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Rumen Methanogens Diversity in Native Cattle under Different Feeding Regimen using 16s Metagenomic Techniques

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ABSTRACT

Background: Pulikulam cattle is an indigenous cattle breed native to south India, Understanding the rumen microbiome is an important step to develop effective strategies to minimize their enteric methane emission. The rumen microbiome of this breed has not been documented. Hence, a study was executed in Pulikulam cattle, fed different diets, to investigate the diversity of rumen methanogens using metagenomic techniques.

Methods: Twenty recently calved Pulikulam cattle with mean body weight of 200 kg were randomly distributed into four treatments, with five animals per treatment. The animals were fed their respective diets as per their treatment for 30 days, after which the rumen liquor was collected. DNA from the samples were extracted and subjected for 16s rRNA gene amplification using region specific primers.

Result: Methanogens belonging to five orders, four classes and eleven families were identified. Order Methanomassiliicoccales was not present in treatment group fed with high energy diet. The methanogens affiliated to the order Methanomicrobiales were predominant in treatment 1 (14%) and treatment 2 (16.30%). Order methanococcales (15%) was predominant in treatment 3 and order Methanosarcinales (18%) was predominant in treatment 4. Archaeal order belonging to Thermoplasmatales were also identified in Pulikulam cattle fed different dietary regimen except in treatment 3. Archaeal family Methanothermaceae belonging to the order Methanobacteriales was identified in Pulikulam cattle. *Methanocorpusculum labreanum* was the predominant species in all the treatment groups. *Sulfolobus acidocaldarius, Sulfolobus islandicus, Sulfolobus solfataricus, Sulfolobus* sp. A20 and *Sulfolobus tokodaii* were identified in treatment 1, which had higher dietary crude protein. It was concluded that methanogen diversity varied under different feeding regimen and predominant methanogen is varied in Pulikulam breed of cattle. *M. labreanum* was the predominant methanogen in Pulikulam cattle fed with different dietary regimen.

Key words: Archaea, Metagenomics, Methanogens, Pulikulam cattle, Rumen liquor.

INTRODUCTION

Rising environmental temperature is a big threat to the whole world. The main reason for environmental temperature rise is emission of green house gases. Methane is one of the important green house gas produced either naturally or by anthropogenic source. Cattle and buffaloes are accounted for 85 per cent of the annual enteric methane emission in India (Bhatta *et al*., 2019). As the country has 13 and 53 per cent of the world's cattle and buffalo population, respectively (Government of India, 2019). Rumen methanogen community is very complex and depending on the substrate being fermented the methane emission varies. Majority of methanogens utilize carbon dioxide and formate while others use methylated compounds for producing methane. Till date, about 155 species of methanogens associated to six orders and twenty-nine genera have been isolated from different ecosystems (Holmes and Smith, 2016). However, only seven species of methanogens have been isolated from the rumen ecosystem (Janssen and Kirs, 2008). Isolated methanogens from the rumen represents less than 10 per cent of the archaeal community (Kim, 2012). Understanding of the rumen microbiome especially rumen archaea is important in order to develop effective strategies for reduction of enteric methane and thus contribute to decreased green house gas emission.

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In India, majority of indigenous cattle are low yielders and are reared through grazing in wastelands or roadside. Pulikulam cattle is one such indigenous cattle breed of south India reared by nomadic grazing system, in a zero input system of management. Bulls of this cattle breed are reared for bull baiting sporting activity which is conducted during paddy harvest season in the state of Tamil Nadu, India. Currently the nomadic farmers show very little interest in rearing this breed and population statistics have shown a decline in their number. Conservation of this cattle breed will have a great impact on the socioeconomic status of the local farming community. Various measures are underway

to ensure the conservation of this breed. One approach is gathering data on the unique rumen fermentation pattern of this breed that has made them resilient in the utilisation of limited nutrient resources that they obtain from the degraded pastures that they graze. The rumen microbiome of this breed has not been studied and this is the first kind of study in Pulikulam cattle breed. Information on diversity of rumen microbes with special regard to methanogens in this breed of cattle will pave a way for their conservation. Hence, a study was carried out using 16s metagenomic techniques to explore the diversity of microbes emphasising on methanogens in the rumen of Pulikulam cattle fed different diets, under similar environmental conditions.

MATERIALS AND METHODS

The study was carried out at Pulikulam Cattle Research Station (PCRS), Manamadurai, Tamil Nadu, India, which is a constituent unit of Tamil Nadu Veterinary and Animal Sciences University. The study period was between November to December, 2021. A total of 20 recently calved Pulikulam cattle having a mean body weight of 200 kg, in first lactation, with average milk yield of 2 litres and milk fat percentage of 4 to 4.5, were selected, dewormed and randomly distributed to each one of the four treatment groups based on body weight, such that each treatment group had five animals. The details of experimental diets provided to the animals in their respective treatments is presented in Table 1. Pulikulam cattle breed have a small stature and have low milk yield, hence the dry matter, energy and protein requirement for diet formulation of this breed were based on the ICAR, (2013) recommendations. Three different energy and protein levels (20% above, same and 20% below) of ICAR were considered for this study. Treatment 1 was control diet followed by some farmers without standard level which was compared with treatment 2, 3 and 4 contains different energy protein levels of 100:80, 120:100 and 100:100 of ICAR, (2013) recommendations.

Rumen liquor collection

The animals in the various treatment groups were fed their respective diets for 30 days after which rumen liquor was collected from three animals per treatment. The collection was carried out early in the morning prior to feeding using a pedal operated vacuum pump (Bioplus®, Pedal suction apparatus BE-SU03, India). During rumen liquor collection, the head of the animal was restrained and rumen liquor was collected by passing the tube using an oral speculum down the oesophagus into the rumen. The tube was gently pushed through the rumen mat to collect ruminal contents. Approximately 180 to 200 cm of the stomach tube was inside the cattle with the remaining portion of tube being outside of cattle, thus providing the flexibility to move the tube and extract ruminal fluid. Approximately 500 mL of initially sampled rumen liquor was discarded due to possible saliva contamination. After discarding the initial volume, an additional 1000 ml of rumen liquor was collected per animal and was used. The collected rumen liquor was transported using prewarmed thermos flask. The rumen liquor was filled

Table 1: Diet composition (DMB Kg/day) and calculated nutritive value of diets offered to animals of different treatment groups.

Ingredient composition	Quantity (kg DMB)			
	Treatment 1	Treatment 2	Treatment 3	Treatment 4
Hybrid cumbu napier grass	2.25	2.90	0.85	2.49
Fodder sorghum	0.75	0	0	0
Paddy straw	0	2.20	3.00	2.00
De oiled rice bran	0.35	1.24	0.90	1.61
Sun flower oil cake	0.17	0	0.78	0.30
Bajra	0.08	0	0	0
Maize	0.30	0	0.88	0
Broken rice	0.17	0	0	0
Copra cake	0.25	0	0	0
Groundnut cake	0.08	0	0	0
Black gram husk	0.20	0	0	0
Mineral mixture	0.03	0.03	0.03	0.03
Salt	0.02	0.01	0.01	0.01
Calcite	0.05	0	0	0
	Calculated nutritive value of rations			
Crude protein (g)	517	398	498	498
TDN (Kg)	2.66	2.94	3.52	2.94
NDF (%)	53.82	62.02	53.34	59.04
ADF (%)	34.98	22.08	30.21	32.25
Forage NDF (%)	43.60	54.60	41.06	47.63
Forage ADF (%)	29.71	19.77	24.82	28.13

up to the neck of the flask to maintain anaerobic condition. The collected rumen liquor was strained through four layer cheese cloth with continuous flushing of carbon dioxide to maintain strict anaerobic conditions and 39°C during transit. In the laboratory the rumen liquor collected from each individual animal of the respective treatment groups were pooled and approximately 50 ml of rumen liquor from the pooled sample for each treatment was stored at -80°C for DNA isolation and 16 s metagenomic study. DNA from the rumen liquor samples was extracted using Qiagen DNeasy Blood and tissue Kit. (Cat#69506). DNA from the samples were subjected for 16s rRNA gene amplification using region specific primers (16s rRNA primer) and PCR reaction was performed. Nanopore sequencing was performed on GridION X5 (Oxford Nanopore Technologies, Oxford, UK) using SpotON flow cell R9.4 (FLO-MIN106) in a 48 hours sequencing protocol.

RESULTS AND DISCUSSION

Comparative biodiversity of microbiome in different treatment groups

The estimated DNA concentration and nanopore read statistics in different treatment groups are presented in Table 2. The comparative microbiome diversity in different treatment groups were studied using Shannon index and rarefaction curve are presented in Fig 1 and 2 respectively, it reveals variation in microbiome community between treatment 1 and the other treatment groups. The results of this study indicate that there is diversity of methanogens in Pulikulam cattle and they vary under different dietary regimen. The rumen microbiota could be affected either by the geographical locations (Guan *et al*., 2008), host (Handerson *et al*., 2015; Guan *et al*., 2008; Roehe *et al*., 2016) or by the diet of the animal. Zhou *et al*. (2009) reported that methanogen composition varied between cattle herds of different feed efficiency. Difference in the rumen microbiota composition was observed between breeds fed on a similar diet (Paz *et al*., 2016). Handerson *et al*. (2015) reported that rumen microbiome varies depending upon the feed.

Effect of different dietary diets on methanogens community

A total of 125,442; 214613; 126123 and 126405 raw reads per sample were generated from treatment 1, treatment 2, treatment 3 and treatment 4 samples respectively. After removal of unclassified reads a total of 139, 428, 110 and 144 archaeal reads were retained for further analysis which is 0.1, 0.3, 0.08 and 0.1 per cent of total microbial reads in

Table 2: Estimated DNA concentration and nanopore read statistics of different treatment groups.

Contents	Treatment 1	Treatment 2	Treatment 3	Treatment 4
DNA concentration (ng/µg)	101.0	213.2	159.2	143.9
Reads generated	125561	215281	126220	126470
Maximum read length	6465	7213	7004	8083
Minimum read length	115	101	110	136
Average read length	1298.2	1080.6	1263.1	1307.8
Median read length	1519	730	933	1591.5
Total reads length	163004266	232630498	159426968	165398850
Reads >=100 bp	125561	215281	126220	126470
Reads >=200 bp	124956	213249	125642	125637
Reads >=500 bp	110612	171443	110651	109023
Reads >=1 Kbp	92697	114049	88463	94683
N50 value	1578	1559	1575	1580

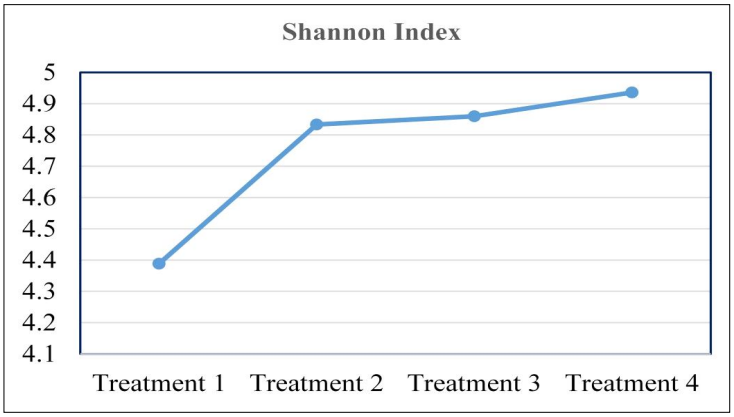


Fig 1: Alpha diversity of ruminal microbiome in different treatment groups.

treatment 1, treatment 2, treatment 3 and treatment 4 respectively. All filtered reads in the present study were affiliated to the rumen archaea. Total rumen archaeal phylum reads in different treatment groups is presented in Table 3. The ruminal archaea community in all the treatments were dominated by phylum Euryarchaeota. The archaea belonging to the other phylum Crenarchaeota and Thaumarchaeota were also present in all the treatments but the representation was lower than phylum Euryarchaeota. Phylum Candidatus Korarchaeota were present in treatment 2 and treatment 4 and phylum Candidatus Micrarchaeota was present only in treatment 2.

These results of this study concur to the previous reports (Abecia *et al*., 2014; Xue *et al*., 2020), where Crenarchaeota methanogens have acknowledged in the rumen. Sulfolobales are physiologically and morphologically distinguished archaea, belonging to the phylum Crenarchaeota which occurs mainly in extreme thermo acidophilic ecosystems (Quehenberger *et al*., 2017). Dias *et al*. (2017) had reported that species *Sulfolobus thuringiensis* was in abundance they reported that *S. thuringiensis* was distributed up to 0.1 per cent of total archaea in dairy calves. Malik *et al*. (2021) also reported the presence of *S. thuringiensis* in livestock. However, the methanogenic capabilities of genera Sulfolobus and their influence to the ruminal methanogensis needs to be explored. Among the eight *Sulfolobus* species established in the literature, *S. islandicus, S. solfataricus* and *S. acidocaldarius* are best defined members of the genus. While *S. islandicus* is used as a model organism for comparative genomics and genetics (Reno *et al*., 2009) and for host virus interactions (Held and Whitaker, 2009). In this study all three *Sulfolobus* species were identified in the Pulikulam cattle.

Ruminal archaeal abundance percentage in different treatment groups at class level is presented in Table 4. Methanogens belonging to a total of four classes were identified in this study. Methanogens associated with the class Methanomicrobia were dominant in all the treatments. Methanogens associated with the class Thermoplasmata was absent in treatment 3.

Ruminal archaeal abundance percentage in different treatment groups at order and family level is presented in Table 5. Methanogens belonging to five orders were identified. The methanogens affiliated to the order Methanomicrobiales were predominant in treatment 1 (14%) and treatment 2 (16.30%). Order methanococcales (15%) was predominant in treatment 3 and order Methanos arcinales (18%) was predominant in treatment 4. Methonogens belong to Methanomassiliicoccales was distributed at lower frequency in treatment 1, 2 and 4 and not present in treatment 3. In treatment 1, Methanococcales was the second most abundant methoanogen (10%) followed by Methanobacteriales (8%) and Methanosarcinales (5%). In treatment 2, Methanobacteriales (11%) was the

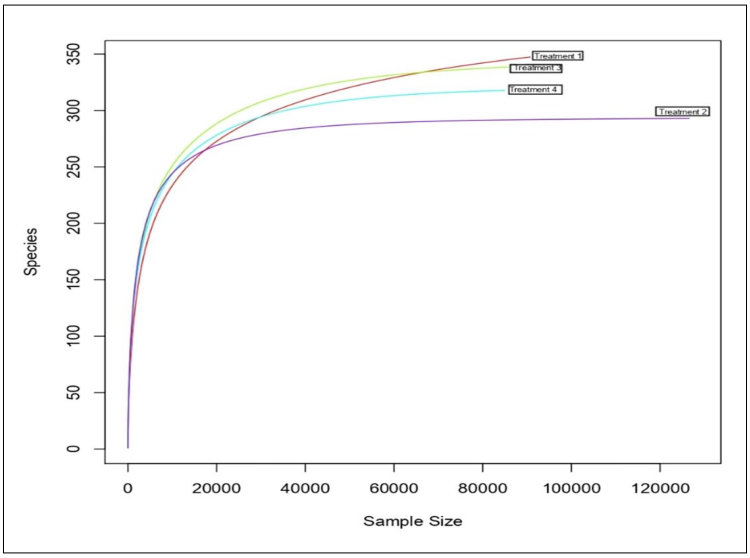


Fig 2: Rarefaction curve of ruminal microbiome in different treatment groups.

Table 3: Total number of rumen archaeal phylum reads in different treatment groups.

Phylum	Treatment 1	Treatment 2	Treatment 3	Treatment 4
Euryarchaeota	52	210	59	84
Crenarchaeota	59	128	15	37
Thaumarchaeota	1	8	4	2
Candidatus korarchaeota	0	4	0	1
Candidatus micrarchaeota	0	1	0	0

second most abundant methoanogen followed by Methanosarcinales (10.6%) and Methanococcales (7%). Methanomicrobiales (12%). In treatment 3 Methanosarcinales and Methanobacteriales abundance were present in equal amount. In treatment 4, Methanomicrobiales (9.6%) was the fourth abundant methonogen. Methanobacteriales and Methanococcales held second and third ranks in the abundance.

The results of this finding is similar to the finding of Sadan *et al*. (2019) in Vechur cattle, another native cattle breed of India. The authors had reported that Methanobactetriales, Methanomassiliicoccales and Methanomicrobiales were the predominant methanogen orders in rumen of Vechur cattle. Xue *et al*. (2016) reported that Methanobacteriales was the predominant archaea of rumen microbiota in the natural qrazing Yak in Sichuan. Other studies have reported an unprecedented greater abundance of Methanomicrobiales in cattle (Tajima *et al*., 2001; Shin *et al*., 2004) and buffaloes (Chaudhary and Sirohi, 2009; Singh *et al*., 2011; Singh *et al*., 2012). Previous studies have also reported that majority of the archaeal sequences retrieved from bovine rumens and cattle dung belonged to order Methanomicrobiales and Methanobacteriales (Rastogi *et al*., 2008; Tatsuoka *et al*., 2004). In contrast to the finding of the present study, Malik *et al*. (2021) reported that Methanomicrobiales proportion was less than 4 per cent of the total archaea in both cattle and buffaloes and the abundance frequency was consistent with the global data sets (Janssen and Kirs 2008; Handerson *et al*., 2015; Zhou *et al*., 2009). This could be due to the different DNA isolation methods, primer sets and animal diets.

Methanomassiliicoccales is a novel group of archaea related to Thermoplasmatales (Horz *et al*., 2012). Methanomassiliicoccales are methylotrophic methanogens that utilize methanol and methylamines for producing methane (Lang *et al*., 2015; Sollinger *et al*., 2016).

In this study methanogen belonging to the order Methanomassiliicoccales was not present in treatment 3, diet of this treatment formulated with high energy contains more amount of maize than the other treatment group's diets. A decrease in rumen pH due to high grain feeding is possibly a general cause for the stimulation or suppression of a particular type of methylotrophs in the rumen (Lana *et al*., 1998). The Methanomassiliicoccales may have specific properties that allowed their survival or inhibition during variable pH. The relative abundance of Methanomass iliicoccales affected by the diet rather than the host. Similarly, Xue *et al*. (2016) reported that methanogen Methanomass iliicoccales was absent in domesticated Yak maintained at China.

In this present study methanogens belonging to Methanosarcinales were present in all the treatment groups. Methanosarcinales grow on a broad range of substrates such as H_2, CO_2, methanol, methylamines and acetate. This is the only methanogen order capable of performing acetoclastic methanogenesis (Kendall and Boone, 2006). Malik *et al*. (2021) reported that methanogens belonging to Methanosarcinales were distributed at very small frequencies in both the cattle and buffaloes, their abundance was significantly greater in cattle compared to in buffaloes. Presence of Methanosarcinales in rumen was reported by (Patterson and Hespell, 1979; Jarvis *et al*., 2000) in both cattle and buffalo. VFA produced during digestion in the rumen are generally absorbed by the rumen epithelium and subsequently converted to animal proteins and therefore not available for utilization as a carbon source by Methanosarcinales residing in rumen. Therefore, abundance of Methanosarcinales was minimal in rumen of cattle

Table 4: Ruminal archaeal abundance (%) in different treatment groups at class level.

Class	Treatment 1	Treatment 2	Treatment 3	Treatment 4
Methanomicrobia	18	24	23	27
Methanococci	10	07	15	12
Methanobacteria	08	11	11	15
Thermoplasmata	3	03	0	03

Table 5: Ruminal archaeal abundance (%) in different treatment groups at order and family level.

Order	Family	Treatment 1	Treatment 2	Treatment 3	Treatment 4
Methanomicrobiales	Methanocorpusculaceae	12	13	11	08
	Methanomicrobiaceae	02	02	0	0.8
	Methanoregulaceae	0	01	01	0
	Methanospirillaceae	0	0.3	0	0.8
Methanosarcinales	Methanosarcinaceae	04	10	08	16ai
	Methanosaetacea	01	0.6	08	02
Methanobacteriales	Methanobacteriaceae	08	11	11	15
	Methanothermaceae	0	0	0	0.8
Methanococcales	Methanococcaceae	07	05	08	10
	Methanocaldococcaceae	03	02	07	02
Methanomassiliicoccales	Methanomassiliicoccaceae	01	03	0	0.8

(Zinder, 1993). H_2/CO_2 is the only carbon source available in plenty for methanogens hence, hydrogenotrophic methanogens (Methanomicrobiales and Methanobacteriales) that are capable of using H_2/CO_2 can multiply easily and their abundance were high in cattle rumen. Similarly, Denman *et al*. (2007) reported that the methanogens belonging to order Methanobacteriales was the major hydrogenotrophic methanogens in rumen of Brahman-crossbred (*Bos indicus*) steers.

In the present study methanogens belonging to 11 families were identified. Rumen archaeal composition at family level in different treatment groups are given in Fig 3, 4, 5 and 6. Families Methanocorpusculaceae, Methanomicro biaceae Methanoregulaceae Methanospirillaceae were affiliated to the order Methanomicrobiales. Methanoregulaceae distributed at lower frequency was present only in treatment 2 and treatment 3. Family Methanospirillaceae was present only in treatment 2 and treatment 4. Orders Methanosarcinales, Methanobacteriales and Methanococcales were characterized by two families of each. Methanothermaceae belonging to the order Methanobacteriales present only in treatment 4. Methanomassiliicoccaceae was absent in treatment 3.

In this study at the species level methanogen *M. labreanum* belonging to order methanomicrobiales was the predominant species in all the treatment groups. The heat map comparison of different archaeal abundance at species level in different treatment groups is given in Fig 7. *Methanococcus maripaludis, Methanococcus aeolicus, Methanobrevibacter millerae* and *Methanobrevibacter olleyae* hold second, third and fourth predominant species in treatment 1. Abundance of species *M. millerae* and *M. olleyae* were similar. On the other hand, in treatment 2, *M. labreanum, M. maripaludis, M. millerae* and *Methanosarcina mazei* were the top four predominant species. Abundance of *M. millerae* and *M. mazei* were similar in this treatment. Abundance of *M. olleyae* was high compared to other treatments. *M. labreanum, M. olleyae, M. maripaludis* and *Methanococcus voltae* were the predominant species in treatment 3. Abundance of *M. maripaludis* and *M. voltae* were similar in this treatment. In treatment 4, *M. labreanum* was the predominant species followed by *Methanococcus vannielii, M maripaludis* and *Methanobrevibacter smithii* hold second and third position. Abundance of species, *M maripaludis* and *M smithii* were similar in this treatment. In the present study, species *Sulfolobus acidocaldarius*, *Sulfolobus islandicus*, *Sulfolobus solfataricus, Sulfolobus* sp. A20 and Sulfolobus *tokodaii* were identified in treatment 1. Species *Ferroplasma acidarmanus*, *Picrophilus torridus*, *Thermoplasma acidophilum*, *Thermoplasma volcanium, Thermoplasmatales archaeon BRNA1* species belonging to order Thermoplasmatales were also identified in the rumen of Pulikulam cattle.

Similar to the finding of this study, in Murrah buffalo of India the majority of methanogens belonged to the genus methanomicrobium (Chaudhary and Sirohi, 2009). Shin *et al*. (2004) reported that 85 per cent of the total clones from the rumen of cattle belong to the order Methanomicrobiales, out of 104 clones 61 clones were resembling *M. mobile*.

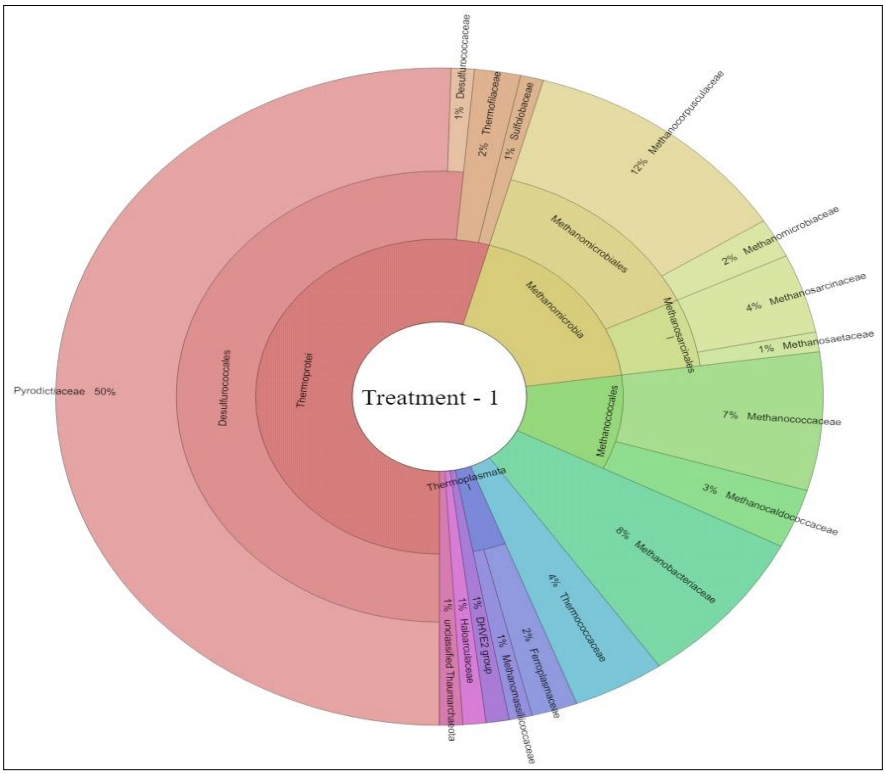


Fig 3: Rumen archaeal composition at family level Treatment 1.

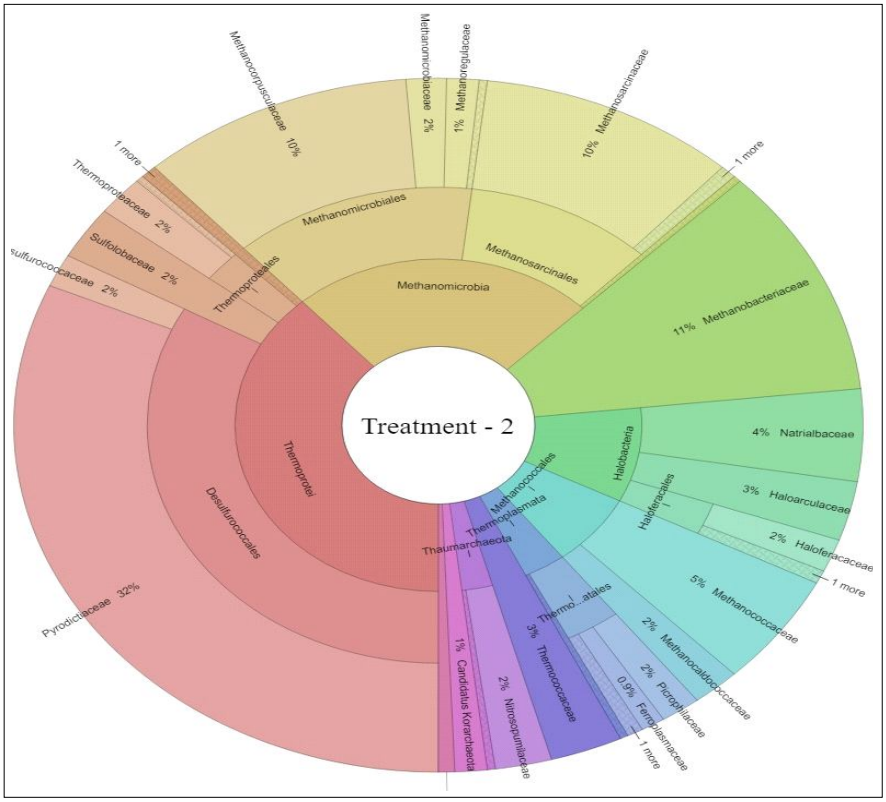


Fig 4: Rumen archaeal composition at family level in Treatment 2.

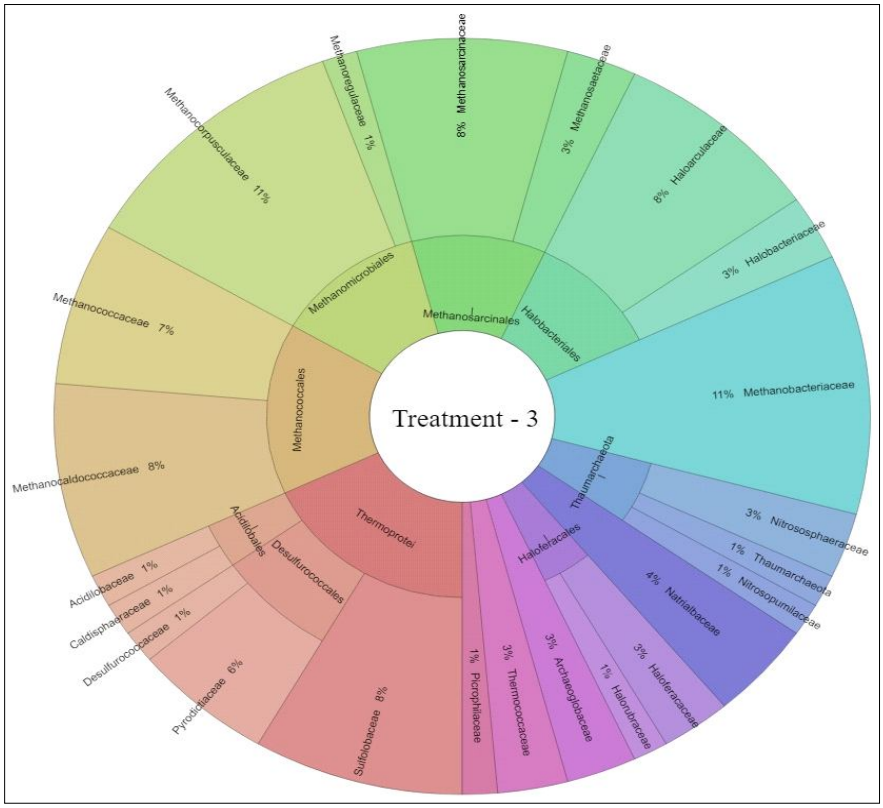


Fig 5: Rumen archaeal composition at family level in Treatment 3.

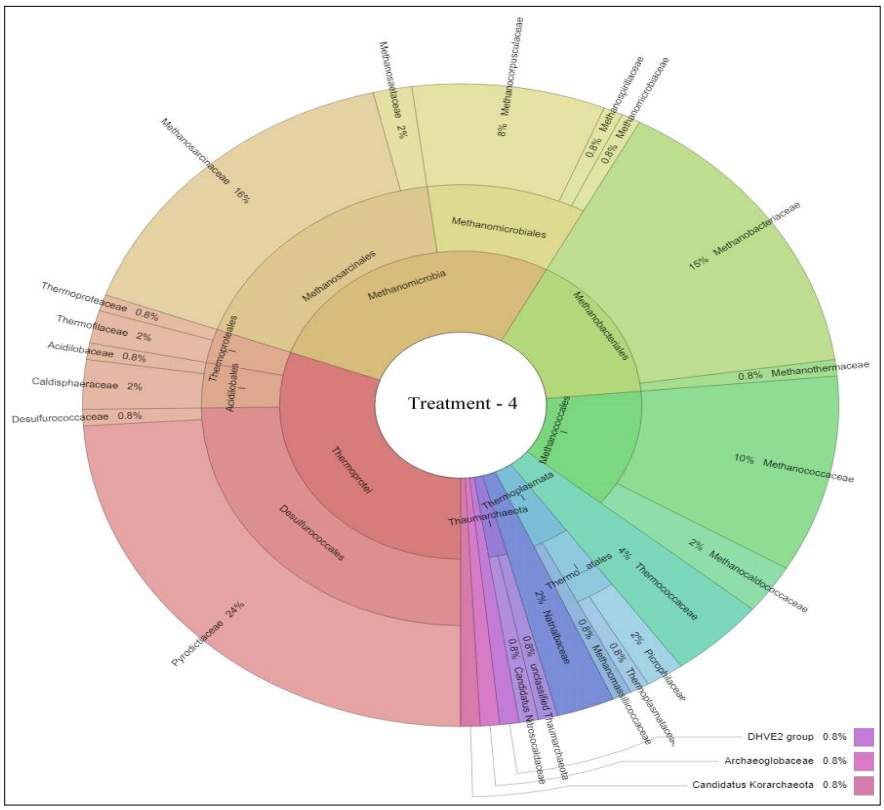


Fig 6: Rumen archaeal composition at family level in Treatment 4.

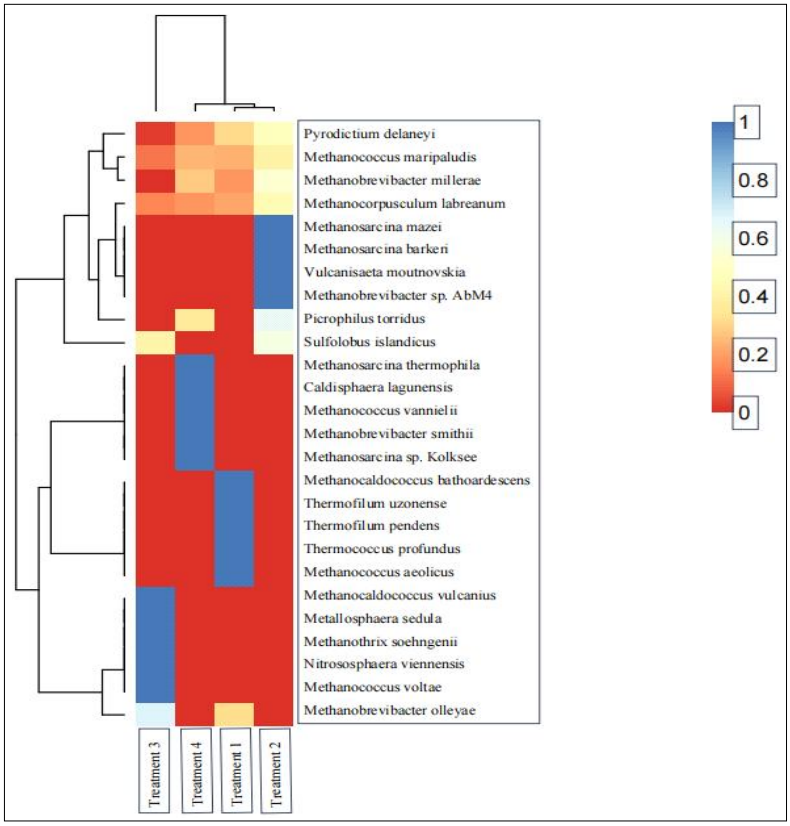


Fig 7: The heat map comparison of different rumen archaeal abundance at species level in different treatment groups.

In contrary, Ozutsumi *et al*. (2012), Lwin *et al*. (2012) and Franzolin *et al.* (2012) have identified Methanobrevibacter as the dominant genus in Holstein cattle, water buffaloes and crossbred buffalo, respectively. In another study Jeyanathan *et al.* (2011) reported that in New Zealand Methanobrevibacter spp. were dominant in cattle. Leahy *et al*. (2013) also reported that in the cow rumen, *Methanobrevibacter* reported to be the dominant genus of the archaeal domain. In the present study *Methanocella arvoryzae* was identified only in treatment 2. Malik *et al.* (2021) reported that species *M. arvoryzae*, was identified in the buffalo rumen at a very low frequency.

CONCLUSION

From the study it is concluded that methanogen *M. labreanum* was the predominant species in Pulikulam cattle fed with different dietary regimen and diversity of methanogen varied in Pulikulam cattle fed with different diets. Methanogens belonging to archaeal family Methanothermaceae was identified in Pulikulam cattle and family Methanomassilii coccaceae was absent in rumen when high grain ration was fed to Pulikulam cattle. Since the predominant methanogen varies in this breed of cattle future studies are needed to explore the amount of enteric methane emission from the rumen of Pulikulam cattle under different feeding regimen and the effects of geographical location in the diversity of methanogen in this breed of cattle.

Conflict of interest

All authors declare that they have no conflict of interest.

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