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Research Article

Effect of Heat Stress on Rumen Microbial Diversity and Fermentation Pattern in Buffalo

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In this study, changes in rumen microbiome and fermentation were examined during heat stress (HS) in buffaloes. Six buffalo heifers were exposed to thermoneutral (THI = 72) and HS (THI = 87 – 90) conditions for 6 h between 10 AM and 4 PM for 21 days in the climatic chamber. A digestibility trial with four-day collection period was conducted at the end of the study, and rumen liquor was collected on day 21. At the phylum level, an increased ($p < 0.05$) abundance of *Firmicutes* and a decreased ($p < 0.05$) abundance of *Proteobacteria* and *Planctomycetes* were observed during HS compared to thermoneutral conditions. The abundance of 6 different genera of phylum *Firmicutes*, *YRC22* (phylum *Bacteroidetes*), and *Stenotrophomonas* (phylum *Proteobacteria*) was increased ($p < 0.05$), whereas *B. firmus*, *Lysinibacillus*, *Bacteroides* unclassified genus (family *Enterobacteriaceae*), and *Methanobrevibacter* decreased ($p < 0.05$) during HS compared to control. Eight new genera from four different phyla were detected after HS exposure, whereas one genus was not detected in the rumen after HS exposure, which was present during thermoneutral conditions. Dry matter intake, volatile fatty acid concentration, and digestibility did not change ($p > 0.05$) during HS. It can be concluded that the resilience of the rumen microbial population invokes adaptive responses by changing their abundance to minimize the adverse effects on fermentation and digestibility in buffalo heifers.

1. Introduction

Variation in bioclimatological attributes is a ubiquitous tenet of the environment, and organisms have differential endurance to ensure their survival, growth, and reproduction. The frequency and magnitude of fluctuations in the last many years and its future predictions have threatened the extinction of one in six animal species [1]. Furthermore, the direct and indirect adverse effects of these extreme fluctuations in terms of climate change on livestock production and health have been well documented [2–4]. Moreover, the investigation of short-term ameliorative measures and long-term adaptation mechanisms to achieve sustainable livestock production in the scenario of climate change is still in progress [5].

Buffalo is a promising future livestock animal, and its population is increasing steadily not only in India but in many parts of the world owing to its better economic traits [6]. Although buffaloes are better adapted than cattle to a hot humid climate, they are more susceptible to heat stress due to less sweat glands and dark coat colour [7]. The effects of heat stress on different physical and physiological homeostatic mechanisms [7–9], production and reproduction performance, and amelioration strategies [10] have been described. However, the effect of heat stress on rumen microbiome changes in buffaloes is yet to be explored.

The gastrointestinal tract, especially the microbial population of the rumen, comprising mainly of bacteria and protozoa, is responsible for the digestion of roughages, synthesizing quality, nutrients, animal performance, immunity development and maintenance, and overall health and welfare of animals. The dynamic balance of the rumen microbiome, host physiology, and diet influences the rumen ecosystems [11]. Heat stress modulates the host physiology and interacts with the rumen ecosystem to reprogram the systemic regulation of animal

production and health [12]. It is pertinent to understand rumen ecosystem responses besides host response to heat stress to optimize the rumen fermentation leading to better nutrient utilization and productivity of ruminants.

Heat-stress mediated decrease in feed intake was reported to be the main reason for changes in volatile fatty acids (VFAs) production, digestibility, and energy utilization [13–17]; however, many researchers proposed that changes in rumen microbial population due to heat stress might be a major factor for changes in rumen fermentation pattern [13, 18]. With the help of metagenomic sequencing, heat stress mediated changes at different levels of taxonomical classification in rumen microbial population in cattle [12, 19–25], goat [26, 27], and sheep [28]; and fecal microbial population in cattle [29] and pigs [30] have been reported. Although a wide variation in the effect of heat stress on microbial diversity exists among different species [31, 32] and among breeds of same species [24]; however, at the phylum level, the most consistent change has been reported in *Firmicutes* and *Bacteroidetes*. Similarly, the effect of heat stress response on the rumen metabolome with respect to breed and species is also very diverse [19, 22, 25, 26, 33]. Furthermore, heat stress was reported to change the expression of genes in rumen microbes, which were related to membrane transport, infectious diseases, immune system modulation, and lipid metabolism [34]. However, it remains unclear how the heat stress-mediated shift in the microbiome, metabolome, and microbial gene expression in ruminants helps the host to better cope with heat stress conditions or compromises animal production and health.

Developing an understanding about changes in rumen microbial population and its functional correlation with animal production and health under shifting global temperature regimes will have the best opportunity for rumen manipulation to ensure sustainable production. The effect of heat stress on rumen microbial diversity and fermentation pattern is yet to be elucidated in buffaloes. Therefore, the present study was carried out to find the effect of heat stress on rumen microbial diversity, VFA production, and digestibility in buffalo heifers.

2. Materials and Methods

2.1. Place of Investigation. The study was conducted at Prof. M.D. Pandey Bio-Climatology Laboratory, Department of Veterinary Physiology, College of Veterinary Science and Animal Husbandry, DUVASU, Mathura, Uttar Pradesh. It is situated at 27°N latitude and 78°E longitude with an elevation of 176 m above mean sea level. The annual temperature ranges from 4 to 46°C (439.2 to 114.8°F) while the relative humidity ranges from 25 to 85%.

2.2. Experimental Animals. The guidelines of The Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA), Government of India were used for experimental procedures as approved by Institutional Animal Ethics Committee, Veterinary University, Mathura (no. IAEC-02/12). Six Murrah buffalo heifers (age, 1.5 to 2.0 years; weight, 250 to 300 kg) were housed in the ante chamber of the climatic chamber. The ante-chamber has the capacity to accommodate ten animals and is well ventilated with pucca floors and has a provision of individual mangers, sprinklers, and fans. The buffaloes were fed with a total mixed ration (TMR) consisting of wheat straw and concentrate mixture to meet or exceed the predicted nutrient requirements (NRC 2001) [35]. Nutrient composition of TMR fed during the experimental period is presented in Table 1. The animals were fed the TMR in such a way that at least 5% refusals were left daily per animal. Deworming of all experimental buffalo heifers was done before the beginning of the experiment by oral administration of fenbendazole bolus (Intas Pharmaceuticals Pvt. Ltd., India) @ 10 mg/kg body weight, and vaccination against food and mouth disease, hemorrhagic septicemia, and black quarter (Triovac, Indian Immunologicals, India) was done.

2.3. Experimental Design. The experiment was conducted on a total of six buffalo heifers. In the first phase, the animals were subjected to an acclimatization period of 10 days followed by thermoneutral conditions for 21 days, while in second phase, the same animals were exposed to an acclimatization period of 10 days followed by heat stress conditions in the climatic chamber. The average maximum and minimum ambient temperature and relative humidity during thermoneutral exposure were 29.8°C and 20.3°C, and 54.8%, respectively, whereas during heat stress exposure, the values were 41.6°C and 28.8°C, and 36.4%, respectively. The combination of temperature and humidity for thermoneutral and heat stress conditions in the climatic chambers was used as standardized in the case of buffalo heifers in our previous study [36]. These combinations were adjusted in such a way that there was a minimum difference between the environmental and climate chamber THI during the entry and exit of the animals from the climate chamber. The temperature and relative humidity of the climatic chamber were automatically controlled with a precision deviation of ±1.0°C and ±1.0%, respectively. The temperature humidity index (THI) was calculated by a standard THI model suited to semiarid environment [37]. The animals were exposed to thermoneutral (THI: 71-72) and heat stress (THI: 87-90) conditions between 10 AM and 4 PM every day in a climatic chamber.

After exposure in the climatic chamber, the animals were kept in the ante-chamber for the rest of the day. The digestibility trial with four-day collection period was conducted from the 18th to 21st day of the experiment. A weighed amount of TMR was offered at 0800 h in the morning, and the residue left in the next day at 0800 h was recorded and used for the calculation of dry matter intake (DMI) of the respective animals. About 5% of thoroughly mixed feces were taken for further analysis of its chemical composition. For fecal N estimation, 1% of the feces were also collected daily in glass containers having 10 ml 25% sulphuric acid solution. Thirty mL rumen liquor was collected on the day 21st, day at 1600 hours by stomach tube method, strained with double-layered muslin cloth, and stored at -80°C for further analysis. A brief experimental design is presented in Figure 1.

2.4. DNA Isolation and Sequencing. After thawing of strained rumen liquor, 5 mL rumen liquor was used for DNA isolation by phenol-chloroform method [38]. Quantification of DNA was done using a biospectrometer (Eppendorf, United

Table 1: Ingredients and chemical composition of TMR (g/kg DM) fed during the experimental period.

	g/kg DM
Ingredient composition	
Wheat straw	500
Maize grain	130
Wheat bran	100
Gram chuni	100
Mustard oil cake (expeller extracted)	160
Mineral and vitamin premix[β]	10
Chemical composition	
DM	895
OM	886
CP	127
EE	25
Ash	114
AIA	357
NDF	569
ADF	319
ADL	59
Ca	75
P	39

[β]Used mineral and vitamin premix was prepared by the Department of Animal Nutrition with the brand name of DUMINAS. Mineral and vitamin premix/kg composed of vitamin A: 10,000,000 IU, vitamin E: 80,000 IU, vitamin D: 1,500,000 IU, Fe: 50 g, Zn: 60 g, Mn: 50 g, Co: 0.1 g, Cu: 12 g, Se: 0.15 g, and I: 0.5 g.

Kingdom), and samples containing more than 100 ng/μL of DNA and an absorbance ratio between 1.8 and 2.0 at 260 nm and 280 nm wavelengths were used for further processing. The quality of the DNA was checked using 1.0% agarose gel electrophoresis.

Amplification of V3-V4 hypervariable regions of the 16S rRNA bacterial genes was performed using PCR. The forward and reverse primers used for amplification were GCCTACGG GNGGCWGCAG and ACTACHVGGGTATCTAATCC, respectively. Using the IlluminaMiSeq platform (Illumina, San Diego, CA, USA), the resultant amplicon libraries were sequenced. QIIME software (a comprehensive software comprising of tools and algorithms) for heuristic-based maximum-likelihood phylogeny inference [39] and RDP classifier for the assignment of taxonomic data using a naive Bayesian classifier [40] was used for sequence analysis. High quality clean reads were obtained using Trimmomatic v0.38 to remove adapter sequences, ambiguous reads (reads with unknown nucleotides "N" larger than 5%), and low-quality sequences (reads with more than 10% quality threshold) along with a sliding window of 10 bp and a minimum length of 100 bp. Stitching of PE data into single end reads was done using FLASH (v1.2.11). Picking of operational taxonomic units (OTUs) was done based on sequence similarity within the reads, and a representative sequence from each OTU was picked against Greengenes database (version 13_8). A taxonomic identity was assigned to the OTU using Greengenes reference databases. Using the taxonomic assignments, the diversity metrics for each sample were calculated, and Shannon and Simpson's index was obtained for comparison of alpha diversity whereas principal coordinate analysis (PCoA) was used for exhibiting beta diversity. The dissimilarity matrix of the Shannon diversity was used to generate heat maps using gplots package in R version 2.13.0.

2.5. VFA Analysis. The VFA content in rumen liquor samples was determined by using gas chromatography (Agilent Technologies 8890 GC system, Santa Clara, USA) in duplicate. GC was operated at 50°C using a separation column (Agilent 19091S-433UI: T341943H) with a dimension of 30 m × 250 μ m × 0.25 μm having a provision of FID detectors. The flow rate of the carrier gas in the column was maintained at 1 mL/min, whereas the average velocity was 26 cm/sec with a holdup time of 1.92 min. For the injector and detector, the temperature was set at 250°C and 280°C, respectively.

2.6. Estimation of Digestibility. A digestion trial with 4-day collection period was conducted at the end of the study for the determination of apparent nutrients digestibility. TMR offered residues left and feces voided during 24 h were collected and measured daily for 4 days. Representative samples of TMR were offered and the residue left and feces voided and were oven dried at 60°C for 72 h and then ground in a Wiley mill to pass through a 1 mm screen. Dry and processed samples were analyzed in duplicate for dry matter (DM; method 973.18c), crude protein (CP; method 4.2.08), ether extract (EE; method 920.85), and total ash (TA; method 923.03) contents as per the methodology described by AOAC [41]. Neutral detergent fiber (NDF), acid detergent fiber (ADF), and acid detergent lignin (ADL) were also determined in duplicate according to the methods of Van Soest [42] using an SOCSPLUS SCS 06 ASDLS Fiber Analyzer (Pelican Equipments, Chennai, India). The NDF fractions in representative samples of feed, fodder, and feces were determined without adding alpha amylase.

2.7. Statistical Analysis. The normality of the data was checked using Shapiro–Wilk test. The digestibility and VFA data were normally distributed, whereas the data of microbial abundance did not show normal distribution. Paired t-test was applied between the data sets which were normally distributed; whereas Wilcoxon signed-rank test was used between the data sets which were not normal to determine the significant differences in the analytical variables and microbial abundance between thermoneutral and heat stress groups. SPSS 20.0 statistical software package was used for the statistical analysis. p values < 0.05 were considered significant.

3. Results

3.1. Acquisition of Sequence and Analysis. A total of 103502 high-quality sequences of the V3-V4 region of the 16S rRNA gene were obtained. The Shannon index (6.33 vs. 6.58; $p > 0.05$) and Simpson's index (0.89 vs. 0.86; $p > 0.05$) obtained for comparison of alpha diversity did not change significantly after heat stress exposure compared to thermoneutral conditions. Based on the unweighted UniFrac distances, principal coordinate analysis (Figure 2) suggested that the beta diversity

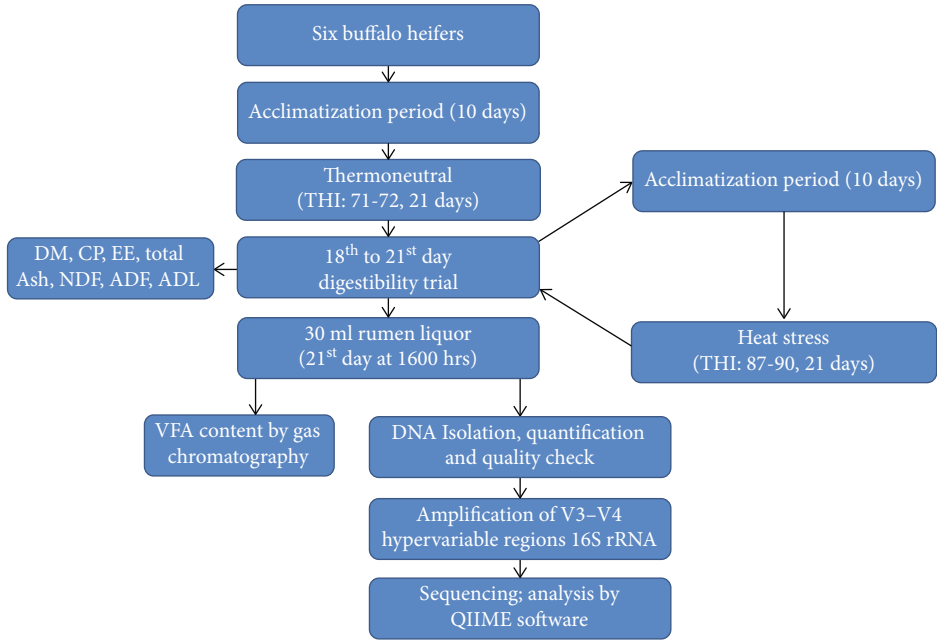

Figure 1: Flowchart of experimental design.

was affected by heat stress. The individual sample's data were presented as supplementary data (Supplementary Table 2-7) for both treatment groups.

3.2. Microbial Biodiversity during Thermoneutral and Heat Stress Conditions. The microbial diversity at the phylum level during thermoneutral and heat stress conditions is presented in the heat map (Figure 3). Irrespective of the heat stress, a total of 26 phyla were identified in rumen liquor samples, in which 25 belong to bacteria and only one belonged to archaea. Similarly, in both experimental groups, the order of the most abundant phyla was *Firmicutes*, *Bacteroidetes*, and *Proteobacteria*, whereas the order of abundance of the other phylum changed after heat stress. Eighteen phyla exhibiting a minimum abundance of 0.001% are presented in Supplementary Table 1. Abundance of phyla *Firmicutes* (47.5 to 63.5%), *Tenericutes* (0.04 to 0.13%), WPS-2 (0.017 to 0.35%), and SR1 (0.002 to 0.115%) was significantly ($p < 0.05$) higher, and abundance of phyla *Proteobacteria* (14.94 to 12.71%), *Planctomycetes* (7.62 to 2.62%), *Lentisphaerae* (0.24 to 0.14%), *Actinobacteria* (9.63 to 0.13%), *Verrucomicrobia* (5.08 to 0.79%), *LD1* (0.04 to 0.008%), *Spirochaetes* (0.02 to 0.0%), and *Deferribacteres* (0.90 to 0.13%) was significantly ($p < 0.05$) lower whereas abundance of phyla *Bacteroidetes*, *Euryarchaeota*, *TM7*, *Chloroflexi*, *Synergistetes*, and *Elusimicrobia* did not change significantly ($p > 0.05$) after exposure to heat stress.

For a better explanation of the effect of heat stress on rumen microbial diversity, the identified sequences were classified at the level of species, but most of them could not be annotated as they were not available in the database. Even at the level of genus, many of the identified sequences were not annotated. Therefore, the effect of heat stress is being presented in terms of phylum, class, order, family, and genus (Figures 4–9).

The abundance of two unclassified genera, belonging to *Lachnospiraceae* and *Ruminococcaceae* families and the genera *Butyrivibrio*, *Clostridium*, *Ruminococcus*, and *Coprococcus*, all belonging to order *Clostridiales* of the phylum *Firmicutes*, increased significantly ($p < 0.05$) in heat stress exposed buffalo heifers (Figure 4). The abundance of genus *YRC22* (Figure 6), *Stenotrophomonas* (Figure 7), and unclassified genus of family *RFP12* (Figure 8) increased significantly ($p < 0.05$) in heat-stressed buffalo heifers.

Genus *Coprococcus* (Figure 4); *YRC22* and unclassified genus of family *BS11* (Figure 6); *Stenotrophomonas*, unclassified genus of family *Moraxellaceae* and *Achromobacter* (Figure 7); and two unclassified genera of phylum *Verrucomicrobia* (Figure 8) were identified only in the heat stressed buffalo heifers. The abundance of *Bacillus* and genus *Lysinibacillus* (phylum: *Firmicutes*) (Figure 5), *Bacteroides* (phylum: *Bacteroidetes*) (Figure 6), unclassified genus (family: *Enterobacteriaceae*), (Figure 7), and *Methanobrevibacter* (Figure 9) decreased significantly ($p < 0.05$), whereas the genus *Streptomyces* (genus: *Actinobacteria*) disappeared in the rumen microbial population after exposure to heat stress in buffalo heifers (Figure 9). The abundance of *Akkermansia* ($p = 0.116$) (Figure 8) and *Mucispirillum* ($p = 0.116$) (Figure 9) tended to decrease in the heat-stressed buffalo heifers population. The population of the genus *Succiniclasticum* and *Oscillospira* (Figure 4), *lactobacillus* (Figure 5), *Prevotella* (Figure 6), *Erwinia*, and *Acinetobacter* (Figure 7) and eight other unclassified genera did not change significantly ($p > 0.05$) in the heat stressed buffalo heifers compared to thermoneutral conditions.

3.3. VFA Concentration, Feed Intake, and Digestibility. DMI, VFA concentration (%) in the rumen liquor, and apparent digestibility of nutrients in experimental buffalo heifers are presented in Table 2. The findings of the present study revealed no effect of heat stress on the feed intake and digestibility of

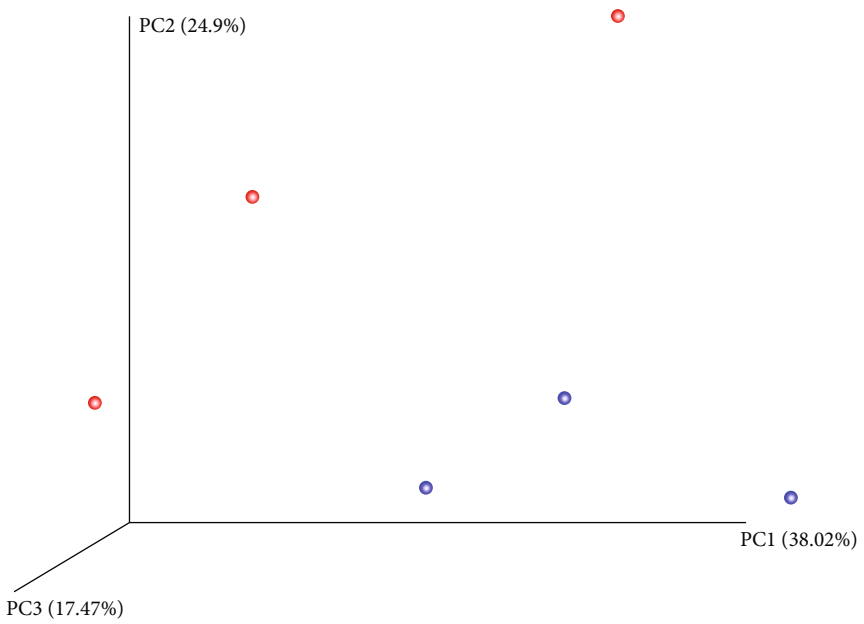

Figure 2: Beta diversity of the rumen microbial population indicated by principal coordinated analysis of all samples using unweighted UniFrac matrix. The variation is explained in percentage in three coordinates. Red points and blue points show the samples of thermoneutral and heat stress condition, respectively.

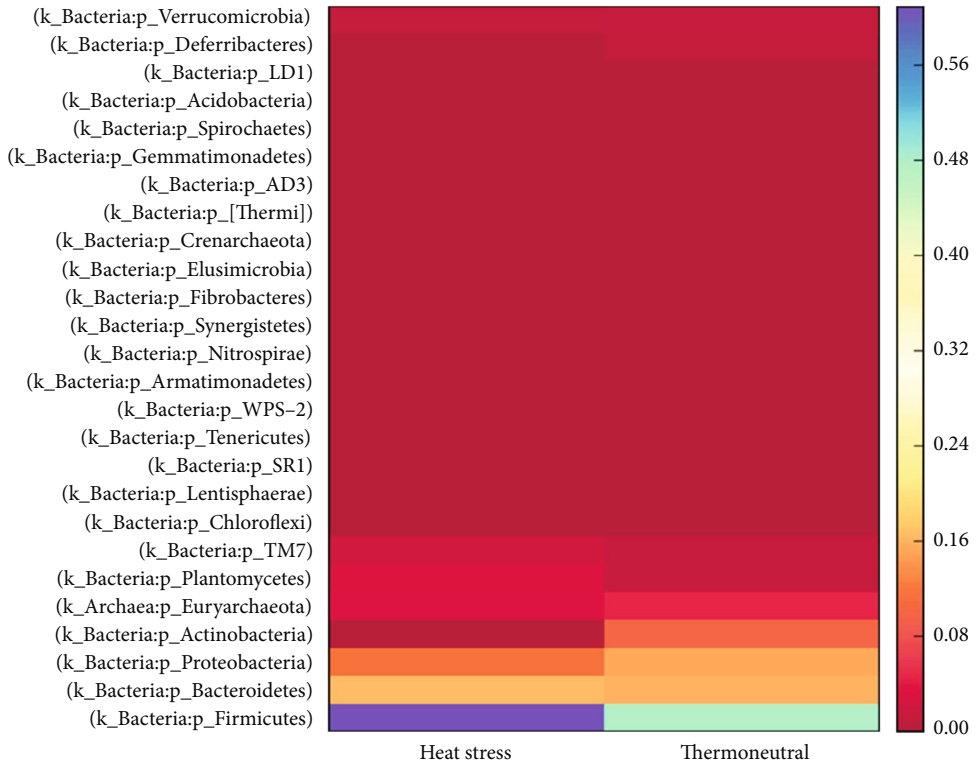

Figure 3: Heat map represents the relative abundance of the top 26 phyla during thermoneutral and heat stress conditions. Scale of the abundance in the form of different colors is presented on the right end of the figure.

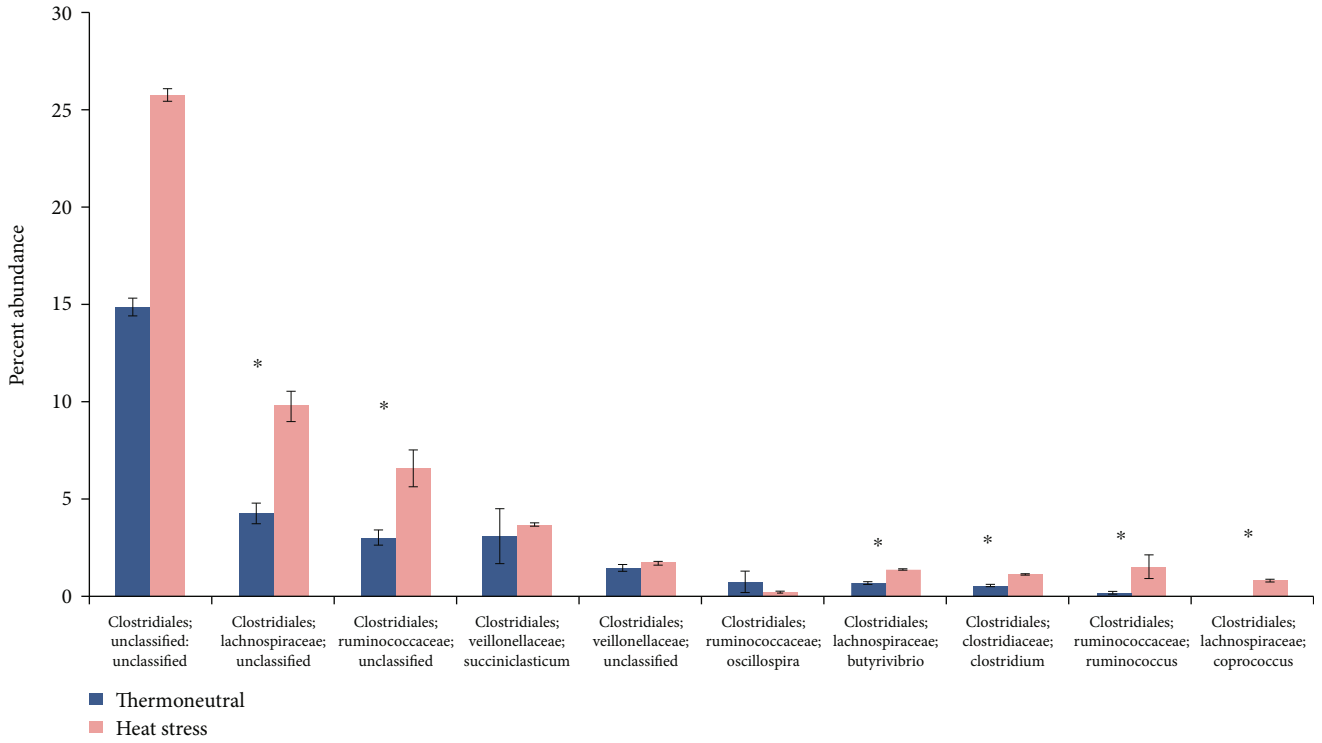

Figure 4: Relative rumen microbial abundance of genera of phylum *Firmicutes* and class *Clostridia* during thermoneutral and heat stress conditions in buffalo heifers. $^{*}p < 0.05$, $^{\#}$sequence of taxonomical classification: order, family, and genus.

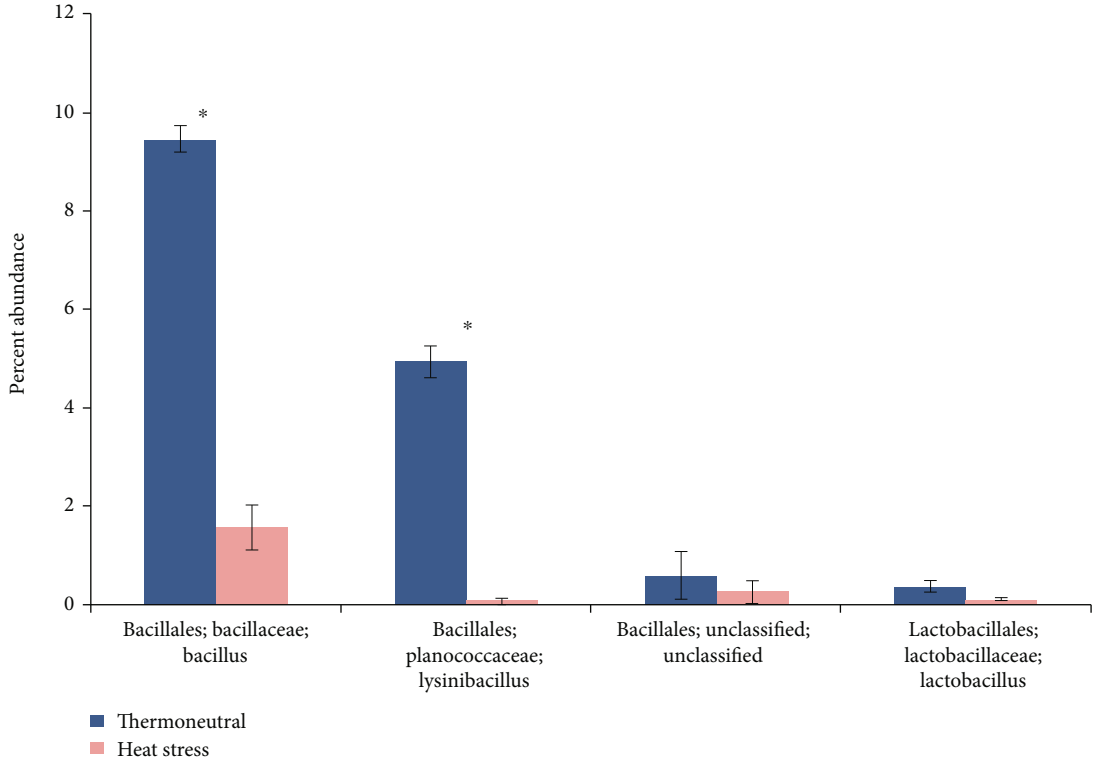

Figure 5: Relative rumen microbial abundance of genera of phylum *Firmicutes* and class *Bacilli* during thermoneutral and heat stress conditions in buffalo heifers ($^{*}p < 0.05$, $^{\#}$sequence of taxonomical classification: order, family, and genus).

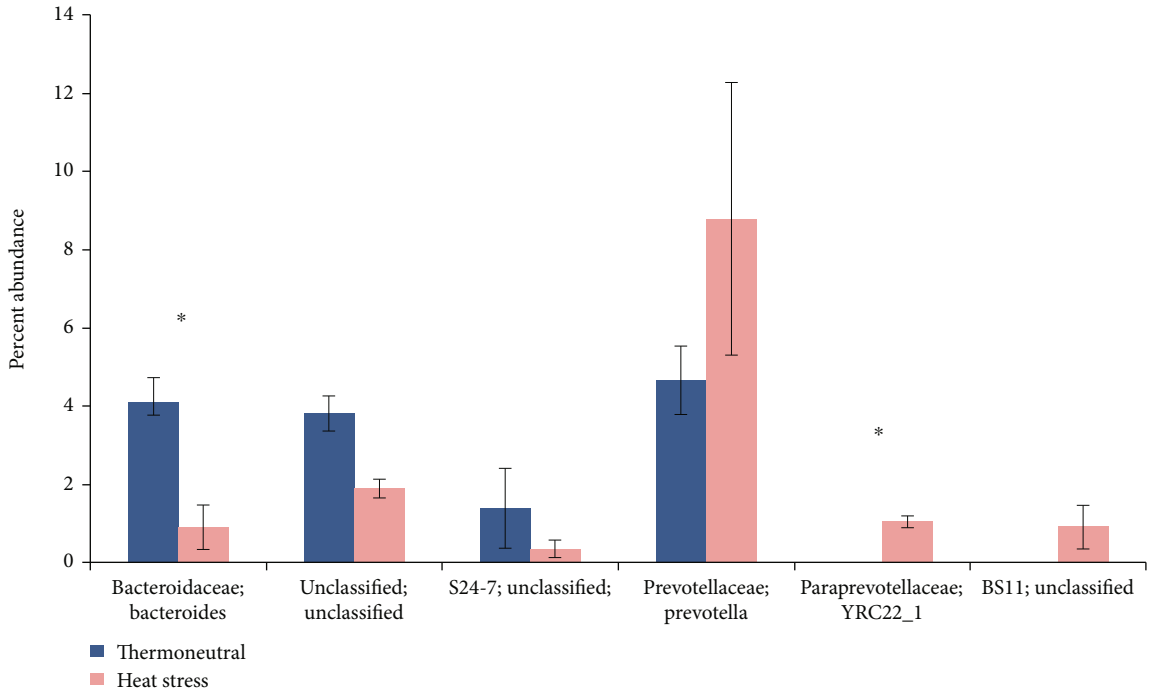

FIGURE 6: Relative rumen microbial abundance of genera of phylum *Bacteroidetes*, class *Bacteroidia*, and Order *Bacteroidales* during thermoneutral and heat stress conditions in buffalo heifers ($^{*}p < 0.05$, $^{\#}$sequence of taxonomical classification: family and genus).

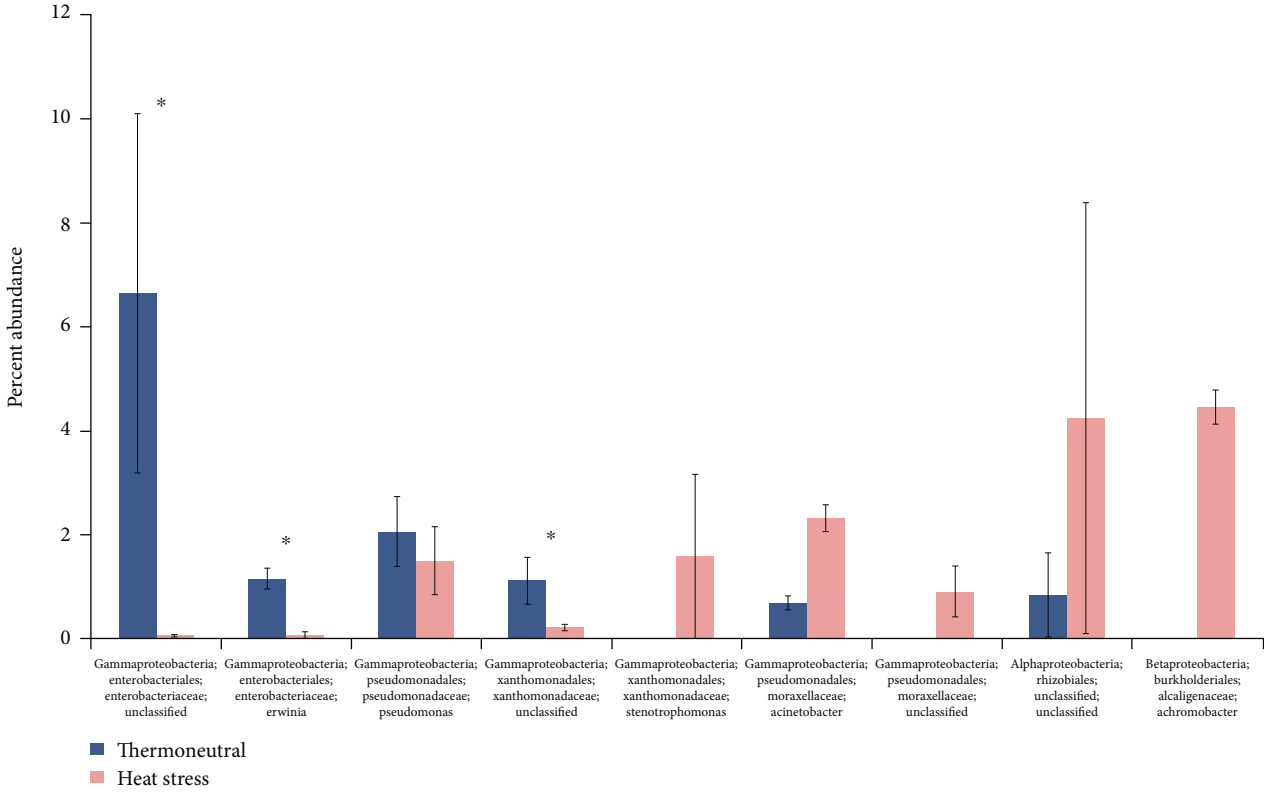

FIGURE 7: Relative rumen microbial abundance of genera of phylum *Proteobacteria* during thermoneutral and heat stress conditions in buffalo heifers ($^{*}p < 0.05$, $^{\#}$sequence of taxonomical classification: class, order, family, and genus).

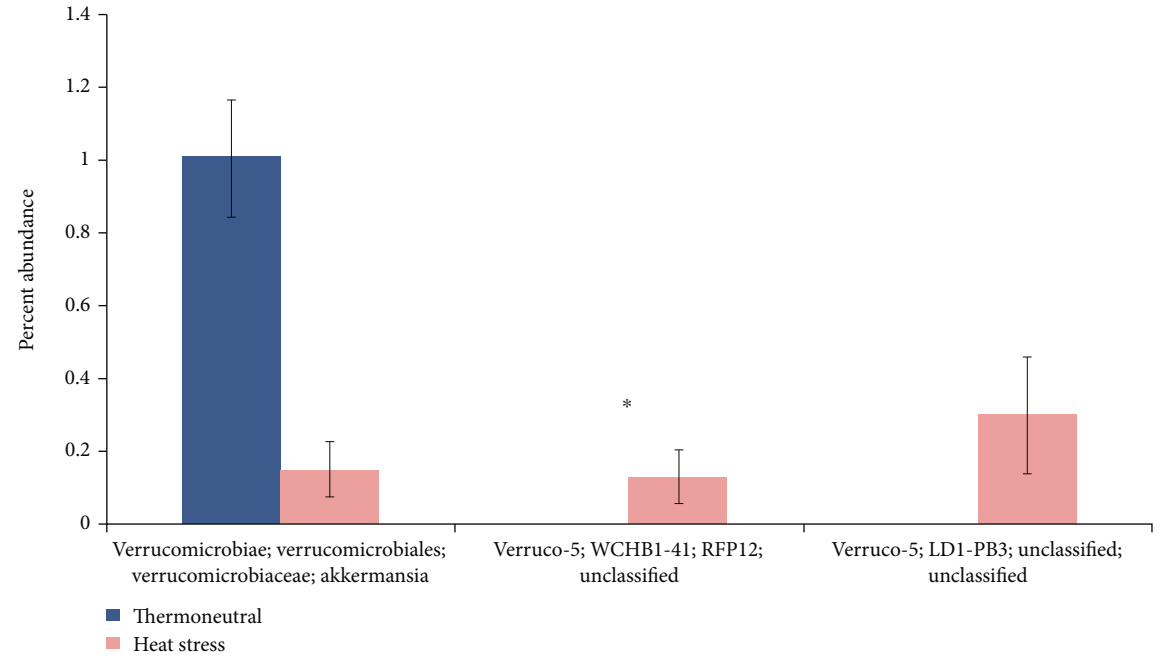

Figure 8: Relative rumen microbial abundance of genera of phylum *Verrucomicrobia* during thermoneutral and heat stress conditions in buffalo heifers ($^{*}p < 0.05$, $^{\#}$sequence of taxonomical classification: class, order, family, and genus).

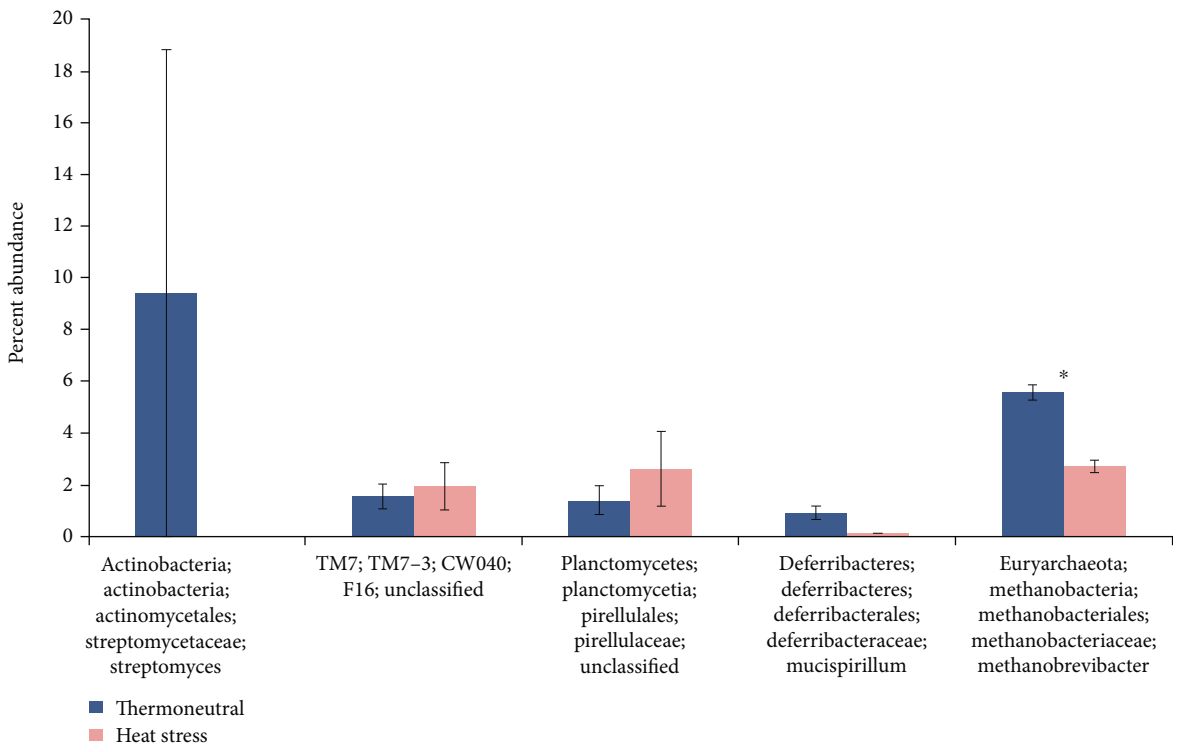

Figure 9: Relative rumen microbial abundance of miscellaneous genera during thermoneutral and heat stress conditions in buffalo heifers ($^{*}p < 0.05$, $^{\#}$sequence of taxonomical classification: phylum, class, order, family, and genus).

Table 2: Effect of heat stress on DMI, digestibility, and VFA concentration.

Attribute	Group		SEM	p value
	Thermoneutral	Heat stress		
DMI (kg/d)	5.30	5.08	0.08	0.137
Apparent nutrient digestibility (g/kg)				
DM	621.53	634.74	24.81	0.403
OM	657.61	665.62	22.60	0.488
CP	721.60	728.14	18.33	0.488
EE	766.34	771.70	15.41	0.488
NFE	655.33	663.42	22.74	0.488
ADF	537.71	539.63	34.33	0.933
NDF	559.60	569.91	29.14	0.488
VFA (%)				
Acetate	47.78	50.86	0.87	0.206
Propionate	24.85	26.10	0.44	0.276
Acetate: propionate ratio	1.92	1.95	0.03	0.826
Butyrate	20.97	18.28	0.69	0.108
Isovalerate	2.22	2.18	0.085	0.838

*$p < 0.05$ shows significant difference between thermoneutral and heat stress groups.

nutrients. Acetate, propionate, and butyrate concentration (%) were not affected ($p > 0.05$) by heat stress.

4. Discussion

Response to heat stress in different livestock species and among breeds of the same species varies [4, 43–45]. Rumen accounts for 1/7th to 1/10th of the animal's body weight and has a volume of 170 to 180 liters with a very complex microbial ecosystem. Functionally, it is partially independent but exists in dynamic equilibrium with host physiology to exhibit a systemic response. Major rumen microbial population consists of prokaryotes which may be less responsive to heat stress-mediated alterations in terms of cytokines and hormones directly, but affect rumen function by modulating the brain-gut axis [12]. However, physiobiochemical changes in the host directly affect rumen microbial diversity, fermentation, and nutrient utilization [15] besides heat stress mediated changes in the rumen itself [19]. In our previous study, we have demonstrated the effect of heat stress on physiobiochemical, redox, endocrine parameters, and miRNAs mediated heat shock protein mRNA regulation [36], and in continuation, the present experiment is aimed at investigating the effect of heat stress on rumen microbiome and fermentation pattern in buffalo heifers.

The results of minimal change in Shannon index during heat stress compared to thermoneutral condition indicated that heat stress did not affect alpha diversity while a change in beta diversity was observed. Similar results were also reported in cattle [19, 25] and goats [27]; however, in the present study, a few bacteria taxa which existed during thermoneutral conditions were not detected, and few bacteria taxa were detected after exposure to heat stress. In the present study, the order of abundance of the three major phyla in the rumen bacterial population was *Firmicutes*, *Bacteroidetes*, and *Proteobacteria*, which were in agreement with the rumen microbial abundance in other species like goats [27, 46, 47] and cattle [12, 19, 24, 25, 48]. On the contrary, the abundance of *Bacteroidetes* was reported to be higher than *Firmicutes* in cattle [21, 49, 50] and lambs [51]. In the present study, the proportion of *Firmicutes* increased, and *Proteobacteria* and *Planctomycetes* decreased, while the proportion of *Bacteroidetes* did not change after exposure to heat stress. Similar to the results of the present study, the percentage of *Firmicutes* increased during summer compared to spring in dairy cattle [29], and population of *Planctomycetes* decreased after 3 days, while the abundance of *Proteobacteria* declined after 6 days of heat stress in Hanwoo steers [49]. In contradiction to the results of the present study, a decrease in *Firmicutes* abundance has been reported in response to heat stress in cattle [12, 19] and goats [27]. Relative abundance of *Firmicutes* was higher in winter, whereas the relative abundance of *Bacteroidetes* was higher in spring and summer as compared to winter [50]. In contrast to the present study, where the *Firmicutes* and *Bacteroidetes* ratio was increased after heat stress, in many studies, the *Firmicutes* and *Bacteroidetes* ratio was decreased. In the present study, the percentage of *Tenericutes* increased, and the relative abundance of *Spirochaetes* decreased during heat stress; however, an opposite trend was reported in dairy cattle [29]. Abundance of *Actinobacteria* increased in lambs during heat stress than thermoneutral condition [28] as opposed to the findings of the present study. A wide variation in heat stress response to rumen microbial population may be attributed to species, breed, age, diet, water quality, farm management, season, health, and stressors like hyperthermia including its intensity and duration [32, 52]. The review of the literature suggested that every host species manifests a distinct microbial response to thermal stress, but *Firmicutes* and *Proteobacteria* showed a consistent shift in heat stress that appeared to be reproducible across the species. In the present study, the effect of heat stress on less abundant phyla such as *Lentisphaerae*, *Chloroflexi*, *LD*, *Spirochaetes*, *WPS2*, *Synergistetes*, *Tenericutes*, and *SR1* remains comparatively vague, as the information

about their respective lower taxonomical classification is insufficiently explicated. Heat stress mediated drastic decrease of *Spirochaetes* to undetectable levels suggesting that heat stress could compromise the fermentation of noncellulosic (xylan, pectin, and arabinogalactan) plant polymers [53]. As the heat stress responses of different genera under the same phyla are distinct and even sometimes exactly opposite, so it would be better to functionally corroborate between heat stress and change in microbial population being done at a lower taxonomical level rather than phyla level. However, in the present study, heat stress responses at the phylum level imply that the rumen microbial population shifts to maintain homeostasis in the rumen fermentation process in order to neutralize the adverse effects of HS [54].

In the present study, two major classes under the phylum *Firmicutes* viz., *Clostridia* and *Bacilli*, exhibited opposite responses to heat stress. The abundance of genera (*Bacillus* and *Lysinibacillus*) and more soeciphically two major species, *Bacillus firmus* and *Lysinibacillus boronitolerans* under class *Bacilli*, decreased during heat stress while the abundance of most of the genera under class *Clostridia* (three unclassified genera, *Ruminococcus, Coprococcus, Butyrivibrio* and *Clostridium*) increased in present study. *B. firmus* and many other species of *Bacillus* genus, isolated from the rumen of North American Moose, have been used as probiotics in lambs as this genus has the capability to synthesize enzymes for the digestion of carboxymethyl cellulose, cellobiose, and starch and can tolerate higher salt concentration [55]. In the present experiment, a decrease in abundance of genus *Bacillus* during heat stress could affect the fermentation and antipathogenic capability of the host-rumen ecosystem. The steep decline in the abundance of *Lysinibacillus boronitolerans* during heat stress requires further studies for functional corroboration. Similar to the observations in the present study, an increased abundance of *Ruminococcus* in cattle [20, 21], and in contrast, a decreased abundance was revealed in goats [27] and cattle [25, 50] during heat stress as compared to thermoneutral conditions. In contrast to the observation in the present study, a decrease in the relative population of bacteria of *Lachnospiraceae* family (*Coprococcus* and one unclassified genus) was reported in cattle [29] and goat [27] which is predominately a starch and fiber degrading family [52]. Similar to the present study, an increase in the genus *Butyrivibrio* (butyrate producing genus) was reported [21], and a corresponding increase in butyrate concentration was observed in ruminal fluid [19–21] in cattle exposed to heat stress; however, no change in butyrate was observed in the present study even though abundance of *Butyrivibrio* was increased significantly. During heat stress, an increase in abundance of the genera *Butyrivibrio*, *Clostridium*, *Ruminococcus*, *Coprococcus*, and two unclassified genera under the families *Lachnospiraceae* and *Ruminococcaceae* (all under the phylum *Firmicutes*) was recorded which are fiber degrading bacteria [56]. On the other hand, the abundance of one of the predominant cellulolytic bacteria of the genus *Streptomyces* was decreased to undetectable levels during heat stress as in Jersey cattle [24]. The results suggested that the rumen microbiome possesses strong heat stress resilience to compensate the functions of each other. In the present study, a 4% decrease (5.30 vs. 5.08 kg/d) in dry matter intake was observed; however, only a minimal change in the digestibility of various fiber components was observed. Under moderate heat stress, an increase in digestibility has been reported, and it was attributed to a decrease in feed intake and increase in retention time of the digesta, alteration in vagal tone, and thyroid activity [20, 21, 24, 57]. Abundance of some of the *Clostridium* species was correlated with IL-1β and TNF-α expression [58] and is a conditional pathogen [59]. Heat stress has been reported to affect fermentation which is attributable to the increase in spores of *Clostridium tyrobutyricum* in feces [60]. An increase in *Clostridium* genus in the present study suggested that heat stress predisposed the gastrointestinal tract to microbial infections.

The bacteria of the families *Lactobacillaceae*, *Bacteroidaceae*, and *Enterobacteriaceae* are known to utilize glucose as an energy source [22]. Unlike an increase in *Lactobacillaceae* during heat stress in cattle [49], a minimal change was observed during heat stress in the present study. Bacteria producing lactate under *Lactobacillaceae* family may cause a decrease in fibrolytic activity of other bacteria; however, no such change was observed during heat stress in the present study. A decreased abundance of the families *Bacteroidaceae* (genus *Bacteroides*) and *Enterobacteriaceae* (unclassified genus) was observed in the present study, contrary to other report [22]; however, a decrease in *Bacteroides* population was reported during heat stress in cattle [50, 57]. The genus *Erwinia* has been known to be pathogenic in both plant and animals [61], although its role in the rumen ecosystem is not yet established. Results indicated that genus *YRC22* and the unclassified genus (family *BS11*) appeared in the rumen only after exposure to heat stress in the present study. In agreement with the present study, an increase in the abundance of *YRC22* during heat stress was reported in cattle [49] which was found to be increased at lower rumen pH. Family *BS11*, which is mainly responsible for hemicellulose fermentation in ruminants [62], was observed to be increased during heat stress in the present study as reported in goats [27]; however, no change was observed in cattle [49]. Increased abundance of family *BS11* in the present study implied that the hemicellulose fermentation ability of the rumen ecosystem was improved during moderate heat stress in buffaloes.

Genus *Acinetobacter* is pathogenic in the environment and acts as a commensal in the ruminant ecosystem and was reported to be positively correlated with milk protein yield and total solids [63]. The abundance of the genus *Acinetobacter* (family *Moraxellaceae*) was found to apparently increase during heat stress in the present study as in cattle [29]. Genus *Stenotrophomonas*, unclassified genus (family: *Moraxellaceae*), and *Achromobacter* of the phylum *Proteobacteria* appeared after exposure to heat stress in the present study. Increased abundance of *Stenotrophomonas* indicated an increase in pathogenic population in the rumen ecosystem as *Stenotrophomonas* population was reported to be correlated with mastitis and subacuteruminal acidosis in dairy cows [64]. Similarly, an increase in abundance of *Moraxellaceae* has been reported during subacuteruminal acidosis [65] which implies that increase in *Moraxellaceae* during heat stress was due to a decrease in pH; however, a minimal change was observed in the bacterial population responsible for lowering pH in the present study. In a review

of the literature, no studies have reported the presence of the genus *Achromobacter* or even the family *Alcaligenaceae* in the rumen; however, it has been reported in the gut of horses [66]. The functional role of *Achromobacter* which appeared after heat stress exposure in the present study needs to be explored. The bacteria of the genus *Akkermansia* are reported to protect gut health by the formation of mucin [67]. Results of the present study suggested that heat stress may hasten the process of pathogenic invasion by decreasing the abundance of genus *Akkermansia* in the rumen ecosystem. Functional importance of the appearance of the unclassified genus of the family *RFP12* and the order *LD1-PB3* after heat exposure in the present study needs to be further explored.

In the present study, *Methanobrevibacter* genus was observed to be the most abundant methane producing bacteria as in many other previous studies in cattle [21, 25, 49]. In the present study, a decrease in abundance of *Methanobrevibacter* was recorded during heat stress, contrary to the previous observations [21, 25]; however, no change in the *Methanobrevibacter* population was reported during heat stress in cattle [49]. Although, in none of the previous studies, methane emission and *Methanobrevibacter* abundance have been correlated; methane emission was reported to decrease during moderate heat stress and increased during severe heat stress in crossbred cattle [18]. As the heat stress in the present study was moderate, it may be speculated that methane emission could have been also decreased, which could be correlated with a decrease in the abundance of *Methanobrevibacter*. On the contrary, an increase in *Methanobrevibacter* abundance was reported during heat stress in cattle [25], and it was speculated that extreme stress could cause an increase in methane emission [18].

VFAs are the major fermentation products in the rumen which alter heat stress [15]. In most of the previous studies, a decrease in total VFA and acetate concentrations and an increase in butyrate/isobutyrate levels were observed during heat stress in different ruminant species [19, 22, 25, 27, 50]. However, heat stress was reported not to affect VFA concentration in cattle [68]. A change in VFA concentration with a change in the level of heat stress has also been reported [19]. Contrary to previous reports, in the present study, both acetate and propionate levels tended to increase, and butyrate levels tended to decrease during heat stress, which was also corroborated with the corresponding changes in the abundance of different bacterial populations. It may be speculated that the rumen bacterial microbial population in buffalo exhibited resilience to heat stress, and the abundance of fiber degrading bacteria increased to optimize the enzyme production in an adverse ruminal environment for fermentation. Acetate production is directly proportional to enteric methane production; however, in the present study, in spite of a minimal increase in acetate production, a decline in the abundance of *Methanobrevibacter* indicated that *Methanobrevibacter* population in the rumen is not only substrate dependent but also dependent upon environmental stress. Results also suggest that under a given heat stress exposure (THI; 87–90), adaptive responses in the rumen ecosystem could not produce detectable harmful effects in the fermentation pattern. In our previous study [36], heat stress exposure exhibited a moderate level of acclimatization responses in terms of hematological, biochemical, oxidative stress, endocrine, and heat shock protein markers which corroborates with the moderate effect of heat stress on the rumen microbial ecosystem and its functional attributes.

5. Conclusions

Heat stress responses to rumen ecosystems differ widely in different ruminant species. In the present study, heat stress (THI; 87–90) induced changes in the microbial population compared to thermoneutral condition (THI; 72). At the phylum level, the abundance of *Firmicutes* increased, and *Proteobacteria* and *Planctomycetes* decreased; whereas *Spirochetes* were not detected after heat stress. The rumen microbiome exhibited resilience to heat stress to minimize changes in fermentation. Decrease in the abundance of antipathogenic and increase in pathogenic bacteria population after heat stress exposure suggested that gut health was compromised during heat stress in buffalo heifers. It can be concluded that under moderate heat stress exposure (THI; 87–90), the resilience of rumen microbial population invoked adaptive responses by suitably changing their abundance to minimize the adverse effects on fermentation and digestibility in buffalo heifers; however, gut health seemed to be compromised.

Data Availability

The required data has been provided in the supplementary files.

Conflicts of Interest

The authors declare that they have no conflicts of interest.

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Supplementary Materials

The data in supplementary table 1 describes the changes in the microbial abundance at phyla level during heat stress as compared to thermoneutral conditions. The supplementary data in tables 2-7 pertains to the microbial abundance at different taxonomical levels in individual animals during both thermoneutral and heat stress conditions. *(Supplementary Materials)*

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