Indian J Microbiol (Apr–June 2017) 57(2):226–234
DOI 10.1007/s12088-016-0635-z

ORIGINAL ARTICLE

Influence of Diet Composition on Cattle Rumen Methanogenesis: A Comparative Metagenomic Analysis in Indian and Exotic Cattle

Nidhi R. Parmar[1] · Prabhakar D. Pandit[2] · Hemant J. Purohit[2] · J. I. Nirmal Kumar[3] · Chaitanya G. Joshi[1]

Received: 26 September 2016 / Accepted: 16 December 2016 / Published online: 16 January 2017

Abstract Comparative metagenomics approach has been used in this study to discriminate colonization of methanogenic population in different breeds of cattle. We compared two Indian cattle breeds (Gir and Kankrej) and two exotic cattle (Holstein and Jersey) breeds. Using a defined dietary plan for selected Indian varieties, the diet dependent shifts in microbial community and abundance of the enzymes associated with methanogenesis were studied. This data has been compared with the available rumen metagenome data from Holstein and Jersey dairy cattle. The abundance of genes for methanogenesis in Holstein and Jersey cattle came from *Methanobacteriales* order whereas, majority of the enzymes for methanogenesis in Gir and Kankrej cattle came from *Methanomicrobiales* order. The study suggested that by using slow/less digestible feed, the propionate levels could be controlled in rumen; and in turn, this would also help in further reducing the hydrogenotrophic production of methane. The study proposes that with the designed diet plan the overall methanogenic microbial pool or the individual methanogens could be targeted for development of methane mitigation strategies.

Keywords Methanogen · Acetate · Formate · Volatile fatty acids · Roughage

Introduction

Livestock and livestock products play a vital role in livelihood of millions of people in developing countries and are such globally demanding that it is projected to increase by 70% by 2050 [1]. However, methane emission by domesticated ruminants has become an integral issue of greenhouse gas (GHG) control policies [2, 3]. There are several natural sources including anthropogenic ones that contribute to global methane emission. Apart from wetlands which is a natural methane emitting source and contribute 31% of total methane emission, enteric fermentation is the second largest source of methane emission contributing 18% of the total methane emission [2] and in turn loss of 5.5 to 9% dietary energy of host livestock. Methane emitted due to enteric fermentation by ruminants as a part of their normal digestive process is about 37% of the all anthropogenic sources.

Among different species of livestock, cattle play a significant role because of its number and potential. Presently, out of a total 108 million cattle, representing about 12% of the global population, India has the highest number of cattle. It is anticipated that livestock population including lactating dairy cattle and buffalo will increase by 3.5 and 5.6 million, and with the result of that, an expected increase in methane emissions will be of ~36 and 17%, respectively by the year 2021 [3]. The amount of methane produced depends on the type of animal, the kind of feed consumed by the animal, feeding frequencies and type of waste management. Thus, the

Electronic supplementary material The online version of this article (doi:10.1007/s12088-016-0635-z) contains supplementary material, which is available to authorized users.

✉ Chaitanya G. Joshi
cgjoshi@rediffmail.com

[1] Department of Animal Biotechnology, College of Veterinary Science and Animal Husbandry, Anand Agricultural University, Anand 388001, Gujarat, India

[2] Environmental Genomics Division, CSIR—National Environmental Engineering Research Institute, Nagpur, Maharashtra, India

[3] Institute of Science and Technology for Advanced Studies and Research, Mota Bazaar, Vallabh Vidyanagar, Anand, Gujarat, India

increased demand brings challenges in terms of resource usage and food security sector. These interlinked issues pose immense pressure on the maintenance of planet's resources. Cultivation and characterisation of rumen methanogens as well as quantitative estimation of methanogen population diversity in cattle is limited. Only a small information is available on total rumen archaeal species quantification [4]. Thus, for assessing the role of the cattle, the animal feeding and its impact on methanogenesis, one must have to understand the role of the methanogens in the rumen of specific breed of cattle and its comparison with other breeds, the shifts in methanogens abundance with diet given to the animal and the abundance of the enzymes involved in methanogenesis pathway followed by different cattle at given diet. There are several molecular approaches like 16 s r-RNA sequencing, RT-qPCR etc. by which we could identify the methanogens. But, these approaches remain to have pitfalls in explaining the functional capabilities of those identified methanogens. With the advancement in next generation sequencing (NGS) technologies, it has become possible to identify the microbial population harbored by an environment and their functional metabolism for maintaining the environment at greater depth.

The metagenomic approach has been used in the present study to explore the total methanogen population and the associated functional abundance of enzymes involved in the process of methanogenesis. The study uses the experimental design wherein the diet dependent shifts were analysed in Indian cattle breeds (Gir and Kankrej) and it was compared with metagenome data of two exotic breeds i.e. Holstein and Jersey cattle. The exotic cattle dataset included a study outcome by Ross et al. [5] using Australian Dairy Holstein cattle (metagenome ID of Holstein 6803–4519874.3 and Holstein 6859–4520066.3) and publically available dataset of Jersey cattle metagenome on MG-RAST (metagenome ID: 4653877.3).

Materials and Methods

Rumen Fluid Sample Collection from Indian Cattle (Gir and Kankrej)

To carry out an experiment, each of four healthy non-lactating adult Kankrej and Gir cattle (3–4 years old) with an average body weight of 350–400 kg were hired at Sardar Krushinagar Dantiwada Agricultural University and Anand Agricultural University, Anand, India, respectively. The animals were maintained on diet as per NRC (National Royal Commission) standards (India) before the start of an experiment [6]. All eight animals were given three treatments based on different proportions of dry roughage and concentrate. The treatments were given in successive manner where, in first treatment (Gir, G1; Kankrej, K1), all eight animals were fed on 50% dry roughage and 50% concentrate for continuous six weeks; in second treatment (Gir, G2; Kankrej, K2), the mixture proportion was 75% dry roughage and 25% concentrate whereas, in the third treatment (Gir, G3; Kankrej, K3), only 100% dry roughage was given. The details on feed composition for Kankrej cattle and Gir cattle is explained in Table 1. At the end of each treatments of six weeks, the samples were collected on the last day, 2 h after the feeding, using flexible stomach tube [7]. After each collection, the collected rumen fluid was fractionated using a muslin cloth and divided into liquid and solid fractions. In total 48 samples were collected and stored at −20 °C till sample processing.

DNA Extraction and Sequencing

From liquid fraction, 350 µl sample was taken for DNA extraction whereas, in case of solid fraction, 1X PBS buffer was added to the sample followed by vortexing about half an hour at 2500 rpm in order to dislodge the fibre adherent microbiota (in supernatant) and pulse spin (1000 g for 30 s) was performed to minimize the DNA from plant fibres (pelleted at bottom). The resulting supernatant (~300 µl) served as an input for DNA extraction. The DNA extraction from both liquid and solid fraction was carried out using QIAamp DNA Stool Mini Kit from QIAGEN [8] as per the manufacturer's protocol. Total DNA concentration was measured using Qubit® dsDNA HS (High Sensitivity) Assay Kit for better accuracy. Library preparation of 48 metagenomic DNA was carried out by Ion Torrent PGM platform where the DNA fragmentation was followed by adaptor ligation, size selection and further library amplification. All 48 libraries were quantified and their size distributions were checked by

Table 1 Proximate analysis of feed given to Kankrej and Gir cattle

Parameters (%)	Kankrej		Gir	
	Dry roughage	Concentrate	Dry roughage	Concentrate
Moisture	6.31	5.74	19.03	8.35
Crude protein	5.32	20.21	3.30	11.77
Crude fat	1.44	1.87	0.64	2.29
Crude fiber	31	12.57	44.93	16.87
Acid insoluble ash	2.53	3.84	6.74	6.72

High Sensitivity DNA kit on bioanalyzer 2100 of Agilent Technologies. The libraries were diluted up to 26 pM as per manufacturer's recommendation and 12 emulsion PCRs were carried out for 48 metagenome libraries where, four libraries were pooled with equimolar concentration in each emulsion PCR setup. Twelve sequencing runs were carried out using 316 chips on Ion Torrent PGM platform.

Processing of Sequencing Data and Bioinformatics Analysis

The sequencing data belonging to individual treatment given to particular cattle was sorted based on the barcodes given to them and then the data was uploaded to MG-RAST web-server where in screening pipeline, *Bos taurus*, UMD v3.0; was used to screen host specific sequences prior to analysis [9]. Further the annotation was performed for Indian cattle metagenome data and it was compared with exotic cattle (Holstein cattle and Jersey cattle) metagenome data.

Taxonomical and Functional Classification

The metagenome data of Indian cattle and exotic cattle was annotated for obtaining taxonomical classification using M5NR database with 60% identity cut off. The taxonomical classification was obtained at domain level and further bacterial domain classification was done up to class level and archaeal domain classified up to genus level. Principal component analysis (PCA) was performed using PAST (Paleontological Statistics) 3.0 [10] software and network formation was carried out using Cytoscape 2.8.0 [11] to evaluate the differences in cattle breeds based on the methanogens that they harbor. Moreover, the major methanogens were identified and their functional capabilities were derived in terms of enzymes involved in methanogenesis pathways using KEGG database [12]. The methanogens abundance and the abundance of the enzymes for various methanogenesis pathway contributed by specific methanogen at different treatments was also correlated. The analysis of similarity (ANOSIM) was performed to evaluate the differences in methanogenic enzyme abundance among cattle breeds ($P < 0.05$) [13].

Results

Total Microbial Abundance in the Rumen of Indian and Exotic Cattle

Total output of the Indian cattle (Kankrej and Gir) metagenome sequencing is explained in Tables S1, S2, S3, S5, S6 and S7. The MG-RAST id of the sequences pertaining to respective sample is listed in Tables S4 and S8. The phylogenetic classification of the sequences from Indian and exotic cattle was carried out in order to determine the diverse microbial makeup of the rumen in both the cattle. We classified the sequences at domain level using M5nr database. We found that, bacterial domain was the most abundant one with an average of 88% in both the breeds (Gir and Kankrej) of Indian cattle, 90% abundance in Holstein cattle and 84% in Jersey cattle (Fig. S1). Archaea was the second prominent domain with an average of 0.83% abundance in Gir cattle, 0.69% in Kankrej cattle, 5.47% in Holstein cattle and 1.81% abundance in Jersey cattle. Eukaryota and viruses were the least abundant domain found in the rumen fluid of all cattle breeds (Fig. S1). Further bacterial domain was sub classified to get comparative depiction of phylum and class level bacterial abundance among cattle breeds. At phylum level, *Bacteroidetes* was found to be most abundant phylum followed by *Firmicutes* in Gir, Kankrej and Holstein cattle (Fig. 1a). Whereas, in Jersey cattle, *Firmicutes* was most abundant phylum as compared to *Bacteroidetes* (Fig. 1a). The abundance of *Proteobacteria* was found to be more in Gir, Kankrej and Jersey cattle as compared to Holstein cattle. The other phyla such as *Chloroflexi*, *Spirochaetes* and *Actinobacteria* were present at very low abundance (Fig. 1a). At class level, more abundance of *Bacteroidia* and *Clostridia* was found as compared to other classes (Fig. 1b). The major difference among cattle breeds was due to different bacterial classes such as *Cytophagia*, *Flavobacteria*, *Alphaproteobacteria*, *Betaproteobacteria*, *Gammaproteobacteria* and *Deltaproteobacteria*, the abundance of which was found to be less in Holstein cattle whereas as class *bacteroidia* dominates comparative to other cattle. (Fig. 1b).

Methanogens Abundance in Comparative Context Among Different Cattle Breeds

To determine the methanogen abundance in all cattle breeds, we sub-classified the Archaeal domain up to genus level. The abundance of methanogen was found varying in different cattle breeds (Fig. S2). In the Indian cattle breeds, Gir and Kankrej, as well as Jersey cattle rumen, the methanogen community was dominated by *Methanobrevibacter* and *Methanosarcina* and along with other 19 genera (Fig. 2). Whereas, the methanogen community of Holstein cattle rumen was dominated by *Methanobrevibacter* and *Methanosphaera* along with other 6 genera only (Fig. 2). *Methanobrevibacter* genus was found abundant in the rumen of all four cattle breeds as compared to other methanogens, but the percent abundance of it varied with different breed type. *Methanosarcina,* the second most abundant methanogen in the rumen of Indian cattle and Jersey cattle, was completely absent in the rumen of Holstein cattle (Fig. 2).

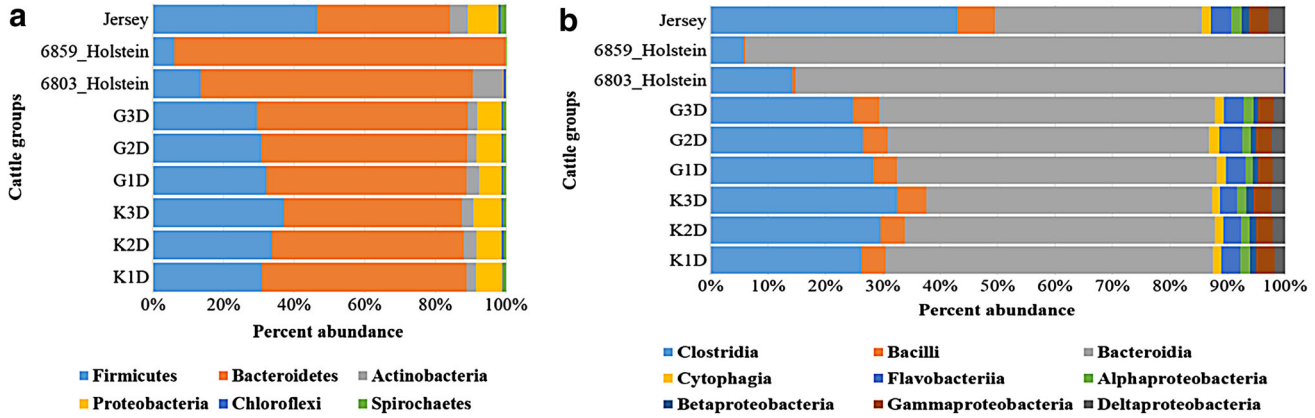

Fig. 1 Phylogenetic classification of the sequences pertaining to bacterial domain at phylum level for respective animal species. *X*-axis: Percent abundance of the sequences assigned to particular phylum, *Y*-axis: Cattle groups

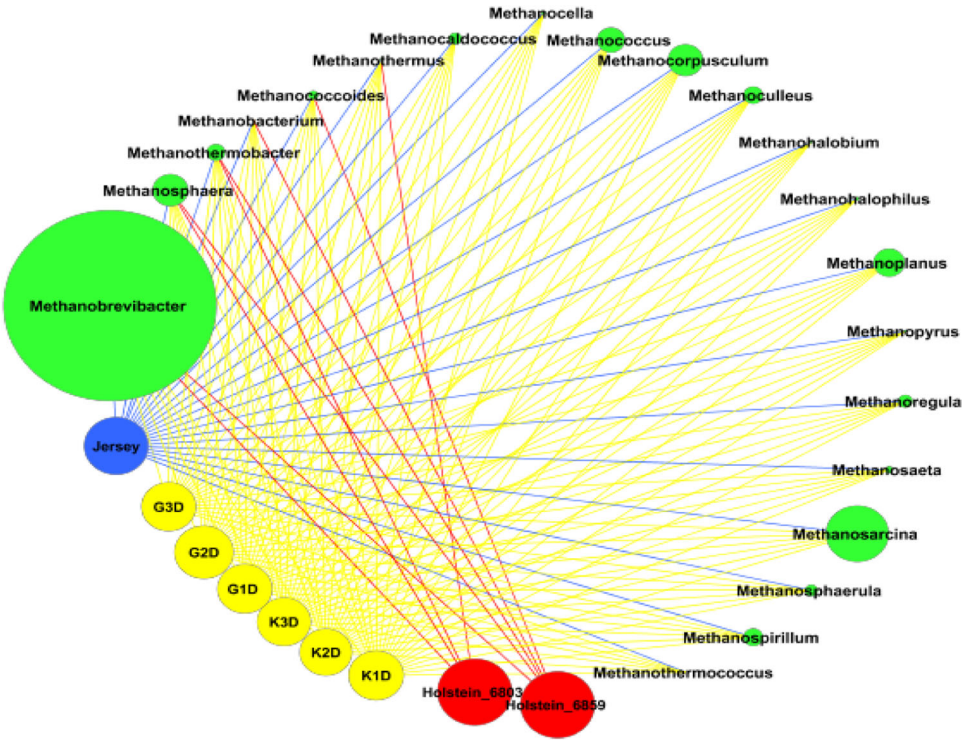

Fig. 2 Network shows the common and unique methanogenic genera present among Indian and exotic animal species. *Yellow* nodes and edges: Indian cattle (Gir and Kankrej), *Red* nodes and edges: Holstein cattle, *Blue* node and edge: Jersey cattle, *Green* nodes: Various genera of methanogens (Node size of the respective genera indicates their comparative abundance) (color figure online)

Unwinding the Abundance of Enzymes Involved in Volatile Fatty Acids (VFAs) and Methane Production

Various pathways of VFA production are represented in Fig. S3. Here, we checked and compared the abundance of the enzymes involved in VFA pathway in our datasets. We also carried out the analysis for total abundant enzymes involved in methanogenesis pathway (Fig. S4).

In case of Gir, Kankrej and Jersey cattle breeds, the genes involved in acetate production were more abundant as compared to propionate and butyrate (Fig. S5a). However, there was no variation in VFA producing genes abundance due to diet treatments given to Gir cattle (Fig. S5a). But, in case of the Kankrej cattle breed, the abundance of the genes involved in acetate and propionate production increased with the increment in the roughage proportion (Fig. S5a). In case of the exotic cattle breed such as Holstein, the genes related to propionate production were dominant, followed by acetate and butyrate (Fig. S5a). We targeted the VFA production genes as there is direct correlation of amount of VFA generated with production of methane.

The results showed more or less similar abundance of the genes involved in acetate to methane pathway and formate to methane production pathway in Gir cattle breed

(Fig. S5b). In Kankrej cattle, more abundance of genes involved in formate to methane production pathway were observed in K1D and K2D treatments whereas, in K3D treatment, more abundance of genes involved in acetate to methane production pathway were perceived (Fig. S5b). In both exotic cattle, the genes involved in formate to methane production were predominant as compared to acetate to methane production (Fig. S5b).

Contribution of Methanogens in Methanogenesis in Indian Cattle Breeds at Different Diet Treatments

To further deeply understand the reason for variation in the abundance of genes for methane production among cattle breeds, we checked the contribution of key role players i.e., methanogens in methane production. Here, we considered specific methanogens such as *Methanobacteriales*, *Methanomicrobiales*, *Methanosarcinales* (*Methanosaetaceae* and *Methanosarcinaceae*) and derived their functional annotation in terms of abundance of enzymes involved in methane production.

In case of Gir cattle, the taxonomic annotation of the sequences based on M5nr database revealed that the percent abundance of *Methanobacteriales* order increased with the increment in the dry roughage (Fig. S6a). If we correlate the enzymes for methanogenesis contributed by this order, we can observe that the abundance of formate to methane producing enzymes increased with the increase in roughage proportion (Fig. S6a). Thus, the diet dependent functional profile shift of *Methanobacteriales* corroborate with its taxonomic profile shift at each treatment. The percent abundance of *Methanomicrobiales* order was found to be more in G2D as compared to G1D and G3D treatments (Fig. S6b). If we correlate the enzymes for methanogenesis contributed by this order, we can observe that the abundance of formate to methane producing enzymes were more in G2D treatment as compared to G1D and G3D treatments (Fig. S6b). The order *Methanosarcinales* is composed of two families i.e., *Methanosaetaceae* and *Methanosarcinaceae*. The percent abundance of *Methanosaetaceae* family was found to decrease with the increment in the dry roughage proportion whereas, the abundance of *Methanosarcinaceae* increased with the increment in the dry roughage proportion (Fig. S6c). If we correlate the enzymes for methanogenesis with that of the taxonomy profile, we can observe that the abundance of the enzymes involved in acetate to methane production pathway showed quite similar profile with *Methanosaetaceae* family (Fig. S6c).

In case of the Kankrej cattle, the percent abundance of *Methanobacteriales* order decreased from K1D to K2D treatment, and then increased in K3D treatment (Fig. S7a). If we correlate the enzymes for methanogenesis contributed by this order, we can observe that the abundance of the enzymes involved in formate to methane production pathway, decreased from K1D to K2D treatment, which further increased in K3D treatment (Fig. S7a). The percent abundance of *Methanomicrobiales* order was found to be more in K1D treatment as compared to K2D and K3D treatments (Fig. S7b). Upon correlating the enzymes for methanogenesis contributed by this order, we observed that the abundance of these enzymes were more in K1D as compared to K2D and K3D treatments (Fig. S7b). The percent abundance of *Methanosaetaceae* family was found to increase with the increment in the dry roughage proportion whereas, the abundance of *Methanosarcinaceae* decreased from K1D to K2D and then increased in K3D treatment (Fig. S7c). If we correlate the enzymes for methanogenesis with that of the taxonomy profile, we can observe that the abundance of the enzymes involved in acetate to methane production pathway showed quite similar profile of the abundance at each treatment with the abundance of *Methanosaetaceae* family (Fig. S7c).

Comparison of Methanogenesis Pathways Contributed by Specific Taxa in Indian (Gir and Kankrej Cattle) and Exotic (Holstein and Jersey) Cattle

Upon comparing the methanogenic enzymes contributed by Indian cattle breeds with exotic cattle breeds, we found that, formate to methane producing enzymes along with shared enzymes contributed by *Methanobacteriales* were more in Australian Holstein cattle and Jersey cattle as compared to Indian Gir and Kankrej cattle (Fig. S8). Whereas, the formate to methane producing enzymes along with shared enzymes contributed by *Methanomicrobiales* were more in Indian origin cattle breeds as compared to Holstein and Jersey breeds. Acetate to methane producing enzymes contributed by *Methanosarcinales* group of organisms showed more representation in Indian cattle and Jersey cattle as compared to Holstein cattle (Fig. S8). In order to further evaluate the differences in the methanogenic enzyme abundance between Indian cattle and two exotic cattle is significant or not, the ANOSIM was performed. The results suggested the significant difference in abundance of methanogenic enzymes was between Indian cattle and Holstein cattle (Table S9). We also performed PCA analysis and found that it corroborates with the results we obtained from ANOSIM analysis, where, more distinct clusters of Holstein and Indian cattle was observed (Fig. S9).

Discussion

In the present study, ruminal digesta samples from Indian cattle breeds viz., Kankrej and Gir that were given three different diet treatments (increasing roughage: concentrate

ratio) were used to discriminate, specifically the methanogen community as well as the enzymes involved in methanogenesis in comparison to breeds like Australian Holstein and Jersey cattle rumen.

The results of phylum and class level bacterial abundance highlighted that the differences in the abundance of *Bacteroidetes* and *Firmicutes* groups in the rumen of different cattle breeds is a consequence of the feeding treatments given to different animals and thus make the rumen a highly dynamic environment. The major difference among cattle breeds in bacterial composition was perceived due to differential abundance of *Clostridium* genus. It has been reported that autotrophic acetogenic bacteria such as *Acetobacterium*, *Acetogenium*, *Eubacterium* and *Clostridium* species are capable of synthesizing acetate by reduction of CO_2 with H_2 or by fermentation of organic compounds [14]. If we correlate the percent abundance of *Clostridium* genus and genes involved in acetate production (Figure S4a) in respective cattle breeds, we could find that higher *Clostridium* abundance in Indian and Jersey cattle breeds correlated with the more abundance of genes involved in acetate production pathway among other VFA pathways, as compared to Holstein cattle, where comparatively negligible abundance of *Clostridium* and more propionate producing genes were observed among other VFAs. Kim et al. [15] also reported that large number of sequences classified within class *Clostridia* in case of rumen. We also looked at the individual VFA producing enzymes and those involved in methanogenesis in the rumen of different cattle breeds to predict the rumen metabolism efficiency so that we can correlate methanogens to cattle metabolism and feed degradation efficiency. Generally, it is observed that the acetate, propionate and butyrate are produced in a ratio varying from approximately 75:15:10–40:40:20 [16], depended primarily upon type of diet, time and quantity fed to animals. If we correlate this report with metagenome analysis, then Indian cattle breeds and Jersey cattle were found to harbor more genes involved in acetate production, followed by propionate and butyrate. However, the same was not observed in case of Holstein cattle where, propionate was the major VFA to be observed followed by acetate and butyrate. The diet given to Indian cattle was *Sorghum bicolor* (as per local availability) which is difficult to digest and the diet given to Australian Holstein dairy cattle (as has been reported) was lucerne which is easily digestible and widely used as a feed for high-producing dairy cows. There are reports which state that rapidly fermentable carbohydrates and highly digestible feed will yield relatively higher propionate as compared to acetate and reverse takes place when less digestible feed is given to animal [17, 18]. Thus, the reason for more acetate to propionate production in Indian cattle might be the consequence of less or slow digestible feed given to them and less acetate to propionate production in Holstein cattle might be due to easily digestible feed given to them. The details of diet given to Jersey cattle is not provided in the metadata submitted in MG-RAST.

The functional abundance of genes for methanogenesis showed similar profile with the taxonomical abundance of methanogens, which further implied that Australian Holstein dairy cattle harbor more methanogens among all other cattle breeds. For gaining deeper insight into the methanogenesis, we targeted two major methanogenesis pathways associated with rumen community via acetate to methane production and/or the formate to methane production pathway. These pathways remained unchanged and were more or less equal in their enzymes abundance during all three diet treatments given to Gir cattle breed. Whereas, in case of Kankrej cattle, in first two diet treatments (different proportion of roughage and concentrate), the enzymes involved in formate to methane production remained significantly active over acetate to methane production, and, in 3rd treatment, where only roughage is given, the enzymes involved in acetate to methane production took over the process of formate to methane production. The difference in abundance of methane producing enzymes between these two ruminal ecosystems can be very well explained based on VFA data. The VFA and methane production are complementary reactions compensating each other in the rumen to maintain ruminal ecosystem. It has been reported that diets promoting fermentation and greater production of VFA also promotes greater levels of milk production [19]. The ruminal ecosystem of Gir cattle is well balanced where methane production is less and efficient VFA is produced and thus this might be the reason for high productivity of Gir cattle. Whereas, in case of Kankrej cattle, total VFA production is less and the methane production is more as compared to Gir cattle and thus this might be the reason why it is less productive as compared to Gir cattle. If we consider the VFA production and methanogenesis in Holstein cattle, then it depicts the active and efficient switch of ruminal organisms towards 2H utilization by more propionate production and methane formation. When there is an excess of 2H in the rumen, it will inhibit the fermentation process and hence removed from the rumen via being utilized in the formation of propionate [20] and methane [21].

Rumen, which acts as a fermenter offering nutrients for animal health maintenance in terms of volatile fatty acids (VFAs), is also one of the efficient producers of methane gas globally. The methanogens, which belong to phylum Euryarcheota, domain Archaea, play a vital role in production of methane in the rumen. Among livestock, methane production is the greatest in ruminants, as

methanogens are able to produce methane freely through the normal process of feed digestion. *Methanobrevibacter* in the rumen has been described as the major group of methanogens in the rumen [22], similar observation was made in our study. However, its abundance profile changed in different animal breeds, but, it remained the major genus of methanogen in the rumen of all four breeds. This being the most abundant genus among methanogens, might be due to its capacity to utilize a wide range of substrates like CO_2, H_2 and formate and convert it into methane. Another dominant genera in Holstein rumen was found to be *Methanosphaera*, the substrate for which is thought to be methanol for methane production [23]. Whereas, in Indian Gir and Kankrej cattle breeds and Jersey breed, *Methanosarcina* genus was found to be the second dominant genus that utilize methanol as a sole source for methane production as its growth is inhibited on hydrogen and carbon dioxide [24].

Thus, to unwind the role of these methanogens in methanogenesis, as we proposed a methanogenic pathway from different substrates like formate, acetate and methanol carried out by methanogens in different cattle breeds, we selected those sequences that were assigned to specific orders such as *Methanobacetriales*, *Methanomicrobiales* and *Methanosarcinales* and then derived functional annotations (in terms of enzymes) contributed by these specific orders. *Methanobacteriales* and *Methanomicrobiales* are the groups of orders that can utilize formate for methane production [24]. Whereas, *Methanosarcinales* constitute two families *Methanosaetaceae* and *Methanosarcinaceae* in which, *Methanosaetaceae* rely on acetate as their sole source for methane production and *Methanosarcinaceae* rely on methanol for methane production [24]. In our data, the functional annotation of the *Methanobacteriales* and *Methanomicrobiales* showed majority of the enzymes involved in formate to methane production. On the other hand, the abundance profile of *Methanosaetaceae* family at each treatment correlated with the abundance of enzymes involved in acetate to methane production pathway at each treatment and thus confirming its role as efficient acetate utilizer for methane production.

The enzymes involved in methanogenesis synthesized by the methanogens has revealed potent activity of methanogens in a particular rumen ecosystem and provided the reason of the observed differences in their methanogenesis activity in different cattle breeds. From Fig. S10, we could observe that the major difference in the Indian cattle and Holstein cattle for methane production is due to type of methanogens involved in methanogenesis. The abundance of methanogenic enzymes contributed by *Methanobacteriales* was more in Holstein and Jersey cattle, however, *Methanomicrobiales* and *Methanosarcinales* did not contributed in methanogenesis in Holstein cattle. Earlier phylogenetic study based on 16S rRNA gene in metagenome of Indian bovine, suggested that more than 50% clones belonged to the order *Methanomicrobiales* [25]. In a study by Shin (2004) the predominant rumen methanogen in the rumen fluid and rumen epithelium were from the family Methanomicrobiaceae [26].

Whereas, one study on Swedish dairy cows based on 16S rRNA qRT-PCR, revealed that the order *Methanobacteriales* accounted for more than 96% of the total number of methanogens and no *Methanomicrobiales* [27]. Another study on Holstein dairy cattle based on 16S rRNA survey, have revealed that, the largest number of clones (24) grouped with *Methanobrevibacter ruminantium*, forming two distinct subclusters and thus suggesting *Methanobrevibacter* as a potent methanogen in the rumen of Holstein dairy cattle [28]. If we compare the total methanogenesis enzyme abundance in Gir and Kankrej cattle, then it was found to be more in Kankrej cattle (Fig. S10a). While looking at the enzyme abundance contributed by specific methanogens in Indian cattle breeds, we could make out that, though total abundance of enzymes was more in Kankrej cattle (Fig. S10b), but when we compare that data with enzymes contributed by specific methanogens, more enzymes were observed in Gir cattle as compared to Kankrej cattle (Fig. S7). This observation leads us to speculation that in Kankrej cattle syntrophic acetate oxidation (SAO) might be taking place. However, with increasing ammonia levels, released during the degradation of protein-rich materials, this acetoclastic methanogens are inhibited and acetate is instead oxidized to H_2 and CO_2 by syntrophic acetate-oxidizing bacteria (SAOB) [29, 30] one of the SAO bacteria is *Clostridium* [29]. Thus, we checked whether the difference in total abundance of methanogenic enzymes and the enzymes contributed by methanogens in Kankrej cattle is due to SAO bacteria that helps in first few steps of acetate to methane production by oxidizing acetate to H_2 and CO_2. To do this, we checked the abundance of enzyme carbon monoxide dehydrogenase (EC: 1.2.99.2) in Gir and Kankrej cattle (Fig. S11). As expected, we found that more abundance of this enzyme was observed in Kankrej cattle as compared to Gir cattle. Thus, this information provides another aspect of methanogenesis that found in Kankrej cattle only.

Conclusion

Results of the experiment indicated that the cattle breed and feed affect colonization of methanogens in rumen. The abundant data for genes involved in VFA production, more in favor of Gir cattle, might be responsible for higher milk production in Gir cattle as compared to Kankrej cattle.

Holstein cattle harbored such a microbial community which is responsible for more propionate production. The study suggested colonization with more abundance of the enzymes involved in methanogenesis in Holstein dairy cattle followed by Jersey and then Indian cattle. Profound study conveys that the enzymes responsible for more methanogenesis in Holstein dairy cattle and Jersey cattle come from *Methanobacteriales* order. Whereas, majority of the enzymes for methanogenesis in Gir cattle come from *Methanomicrobiales* order. This study also supported that total methanogens are not crucial in case of rumen methanogenesis but the differences in individual methanogenic microbial groups and pathways involved in methanogenesis plays crucial role when we change diet concentration in individual cattle. This should be taken into consideration while developing the methane mitigation strategies.

Acknowledgements Authors are thankful to Indian Council of Agricultural Research (ICAR) for providing the fund and also to the field veterinarians of Sardar Krushinagar Dantiwada Agricultural University and Anand Agricultural University (India) for their co-operation in this study.

Compliance with Ethical Standards

Competing interests The authors declare that they have no competing interests.

References

1. Herrero M, Grace D, Njuki J, Johnson N, Enahoro D, Silvestri S, Rufino MC (2013) The roles of livestock in developing countries. Animal 7:3–18. doi:10.1017/S1751731112001954
2. Scheehle EA, Kruger D (2006) Global anthropogenic methane and nitrous oxide emissions. Energy J 3:33–44
3. Chhabra A, Manjunath KR, Panigrahy S (2007) Assessing the role of Indian livestock in climate change. Int Arch Photogramm Remote Sens Spat Inform Sci XXXVIII Part 8:W3
4. Janssen PH, Kirs M (2008) Structure of the archaeal community of the rumen. Appl Environ Microbiol 74:3619–3625. doi:10.1128/AEM.02812-07
5. Ross EM, Moate PJ, Marett LC, Cocks BG, Hayes BJ (2013) Metagenomic predictions: from microbiome to complex health and environmental phenotypes in humans and cattle. PLoS ONE 8:e73056. doi:10.1371/journal.pone.0073056
6. Patel V, Patel AK, Parmar NR, Patel AB, Reddy B, Joshi CG (2014) Characterization of the rumen microbiome of Indian Kankrej cattle (*Bos indicus*) adapted to different forage diet. Appl Microbiol Biotechnol 98:9749–9761. doi:10.1007/s00253-014-6153-1
7. Parmar NR, Solanki JV, Patel AB, Shah TM, Patel AK, Parnerkar S, Kumar J, Joshi CG (2014) Metagenome of Mehsani buffalo rumen microbiota: an assessment of variation in feed-dependent phylogenetic and functional classification. J Mol Microbiol Biotechnol 24:249–261. doi:10.1159/000365054
8. Yuan S, Cohen DB, Ravel J, Abdo Z, Forney LJ (2012) Evaluation of methods for the extraction and purification of DNA from the human microbiome. PLoS ONE 7:e33865. doi:10.1371/journal.pone.0033865
9. Glass EM, Wilkening J, Wilke A, Antonopoulos D, Meyer F (2010) Using the metagenomics RAST server (MG-RAST) for analyzing shotgun metagenomes. Cold Spring Harb Protoc 2010:prot5368. doi:10.1101/pdb.prot5368
10. Hammer Ø, Harper DAT, Ryan PD (2001) Past: paleontological statistics software package for education and data analysis. Palaeontol Electron 4(1):4–9. http://palaeo-electronica.org/2001_1/past/issue1_01.htm
11. Shannon P, Markiel A, Ozier O, Baliga NS, Wang JT, Ramage D, Amin N, Schwikowski B, Ideker T (2003) Cytoscape: a software environment for integrated models of biomolecular interaction networks. Gen Res 13:2498–2504. doi:10.1101/gr.1239303
12. Kanehisa M (2002) The KEGG database. In: Bock G, Goode JA (eds) 'In Silico' simulation of biological processes: novartis foundation symposium, vol 247. Wiley, Chichester. doi:10.1002/0470857897.ch8
13. Thomas T, Gilbert J, Meyer F (2012) Metagenomics—a guide from sampling to data analysis. Microb Inform Exp 2:3. doi:10.1186/2042-5783-2-3
14. Westermann P, Ahring BK, Mah RA (1989) Acetate production by methanogenic bacteria. Appl Environ Microbiol 55:2257–2261
15. Kim M, Morrison M, Yu Z (2011) Status of the phylogenetic diversity census of ruminal microbiomes. FEMS Microbiol Ecol 76:49–63. doi:10.1111/j.1574-6941.2010.01029.x
16. Bergman E (1990) Energy contributions of volatile fatty acids from the gastrointestinal tract in various species. Physiol Rev 70:567–590
17. Rahman MM, Salleh MA, Sultana N, Kim MJ, Ra CS (2013) Estimation of total volatile fatty acid (VFA) from total organic carbons (TOCs) assessment through in vitro fermentation of livestock feeds. Afr J Microbiol Res 7:1378–1384. doi:10.5897/AJMR12.1694
18. Bell MJ, Eckard RJ (2012) Reducing enteric methane losses from ruminant livestock—its measurement, prediction and the influence of diet. In: Khalid J (ed) Livestock production. InTech, Trichy. doi:10.5772/50394
19. Jami E, White BA, Mizrahi I (2014) Potential role of the bovine rumen microbiome in modulating milk composition and feed efficiency. PLoS ONE 9:e85423. doi:10.1371/journal.pone.0085423
20. Hegarty RS, Nolan JV (2007) Estimation of ruminal methane production from measurement of volatile fatty acid production. In: Makkar HPS, Vercoe PE (eds) Measuring methane production from ruminants. Springer, Dordrecht, pp 69–92. doi:10.1007/978-1-4020-6133-2_4
21. Hawkes F, Dinsdale R, Hawkes D, Hussy I (2002) Sustainable fermentative hydrogen production: challenges for process optimisation. Int J Hydrog Energy 27:1339–1347. doi:10.1016/S0360-3199(02)00090-3
22. Carberry CA, Waters SM, Kenny DA, Creevey CJ (2014) Rumen methanogenic genotypes differ in abundance according to host residual feed intake phenotype and diet type. Appl Environ Microbiol 80:586–594. doi:10.1128/AEM.03131-13
23. Balch WE, Fox G, Magrum L, Woese C, Wolfe R (1979) Methanogens: reevaluation of a unique biological group. Microbiol Rev 43:260
24. Hook SE, Wright A-DG, McBride BW (2010) Methanogens: methane producers of the rumen and mitigation strategies. Archaea. doi:10.1155/2010/945785
25. Singh KM, Pandya PR, Parnerkar S, Tripathi AK, Rank DN, Kothari RK, Joshi CG (2011) Molecular identification of methanogenic archaea from surti buffaloes (*Bubalus bubalis*),

reveals more hydrogenotrophic methanogens phylotypes. Braz J Microbiol 42:132–139. doi:10.1590/s1517-83822011000100017
26. Shin EC, Choi BR, Lim WJ, Hong SY, An CL, Cho KM, Kim YK, An JM, Kang JM, Lee SS (2004) Phylogenetic analysis of archaea in three fractions of cow rumen based on the 16S rDNA sequence. Anaerobe 10:313–319. doi:10.1016/j.anaerobe.2004.08.002
27. Danielsson R, Schnürer A, Arthurson V, Bertilsson J (2012) Methanogenic population and CH_4 production in Swedish dairy cows fed different levels of forage. Appl Environ Microbiol 78:6172–6179. doi:10.1128/aem.00675-12
28. Whitford MF, Teather RM, Forster RJ (2001) Phylogenetic analysis of methanogens from the bovine rumen. BMC Microbiol 1:5. doi:10.1186/1471-2180-1-5
29. Schnürer A, Nordberg Å (2008) Ammonia, a selective agent for methane production by syntrophic acetate oxidation at mesophilic temperature. Water Sci Technol 57:735–740. doi:10.2166/wst.2008.097
30. Westerholm M, Levén L, Schnürer A (2012) Bioaugmentation of syntrophic acetate-oxidizing culture in biogas reactors exposed to increasing levels of ammonia. Appl Environ Microbiol 78:7619–7625. doi:10.1128/aem.01637-12