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RUMEN MICROBES ASSOCIATED POTENTIAL TO ESTABLISH CLIMATE RESILIENCE IN RUMINANTS – A REVIEW

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Abstract

Ruminants are stated to play a pivotal role of converting feed resources into nutritious outputs suitable for human consumption, which primarily is attributed to the diversified rumen microbiota they harbor. The rumen microbial niche is however not explored to its complete potential due to several limitations, the crucial one being the inability to culture more than 80% of the rumen microbes. Advances in next generation sequencing approaches have made promising inroads to bridge this gap. The adoption of techniques like 16S rRNA sequencing and whole genome shotgun metagenomics has now made it possible to assess the structural and functional dynamics of rumen microbiota. Nevertheless, there is a dearth of information pertaining to the impact of climate change on rumen microbe. This review was therefore an attempt to highlight the potential role of rumen microbiome during heat stress and also to emphasize on the significance of adopting rumen metagenomics to link climate change impact with altered productive potential in ruminants. Changes in ruminal microflora, like alterations in *Firmicutes-Bacteroidetes* ratio (highly correlated with milk fat yield), altered abundance of *Fibrobacteres* group (potentially heat generating microbes), *Actinobacteria* group (acetate producing microbes), *Fusobacteria*, *Cyanobacteria* and so on were observed in ruminants during heat stress. Furthermore, it was also possible to link these alterations with several functional and metabolic pathways, like energy production and conversion, defense mechanism, lipid transport and metabolism, coenzyme transport and metabolism, cell cycle control, cell division and a few more. Employing rumen metagenomics could also aid in understanding the differences in heat stress adaptive strategies between breeds. Therefore, intensifying the application of this methodology with heat stress in ruminants and extending it to assess the effectiveness of nutritional amelioration strategies could start a new journey towards heat stress mitigation in ruminants.

Key words: climate change, functional analysis, heat stress, metagenomics, rumen microflora

Climate change is predicted to have detrimental effects on all life forms on the globe. The agricultural sector, which acts as a backbone to support the gross domestic product (GDP) especially in developing countries, is also going to be severely affected by climate change (Sejian et al., 2021 a). The direct and/or indirect impact of climate change on agriculture and livestock production is mainly due to impaired fodder production, changes in pasture composition, poor herbage quality, increasing conflicts on land usage for agriculture, increased productive losses in livestock, emerging and widespread disease outbreaks and so on (Naqvi and Sejian, 2011).

Heat stress, one of the major consequences of climate change, has been proven to impose serious negative impacts on livestock production and welfare globally (Sejian et al., 2021 b). Heat stress can be understood to be a condition that occurs when the animal's body temperature increases beyond a threshold limit beyond which the individual cannot dissipate body heat adequately so as to maintain thermal equilibrium (Morrison, 1983; Bernabucci et al., 2010). An elevation in ambient temperature above thermoneutral zone along with high humidity, slow air movement and other environmental variables are the primary factors contributing towards heat stress (Morrison, 1983; Bernabucci et al., 2010; Wang et al., 2020). The thermoneutral zone can be defined as "the range of environmental temperature within which a constant body temperature is maintained, with minimal effort from thermoregulatory mechanism" (Krishnan et al., 2023). Defining the critical/threshold temperatures is difficult as it varies among species, breed, age group and production cycle of the animals, region, etc.

Livestock experience a series of behavioral and physiological alterations due to heat stress, all of which

impairs their productive potential, reproductive ability, immune status and well-being (Sejian et al., 2021 b). Extensive studies have been conducted to assess the impact of climate change, especially heat stress on ruminants and to assess differences in heat stress responses among them (Lees et al., 2018; Pragna et al., 2018). While genetics and environment describe most of the differences exhibited by various species, breeds, and individuals to heat stress, one potential factor gaining significance is the rumen microbiome (Kim et al., 2020). As an evident fact, the rumen hosts a highly dense and diversified group of microbial populations, which in itself is a separate niche. The richness, composition, and harmony of the ruminal ecosystem is governed by several factors, environment being one of them (Kim et al., 2020). The importance of the rumen microbiome in overall animal health is attracting the attention of livestock researchers working on the impacts of climate change, to unravel the ruminal dynamics in heat stressed animals.

Several reports on altered ruminal variables during heat stress have been published in cattle (Lees et al., 2018), buffalo (Gaafar et al., 2009), sheep (Bernabucci et al., 2009) and goat (Chaidanya et al., 2017). However, there are numerous unexplored paths in this ecosystem. It should be noted that only 20% of the rumen microbiota have been reported to be cultivable using standard techniques (Krause et al., 2013). Hence the recent advances in rumen microbiome and feed fermentation research focuses on "metaomic" methodologies like metagenomics, metatranscriptomics, metabolomics and metaproteomics (Islam and Lee, 2018). Metagenomics approaches will help to establish the significant role of how the rumen microbiome influences productive returns in ruminants (Islam and Lee, 2018). This methodology also makes it possible to assess the microbial community composition and dynamics among species, breeds and animals subjected to different environmental and management conditions (Islam and Lee, 2018; Kim et al., 2019). While most of these applications have gained an appreciable momentum, their adoption to assess heat stressed rumen microbiome is limited. This review is therefore an attempt to highlight the significance of understanding the impact of climate change on rumen microbiota at its structural, genetic, and functional level focusing primarily on rumen metagenomics.

Farm animals' significance from food security perspectives

Climate change will negatively influence global animal production systems unless we implement quick and efficient actions. These could begin by gaining an understanding of the mechanism of adaptability in animals to a changing climate. Furthermore, improving the production from local breeds through improved breeding models or biotechnology tools is also a wise strategy (Sejian et al., 2021 c). However, more research efforts are needed with the support of policy makers to improve the efficiency of livestock production systems to ensure sustainable livestock farming into the future. Livestock has the potential to fulfil the calorific and protein demand of current and future populations provided improvements in production systems, processing and storage of livestock products are improvised, keeping in view the present and future climate change scenario.

Significance of understanding the hidden intricacies of livestock adaptation

Livestock face a variety of environmental stressors, of which the most captivating component influencing its productivity in the constantly shifting climate scenario is the thermal stress. Further, arid to semi-arid tropical environments which harbor a significant proportion of the global livestock populations, face severe climatic fluctuations, water scarcity and low biomass productivity (Sejian et al., 2010). For generations, livestock husbandry has been practiced under harsh and extreme environmental conditions. In order to cope with the stressors in the challenging environmental condition, animals have acquired a variety of adaptable traits that improve their chances of surviving (Naskar et al., 2012).

Animals need to be genetically compatible and produce optimally in order to thrive in a variety of adverse conditions in a climate challenging environment. Livestock may adapt to a variety of environmental stressors through a dynamic symphony of a wide range of morphological, behavioral, physiological, biochemical, endocrine, cellular, and molecular responses (Niyas et al., 2015). The morphological adaptive changes include the allometric features of the body such as body size and form, extremity organization, skin characteristics like colour, thickness, sweat gland distribution pattern, and features of the hair coat like colour, thickness, absorption, reflectivity, and angle of hair arrangement to skin (Sejian et al., 2017).

Behavioral adaptations are the primary response exhibited by an animal to heat stress (Shilja et al., 2016). Moreover, the key physiological adaptive mechanisms that assist in preserving homeostasis and heat balance in stressed animals are respiration rate (RR), pulse rate (PR), skin temperature (ST), and rectal temperature (RT). In addition, it has been established that there tends to be a strong variation in the physiological adaptability to harsh environments across breed (Rashamol et al., 2019). Blood-biochemical and endocrine markers e.g., tri-iodothyronine, thyroxine, cortisol, non-esterified fatty acids, hemoglobin, hematocrit, packed cell volume and many more along with cellular responses like heat shock proteins, cytokines, toll-like receptors and apoptotic genes act synergistically to maintain homeostasis in animals (Sejian et al., 2018).

The adaptive responses can thus be used as ideal indicators for quantification of the heat stress response in livestock. Moreover, comprehending the adaptive mechanism involved in adjusting to heat stress might facilitate our understanding of the alterations in livestock productive variables. It can also assist in improving animal

welfare and in framing mitigation strategies to earn good returns and ensure optimal production in the climate change scenario.

Livestock adaptation to hot environment is mediated by a complicated network of genes (Mwacharo, 2016). Moreover, indigenous livestock animals developed in demanding tropical habitats exhibit diverse adaptive traits. A study established that these tropically adapted breeds may also thrive and procreate under adverse seasonal conditions with inadequate nutrition and increased disease and parasite loads (Renaudeau et al., 2012). Thus, an improved comprehension of this native livestock adaptation to climate challenging environment will aid in framing the selection criteria of the animals used across breeding programs.

Most often, heat stress adaptation studies focus on the previously described mechanisms *viz*, behavioral, physiological, biochemical, endocrine, cellular and molecular. However the dynamics of rumen microbiota and its associated implications are less explored in relation with susceptibility/resilience to heat stress. Researchers were successful in establishing alteration in rumen microbiota is response to heat stress in ruminants (Chen et al., 2018; Zhao et al., 2019). Furthermore, heat stress influenced rumen microbial dynamics were also stated to affect physiological responses, feed intake and production efficiency (Chen et al., 2018; Kim et al., 2022). Encouraging further research in such area, may aid to strengthen the knowledge on rumen microbial dynamics in response to heat stress. Associating alterations in rumen microflora with established heat stress response and/or adaptation traits can play a vital role towards defining the rumen microbiota influenced adaptive mechanisms in animals.

Therefore it is vital to understand livestock adaptation as it plays a crucial role in incorporation of beneficial traits while strategizing breeding programs. Thereby, identification of adaptable breeds in specific agro-climatic zones is possible and this further will assist in assuring climate resilient livestock production.

Significance of rumen microbes in ruminant species

The rumen microbiome is a complex community of various microbial species comprising of bacteria, fungi, protozoa, amoeba and bacteriophages (Mackie et al., 2002). Rumen microbes play a critical role in the digestive process and overall health of the ruminants by modifying the ruminal environment and participating in various metabolic activities to provide energy for the host (Sanjorjo et al., 2023). However, there are several factors like diet, host genetics, nutrition, and early life interventions that alter the rumen microbiota and fermentation process (Sanjorjo et al., 2023). These microbes contribute a significant portion of the total energy that ruminants derive from their diet. The energy contribution can vary based on factors such as the composition of the diet (e.g., types of forages, grains, and supplements), the efficiency of microbial fermentation, and the nutritional requirements of the ruminant. Generally, rumen microbes contribute approximately 50–70% of the total energy obtained from the digestion of plant materials in ruminants (Niwinska, 2012). Thus, regulating the rumen microbiota optimizes the nutritional value, thereby improve the productivity.

Apart from maintaining homeostasis in ruminants, the rumen microbes, particularly methanogenic archaea, play a critical role in methane production in ruminant animals through their metabolism of hydrogen and other substrates derived from rumen fermentation (Bodas et al., 2012). Therefore, it is necessary to understand the interactions and biochemical pathways involved in methanogenesis to develop effective strategies to mitigate methane emissions and reduce the environmental impact of ruminant production systems.

Research using advanced molecular techniques, such as metagenomics and 16S rRNA gene sequencing, has provided more detailed insights into the microbial community structure within the rumen, including the relative abundances of bacteria and archaea (Walsh et al., 2017). This will help in altering the microbial population in the rumen of ruminants which can significantly increase productivity through several mechanisms that optimize nutrient utilization, enhance digestive efficiency, and improve overall animal health. In essence, the symbiotic relationship between ruminants and their rumen microbes are pivotal to the digestive physiology of ruminants, facilitating the breakdown of complex carbohydrates, production of energy-rich VFAs, synthesis of essential nutrients like microbial protein and vitamins, maintenance of optimal rumen pH, adaptation to dietary changes, and promotion of immune function and gut health (Kamra, 2005). Their intricate interactions and metabolic activities make them indispensable partners in the nutrition and well-being of ruminant animals.

Significant impact of heat stress on rumen microbiome has been established by various researchers (Chen et al., 2018; Zhao et al., 2019). The significant decline in dry matter intake and production was associated with heat stress in animals. Such implications subsequently affected the ruminal variables, for instance significant reduction in rumen pH, rumen acetate. Likewise, increased propionate and butyrate and lactate concentrations were also reported by various researchers in heat stressed animals (Nonaka et al., 2008; Zhao et al., 2019).

Nevertheless, the implications of microbial alteration with resilience to heat stress is scarcely addressed. Decreased ruminal pH in heat stressed ruminants was stated to impact the ruminal dynamics. Lactate producing and soluble carbohydrate utilizers were observed to have increased abundance in heat stress ruminants while the abundance of acetate producing bacteria decreases under heat stress. Furthermore, due to its higher heat resistance, the abundance of cellulolytic bacteria especially *Fibrobacteres*, are reported to increase during heat stress (Kim et al., 2022). Such alterations in rumen microbiota were observed to alter the rumen metabolite concentrations. In a recent report, using rumen metagenomics and metabolomics analysis Wang et al. (2022 a) were successful in identifying novel

microbes and metabolites that potentially confer thermal adaptability in Holstein Friesian cows. Ruminal microbes *viz*, *Rikenellaceae*, *Christensenellaceae*, *Succiniclasticum*, and *Ruminococcaceae NK4A214* group were among the identified ruminal flora stated to be associated with thermal adaptation. Likewise, metabolites, maltose, glycerol, and mannitol were also proposed to be potential biomarkers for heat stress adaptation in cattle.

Differences in microbial diversity based on Shannon and Chao1 indices

The rumen nurtures diverse and highly intense microbial populations that play a crucial role in ensuring metabolism, optimum production and health in animals (Kim et al., 2020). However, there are a number of factors which influence the diversity and function of the ruminal microbial niche (Kim et al., 2020). Assessing an alteration in such microbial diversities may give an insight into the impact of external factor on rumen ecology. Several such indices (e.g., alpha diversity metrics) have been established which aid in our understanding of the structure of the microbial community. Alpha diversity indices describe the microbial structure focusing on its richness and evenness or both (Willis, 2019). The richness gives an overview of the number or types of taxonomic groups in the sample (Willis, 2019), higher index indicating more rich community (Thukral, 2017). The evenness or equitability indices describes relative abundance of different species in a sample with respect to their evenness in distribution (Thukral, 2017). Higher equitability indices indicate an evenly distributed community while lower values reflect dominance by one or few abundant species (Thukral, 2017). Chao, Shannon, Fisher, Simpson, Abundance-based coverage estimator (ACE), are some of the frequently used alpha diversity indices (Biscarini et al., 2018).

Islam et al. (2021) led a study to assess the seasonal influence on rumen microbiome in Holstein and Jersey steers, by considering a number of variables. On assessing the alterations at the microbiome diversity using V3-V4 metagenomics approaches, the authors observed seasonal variations in the operational taxonomic units (OTUs) and Chao1 estimates. While the highest number of OTUs and Chao1 index were observed during spring, lower and highly unstable estimates were observed in cattle, irrespective of their breed, during the summer season. Such reductions and alterations in Chao1 index during summer season, reflecting impaired species richness, was thought to be due to the impact of heat stress on the rumen microbiome. Furthermore, the study also revealed the highest Shannon diversity index during summer while the lowest values were recorded during winter. The authors suggested that the higher index during summer was a result of climatic conditions that favored a diversity of microbial groups in the rumen. Additionally, the lower diversity during winter could reflect selective proliferation of metabolic heat production associated microbes. Thereby such seasonal impact on rumen microbiome highlights the potential of animals to be affected due to climate change and adopt adaptive mechanisms to maintain homeothermy (Islam et al., 2021).

In another study led by Correia Sales et al. (2021), heat stress was observed to significantly alter the rumen microflora in the Nellore cattle. The rumen microbiome of heifers under heat stress had a significantly lower richness (Chao1 and Menhinick indices) when compared to those under thermoneutral conditions while their diversity remained unaltered. Based on the findings from this study, a significant alteration of the rumen ecology was established due to heat stress, which was also reflected at the functional level. However, some studies have also reported a significant impact of heat stress on rumen bacterial composition with minimal alterations observed via the diversity indices (Zhao et al., 2019; Kim et al., 2020). In a study on Holstein dairy cows, heat stress did not significantly influence the alpha and beta diversity of ruminal microflora with the exception of altered richness, with Chao1 being higher in heat stressed group (Zhao et al., 2019). Having said that, heat stress significantly altered the rumen microbial composition wherein an increased abundance of lactate and decreased abundance of acetate producing bacteria were observed when compared to non-heat stressed cows. In another study, which assessed the rumen microbiome in heat stressed Holstein and Jersey cows, no significant differences in Shannon and Simpson indices were observed (Kim et al., 2020). However, heat stress was observed to significantly alter the rumen bacterial taxa and functional gene abundance in both dairy breeds. The authors also reported variations in response to heat stress between the two breeds based on their rumen metagenomics and metabolomics profile (Kim et al., 2020). An overview of the impact of heat stress on rumen microbial diversity is presented in Table 1.

Differences in heat stress associated distribution of microbes based on principal component analysis in ruminants

Heat stress is one of the several factors that deleteriously affect rumen microbial distribution. Alterations in the ruminal microbiome can directly impair rumen fermentation, volatile fatty acid production and microbial protein synthesis, all of which culminate in reduced production, growth, reproduction and health of ruminants (Zhao et al., 2019).

The principal component analysis (PCA) is one of the oldest, yet most widely used multivariate statistical methods to assess large datasets while preserving as much variability as possible (Jolliffe and Cadima, 2016). In PCA analysis, the results are presented in a graphical plot. Therefore, pairwise score plots resulting from PCA are useful to find similarities and dissimilarities between samples, while associations between variables can be identified in loading plots (Fievez et al., 2003). PCA could reveal a discrete clustering of the microbial communities depending on ruminant species. The PCA and Bray-Curtis distance analysis clearly indicated the variations in bacterial, methanogen and protozoal OTUs, abundance and community compositions between Jersey cows and buffalo (Iqbal et al., 2018).

Table 1. Impact of heat stress on rumen microbial diversity and composition

NGS approach	Species/Breed	Influence of heat stress/season on rumen microbiome	Conclusive inference	Reference
16s V3-V4 rRNA sequencing	Holstein and Jersey steers	• Lower and highly unstable Chao1 index during summer • Highest Shannon diversity index of rumen microbiome, reflecting increased diversity during summer in comparison to winter	Higher diversity and decreased richness of rumen microbiome during summer could indicate decreased abundance of the active rumen microbes that indirectly were also responsible for increasing metabolic heat production.	Islam et al., 2021
16s V3-V4 rRNA sequencing	Nellore beef cattle	• Significantly lower richness of rumen microbiome in heat stressed beef cattle. However, no significant difference in rumen microbial diversity was observed • The rumen microbiota of heat stressed heifers had relatively lower abundance of genera *Flavonifractor* (1.4%), *Treponema* (0.6%) and *Ruminococcus* (0.9%) in comparison to the control group • Heat stress significantly increased the relative abundance of the genus *Carnobacterium* in beef cattle		Sales et al., 2021
16s V3-V4 rRNA sequencing	Holstein dairy cows	• Heat stress significantly altered only the Chao1 index (higher in heat stress group) while the remaining alpha and beta diversity indices were not significantly altered • Significant increase in *Ruminobacter, Streptococcus, Enterobacteriaceae, Treponema* abundance		Zhao et al., 2019
16s V3-V4 rRNA sequencing and whole genome shotgun sequencing	Holstein and Jersey cows	• No significant difference in Shannon and Simpson indices • Distinctive variation in heat stress responses between cattle breeds • 6 taxa that were distinguishing the ruminal flora in Holstein cows under normal and heat stress conditions while the same for Jersey cows were 29 • Increased abundance of *Fibrobacteres* group in Holstein cows in contrast to reducing abundance of *Actinobacteria* group in Jersey cows Increased abundance of *Fusobacteria* and *Cyanobacteria* • Significant reduction in the *Acetobacter*	Though heat stress did not significantly change the diversity indices, significant impact of heat stress was recorded in the structure and function of rumen microbes.	Kim et al., 2020
16s V3-V4 rRNA sequencing	Boer goats	• Transition of rumen microbes from *Firmicutes* to *Bacteroidetes* with increasing THI • Relative abundance of the genus *Prevotella* increased with rising THI gradually becoming the dominant genera • Increased abundance of pathogenic bacteria like *Erysipelotrichaceae_UCG-004* and *Treponema_2* due to heat stress	Apart from impairing production, heat stress could also increase the abundance of potentially pathogenic bacteria in caprine rumen at the expense of beneficial bacteria abundance, thereby causing an imbalance in the rumen micro-ecology.	
RNA-based method (sequence-specific small-subunit (SSU) rRNA cleavage method)	Holstein heifers	• Increased abundance of ruminal *Clostridium coccoides-Eubacterium rectale* group, and the genus *Streptococcus* with decline in *Fibrobacteres* as the temperature progressed		Uyeno et al., 2010
16s V3-V4 rRNA sequencing	Osmanabadi, Malabari and Salem Black goats	• Lower *Firmicutes* and higher *Bacteroides* microbes in the Salem Black goat		

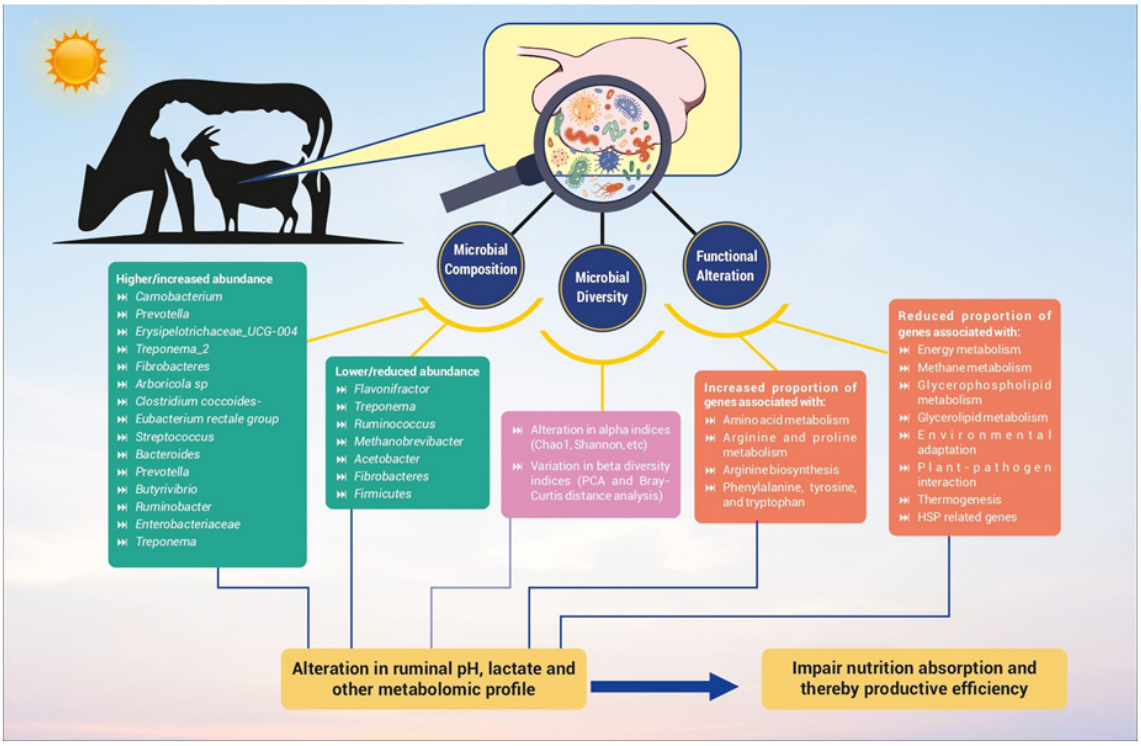

Figure 1. Direct and indirect impacts of heat stress on rumen microbiome

Correia Sales et al. (2021) reported the changes in the abundance of rumen microbiota in Nellore heifers subjected to heat stress, which was analyzed by using PCA. The results showed that the animals exposed to heat stress had lowest richness of prokaryotes compared to animals under thermoneutral conditions. The relative abundance of genera *Flavonifractor* (1.4%), *Treponema* (0.6%) and *Ruminococcus* (0.9%) exhibited the lowest ($P<0.04$) while *Carnobacterium* (7.7%) showed the highest ($P=0.02$) relative abundance in rumen of the animals subjected to heat stress as compared to the animals in the thermoneutral conditions. At the same time, there was a significant increase in the relative abundance of *Methanobrevibacter* in the animals subjected to heat stress and provided *ad libitum* feeding.

Heat stress impact on rumen microbes at different taxonomic levels

In ruminants, the rumen microbiome is closely linked to heat stress as the metabolic heat generated during digestive fermentation is among the crucial factors contributing to heat load especially in cattle (Uyeno, 2021). In addition to this, heat stress is also reported to significantly alter the rumen microbial composition and structure which is mainly due to reduced feed intake and variations in eating times (Uyeno, 2021). Impairment of the rumen microbiome can adversely affect the animals' productivity, metabolic activity, health status and may also alter the enteric methane production (Uyeno, 2021; Cholewińska et al., 2021). The rumen microbiome is comprised of a diverse population of bacteria, archaea, protozoa, fungi, bacteriophage and viruses (Matthews et al., 2019). Among these, *Firmicutes*, *Bacteroidetes* and *Proteobacteria* are the most prevalent ruminal bacterial phyla (Zhong et al., 2019). Heat stress was reported to significantly alter the rumen microbiome mainly by having an impact on fiber decomposing and starch decomposing bacteria (Kocherginskaya et al., 2001; Tajima et al., 2001)

A study to assess the continuous dynamic alterations in rumen microbiome of Boer goats under 16 gradually increasing temperature and humidity indexes (THI) using 16S V3-V4 rRNA sequencing was reported by Zhong et al. (2019). The study revealed transition of the dominant phylum from *Firmicutes* to *Bacteroidetes* with increasing THI in caprine rumen, which existed in co-exclusion. It should be noted that the *Firmicutes-Bacteroidetes* ratio was highly correlated with milk fat yield in cows (Jami et al., 2014). Lower abundance of *Bacteroidetes* when compared to *Firmicutes* was associated with increased milk fat in lactating Israeli Holstein Friesian cows (Jami et al., 2014). Therefore, alterations in *Firmicutes-Bacteroidetes* ratio in ruminants during heat stress can impair their productivity. In the same study stated above, Zhong et al. (2019) also observed that relative abundance of the genus *Prevotella* increased with rising THI which gradually became the dominant genera. Furthermore, the study also reported increased abundance of pathogenic bacteria like *Erysipelotrichaceae_UCG-004* and *Treponema_2* due to heat stress in caprine rumen. Therefore, apart from impairing production, heat stress could also increase the abundance of potentially pathogenic bacteria in caprine

rumen at the expense of beneficial bacteria abundance, thereby causing an imbalance in the rumen micro-ecology (Zhong et al., 2019). The intense association of rumen microbiota with animals' health status highlights the potential risk associated due to heat stress in ruminants. Figure 1 describes the direct and indirect impacts of heat stress on rumen microbiome.

The impact of heat stress on rumen microbiota was also reported in cattle (Kim et al., 2019, 2020). Significant alterations in the rumen microbial composition at phylum, genus and species levels was observed in Holstein Friesian cows on exposure to heat stress in Korea (Kim et al., 2019). In another study from the same research group, the heat stress response with respect to rumen microbial diversity was compared between Holstein Friesian and Jersey dairy cows (Kim et al., 2020). Distinctive variations in breed responses were observed indicating that Jersey cows were relatively more sensitive to heat stress when compared to Holstein cows based on the higher alterations in ruminal microbiota in Jersey cows. The authors identified 6 taxa that were distinguishing the ruminal flora in Holstein cows under normal and heat stress conditions while for Jersey cows there were 29. Only one taxon, *Ruminococcaceae bacterium P7* sp. was enriched in the normal condition in Holstein cows. While under heat stress, *Fibrobacteres* (phylum), *Fibrobacteria* (class), *Fibrobacterales* (order), *Fibrobacteraceae* (family) and *Arboricola* sp. were observed to be enriched in the Holstein rumen. The authors associated the variation in rumen microbial alteration due to heat stress in the two cattle breeds to the differences in the adaptive mechanisms being adopted. In another study, Uyeno et al. (2010) had reported an increased abundance of ruminal *Clostridium coccoides-Eubacterium* rectale group, and the genus *Streptococcus* with decline in *Fibrobacteres* in Holstein heifers, as the temperature progressed. Such alterations in rumen microbiota during heat stress, however, vary among species, breeds, and individuals.

Researchers have also assessed the impact of heat stress on rumen microbiota of beef cattle (Correia Sales et al., 2021). The rumen microbiota of heat stressed Nellore beef heifers were observed to have relatively lower abundance of the genera *Flavonifractor* (1.4%), *Treponema* (0.6%) and *Ruminococcus* (0.9%) in comparison to their control group animals (Correia Sales et al., 2021). Additionally, the authors also observed that heat stress significantly increased the relative abundance of the genus *Carnobacterium* in the rumen of beef cattle. Metagenomic analysis to assess the alteration in the rumen microbiome in buffaloes has also been conducted, however there are limited data pertaining to heat stress in buffaloes.

There was no evidence directly linking heat stress responses to any specific rumen bacterial population, and very scarce information revealing the heat production by specific rumen microbe (Kim et al., 2020). Therefore, it is necessary to assess the responses exhibited by each microbial taxa. Doing so would provide a much better understanding of rumen dynamics during periods of heat stress. For instance, in the previously stated study by Kim et al. (2020), heat stress was observed to significantly increase the abundance of *Fibrobacteres* group (phylum to order) in Holstein cows. This group was stated to have stronger heat resistance in comparison to other ruminal bacteria in addition to be associated with cellulolytic activity (Kim et al., 2020). The authors suggest that there could be a rise in ruminal heat production in Holstein cattle due to increased abundance of *Fibrobacteres* group. Furthermore, in contrast to Holstein cattle, a completely different rumen microbial profile was observed in heat stressed Jersey cows. This indicates that there are differences in selection process exhibited by the two breeds in response to heat stress (Kim et al., 2020). Advanced next generation sequencing approaches will aid in assessing the deeper impact of heat stress on rumen microbiome in livestock. Table 1 enlists an overview of the impact of heat stress on rumen microbial composition based on some of the recent reports. Figure 1 describes both the direct and indirect impacts of climate change on rumen microbiome.

Applications of 16s rRNA gene sequencing of V3-V4 region technology to study heat stress associated changes in rumen microbial diversity

Various studies documented the importance of the microbiome in cattle health and associated productivity. The primary function of ruminal microbes is to produce energy by producing short-chain fatty acids from undigested dietary polysaccharides in ruminants (Flint et al., 2008). To comprehend the energy production processes in ruminants, a thorough understanding of the ecology of the rumen microbes and their interaction becomes vital in the present climate-changing scenario. To implement any ameliorative nutritional strategies to counter the heat stress in ruminants and increase productivity, technologies like sequencing of bacterial 16s rRNA gene as phylogenetic markers play an important role. Bacterial identification and genetic evolution studies in ruminants rely on 16s as the most stable target. There are other methods to identify the taxonomical assignment and relative abundance of numerous microbes in rumen like shotgun sequencing, meta-proteomics and targeted quantification. Both amplicon sequencing (16s rRNA) and shotgun sequencing have taxonomic assignments. Shotgun sequencing reported higher resolution of taxa of the bacterial and archaeal population in rumen samples whereas; amplicon sequencing outclassed the other method by revealing the protozoal composition as well (Zhou et al., 2021). Moreover, the 16s rRNA sequencing of rumen microbiome remains the most cost-effective and simple method for comparing the microbial diversity in ruminants (McGovern et al., 2018). Thus, through 16s rRNA sequencing, identifying various microbes that were unidentified in the rumen can be easy and further leads researchers to appreciate the diversity and complexity of the rumen microbiome, especially when sub-

jected to heat stress.

Sejian et al. (2021 c) reported the adaptation of goat breeds to heat stress through the rumen microbial approach. The researchers identified lower *Firmicutes* and higher *Bacteroides* microbes in the Salem Black goat breed, depicting the role of those bacteria in breaking down complex lignocellulose feed and polysaccharides in the plant cell wall, implicating the superior adaptability to heat stress compared to the other breeds. Further, the study validated that microbes like *Prevotella* (which degrades non-structural carbohydrates and proteins, amino acid metabolism and energy metabolism), *Butyrivibrio* (secretion of enzymes and degradation of polysaccharide in the rumen) were abundant in Salem Black goat. This signifies the adaptive capability of the breed to heat stress by keeping microbial composition intact and facilitating the necessary nutrients during heat stress.

Other research (Kim et al., 2020) validated the ruminal microbial adaptation between Jersey and Holstein cows exposed to heat stress. During the assessment, researchers witnessed that Holstein and Jersey cows endure an utterly different selection and production process of rumen microbes in response to heat stress. In Holstein cows, the impact of heat stress significantly increased the abundance of *Fibrobacteres* phylum. The ability of the *Fibrobacteres* phylum to withstand higher temperatures than other ruminal bacteria is highly regarded. All microbes prefer a specific temperature range for optimal growth and function. Using the 16s rRNA sequencing methodology, the authors were able to depict the ruminal microbial dynamics. Heat stress was established to increase abundance of the potentially heat generating *Fibrobacteres* group in Holstein cows in contrast to reducing abundance of the acetate producing *Actinobacteria* group in Jersey cows. In addition to this, the abundance of *Fusobacteria* and *Cyanobacteria* were increased which so far were not included in the list of bacteria that break down cellulose. Thus, such an overview through 16s rRNA sequencing may provide additional insight into Jersey cows' increased heat tolerance and lower metabolic heat output. However, if Jersey cows compromise cellulose's digestion during heat stress, how does it maintain the production of necessary volatile fatty acids to ensure that essential functions remain optimized? Additional research might add more insights towards the fascinating mechanisms involved in Jersey cows to ameliorate heat stress.

Similar studies were undertaken by Zhao et al. (2019), where Holstein cows were subjected to heat stress and their impact on rumen microbiota was assessed through 16s rRNA sequencing. Significant increases in *Ruminobacter*, *Streptococcus*, *Enterobacteriaceae*, *Treponema* abundance were identified. To validate the results, there was an increase in lactate levels and a reduction in pH levels in the rumen of heat stressed cows. Wang et al. (2012) observed comparable results, where the increase in the Streptococcus population increased lactate and reduced pH in the rumen. Therefore, it could be inferred that cows exposed to heat stress develop increased lactate production in the rumen.

Further, a significant reduction in the *Acetobacter* population have also been established in heat stressed cows using 16s rRNA sequencing (Kim et al., 2020). *Acetobacter* is primarily acetate producing bacteria that can further lead to a decrease in the acetate production in rumen fluid. As previously discussed, acetate produces more ATP than volatile fatty acids, therefore reduction in this group could be associated with reduced metabolic heat generation (Kim et al., 2020). The application of 16s rRNA sequencing is not restricted to rumen; further, it can be utilized even in identifying the fecal microbiota of cows exposed to heat stress. A study conducted by Czech et al. (2022) revealed changes in fecal microbes that were associated with an increase in rectal temperature. Intriguingly, the abundance of bacteria such as *Helcococcus* increased proportionately to the upsurge in rectal temperature. Additionally, numerous microbes associated with rectal temperature were upregulated, implying that increasing rectal temperature has an impact on the fecal microbiota. Conversely, the abundance of *Fibrobacteres* was decreased due to heat stress.

Shotgun whole metagenome sequencing applications to study differences at functional level in heat stressed ruminants

Next generation sequencing approaches have eased the process of assessing rumen microbiota wherein 16S rRNA sequencing and whole genome shotgun sequencing (WGS) are among the recent and most used approaches (Ranjan et al., 2016). While the 16S rRNA sequencing adopts sequencing of only the highly conserved regions of the 16S rRNA gene (mostly V3-V4), the whole genome of the microbial DNA is sequenced in WGS (Sanschagrin and Yergeau, 2014). The major limitation of the 16S rRNA sequencing is that the annotations are made based on putative association of the amplified region with taxa that are defined as operational taxonomic units (OTUs). With the existence of numerous bacterial strains and horizontal gene transfer, a lack of direct gene sequencing potentially limits the proper assessment of a microbe (Poretsky et al., 2014; Ranjan et al., 2016). This further makes it difficult to predict the role of the microbes at the functional levels. The major advantage of WGS is that it enables more accurate annotation of the taxa at the species level with even better prediction of functional analysis (Ranjan et al., 2016). As the complete genomic sequence of microbial community is available on shotgun whole metagenomics, the identification of genes and its associated functional profile and metabolic pathways are more accurate (Pérez-Cobas et al., 2020). However, WGS metagenomics approach is more expensive than 16S rRNA sequencing.

Despite its high cost, WGS metagenomics has been widely adopted by researchers across the globe to assess functional responses in livestock. Studies pertaining to genetic and functional analysis of microbes prevailing in

the gut/rumen (Reddy et al., 2014), milk (Hoque et al., 2019), uterus (Bicalho et al., 2017), udder (Dean et al., 2021), nasal track (Cui et al., 2021) and feces (Liu et al., 2019) have been reported in several livestock species. However, reports on adoption of this methodology to assess heat stress impact in ruminants is scarce.

In another study, Reddy et al. (2014) adopted shotgun metagenomics to comparatively profile the phylogenetic and functional potential of stress, resistome and mobilome response genes in buffaloes. Their approach could identify rumen microbial genes coding functional responses like oxidative stress, carbon starvation, heat shock, osmotic stress, resistance to antibiotics and toxic compounds, transposable elements, phages and so on. Therefore, extending shotgun metagenomics studies to heat stressed livestock could unravel the hidden intricacies involving the impact, adaptation, and resilience of rumen microbiota to heat stress.

Heat stress associated changes at Cluster of Orthologous Gene level

Functional annotation of genomes is a vital step in genomics studies (Natale et al., 2000). Therefore, employing particular methodology to annotate genome function is necessary to obtain the functional information from characterized genes (proteins) to their respective uncharacterized homologs in newly sequenced genomes (Galperin and Koonin, 2000). Clusters of orthologous genes (COG) analysis is the widely used method for classification of the gene functions. The COG proteins system (COG, http://www.ncbi.nlm.nih.gov/COG/) was helpful for the functional annotation of genes from new genomes and for research into genome evolution (Li et al., 2017).

The eggNOG (evolutionary genealogy of genes: Non-supervised Orthologous Groups) database is useful to annotate any new sequences identified and their corresponding orthologous groups. The annotated new transcripts are classified into functional categories based on their putative functions. Kim et al. (2020) used the eggNOG database to find out the distribution of clusters of orthologous groups (COGs) of protein in the rumen sample. This enabled the authors to identify the genes linked with energy production and conversion, which were significantly decreased in heat stressed Jersey cows as compared to control cows in which they increased. They also observed a significant increase of genes associated with the defense mechanism, lipid transport and metabolism, coenzyme transport and metabolism, cell cycle control, cell division, and chromosome partitioning in Holstein cows exposed to heat stress. However, there was no significant difference observed in relative abundance of genes in both normal and heat stressed Jersey cows. The relative abundance of genes associated with cell motility and extracellular structures was decreased in heat stressed Holstein cows.

The COG functional annotation revealed the abundance of COG genes in the ruminant GIT regional microbiome. The functional analysis showed that the predicted COGs gene associated with digestion of dietary carbohydrates, proteins and lipids were enriched in the stomach, nucleic acid metabolism in the small intestine, and protein synthesis in the large intestine (Xie et al., 2021).

Sejian et al. (2021 c) observed the abundance of different COGs in both the control and heat stress groups in three indigenous goat breeds. This COGs annotation was consistent with the KEGG (Kyoto Encyclopedia of Genes and Genomes) pathway analysis. They did not find any difference in the level of richness of most of the COGs between the control and heat stress groups in Osmanabadi and Malabari breeds. However, the abundance of most of the COGs was reported to have decreased in heat stress group of Salem Black breed as compared to that of control group.

Liu et al. (2020) used PICRUSt software to predict gene function and identified a total of 25 COG gene families in the 16S rRNA gene sequencing data from rumen samples of Tibetan sheep. Among the COG gene predictions, 13 gene families exhibited significant variations between the cold and warm seasons. There was a decrease of COG gene families linked to carbohydrate transport and metabolism and an increase of genes associated with energy production and conversion to meet the energy metabolism needs of the sheep during the cold season. This trend was consistent with the KEGG pathway analysis. Those COG gene families related to sugar biosynthesis and metabolism also increased during the cold season.

The COG functional analysis of rumen microbiota showed the functional classification of the microbiome broadly within the area of metabolism, particularly amino acid, carbohydrate, and lipid metabolism and transport. The COG gene families related to amino acid transport and metabolism showed 51.6% increases in abundance, 43.8% of COG families showed increases in abundance pertaining to carbohydrate transport and metabolism, while 50.0% of COG families related to lipid transport and metabolism showed significant increases in abundance (Mayorga et al., 2016).

A differential COG function prediction analysis of rumen microbiota data identified about 25 COG functional genes during the month of April and August. Nine differential COG functional genes predicted were related to metabolism pathway, which accounted for 13.67%. The COG genes associated with amino acid transport and metabolism pathway (7.63%) were significantly higher in April compared with December (Lv et al., 2021).

The COG analyses showed that gene functions associated with carbohydrate metabolism and energy production were significantly increased in the grazing and supplementary feeding group of cattle-yak during cold season. Among the 25 COG gene families identified, genes related to transcription were significantly enriched in 16S rRNA gene sequencing from rumen sample of cattle-yak. Additionally, the most abundant COG gene families associated with carbohydrate transport and metabolism, amino acid transport and metabolism, and en-

ergy production and conversion were significantly varied across the two groups of cattle-yak. The COG functional categories related to carbohydrate transport, amino acid transport and metabolism were significantly higher while genes linked with energy production and conversion were lower in grazing group than the grazing and supplementary feeding groups of cattle-yak during cold season (Sha et al., 2021).

Heat stress associated changes in stress pathways at different KEGG levels in ruminants

In brief KEGG is a database where the interactions between various genes, molecular pathways and resulting functional proteins in the biological system can be known (Kanehisa, 2000). As heat stress affects metabolism, immune functions, and gene expression in ruminants, there would be associated cellular and epigenetics changes, which could be explored with the aid of KEGG database.

In the previously discussed study by Kim et al. (2020), the rumen microbial functions were characterized using whole genome shotgun metagenomics. Level two KEGG pathway analysis revealed differences in proportion of gene functions both in Holstein and in Jersey rumen due to heat stress. In Holstein cows, heat stress resulted in increased proportion of genes associated with amino acid metabolism; while the same condition led to decreased expression of energy metabolism associated genes in Jersey cows. To gain a further understanding of the heat production by ruminal fermentation, level three KEGG pathways were assessed. For energy metabolism, heat stress significantly reduced the proportion of genes associated with methane metabolism, glycerophospholipid metabolism and glycerolipid metabolism in Jersey cows. Likewise, by looking into the amino acid metabolism associated pathway, heat stress was reported to significantly alter the genes link to this pathway in Holstein cows. Significantly higher proportion of genes associated with arginine and proline metabolism, arginine biosynthesis and phenylalanine, tyrosine, and tryptophan were observed in Holstein cows while in Jersey they remained unaffected. This study (Kim et al., 2020) could also establish the functional dynamics of bovine rumen microbiome as a consequence to heat stress response via the increased abundance of genes linked to environmental adaptation. KEGG analysis revealed that genes for plant-pathogen interaction and thermogenesis were increased unanimously due to heat stress, in both Holstein and Jersey cows. Lastly, heat stress also increased the percentage of HSP related genes in Holstein rumen, which remained unaltered in Jersey cows.

In another study led by Zhong et al. (2019), changes in rumen microbial composition of Boer crossbred goats under different THI was established using 16s RNA sequencing. Using PICRUSt analysis the authors were able to predict the functional distribution of caprine ruminal microbes depending on their KEGG pathway categorization. Functional information of rumen microbiota based on three levels of KEGG pathways were acquired based on the relative OTUs wherein KEGG level one was categorized into 5 groups. A total of 39 gene families were observed at KEGG level two while 232 KEGG orthology (KO) pathways were reported at KEGG level three. Genes associated with carbohydrate metabolism exhibited marked variations across the THI groups. Furthermore, the authors also performed correlation analysis between the topmost abundant rumen microbial phyla and core KO pathways among different THI groups. This established the negative correlation of *Firmicutes* with amino sugar and nucleotide sugar metabolism. Similarly, *Bacteroidetes* was also reported to have significant positive correlation with purine metabolism while the same was also negatively correlated with ABC transporters and aminoacyl-tRNA biosynthesis.

In another recent report, Li et al. (2021) adopted 16S rDNA sequencing (similar to 16S rRNA sequencing) and metaproteomics methodologies to unravel the potential benefit of dietary creatine pyruvate supplementation on rumen microbial protein function in heat stressed beef cattle. The authors were able to understand the detailed regulatory mechanism of creatine pyruvate on bovine rumen fermentation. Therefore, rumen metagenomics can be a potential tool that could provide access into the unknown intricate mechanisms prevailing in the rumen microbiome. Utilizing this methodology to characterize the rumen microbes and understand their functional roles can effectively support heat stress studies in ruminants.

Application of metabolomics in assessing heat stress response in ruminants

The rumen microbiome and its metabolome have a crucial role in determining the performance of the ruminants including production (Weng et al., 2024), immune response (Eom et al., 2022) and adaptative potential (Wang et al., 2022 a). The rumen microbiota plays a crucial role in regulating host metabolism by producing various metabolites, and its activity is influenced by factors such as the host, environment, and the microbiota itself (He et al., 2024). Under heat stress conditions, the rumen microbiome undergoes substantial changes, which lead to alterations in normal metabolic pathways and, consequently, the production of metabolites (Wang et al., 2022 a). This affects the overall health and productivity of the animals. Therefore, understanding the rumen bacteria and metabolome can help elucidate the impact of heat stress on animal productivity, physiology, and immune function.

Several production and adaptation related metabolic pathways have been altered due to variation in the metabolites produced during heat stress. Weng et al. (2024) compared the lactation performance of Montbéliarde × Holstein and Holstein cows under heat stress, focusing on the rumen microbiome and metabolites. The study revealed significantly higher levels of ruminal metabolites with antioxidant and anti-inflammatory properties in the crossbred cows. These metabolites likely played a key

role in enhancing lactation by improving the cows' ability to cope with oxidative stress and inflammation caused by heat stress. The results suggest that the superior lactation performance and metabolic efficiency in Montbéliarde × Holstein cows may be linked to their ruminal metabolites, which aided in better heat stress adaptation and lactation performance. In a study by Contreras-Jodar et al. (2019) on dairy goats, alterations in urinary metabolites were observed due to changes in the gastrointestinal microbiome. These changes negatively impacted milk quality by disrupting the synthesis of neurotransmitters and thyroid hormones. Additionally, numerous studies have explored how heat stress causes a drop in milk production by focusing on changes in metabolites in milk (Tian et al., 2015) and plasma (Yue et al., 2020), further emphasizing the metabolic disruptions caused by heat stress.

Wang et al. (2022 b) in their study on the ruminal fluid of heat stressed buffaloes observed significant alterations in the rumen microbiome and identified thirty-two metabolites involved in gluconeogenesis that were closely associated with heat stress, reflecting metabolic changes triggered by elevated temperatures. Additionally, they found that heat stress impacted four key metabolic pathways: D-alanine metabolism, lysine degradation, galactose metabolism, and the biosynthesis of tropane, piperidine, and pyridine alkaloids (Wang et al., 2022 b). In another similar study examining heat-stressed and heat-tolerant cows, the authors found changes in metabolites related to carbohydrate and lipid metabolism, as well as membrane transport, which contributed to the enhanced adaptive capacity of the heat-tolerant animals (Wang et al., 2022 a). Eom et al. (2022) highlighted the impact of heat stress on the rumen metabolites and immune response in steers. They identified the four key metabolites as potential markers and also revealed that heat stress affects immune responses by downregulating pathways like the hypoxia-inducible factor-1 signaling pathway. These findings highlight the link between rumen metabolism and immune function in heat-stressed ruminants. These studies prove that alterations in key metabolites, play a significant role in the enhanced adaptation of ruminants to heat stress. These metabolic changes contribute to better heat stress tolerance, improved lactation performance, and overall resilience in animals.

Metabolomic studies have been effectively used to identify biomarkers related to milk quality, energy metabolism, and rumen health in ruminants (Wang et al., 2022 a). A metabolomic study on the ruminal fluid of dairy cows revealed distinct alterations in the ruminal microbiome and metabolites. They indicated mannitol, maltose and glycerol could be candidate markers to determine the heat-resistant animals (Wang et al., 2022 a). In another study, Eom et al. (2022) identified key rumen metabolites, including isocitrate, formate, and creatine, as potential biomarkers for heat stress in steers. Several studies have identified novel metabolomic markers for assessing heat stress in ruminants, but research focusing specifically on metabolomic markers derived from ruminal fluid is relatively limited. Therefore, further research into ruminal fluid-based metabolomic markers could indeed offer a promising method for evaluating heat stress in ruminants as it can provide valuable insights into the ruminant's physiological response to heat stress.

With the increasing concerns on global climate change and food security, there is an increasing urge to target towards breeding for climate resilient livestock population. Though an easier stated term, achieving it involves dedicated and persistent multi-institutional and multi-disciplinary research that considers a holistic approach to identify resilient animals, breed them and mitigate them to the larger masses. This would involve forming a system road map considering all the vital information to identify resilient animals using biomarkers and other assisted tools, incorporating technology development and information transfer, involving all the stakeholders, etc. One may also emphasize on promoting livestock breeding and management practices that would be agro-ecological zone specific (Sejian et al., 2021 c). Understanding the ruminal dynamics and its underlying mechanisms can pave ways in identifying potential biomarkers for thermal adaptation/resilience in ruminants.

Conclusion

Impact of climate change on rumen microbiome is significant, assessing the impact could provide the key to crack the quest towards the development of climate resilient livestock. Advances in next generation sequencing approaches like 16S rRNA sequencing and whole genome shotgun metagenomics has opened new pathways to assess the structural, genetic and functional dynamics of the ruminal ecosystem. Rumen metagenomics aided researchers across the globe to assess impact of heat stress on rumen microbiome that included alteration in rumen microbial richness, diversity, *Firmicutes-Bacteroidetes* ratio, altered abundance of several microbes involved in functional pathways (like *Fibrobacteres* group, *Actinobacteria* group, *Fusobacteria*, *Cyanobacteria*, *Flavonifractor*, *Treponema*, *Ruminococcus*, etc) and many more. Using metagenomics it was also possible to assess changes at the functional levels, some of which were associated with pathways like energy production and conversion, defense mechanism, lipid transport and metabolism, coenzyme transport and metabolism, cell cycle control, cell division and a few more. Very few researchers were also able to comparatively assess the impact of heat stress on rumen microbiome between breeds and thereby understand differences in adaptive strategies adopted by them. However, despite having such significant applications, there is a dearth of information on the dynamics of the rumen microbiome during heat stress in ruminants. Therefore, it is of prime importance to encourage the usage of such advanced methodologies that hold the potential to provide several answers towards path-breaking research. Furthermore, rumen metagenomics can also be extended towards studies focusing on

nutritive mitigation strategies for climate change in ruminants. Another vital applicability of rumen metagenomics could be exploring enteric methane emission, especially during heat stress in ruminants. Intense application of rumen metagenomics could also aid to identify markers and/or breeds or individuals that could be considered for improvised breeding strategies.

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