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Microbiota composition, gene pool and its expression in Gir cattle (*Bos indicus*) rumen under different forage diets using metagenomic and metatranscriptomic approaches

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ABSTRACT

Zebu (*Bos indicus*) is a domestic cattle species originating from the Indian subcontinent and now widely domesticated on several continents. In this study, we were particularly interested in understanding the functionally active rumen microbiota of an important Zebu breed, the Gir, under different dietary regimes. Metagenomic and metatranscriptomic data were compared at various taxonomic levels to elucidate the differential microbial population and its functional dynamics in Gir cattle rumen under different roughage dietary regimes. Different proportions of roughage rather than the type of roughage (dry or green) modulated microbiome composition and the expression of its gene pool. Fibre degrading bacteria (i.e. *Clostridium*, *Ruminococcus*, *Eubacterium*, *Butyrivibrio*, *Bacillus* and *Roseburia*) were higher in the solid fraction of rumen ($P < 0.01$) compared to the liquid fraction, whereas bacteria considered to be utilizers of the degraded product (i.e. *Prevotella*, *Bacteroides*, *Parabacteroides*, *Paludibacter* and *Victivallis*) were dominant in the liquid fraction ($P < 0.05$). Likewise, expression of fibre degrading enzymes and related carbohydrate binding modules (CBMs) occurred in the solid fraction. When metagenomic and metatranscriptomic data were compared, it was found that some genera and species were transcriptionally more active, although they were in low abundance, making an important contribution to fibre degradation and its further metabolism in the rumen. This study also identified some of the transcriptionally active genera, such as *Caldicellulosiruptor* and *Paludibacter*, whose potential has been less-explored in rumen. Overall, the comparison of metagenomic shotgun and metatranscriptomic sequencing appeared to be a much richer source of information compared to conventional metagenomic analysis.

Introduction

Zebu (*Bos indicus*) is one of the important domesticated cattle species worldwide, especially in tropical countries. *Bos indicus* compared to *Bos taurus* is characterized by its heat tolerance [12], as well as resistance and resilience to parasites [40,73]. India harbours a rich genetic diversity of zebu cattle with 40 breeds currently registered at the National Bureau of Animal Genetic Resources (NBAGR, India). Among these, Gir is one of the best milk producing indigenous cattle breeds [9]. As a result of such profitable qualities, Gir cattle were used initially for improvement of breeds in Brazil [49] and then globally, mainly in African and Southeast Asian countries, as well as the United States [61]. Despite such valued characteristics, scientific studies targeting the microbial diversity and their function in Gir cattle rumen are missing from the literature.

Ruminants fulfil their nutritional requirements through grazing. However, the enzymes required for breakdown of plant constituents are absent in these animals and therefore they rely on the microbial symbionts residing in their rumen, an anaerobic fermentation sack, where breakdown of complex plant polymers occurs by the enzymatic processes of various microbes [23]. The rumen harbours a unique consortium of microbes that has evolved into a complex and efficient system of lignocellulose degradation. This panel of enzymes are collectively known as Carbohydrate-Active

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enZYmes (CAZYmes) and have been studied in cattle and buffalo rumen [20,21,68,74]. However, it is crucial to understand the expression of such enzymes under different dietary regimes, especially in animals that thrive on high roughage diets. Among the microbial symbionts, bacteria contribute much more to ruminal fermentation than fungi and protozoa. The rumen microbial diversity has been extensively studied using amplicon [19,35,41,53,57] and shotgun sequencing approaches [6,52,58,72]. Considering the microbial composition, diet exerts a significant impact on the rumen microbial population [13,14]. Moreover, the bacterial fermentation products in the rumen, especially volatile fatty acids (VFAs), have a direct correlation with milk production [16,63].

Due to certain limitations of amplicon and shotgun sequencing approaches, it is difficult to determine which bacteria are actively engaged in fibre degradation and fermentation, and which genes are transcribed. Metatranscriptome sequencing allows an understanding of the functional dynamics of the microbial communities. To this date, only a limited number of studies focusing on the understanding of actively transcribed genes in rumen microbial populations have been carried out [5,10,55,65]. The energy harvesting capacity of the ruminants depends on specific microbial symbionts [64] and it is also possible to modulate this population for increased feed efficiency [38] and milk production [18,75].

We have previously characterized the rumen microbiome of Kankrej cattle, as well as Jaffrabadi and Mehsani buffalo, fed with different roughage concentrations in the diet using amplicon and shotgun sequencing methods, and demonstrated that the bacterial communities in the liquid and solid fractions of rumen are diverse, and different proportions of roughage in the diet exerted a significant impact on rumen bacterial populations [42,45,48,53]. In the present study, it was further hypothesised that the importance of the role of bacterial species in the rumen would not only be determined by their abundance but how functionally active they were. Therefore, the main objectives of this study were to: (a) understand the active bacterial populations and their dynamics by comparing metagenome and metatranscriptome data from rumen adapted to different roughage proportions in their diet; (b) study the differentially expressed Carbohydrate-Active enZYmes (CAZYmes) during different feed treatments; and (c) identify feed-associated biomarkers of the rumen microbiome.

Materials and methods

Ethics statement

All experimental procedures were approved by the Institutional Animal Ethical Committee of the College of Veterinary Science and Animal Husbandry, Anand Agricultural University, Anand, Gujarat, India *vide* letter no. AAU/GVC/CPCSEA-IAEC/108/2013 dated 05/10/2013.

Dietary treatments and sample collection

The experimental design and nutritive value of feed were similar to that described in our previous studies [42,48,53]. In brief, eight healthy, female, non-pregnant and non-lactating 3–4 year old Gir cattle with an average weight of 250–300 kg were reared at the Livestock Research Station, Anand Agricultural University, Anand, Gujarat, India (Latitude: 22.527413, Longitude: 72.97065). Before commencement of the experiment, the animals were fed with a diet as per National Royal Commission (NRC) [59] standards, India. The animals were divided into two groups with four animals in each group, and one was nourished on green fodder as roughage and the other on dry fodder as roughage both combined with a commercially available concentrate mixture. The composition of the concentrate mixture used was the same as described previously by Parmar et al. [45], except that *Sorghum bicolor* was used as fodder. Briefly, during the experiment, animals were subjected to three dietary treatments comprised of different proportions of roughage and concentrate mixture with access to fresh water. During the first treatment (G1), animals were fed with 50% roughage of the respective fodder (green or dry) and a 50% concentrate mixture; during the second treatment (G2) with 75% roughage and 25% concentrate mixture; and during the third treatment (G3) with only 100% roughage as the diet. This dietary schedule was followed twice a day, in the morning and afternoon. Each dietary treatment continued for six weeks and was then switched to the next treatment. Before starting the next treatment, 500–700 mL of rumen digesta were collected 3 h post-morning feeding using a flexible stomach tube attached to a vacuum pump. The rumen liquor was fractionated into solid (fibre) and liquid components by filtering through two-layered muslin cloth [30]. Each fraction was collected separately into sterile 2 mL cryovials containing 1 mL RNAprotect Bacteria Reagent (QIAGEN, USA), transported to the laboratory at −20 °C, and stored at −80 °C until further processing. A total of 48 samples were collected and further processed for sequencing. A schematic representation of the experimental design is shown in Supplementary Fig. S1.

DNA and RNA isolation

Total DNA from 600 μL liquid or ∼0.5 g solid samples was extracted using the QIAamp DNA Stool Mini Kit (Qiagen, Germany), according to the manufacturer's instructions. A few modifications to the solid fraction were implemented as described in our previous study [45]. DNA quantity and quality were examined using a Qubit 2.0 fluorometer (ThermoFisher Scientific, USA) and gel electrophoresis, respectively. For metatranscriptome sequencing, RNA was extracted using the RNeasy Mini Kit (Qiagen, Germany), according to the manufacturer's instructions but with a few modifications in the solid fraction samples. These modifications have been described in our previous study [15]. The quantity and quality of RNA were estimated using a Qubit 2.0 fluorometer and the RNA 6000 Nano Kit on an Agilent 2100 Bioanalyzer (Agilent Technologies, CA), respectively. Samples with an RNA Integrity Number (RIN) > 6 were further processed for rRNA depletion using RiboMinus™ (bacterial module) (ThermoFisher Scientific, USA) following the manufacturer's instructions.

Metagenomic shotgun and metatranscriptome library preparation and sequencing

A total of 48 samples were subjected to shotgun (referred to as MG here after) and metatranscriptome (referred to as MT here after) barcoded library preparation using a standard 400 bp Ion Plus Fragment Library Kit and Ion Total RNA-Seq Kit v2 (ThermoFisher Scientific, USA), respectively, following the manufacturer's instructions. Approximately 500 ng of DNA and 250 ng of rRNA-depleted mRNA were used for each respective library preparation. The quantity and quality of libraries were determined using a Qubit 2.0 fluorometer (ThermoFisher Scientific, USA) and an Agilent 2100 Bioanalyzer (DNA high sensitivity assay kit, Agilent Technology, CA), respectively. A total of 24 sequencing runs (12 metatranscriptome and 12 shotgun) were carried out on an Ion Torrent PGM (ThermoFisher Scientific, USA) using an Ion 316 chip, wherein each run contained four barcoded libraries that represented biological replicates for each treatment.

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