by the feeds used. Bracharia had the most significant effect on the abundance of different phyla followed by Azolla and Cassava leaf meal.

Discussion

At the phylum level, Bacteroidetes and Firmicutes were the most common. This was similar to work by Edwards et al (2004) that showed them to dominant in the rumen. There are other large groups of bacteria that are not classified including Clostridiales, Lachnospiraceae, and, Ruminococcaceae, which are likely to be the bacteria that are most dominant in the rumen. Together, members of Bacteroidetes and Firmicutes are most of the times the most abundant bacteria detected in the rumen by culture-independent methods. In this study the prevalence of Firmicutes was found to be 82.1% which is one of the highest values reported for the rumen. Other studies of reported values of 90.2% and 95% of sequences assigned to Firmicutes in Holstein cows on a high roughage and grain diets, respectively (Tajima et al., 2000), however, most studies reports values are less than 70%.

In addition to the mentioned phyla, Cyanobacteria was also detected in the samples irrespective of diet. This phylum is known for its photosynthetic capability, but recent studies demonstrated the presence of non-photosynthetic members in the stomach of humans and underground water (Di Rienzi et al., 2013). Their analysis demonstrated that the order YS2, present in our data, has many roles such as obligate anaerobic fermentation, fixation of nitrogen, production of hydrogen syntrophically and the manufacture of vitamins K and B. YS2 has been suggested as possibly a new phylum, "Melainabacteria" (Di Rienzi et al., 2013). Other reports have also showed its presence in the gut of mammals (Soo et al., 2014; Zeng et al., 2015). Currently there is limited work describing the Zebu heifers' microbiome through the use of high-throughput sequencing, thus more studies are necessary. Recent work by Pitta et al (2016), showed the abundance of Bacteroidetes, , Firmicutes and Proteobacteria to be at 70%, 15-20% and 7% respectively and this dominance was influenced by diet and age of the cow. Additionally, previous studies by Alzahal et al. (2017) demonstrated (using pyrosequencing on samples collected via the stomach tubing method) that there exists both structural and composition differences in the bacterial microbiome between dry and early lactating cows. Lactating cows had greater proportion of Proteobacteria, lower Firmicutes, and no change in the proportion of Bacteroidetes.

The results showed that the rumen microbes in Zebu cattle have a high diversity and the abundance of these microbes is affected by the feeds consumed. Time after feeding also affected the structure of the bacterial community but not their diversity. Generally, in the world, there is an increasing demand for cattle products as a result of the continued population growth and improving standards of living in the developing countries. There is therefore need to improve the efficiency of feed utilization Rumen microbiome studies can inform identification of new feeding ways to ensure efficient use of feed and improved animal health.

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