ASSESSMENT OF RUMINAL METHANOGEN COMPOSITION AND METHANE EMISSION LEVELS IN CROSSBRED AND VECHUR COWS UNDER THE SAME DIETARY REGIMEN*

Tina Sadan[1], T.V. Aravindakshan[2], G. Radhika[3], Lali F Anand[4] and K. Ally[5]
Department of Animal Breeding Genetics and Biostatistics
College of Veterinary and Animal Sciences
Mannuthy, Thrissur, 680 651

Received: 21.08.2018
Accepted: 23.10.2018

Abstract

The rumen microbial community plays a critical role in methane emission from ruminants. However, there is a lack of data comparing the composition of the rumen methanogenic community of cattle breeds of Kerala. Present study was undertaken with the objective of assessing rumen methanogen composition and methane emission levels of crossbred and Vechur cattle. All the animals were fed with ration of 50:50 (forage: concentrate) diet on dry matter basis for a period of three weeks. Rumen liquor and rumen gas samples were collected. DNA isolated from rumen liquor using standard procedure were pooled genetic group wise and subjected to whole metagenome sequencing and further bioinformatics analysis. The concentrations of methane (percentage) in gas samples were determined using a methane analyser. Research findings revealed that bacteria was the most dominant and archaea was the second prominent domain found in rumen of both genetic groups. Phylum Euryarchaeota of the domain Archaea constitute methanogens. At family level, Methanomassiliicoccaceae, Methanomicrobiaceae, Methanobacteriaceae were the predominant methanogens in crossbred and Vechur rumen. Population of specific methanogens were found to be significantly different between genetic groups. Biodiversity indices displayed higher richness, evenness and diversity for rumen methanogens in Vechur cows compared to crossbred. Comparative analysis of methane emission levels in crossbred and Vechur confirmed the effect of genetic group on methane emission from rumen.

Keywords: *Rumen metagenome, methane emission, methanogen composition, Vechur cattle, crossbred cattle*

Methane emission by domesticated ruminants has become an important efficiency trait due to its negative impact on animal production and its contribution to climate change. Methane is one of the most potent greenhouse gases with global warming potentials (GWPs)

** Part of M.V.Sc thesis of first author submitted to Kerala Veterinary and Animal Sciences University, Pookode, Wayanad, Kerala.*

1. M.V.Sc Scholar, tinasadan@gmail.com, Mob: 9400675241

2. Professor and Head

3. Assistant Professor

4. Assistant Professor

5 Professor and Head, Department of Animal Nutrition

about 23 times greater than carbon dioxide. According to Global greenhouse gas emissions data by IPCC, (2015) methane accounted for about 16% of total greenhouse gas emission. Food and Agriculture Organization of the United Nations, (2006) identified that farm animal production sector is responsible for about 18% or around one-fifth of human-induced GHG emission. Methanogens belonging to the phylum *Euryarchaeota* of the domain Archaea uses hydrogen and carbon dioxide produced by fermentation as substrates for methane production and causes a loss of 5.5 to 9% dietary energy of host livestock. Consequences of methanogenesis in the rumen include low productivity and a negative impact on the sustainability of the ruminant's production. However, information on the diversity of methanogens population in the rumen of dairy cattle is very limited. Therefore, culture-independent detection methods are used to identify and compare the population structures of rumen methanogens.

In this study, the metagenomic approach has been used to explore the total methanogens population in the rumen of crossbred and Vechur cows that were fed the same diet and maintained under the same environmental conditions. The specific objectives of our study were (i) to identify methanogens that reside in the rumens of crossbred and Vechur cattle (ii) to investigate whether methanogens population structures vary significantly between genetic groups (iii) to estimate methanogens diversity measures and compare between genetic groups and (iv) to compare methane emission levels in crossbred and Vechur cattle.

Material and Methods

Animals, Diets and Sampling

A total of 12 adult animals, six each from crossbred and Vechur cattle maintained at University Livestock Farm and Vechur cattle Conservation unit, Mannuthy were used in the experiment. During the experimental period all animals were fed with forage: concentrate ratio of 50:50 on dry matter basis as per dietary recommendation of ICAR (2013). After an adaptation period of 21 days, 200 ml of rumen fluid samples were collected by stomach tube from five crossbred and five Vechur cattle, filtered through four layers of muslin cloth and were stored at -80°C until DNA extraction.

DNA Extraction and Sequencing

The DNA was isolated from rumen liquor using CTAB based buffer for cell lysis and further purification with phenol: chloroform: isoamyl alcohol. The metagenomic DNA samples were pooled genetic group wise before subjecting to library preparation by NEB Next Ultra DNA Library preparation Kit (New England Biolabs, USA), where in DNA fragmentation was followed by adaptor ligation, size selection and further library amplification. All the samples were submitted for deep sequencing using the Illumina HiSeq 2500 platform (AgriGenome, Pvt Ltd., Kochi).

Taxonomic classification

The rumen metagenome data of crossbred and Vechur cows were annotated for obtaining taxonomical classification using NCBI taxonomy data sets. Sequenced data was uploaded to MEGAN5 (MEtaGenomeANalyzer) software (Huson *et al*., 2007) and analysis was done at taxonomical level.A phylogenetic tree was constructed based on neighbour-joining method with MEGAN5. Classification was done up to species level.

Methane estimation

Rumen gas samples were collected in 50 ml sterile syringe by rumen puncture using 16 gauge needles on 21st day of diet regimen from six crossbred and six Vechur cattle. Samples were collected at four hours after feeding. The concentration of methane (percentage) in the total gas produced was determined by using a methane analyser.

Statistical Analysis

The significance of the total abundance of methanogen species using Mann-Whitney test, methanogen diversity measures and comparison of methane emission levels of crossbred and Vechur cattle using t test were estimated by PAST v3.18 software

package (Hammer *et al*., 2001). Significance of individual taxa was tested using G-test or Fischer's test with Bonferroni correction in STAMP v2.1.3software package (Parks *et al*., 2014).

Result and Discussion

The rumen metagenome sequencing generated 26,711,915 reads for crossbred and 27,498,309 reads for Vechur samples. In this, a total of 12992 and 11218 reads were assigned to Archaeal domain for crossbred and Vechur samples, respectively. Archaea was the second prominent domain found in rumen of both genetic groups similar to the previous studies reported by Parmar *et al*., (2017) in Gir, Kankrej, Holstein cattle and Jersey cattle. It was observed that phylum Euryarchaeota was more abundant in Archaeal domain, which consisted of methanogens and accounted for approximately 98% of total Archaeal reads (Table 1). The proportion of phylum Euryarchaeota differed significantly between genetic groups (P< 0.01) indicating methanogenic archaeal variation between genetic groups.

Comparative analysis of rumen methanogens between crossbred and Vechur cattle

In this study, archaeal domain was sub-classified up to species level and determined the methanogen abundance in percentage of total archaeal reads in crossbred and Vechur cows. Most of the methanogens were found to be varying in their proportions between genetic groups (Table 2).

Methanobacetriales, *Methano-massiliicoccales* and *Methano-microbiales* were the predominant methanogen orders in crossbred and Vechur rumen. Among these orders, the proportions of *Methanobacetriales*, and *Methanomicrobiales* differed significantly between genetic groups (Fig. 1). Present results are comparable with Shin *et al*. (2004) who conducted phylogenetic analysis of archaea in Korean cow (Hanwoo) rumen. The archaeal species belonging to these two orders were known to be associated with utilization of formate for methane production and synthesis of majority of the enzymes involved in conversion of formate to methane (Parmar *et al*., 2017).

In Vechur cattle, *Methanomass-iliicoccaceae* family was found to be more abundant compared to other methanogens, whereas, *Methanomicrobiaceae* family was found to be abundant in the crossbred cattle. The proportions of *Methanosarcinaceae* and *Methanosaetaceae* families among total archaeal reads were found to be 1.40 and 0.71 in crossbred and 1.35 and 0.69 in Vechur cattle. The members of the *Methanosaetaceae* family use acetate as their sole source carbon for methane production whereas the members of the *Methanosarcinaceae* family rely on methanol for methane production (Hook *et al*., 2010). Knowledge of methanogen abundance and its involvement in methanogenic pathways aid in development of manipulation strategies for lowering methane production. Out of 14 families identified through rumen metagenome analysis, the proportions of only two families, namely, *Methanomicrobiaceae* and *Methanobacteriaceae* were found to be differing

Table 1. Percentage abundances of Archaeal phyla in crossbred and Vechur cattle

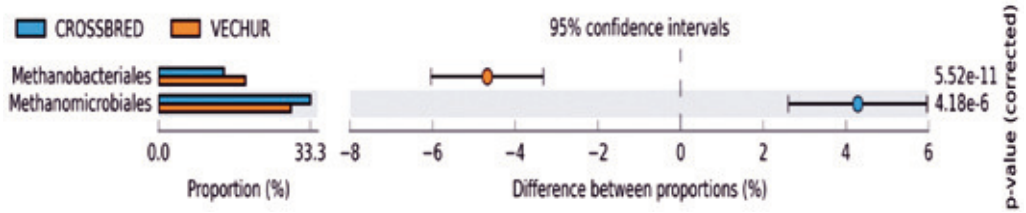
Phylum	Crossbred (%)	Vechur (%)	P - value
Aenigmarchaeota	0	0.02	0.128
Crenarchaeota	0.39	0.49	0.251
Diapherotrites	0.03	0.02	0.523
Euryarchaeota	97.93	97.36	0.003**
Korarchaeota	0.02	0.06	0.133
Nanoarchaeota	0.02	0.04	0.318
Nanohaloarchaeota	0.01	0.02	0.480
Parvarchaeota	0.03	0.01	0.238
Thaumarchaeota	0.35	0.46	0.181

(** Significant at P value< 0.01)

significantly (P< 0.01) between crossbred and Vechur cattle.

Methanogen community in crossbred and Vechur cattle rumen was dominated by members of the genus *Methanobrevibacter* and *Methanomicrobium* and their abundance differed significantly between two genetic groups (Fig. 2). The species of archaea coming under the genus *Methanobrevibacter* are considered as potent methane emitters in the rumen of cattle (Whitford *et al*., 2001) and they are more frequently observed in high methane emitters (Wallace *et al*., 2015). They accounted for 5.77% abundance in Vechur and 8.09% abundance in crossbred which partially explains high methane emission levels in Vechur cattle.

A comparative analysis revealed that crossbred and Vechur cattle have only nine methanogens species in common with 53 and 49 species found exclusively in crossbred and Vechur cattle, respectively. In both genetic groups, the methanogen community was dominated by *Methanomicrobium mobile* and *Methanobrevibacter ruminantium* species. A previous study on comparison of methanogen species abundance in two Indian



. **Fig. 1** Relative abundance of archaeal orders differing significantly in crossbred and Vechur cattle rumen metagenome.

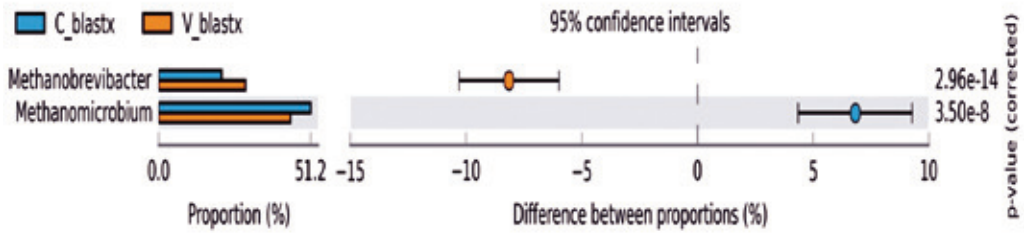


Fig.2 Relative abundance of archaeal genus differing significantly in crossbred and Vechur cattle rumen metagenome.

Table 2. Percentage of taxonomic groups in archaeal domain in crossbred and Vechur cattle

Taxa	Crossbred cattle	Vechur cattle
Class		
Methanomicrobia	19.52	17.56
Methanobacteria	7.16	9.68
Methanococci	0.38	0.37
Methanopyri	0.01	0.01
Order		
Methanomicrobiales	16.61	14.74
Methanomassiliicoccales	15.46	14.86
Methanobacteriales	7.17	9.68
Methanosarcinales	2.37	2.33
Methanococcales	0.38	0.37
Methanocellales	0.18	0.21
Methanopyrales	0.02	0.02

Table 2. Continued

Taxa	Crossbred cattle	Vechur cattle
Family		
Methanomassiliicoccaceae	15.46	14.86
Methanomicrobiaceae	15.75	13.98
Methanobacteriaceae	7.08	9.56
Methanosarcinaceae	1.40	1.35
Methanosaetaceae	0.71	0.67
Methanocellaceae	0.18	0.21
Methanocorpusculaceae	0.22	0.20
Methanocaldococcaceae	0.12	0.14
Methanococcaceae	0.14	0.14
Methanospirillaceae	0.08	0.12
Methanoregulaceae	0.12	0.12
Methermicoccaceae	0.02	0.04
Methanothermaceae	0.02	0.03
Methanopyraceae	0.02	0.02
Genus		
Methanomicrobium	13.95	12.24
Methanobrevibacter	5.77	8.09
Methanomassiliicoccus	3.14	2.96
Methanobacterium	0.77	0.73
Methanoculleus	0.75	0.69
Methanosaeta	0.71	0.67
Methanosarcina	0.55	0.68
Methanocorpusculum	0.22	0.20
Methanolobus	0.19	0.16
Methanocella	0.18	0.21
Methanoplanus	0.13	0.05
Methanolacinia	0.12	0.09
Methanomethylovorans	0.09	0.06
Methanospirillum	0.08	0.12
Methanocaldococcus	0.08	0.10
Methanoregula	0.08	0.09
Methanococcus	0.07	0.07
Methanococcoides	0.06	0.03
Methanofollis	0.06	0.06
Methanosphaerula	0.05	0.03
Methanothermobacter	0.04	0.05
Methanosphaera	0.03	0.05
Methanothermococcus	0.03	0.03
Methanohalophilus	0.02	0.04
Methanothermus	0.02	0.03
Methanopyrus	0.02	0.02
Methanohalobium	0.01	0.01

cattle (Gir and Kankrej) and two exotic cattle (Holstein and Jersey) breeds showed that *Methanobrevibacter ruminatium* was found in the rumen of all four cattle breeds with different levels of abundance (Parmar *et al*., 2017). The relative abundance of Archaeal species genus differing significantly in crossbred and Vechur cattle rumen metagenome is presented in

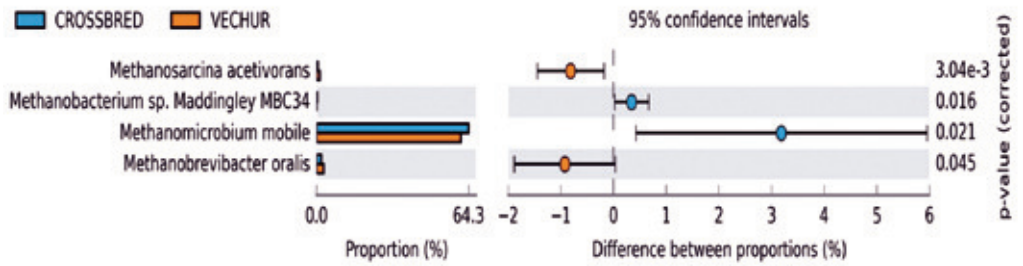


Fig. 3 Relative abundance of archaeal species differing significantly in crossbred and Vechur cattle rumen metagenome

Fig 3.

Overall, these results showed that only population of specific methanogenic structures (species, genus and family) differed significantly between genetic groups and there was no difference in the total methanogen abundance between them.

Diversity analysis

Diversity analysis of methanogen community showed that Vechur rumen had higher species richness and evenness than crossbred rumen. Alpha diversity metrics, Shannon index and Simspon index displayed higher microbial diversity in Vechur cattle compared to crossbred cattle (Table 3). In addition, Simpson diversity index were found to be significantly different between the genetic groups using diversity t-test. King *et al*., (2011) also observed significant difference in methanogen diversity in Jersey and Holstein dairy cattle. Abundance and diversity of methanogens and their constituent genes corresponds strongly with methane emissions by the host animal (Janssen, 2010; Wallace *et al*., 2015). Methanogenesis is the only mechanism of ATP synthesis available to the Archaeal methanogens (Thauer *et al*., 2008). Therefore higher methanogen diversity of Vechur indicated higher methane emission in Vechur cattle.

Methane emission levels

The amount of methane produced in the rumen depends on the type of animal, level of intake, type and quality of feeds and number of other environmental factors (McAllister *et* al., 1996; Kumar *et al.,* 2009; Shibata and Terada, 2010). Present study was undertaken to find out the effect of genetic group on methane emission levels in crossbred and Vechur cattle under the same diet regimen and management conditions. It was observed that genetic group had significant influence on the methane emission levels and a higher mean percent of methane was reported in Vechur cows (Table 4).

On summarizing the overall results of the study, it could be inferred that genetic group has a significant effect on specific methanogen abundance and methane emission levels from the rumen. This study forms the basis for developing selection criteria and dietary manipulation strategies to mitigate methane emission without compromising the production performance.

References

Table 3. Different biodiversity measures observed in crossbred and Vechur cattle rumen

Biodiversity Measures	Crossbred	Vechur
Simpson index	0.58	0.61
Shannon index	1.77	1.85
Buzas and Gibson's evenness	1.73	1.80
Brillouin's evenness	0.09	0.11
Menhinick's richness	1.13	1.18
Margalef's richness	7.43	7.13

Table 4. Mean per cent of methane emission levels in crossbred and Vechur cattle

Variable	Crossbred	Vechur	F- value	P- value
	Mean ± SE	Mean ± SE		
Methane emission	25.47± 0.44	27.34± 0.52	7.471*	0.021

(* Significant at P< 0.05)

FAO [Food and Agriculture Organization of the United Nations]. Livestock a Major Threat to the Environment: Remedies Urgently Needed. 2006. Available:http://www.fao.org/newsroom/en/news/2006/1000448/index.html.

Hammer, Harper, D.A.T., Ryan, P. D. 2001. PAST: Paleontological Statistics Software Package for Education and Data Analysis. *Palaeontologia Electronica.* **4**:1- 9.

Hook, S.E., Wright, A.D.G. and McBride, B.W. 2010. Methanogens: methane producers of the rumen and mitigation strategies. *Archaea*. [on line]. **2010.** Available: doi:10.1155/2010/945785. [7 Dec 2010].

Huson, D.H., Auch, A.F., Qi, J. and Schuster, S.C. 2007. MEGAN analysis of metagenomic data. *Genome Res*. [on line]. **17**(3). Available: doi:10.1101/gr.5969107. [25 Jan 2007].

ICAR [Indian Council of Agricultural Research]. 2013. *Nutrient requirements of Animals-Cattle and Buffalo.* (3rd Ed.). Indian Council of Agricultural Research, New Delhi. 24p.

IPCC (Intergovernmental Panel on Climate Change). 2015. Climate change 2014. *Mitigation of climate change.* (Vol. 3). Cambridge, University Press.

Janssen, P.H. 2010. Influence of hydrogen on rumen methane formation and fermentation balances through microbial growth kinetics and fermentation thermodynamics. *Anim. Feed Sci. Tech*. **160**:1-22.

King, E.E., Smith, R.P., St-Pierre, B. and Wright, A.D.G. 2011. Differences in the rumen methanogen population of lactating Jersey and Holstein dairy cows under the same diet regimen. *Appl. Enviro. microbiol*. [on line]. Available: doi: 10.1128/AEM.05130-11. [24 June 2011].

Kumar, S., Puniya, A.K., Puniya, M., Dagar, S.S., Sirohi, S.K., Singh, K. and Griffith, G.W. 2009.Factors affecting rumen methanogens and methane mitigation strategies. *World J. Microbio.Biotech*. **25:**1557-1566.

McAllister, T.A., Cheng, K.J., Okine, E.K. and Mathison, G.W. 1996. Dietary, environmental and microbiological aspects of methane production in ruminants. *Canadian J. Anim. Sci*.**76:**231-243.

Parmar, N.R., Pandit, P.D., Purohit, H.J., Kumar, J.N. and Joshi, C.G. 2017. Influence of diet composition on cattle rumen methanogenesis: a comparative metagenomic analysis in Indian and exotic cattle. *Indian J. Microbiol*.**57**: 226-234.

Parks, D.H., Tyson, G.W., Hugenholtz, P. and Beiko, R.G. 2014. STAMP: statistical analysis of taxonomic and functional profiles. *Bioinformatics*.**30:** 3123-3124.

Shibata, M. and Terada, F. 2010.Factors affecting methane production and mitigation in ruminants. *Anim. Sci. J.* **81(1):** 2-10.

Shin, E.C., Choi, B.R., Lim, W.J., Hong, S.Y., An, C.L., Cho, K.M., Kim, Y.K., An, J.M., Kang, J.M., Lee, S.S. and Kim, H. 2004. Phylogenetic analysis of archaea in three fractions of cow rumen based on the 16S rDNA sequence. *Anaerobe*.**10**:

313-319.

Thauer, R.K., Kaster, A.K., Seedorf, H., Buckel, W. and Hedderich, R. 2008. Methanogenicarchaea: ecologically relevant differences in energy conservation. *Nat. Rev. Microbiol*. **6**: 579-591.

Wallace, R.J., Rooke, J.A., McKain, N., Duthie, C.A., Hyslop, J.J., Ross, D.W., Waterhouse, A., Watson, M. and Roehe, R. 2015. The rumen microbial metagenome associated with high methane production in cattle. *BMC genomics*. [on line]. **16**(1). Available: https://doi.org/10.1186/s12864-015-2032-0. [23 Oct 2015].

Whitford, M.F., Teather, R.M. and Forster, R.J. 2001. Phylogenetic analysis of methanogens from the bovine rumen. BMC Microbiol. [on line]. 1(1): Available: https://doi.org/10.1186/1471-2180-1-5. [16 May 2001]. ■