Heat Stress and Rumen Microbial Ecology: Novel Insights Into Goat Adaptation

Sejian Veerasamy (✉ drsejian@gmail.com)

National Institute of Animal Nutrition and Physiology <https://orcid.org/0000-0002-8224-4521>

Silpa MV

Justus Liebig Universitat Giessen

Shraddha Trivedi

NIANP: National Institute of Animal Nutrition and Physiology

Reshma Nair MR

Kerala Agricultural University

Bagath Madijagan

NIANP: National Institute of Animal Nutrition and Physiology

Krishnan Govindan

NIANP: National Institute of Animal Nutrition and Physiology

Devaraj Chinnasamy

NIANP: National Institute of Animal Nutrition and Physiology

Manjunathareddy GB

NIVEDI: National Institute of Veterinary Epidemiology and Disease Informatics

Malik PK

NIANP: National Institute of Animal Nutrition and Physiology

Soren NM

NIANP: National Institute of Animal Nutrition and Physiology

Giridhar K

NIANP: National Institute of Animal Nutrition and Physiology

Raghavendra Bhatta

NIANP: National Institute of Animal Nutrition and Physiology

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Abstract

Establishing the functional significance of rumen microbiome during heat stress may provide crucial information for elucidating climate resilience in goat. The current study was thus designed with an aim to assess the impact of heat stress on the rumen microbiome across three indigenous goat breeds. To prove this hypothesis, six experimental groups were chosen as follows: Osmanabadi Control (n=6), Osmanabadi Heat (n=6), Malabari Control (n=6), Malabari Heat (n=6), Salem Black Control (n=6) and Salem Black Heat (n=6). The study, being first of its kind, provides novel insights into influence of both genetic and environmental interaction on rumen microbial diversity in goats. An evident variation in the rumen microbial abundance was established during heat stress across all three goat breeds. The decreased *Firmicutes* and higher *Bacteroides* abundance in Salem Black goats could indicate the better protein and polysaccharide degrading ability of Salem Black breed. Further, the number of unique OTUs in the Salem Black heat group was higher as compared to both Osmanabadi and Malabari heat groups. In addition, the lower abundance of both KEGG as well as COG pathways indicated the sub-threshold level of heat stress experienced by this breed. Moreover, the histomorphometric changes in the rumen also pointed towards the better adaptive ability of Salem Black breed. This result point towards the genetic factor governing the rumen microbial diversity in goats during heat stress exposure. The findings provide substantial evidence on the genetic superiority of Salem Black breed in altering the rumen microbial population which could impart them the potential to thrive well in harsh environmental conditions.

1. Introduction

Livestock possess various adaptive mechanisms to survive during harsh environmental conditions which vary from species to breed up to individual level. Irrespective of the mechanisms adopted, they deter the productive ability of the animals (Sejian et al. 2018). Among the livestock species, goat is considered as an ideal climate change animal as this species survive well across all agro-ecological zones. Their essential feature of low input and ensured output is the most significant quality supporting many marginal small scale farmers in tropical regions (Kumar 2007). These animals, apart from being small and compact, have morphological characteristics like loose skin and floppy ears. Additionally, they possess excellent browsing behavior and have unique qualities like high digestive efficiency, low feed and water requirement and disease resistance (Gaughan et al. 2019). These are just few of the potent features of goats which represent only the tip of an iceberg as these species encompass a long list of influential characteristics. Time immemorial, goats have always proved their worth which somewhere down the line was neglected. However in the current climate change scenario, having known the impact and future consequences of climate change, it is of utmost importance to propagate goat farming as they are predicted to sustain production.

One of the preliminary responses to heat stress in farm animals is declined feed intake (Sejian et al. 2018). Reduction in feed intake is an adaptive strategy adopted by animals so as to reduce their metabolic heat generation. Additionally, Tajima et al. (2007) have also reported that decreased growth performance of animals during higher environmental temperatures are not only due to increased

maintenance energy expenditure but also due to shrinking carbon and energy supply obtained through microbial fermentation. A number of studies have been conducted on nutritional interventions, to ameliorate the adverse impact of heat stress on livestock (Hamzaoui et al. 2013; Sejian et al. 2014). Most of these studies succeeded in identifying suitable nutritional strategies to maintain production during such stressful situations. However, a vital component which was overlooked while conducting both impact assessment and ameliorative studies in heat stressed ruminants was the rumen microbiome.

The major component in ruminants playing a crucial role in their productivity is the rumen microbiome. This is characterized by its high population density, wide diversity and presence of myriad microbial interactions (Ghazanfar and Azim 2009). The rumen microbial composition plays an indispensable role in ruminant physiology, nutrition, pathology and host immunity (Li et al. 2015). Over the past few years, metagenomics has emerged as a powerful tool to study the rumen microbiome (Handelsman et al. 1998). Recent studies identifying rumen microbial community for novel enzymes, uncultured methanogens, and other metabolic pathways have opened new insights in this area. However, there are very few metagenomics studies conducted on heat stressed animals. This also is a potential area of research which could open up new pathways towards amelioration and mitigation of heat stress in farm animals.

Rumen microbiome plays a vital role in ruminants to convert low-quality plant feed into high-quality livestock products. Although the rumen microbial community is largely stable throughout the entire lifespan of the animal, it is highly responsive to diet, development stage, genetics and environmental stressors (Morgavi et al. 2012; Lei et al. 2019). There is a paucity of information on the impact of heat stress on rumen microbiome. Thus, this study holds a novelty for evaluating the variation in rumen microbial community during heat stress exposure in indigenous Osmanabadi, Malabari and Salem Black goat breeds. Additionally, this study is the first of its kind for assessing the breed variation in rumen microbial diversity during heat stress exposure.

2. Materials And Methods

2.1. Ethics Statement

The authors declare that all operations to animals in this study followed the standard practices of animal care and use. The study was conducted after obtaining approval from the Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA), Ministry of Environment, Forest and Climate Change, Animal welfare Department, Government of India.

2.2. Animal Experimentation and Sampling

Thirty six adult female goats (10–12 months age) weighing between 12 to 19 kg were selected for the study belonging to three indigenous breeds, Osmanabadi, Malabari and Salem Black with twelve animals in each breed. Though all the three were meat type goat breeds, they were from different native tracts located across three states of Southern India. Osmanabadi goats selected for the study were native to Karnataka, while Malabari and Salem Black goats belong to the hot humid states of Kerala and Tamil

Nadu states, respectively. All the animals were acclimatized for 45 days at the study location before the onset of the experiment. Additionally, standard management practices were followed at the experimental livestock unit wherein the animals were housed in well ventilated sheds throughout the duration of the study.

Twelve goats from each breed were further allocated randomly into two groups of six animals each thereby attaining six experimental groups; Osmanabadi Control (n = 6), Osmanabadi Heat (n = 6), Malabari Control (n = 6), Malabari Heat (n = 6), Salem Black Control (n = 6) and Salem Black Heat (n = 6). Through the entire 45 day study period all the animals were fed with a standard diet of 60 per cent Hybrid Napier (roughage) and the rest 40 per cent concentrate mesh comprising of maize (36 %), wheat bran (37 %), soyabean meal (25 %), mineral mixture (1.5 %) and common salt (0.5 %). All the control group animals were housed inside the shed without exposure to outside environment while the heat stress group animals were exposed to summer heat stress by keeping them outside the shed from 10:00 h to 16:00h. During the entire study period, all the animals were fed and watered once as per their individual requirement. The control group animals were fed and watered within the shed while the heat stress goats were provided the same outside the shed, during their exposure to heat stress.

2.3. Environmental variables

All the prime weather variables were recorded both inside and outside the shed, diurnally at 08:00 h and 14:00 h on a daily basis. The maximum (max T) and minimum temperature (Min T), ambient temperature (AT) and relative humidity (RH) were recorded with a thermo-hygrometer while the dry (DBT) and wet bulb temperatures (WBT) were recorded using a dry bulb and wet bulb thermometer. Additionally, the pen surface temperatures (PST) were also recorded by using an infrared thermometer. McDowell (1972) methodology was followed for calculating the temperature humidity index (THI). The equation as per this methodology to derive at THI values is as follows: $THI = 0.72 (T_{db} + T_{wb}) + 40.6$ where, T_{db} = Dry bulb temperature in °C; T_{wb} = Wet bulb temperature in °C.

2.4. Panting Score

The panting score for all experimental groups were recorded during 14.00 h at fortnightly intervals. The panting score was calculated as per method described by Darcan et al. (2007). The description and scoring pattern of panting behavior in goats was provided in supplementary table 1.

2.5. Rumen liquor samples

Rumen liquor was collected from three animals per experimental group randomly, on the last day of the study using a stomach tube connected to a hand help pump (Pragna et al. 2017). The collected rumen liquor samples were stored immediately at 4° C until DNA extraction.

2.6. Metagenomic DNA extraction and sequencing of 16s rRNA gene

Metagenomic DNA was extracted from the collected rumen liquor samples using cetyltrimethylammonium bromide (c-TAB) and Phenol: Chloroform extraction method. The isolated DNA was also subjected to RNase A treatment and its final quantity was estimated using the NanoDrop. After the quality check, the DNA samples were submitted to M/s Eurofins Genomics India, Bengaluru (www.eurofinsgenomics.co.in) for 16s rRNA (V3-V4) gene sequencing. Amplicon libraries were prepared using Nextera XT Index Kit (Illumina Inc.) as per the 16S metagenomic sequencing library preparation protocol (Part # 15044223 Rev. B). The V3-V4 16s rRNA region was amplified using region specific primers followed by amplification with dual index primers. The amplicon libraries were purified by AMPure XP beads and quantified using Qubit Fluorometer. On completion of the quantity and quality check on Agilent 4200 Tape station, the libraries were sequenced using the Illumina MiSeq platform.

2.7. Microbial community analysis/ Sequence and diversity analysis

The downstream processing of the sequence reads followed a series analysis including obtaining high quality clean pair end reads from the raw reads using Trimmomatic v0.38. The FLASH (v1.2.11) was used to stitch paired end data into single end reads which were then subjected to QIIME analysis. The quality filtered reads were joined using FLASH tool v1.2.11 (Magoč and Salzberg 2011) and then imported into QIIME for further analysis. The high quality sequences were clustered into operational taxonomic units (OTUs) using UCLUST at 97% sequence similarity followed by picking of representative OTU sequences. The representative sequences were used for taxonomical classification using UCLUST at 90% threshold against Greengenes database v13_8. The OTU count table along with the taxonomical classification obtained from QIIME was imported into Microbiome Analyst for further analysis including diversity, statistical and differential analysis.

Alpha (Shannon and Chao1 indices) and beta diversity analyses (Bray-Curtis index) of the data were rarefied to the minimum library size corresponding to ~ 54,000 sequences. The Venn diagram was plotted using the group wise OTU's which were picked using QIIME. Based on OTU's generated in their respective taxonomic level, phylum, class and genus, the Venn diagram was plotted using Jvenn. Additionally, heatmaps were generated using gplots package in R version 2.13.0.

2.8. Functional prediction of rumen microbiome

Functional capacity of the caprine rumen microbiota was predicted using the default parameters of the PICRUSt (Phylogenetic Investigation of Communities by Reconstruction of Unobserved States) software version 1.1.0. The Greengene picked OTUs generated during the sequence analysis pipeline was used in PICRUSt which was converted into a BIOM (Biological Observation Matrix) table. This was the input data for PICRUSt from which the relative Kyoto Encyclopedia of Genes and Genomes (KEGG) and Clusters of Orthologous Groups of proteins (COG) abundance information were derived.

2.9. Histomorphometric changes in the rumen

Ruminal tissues were collected from three animals in each group in 10% buffered formal saline solution. After fixation the tissues were processed to obtain 4 micron sections and were stained with Haematoxylin and Eosin. The stained sections were subjected for histomorphometric analysis using Nikon Trinocular Microscope Model Ci-L (Tokyo, Japan) with NIS Elements image analysis software to measure the ruminal papillae height and width. In every section at least five papillae were measured as described by Wang et al. (2009).

2.10. Statistical analysis

The diurnal variations in all the weather variables and THI were estimated by t-test. The panting scores data were analyzed using repeated measures analysis of variants using SPSS software (Version 18). The differentially abundant bacterial phyla between the control and heat-stress groups were determined using metagenomeSeq tool (using the *zero-inflated Gaussian fit* statistical model) integrated within Microbiome Analyst. Univariate T-test was performed to identify significant differentially abundant genera between the control and heat-stressed groups. Histomorphometric changes in the rumen were analyzed by general linear model univariate analysis using SPSS software version 18.

3. Results

3.1. Climatological Variables

Supplementary Table 2 describes the diurnal variation in average weather variables and THI for the entire study duration both inside and outside the shed. The DBT, WBT, Max T, Min T, RH, AT and PST were significantly ($P < 0.01$) higher outside the shed than inside the shed. Similarly, all these variables were significantly ($P < 0.01$) higher at 14.00 h when compared to 8.00 h. Similar pattern was also established for the calculated values of THI. The THI values outside the shed were significantly ($P < 0.01$) higher throughout the study period and falls in the category of extremely severe heat stress as per the McDowell (1972) formula.

3.2. Panting Score

Figure 1. describes the average panting score for the entire study duration in the heat stress groups of Osmanabadi, Malabari and Salem Black goat breeds. The animals in the control groups of all the three breeds did not show any panting. Although the panting score increased in all the three breeds' heat stress groups, still the level of panting in Salem Black breed was significantly ($P < 0.01$) lower than the other two breeds. However, the panting scores were comparable between the heat stress groups of both Osmanabadi and Malabari breeds.

3.3. Sequence acquisitions, OTU's and ruminal microbial diversity

A total of 4,239,575 reads were obtained on sequencing the V3-V4 region of 16S rRNA of the rumen microflora from 18 goats with an average of 235,532 reads per sample. From the 18 ruminal DNA

samples 39304 OTU's were detected which based on 97% identity between reads and having a range from 1123 to 2859 OTU's. The alpha diversity indices, Chao1 and Shannon index, were used to estimate the microbial diversity both between the overall and breed-wise control and heat stress groups. While heat stress increased the bacterial Chao1 diversity on Osmanabadi goats, the same was reduced in Salem Black goats (Supplementary Fig. 1). However the opposite trend was observed for Shannon index with lower values in heat stressed Osmanabadi goats and higher in Salem Black heat stress when compared to their respective control (Supplementary Fig. 2).

The PCoA resulted in a 3-dimensional solution (Supplementary Fig. 3) in which, PC1 accounted for 41% of the variation and PC2 and PC3 for 13.6 and 11.3%, respectively. Overall the results indicated a clear division between all the six experimental groups.

3.4. Rumen microbial composition of control and heat-stressed goats based on 16s rRNA gene sequencing

Heat stress was found to influence the composition of rumen microbial phyla at the breed level when compared to their respective control groups (Fig. 2a). While the heat stressed Osmanabadi goats had a relatively higher abundance of *Firmicutes* (40.14–50.04%), the same phyla had a lower abundance in Salem Black (49.15–42.99%) and Malabari (51.33–50.62%) goats under heat stress. Additionally, a substantial decline in the relative abundance of *Proteobacteria* (21.06–15.76%), *Planctomycetes* (9.51–3.42%) and *TM7* (2.51% to 0.70) was observed in heat stressed Osmanabadi goats. Additionally, an increased abundance of *Planctomycetes* was observed in heat stressed Malabari goats (3.76–5.61%) while the same had a lower abundance in Salem Black goats (4.69–2.98%).

Further, an appreciable microbial abundance at the class level was observed between all the heat stressed breeds when compared with their respective control groups (Fig. 2b). The abundance of several rumen microbial classes were altered as a consequence of heat stress within each breed group thereby indicating the impact of heat stress on the rumen microbial ecology.

The variation in the ruminal microbial genera at the breed level was also evaluated depicting both within breed and within treatment variations (Fig. 2c). The genera exhibiting a highlighting abundance in these groups were *SHD_231*, *Pseudomonas*, *Butyrivibrio*, *Methanobrevibacter* and *Ruminococcus*. While the relative abundance of *Pseudomonas* was similar both in Salem Black control and heat stress goats, a drastic reduction was noticed in Osmanabadi (14.79–0.82%) and Malabari (6.16–1.23%) heat stressed goats than their respective control goats. Though the relative abundance of *Pseudomonas* was alike across all the breeds subjected to heat stress, the proportion of its reduction highly varied; the maximum reduction noticed in Osmanabadi (-13.97%) than the Malabari goats (-4.93%). The relative abundance of *SHD_231* was higher in heat stressed Osmanabadi goats (2.9–15.03%) while a decline was observed in heat stressed Salem Black goats (2.40–0.91%) as compared to control. Comparing across the three heat stressed breeds, Osmanabadi goats had the highest relative abundance of *SHD_231* while Salem Black had the least (15.03% and 0.91%, respectively). Another highly variable relative abundance was observed in the genus *Methanobrevibacter*. While the relative abundance of this genus declined in Malabari goats

(1.32–0.66%), a substantial increase was noticed in Osmanabadi (1.72–3.26%) and Salem Black (0.41–1.98%) heat stressed goats. The genus *Butyrivibrio* and *Ruminococcus* also exhibited varied relative abundance across the breeds and treatment groups. The relative abundance of *Butyrivibrio* was higher in heat stressed Osmanabadi (1.63–2.66%) and Salem Black (1.79–5.78%) goats than the control with a highlighting increase in the later breed. The relative abundance of *Ruminococcus* was also drastically reduced in Salem Black (4.96–2.06%) goats subjected to heat stress compared to its control goats. The relative abundance of these two genera was almost similar in Malabari goats.

Additionally, the heat maps representing the microbiota at the phylum, class and genera level for all the control and heat stress groups also depicts a distinctive variation (Supplementary Figs. 4, 5, 6). Heat stress could be found to have an influence in altering the rumen microbial composition in these animals. Variation in the rumen microbiota among the control groups of the three breeds could be attributed to the genetic differences prevailing in these breeds. However, the variations between and among the treatment groups highlights the impact of heat stress.

3.5 Common and unique bacterial phyla, class and genus levels among three goat breeds under control and heat-stress conditions

The Venn diagram depicting the OTU distribution at phyla level was described in supplementary Fig. 7. At phyla level, a total of 1624 OTUs were found to be common among all the treatment groups (Supplementary Fig. 7). The supplementary Fig. 8 depicts the Venn diagram for OTU distribution at class level wherein a total of 1621 OTUs were found to be common among all the treatment groups. The OTUs unique to each group was also depicted in this figure.

The Venn diagram depicting the OTU distribution at genus level was described in Fig. 9. At the genus level, a total of 890 OTUs were found to be common among all the treatment groups (Supplementary Fig. 9). The unique OTUs possessed by Osmanabadi control, Osmanabadi heat, Malabari control, Malabari heat, Salem Black control and Salem Black heat were 108, 49, 43, 59, 78, and 89, respectively. Forty four OTUs representing the bacterial genera were found to be common in the rumen of the Osmanabadi control and heat group goats likewise the same for Malabari and Salem Black goats were 44 and 67 respectively (Supplementary Fig. 9).

3.6. Differential analysis of microbial taxa between control and heat-stressed groups

Differential analysis of the microbial taxa resulted in a total of 3 significantly differential phyla between the heat-stress and control groups across three breeds. *Chroloflexi* was found to be significantly higher in abundance ($P = 0.02$) in the heat-stressed Osmanabadi breed compared to the control (Fig. 4). The bacterial phyla *WPS_2* ($P < 0.01$) and *Tenericutes* ($P = 0.02$) were significantly higher in Malabari heat-stressed condition (Fig. 10), whereas, *WPS_2* had significantly ($P = 0.039$) lower abundance in the heat-

stressed Salem black breed compared to their respective control group (Fig. 10). Similarly, at the genus level, 7 bacterial taxa were significantly abundant between the heat stress and control goats across all the breeds (Fig. 10). Among these, *Carnobacterium* and *L7A_E11* were most significant in the Osmanabadi (Fig. 10) and Malabari (Fig. 10) breeds, respectively and both had lower abundance in the heat-stressed condition. *Devosia* was the only significant genus, and had a higher abundance in the heat-stressed condition in Salem black breed (Fig. 10). The other genera that were significantly different between the heat-stress and control conditions were *Bdellovibrio* in Osmanabadi (Fig. 10), and *Larkinella*, *Rubricoccus* and *Truepera* in Malabari breeds (Fig. 10).

3.7. Functional prediction of rumen microbiome

Figure 4a describes the most abundant predicted functional rumen metagenome pathways at KEGG level-01 across the groups. The pathway metabolism is the major functional attribute of the caprine rumen microbiome irrespective of breed and heat stress treatment followed by genetic information processing, environmental information processing, cellular processes and organismal systems respectively. In Osmanabadi and Malabari breeds all these abundant metagenome pathways are similar between their respective control and heat stress groups. However, in Salem Black breed all these pathways abundance got reduced in heat stress group as compared to its control group. Figure 4b describes the most abundant predicted functional rumen metagenome pathways at KEGG level-02 across the groups. The predominant top five functional attributes of level 02 KEGG pathways identified in the caprine rumen microbiome are membrane transport, carbohydrate metabolism, energy metabolism, metabolism of cofactors and vitamins, and lipid metabolism. In Osmanabadi and Malabari breeds all these abundant level 02 KEGG metagenome pathways are similar between their respective control and heat stress groups. However, in Salem Black breed all these level 02 KEGG pathways abundance got reduced in heat stress group as compared to its control group.

Figure 4c describes the abundance of different COGs across the control and heat stress groups in three goat breeds. The COGs also showed similar trend as that of KEGG pathways. The levels of abundance of most of the COGs across the control and heat stress groups in Osmanabadi and Malabari breeds are similar. However, in Salem Black breed the abundance of most of the COGs got reduced in heat stress group as compared to its control group.

3.8. Histomorphometric changes in the rumen

Impact of heat stress on the rumen histomorphometric changes in three indigenous goat breeds was depicted in Fig. 5a. Further, the impact of heat stress on the ruminal papillae height and width recorded in three indigenous goat breeds are described in Fig. 5b. Heat stress significantly reduced ($P < 0.01$) both the height and width of papillae in all three breeds during heat stress exposure. Among the heat stress groups, the magnitude of decrease in papillae height was more in Osmanabadi and Malabari breeds as compared to Salem Black breed. Further, increased levels of keratinization and sloughing off of squamous cells of papillae was noticed in the heat stress groups of Osmanabadi and Malabari breed as compared to Salem Black breed.

4. Discussion

Research efforts pertaining to identifying superior thermo-tolerant indigenous breeds are gaining significance as the approach may help to sustain livestock production in the changing climate scenario. The present study being first of its kind, is one such attempt in trying to establish thermo-tolerance in three different indigenous goat breeds based on changes associated with rumen microbiome. The results from the study revealed that the animals were exposed to extremely severe heat stress as reflected by the weather variables and THI. Further, the panting score also revealed that the animals experienced the heat stress. In addition, heat stress was found to influence rumen microbiome at various levels such as phyla, class and genera.

The weather variables and THI indicated that the animals in the shed were at comfort condition while the animals exposed to outside environment were subjected to extremely severe heat stress. As per McDowell (1972) THI model any values above 78 is considered extremely severe heat stress and the average fortnightly THI values of 86.5, 86.3 and 86.8 in outside environment reflected that the heat stress group animals were exposed to extremely severe heat stress throughout the study duration. Similar observations using the same THI was also established by other researchers (Shaji et al. 2016; Aleena et al. 2019). Likewise the panting score established among the heat stress groups reflected the differences in the level of heat stress being experienced across the breeds. Gaughan (2003) established that panting score has the potential to be used in the assessment of the welfare status of animals. Although all the three breeds were experiencing severe heat stress as reflected by their panting score but the magnitude of this effect was less in Salem Black breed as compared to both Osmanabadi and Malabari breeds. The panting score in the heat stress groups of Osmanabadi and Malabari breeds were comparable. Similar observations of validating the magnitude of heat stress experienced by goats based on panting score changes was established by Darcan et al. (2007) and Fioni (2014). In a recent report Reddy et al. (2019) has demonstrated that panting score could be a reliable easy to use methodology to assess thermal resilience capacity of goats.

Firmicutes, *Proteobacteria* and *Bacteroidetes* were among the most abundant phyla in all the breeds irrespective of heat stress, which was in line with the findings reported by previous studies revealed by rumen metagenomics in goats (Wang et al. 2016; Do et al. 2017). Furthermore, *Firmicutes* and *Bacteroidetes* were found to be the dominant bacterial phyla in rumen fluid of buffalo and Jersey cows; indicating that these phyla could be the dominant core microbiome in ruminant species (Henderson et al. 2015). Similar prevalence of dominant phyla *Firmicutes*, *Proteobacteria* and *Bacteroidetes* were also reported by Zhong et al. (2019) in Boer goats subjected to heat stress. *Firmicutes* are primarily composed of gram positive low GC content bacteria (Boone et al. 2001). Previous studies have indicated that the abundance of *Firmicutes* is related to the feed utilization efficiency and found to be higher in gut of efficient feed utilizing animals (Myer et al. 2015). *Firmicutes* are the largest bacterial phylum and their higher abundance may increase carbohydrate fermentation in the rumen (Fernando et al. 2010). Thus, higher abundance of *Firmicutes* in Malabari and Salem Black breed control groups in the present study indicates that these breeds are more efficient in breaking down of complex lignocellulosic feeds than

Osmanabadi. The phylum, *Firmicutes*, includes a majority of butyrate-producing bacteria (Li, 2015), which has the least abundance in Salem Black heat stressed goats while highest in Osmanabadi heat stressed goats. However, the phylum *Bacteroidetes* is known as good degraders of complex polysaccharides present in the plant cell wall than *Firmicutes* (Khafipour et al. 2016). This phylum has the highest abundance in Salem Black goats subjected to heat stress than the other two breeds. Thus the decreased *Firmicutes* and higher *Bacteroidetes* abundance in Salem Black goats could indicate the better adaptation of this breed to heat stress when compared to the other two breeds of goat. Further, it is an established fact that *Firmicutes* phylum also has high abundance of known H₂ producer species (Stewart et al. 1997). Therefore, the lower *Firmicutes* phylum in the heat stress groups of Salem Black breed establishes the advantage of this breed over other two breeds in terms of contributing low H₂ in the rumen environment. Nearly similar alteration of rumen microbiome at class level was noticed in heat stressed Osmanabadi, Malabari and Salem Black goats when compared to their control. This could be attributed to the indigenous nature of these breeds.

Previous studies have shown that *Bacteroidetes* possesses a strong ability to degrade protein and polysaccharides (Huo et al. 2014; Pitta et al. 2016). Moreover, *Bacteroidetes* are considered net H₂ (hydrogen) utilizers (Stewart et al. 1997). As evident from the present study, the *Bacteroidetes* was reported to be the third abundant phylum, with highest abundance in Salem Black breed heat stress group. Hence, this breed could possess better protein and polysaccharide degrading ability in addition to increased utilization of H₂. Thus, Salem Black breed could have the dual advantage of increased production and low methane emission potential.

Güllert et al. (2016) suggested that low ruminal *Firmicutes* to *Bacteroidetes* ratio would increase the capacity of biomass degradation as the *Bacteroidetes* phylum had higher glycoside hydrolase enzymes. When comparing the three goat breeds subjected to heat stress, this ratio was least for Salem Black goats and highest for Osmanabadi goats which further add on to the potential superiority of Salem Black goats to adapt to heat stress. Though the relative abundance of several other phyla varied as a consequence of heat stress, there is paucity of information on their functional role in the rumen.

Generally, the genus *Prevotella* was found to degrade nonstructural carbohydrates and protein (Belanche et al. 2012; Thompson et al. 2015). Further, this genus also involves in amino acid metabolism, nucleotide metabolism, energy metabolism, and glycan biosynthesis (Hook et al. 2011). However, the present study indicated no significant change in this genus in control and heat stress groups across all the breeds with a slightly higher prevalence in Salem Black breed as compared to other breeds indicating the better feed degradation and nutrient utilizing ability by this breed.

The *Butyrivibrio* genus was known to modulate secretion of hemicelluloses-degrading enzymes thereby proposed to play an important role by contributing polysaccharide degradation in rumen (Dunne et al. 2012). The significantly increased abundance of this particular genus in Salem Black breed heat stress groups signifies the better digestion efficiency of this breed over other breeds. *Ruminococcus* is one of the most predominant fibrolytic bacteria in the rumen (Hespell et al. 1997) and their concentration was

expected to increase for the breakdown of generally increased cellulose and hemicellulose content in ruminant feed (Biddle et al. 2013). In the present study, the abundance of *Ruminicoccus* was found to decrease in heat stress group of Salem Black breed as compared to control, indicating the sensitivity of this genus to heat stress in this particular breed.

Bifidobacteria are known to be the beneficial gut bacteria, as they act as probiotic and helps maintain healthy internal environment of the host (Song et al. 2012). In the present study, we did not observe any reduction in beneficial rumen bacteria (probiotic) like *Bifidobacteria* in control and heat stress groups, respectively across all breeds. This could be attributed to the indigenous nature of all three breeds. However, the overall increased concentration of this particular genus in Salem Black breed indicates the better adaptability of this breed for heat stress.

Butyrivibrio group is known to be a fibrolytic bacterium (Fernando et al. 2010) and are the most active bacterial species involved in the biohydrogenation of C18 unsaturated FA (Durmic et al. 2008). The increased concentration of *Butyrivibrio* in all heat stress groups across the breeds indicates that these breeds are much tolerant and well adapted to degrade unsaturated fatty acids efficiently even in heat stress condition. However, the evident increase in concentration of this particular genus in Salem Black heat stress group over other breeds establishes the better adaptability of this breed to heat stress challenges.

Additionally, an evident increase in *Methanobrevibacter*, having a significant role in contributing towards methane production was observed in heat stress groups of both Osmanabadi and Salem Black goats as compared to Malabari breed. However, this particular result needs to be validated by measuring methane production in addition to specifically targeting metagenomics analysis for 16s archeal microbial population to have a clear picture of this association. This is because as per the results of the previous studies, it has been proved Salem Black breed to be well adapted and also maintain its production during heat stress when compared to Osmanabadi and Malabari goats (Aleena et al. 2018; Pragna et al. 2018). Further, Denman et al. (2015) reported that abundance of *Prevotella* was stimulated when methanogenesis (which is a hydrogen-consuming process) was inhibited. However, the present study turned out to be a deviation of this previous finding (Denman et al. 2015); we reported a higher abundance of *Methanobrevibacter* as well as slight increase in *Prevotella* in heat stress groups of Osmanabadi and Salem Black breed.

The very less number of significantly abundant microbes, due to heat stress, at all taxonomic levels in the Salem Black goat indicates the sub-threshold level of heat stress in this breed to induce changes in the microbial composition unlike Osmanabadi and Malabari. These findings again establish the immense adaptive potential of Salem Black breed to keep intact the rumen microbial composition probably to maintain the digestive functions.

At each taxonomic (phyla, class and genera) level, the number of OTUs in common between the control and heat stress groups was higher in Salem Black breed as compared to both Osmanabadi and Malabari breeds indicating the lesser impact of heat stress on the microbial diversity in Salem Black breed. Further,

the number of unique OTUs in the Salem Black heat group was higher as compared to both Osmanabadi and Malabari heat groups. This shows that the level of heat stress in the study was of lower threshold to induce higher impact on rumen microbial population in Salem Black breed. These findings points towards the superior ability of Salem Black breed over the other two breeds to maintain rumen microbial population to elicit important biological functions.

Both level 01 and level 02 KEGG analysis as well as COG analysis revealed that heat stress did not influence the abundance of predicted functional rumen metagenome pathways in Osmanabadi and Malabari breeds. However in Salem Black breed, the heat stress group showed less abundance of all the rumen metagenome pathways. This indicates that the magnitude of heat stress did not elicit stress response to the level to influence predicted functional metagenomics pathways in Salem Black breed. The probable reason for the reduced abundance of all these pathways in heat stressed Salem Black breed could be attributed to the sub-threshold level of the heat stress experienced by these animals. Further, this hypothesis was justified by the lower level of panting score recorded in the heat stress group animals of this breed in contrast to other two breeds. Generally, the metabolic activities are expected to be higher in breeds wherein there is more requirement of energy to support life sustaining activities to cope with heat stress. Since the heat stress level was sub-threshold in Salem Black breed, the metabolic and other cellular pathways associated with adaptive mechanisms are of less abundance in this breed. In a similar study conducted on the Salem Black breed, lower levels of expression was established for cellular heat stress markers such as HSP70 and HSP90 and the authors attributed this to the sub-threshold level of the heat stress experienced by this breed (Madhusoodhan et al. 2020). One should never overlook the fact that the THI established in this study falls in the category of extremely severe heat stress. Further, the higher THI induced significantly increased levels of stress response in heat stress groups of both Osmanabadi and Malabari breed as evident from the higher magnitude of panting scores recorded in these breeds. This indicates that the higher threshold stress level in Osmanabadi and Malabari breed was of sub-threshold level in Salem Black breed reflecting the inherent ability of this particular breed to cope with heat stress of higher magnitude without altering much of its adaptive pathways.

The morphometric characteristics of ruminal papillae in terms of height and width has significant role in adaptation of animals to heat stress challenges (Yan-fen et al. 2013). These anatomical structures in the rumen have implications in determining the digestion efficiency of animals during heat stress in tropical countries. In our study it was observed among the three breeds, the Salem Black breed showed lesser degree of changes in ruminal mucosa than the other two breeds indicating the better genetic potential of this breed in adapting to adverse environment. These findings in the histological changes in the rumen among the breeds further supports the superior genetic potential of Salem Black breed to counter heat stress challenges. Further, studies are required though to document in details the heat stress associated changes in the rumen histology.

5. Conclusion

The study presents some unique findings which provide clear insight into the heat stress associated changes in rumen microbiome across three indigenous goat breeds. The THI index used in the study reflected that the animals were subjected to extremely severe heat stress. The phenotypic trait panting score indicated that Salem Black goat breed to be the better climate resilient breed as compared to Osmanabadi and Malabari breeds. The results obtained in the study revealed a significant alteration in the rumen bacterial community at all taxonomic levels across control and heat stress groups of all the three targeted breeds. The findings from the study thus provided answer from the different angle of rumen microbiome for better climate resilience in Salem Black goat breed. Though there are several approaches for ameliorating the heat stress effects in livestock, one crucial aspect often neglected is the rumen microbiome. In this context, this preliminary study provided some crucial initial information on the relationship between heat stress and the ruminal microbiome which could help in identifying novel strategies to relieve the effect of heat stress by manipulating or altering the ruminal microbial composition. Such an approach could open up new concepts to tackle climate change associated production loss in ruminants.

Declarations

Ethics Statement

The authors declare that all operations to animals in this study followed the standard practices of animal care and use. The study was conducted after obtaining approval from the Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA), Ministry of Environment, Forest and Climate Change, Animal welfare Department, Government of India (NIANP/IAEC/2/2017).

Consent to publish

There no any such material in this manuscript to get consent to publish

Authors' contributions

VS and RB conceived the work; all authors designed the experiments, MVS, ST, MRRN, PKM, and NMS analyzed the data, all authors interpreted the data; VS, MVS, MB, GK, CD, GBM, KG and RB wrote the manuscript. All authors contributed to the final writing of the paper. All authors read and approved the final manuscript.

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Competing Interests

The authors declare that there is no any conflict of interest for this manuscript

Availability of data and material

The dataset collected for the analysis is available upon reasonable request to the authors.

Sequence Code Availability

16s V3-V4 rRNA metagenome sequences generated were deposited into the National Center for Biotechnology Information (NCBI) Sequence Read Archive (SRA) database under the BioProject ID PRJNA655551.

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Figures

Fig. 1

Figure 1

The average panting score during the study period (45 days) in heat stress groups of Osmanabadi, Malabari and Salem Black goat breeds. Values bearing different alphabets across the breeds differ significantly with each other at $P < 0.01$.

Figure 2

(a) Distribution of most dominant rumen microbial phyla within and between the three indigenous goat breeds subjected to heat stress and their respective control groups (those phyla with relative abundance $<0.1\%$ were combined as 'Others'). Relative abundance of different microbial phyla varied within all the three goat breeds during heat stress and also a significant variation in the abundance was noticed between the breeds. (b) Distribution of the most abundance rumen microbial class among all the six experimental groups, Osmanabadi control, Osmanabadi heat stress, Malabari control, Malabari heat stress, Salem Black control and Salem Black heat stress. Classes with relative abundance $<0.1\%$ were grouped as 'Others'. Microbial classes varied both across breeds and also across the experimental groups indicating and impact of heat stress in rumen microbiota. (c) Genus level microbial composition of Osmanabadi, Malabari and Salem Black goats subjected to heat stress along with their control groups. 'Others' refers to genera with an abundance of $<0.1\%$. The relative abundance of several vital microbial genera was altered in Osmanabadi, Malabari and Salem Black goats, under heat stress, when compared with their respective control groups. Additionally a significant variation was also noticed between the breeds

Fig.3

Figure 3

Significantly abundant taxa composition across different breeds. Both in Osmanabadi and Malabari breeds there were more significantly abundant microbes at different taxonomic levels as compared to Salem Black breed. This result clearly indicates that Salem Black breed was genetically superior over the other two breeds in terms of maintaining the rumen microbial diversity during heat stress exposure.

Figure 4

a. PICRUSt derived abundance values of predicted functional rumen metagenome at KEGG level-01. The predominant functional attributes of KEGG pathways identified in the caprine rumen microbiome are metabolism, genetic information processing, environmental information processing, cellular processes and organismal systems. The levels of abundance of these functional attributes are similar in Osmanabadi and Malabari breeds between their respective control and heat stress groups while in Salem Black breed the heat stress treatment reduced the abundance of all the pathways as compared to its control group. b. PICRUSt derived abundance values of predicted functional rumen metagenome pathways at KEGG level-02. The predominant top five functional attributes of level 02 KEGG pathways identified in the caprine rumen microbiome are membrane transport, carbohydrate metabolism, energy metabolism, metabolism of cofactors and vitamins, and lipid metabolism. The levels of abundance of these KEGG level 02 functional attributes are similar in Osmanabadi and Malabari breeds between their respective control and heat stress groups while in Salem Black breed the heat stress treatment reduced the abundance of all the pathways as compared to its control group. c. PICRUSt derived abundance of different COG categories for all the treatment groups. The abundance levels of COGs are similar between the control and heat stress group in both Osmanabadi and Malabari breeds while in Salem Black the abundance of COGs was lower in heat stress group as compared to its control group.

Fig. 5(a)

Fig. 5(b)

Figure 5

a. Impact of heat stress on the rumen histopathological changes in three indigenous goat breeds. The histological changes associated with heat stress pertaining to rumen papillae were evident in all three breeds. Further, increased keratinization and sloughing off of squamous cells of papillae was noticed in the heat stress groups of Osmanabadi and Malabari breeds as compared to Salem Black breed. b. Effect of heat stress on the rumen papillary height and width in all three indigenous goat breeds. Both papillary height and width showed significant difference for heat stress in all three breeds. Means bearing different superscript with the rumen variables differ significantly at $P < 0.01$.

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