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Ecology/environmental microbiology

Influence of high temperature and humidity on rumen bacterial diversity in Holstein heifers

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Abstract

The effect of heat and humidity stresses on the rumen bacterial molecular diversity of heifers was studied. No statistically significant changes in the rumen microbiota composition were found in the first experiment (average body mass 250 kg) while in the second and third experiments (additional variables included the relative humidity and body weight), the microbiota composition was significantly different at elevated environmental temperatures and humidity. These shifts were accompanied by the decrease in concentration of short-chain fatty acids in the rumen.
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Keywords: Heat stress; Rumen bacteria; Holstein heifers

1. Introduction

According to the IPCC Third Assessment Report, because of the increasing green-house gases emission, the environmental temperatures projected may rise anywhere from 1.4 to 5.8 °C during the period from 1990 to 2100 [1]. Stainforth with coauthors suggested that the average environmental temperature may increase up to 11 °C if the concentration of CO_2 will increase 2-fold in the atmosphere [2]. There are several reports describing the influence of heat stress on domestic animals [3–12]. It is well known that the ruminant animals are least tolerant to the heat stress because the ruminal fermentation produces excessive heat, which needs to be efficiently dissipated in order to maintain the constant body temperature. The negative correlation between temperature–humidity index (THI) and dry matter (DM) intake was reported for cows kept in the southeastern United States, which is classified as a subtropical area [13]. The milk yield was also found be decreasing with the increase of THI, when the certain threshold level is reached [14].

Heifers are considered to be more tolerant to the heat stress than the dairy cows and mature cattle, because the metabolic heat production rate in heifers is lower than that in cows. In addition, heifers have a greater body surface area relative to the body mass thus allowing more efficient dissipation of the body heat [12]. But still, Colditz and Kellaway [15] have found the substantial effect of the environmental temperatures on the Friesians, Brahmans and F1 cross-heifers. Feed intake of Friesians and Brahmans declined for about 17% and 12%, respectively, when the animals were maintained at higher temperatures (17.2 vs. 37.8 °C). Previously, we also reported the variations in physiological status, nutritional intake and body weight gain of Holstein heifers, which were maintained at three environmental temperatures, at 20, 28, and 33 °C [16]. To the best of our knowledge, there is no literature describing the effect of environmental conditions such as temperature and humidity on the rumen microbial diversity. Thus, the main aim of this work was to characterize molecular bacterial diversity in the rumen of

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Holstein heifers, maintained at different environmental temperatures and humidity.

2. Materials and methods

2.1. Experimental design

Three sets of experiments were carried out. In the first set, heifers with the average body weight of 250 kg were maintained at three environmental temperatures, 20, 28, and 33 °C and at 60% of relative humidity. In the second set of experiments, the body weight of animals and temperature conditions were the same, but the relative humidity was set at 80%. In the third set of experiments, the environmental variables were the same as in the first set but the average body weight of the heifers was 430 kg. Each set of experiments was performed in a similar manner, with four heifers being kept at 20 °C for 2 weeks, with the first week allowing the animals to adapt to the environmental conditions and the feeding regimen. In the next step, the experimental temperature was raised to 28 °C and the experiment was continued for another two weeks. After that, the temperature was changed to 33 °C and experiment was continued for another 2 weeks. All experiments were carried out in a environmentally controlled laboratory.

2.2. Animals and diet

Totally eight heads of Holstein heifers were used. Experimental diet consisted of 50% concentrate, 45% Italian ryegrass silage, and 5% alfalfa hay cubes in the first and second set, and 50% concentrate and 50% Italian ryegrass silage in the third set. Animals were fed twice a day (10 a.m. and 4 p.m.) in quantities that are sufficient to cover the heifer's energy and protein requirements according to the Japanese Feeding Standards [17]. Water and mineral salts were given ad libitum.

2.3. Sampling and assay

At the end of each 2-week temperature regimen, the rumen fluid was sampled before morning feeding via oral cavity with stomach-tube (experiment 1) or rumen fistula (experiments 2 and 3). In all experiments, the first 30 ml of rumen fluid was discarded to reduce contamination by oral or body surface bacteria. After that, the samples were collected and squeezed though two layers of cheesecloth and immediately centrifuged at 15,000 rpm for 5 min at 4 °C. The pellets obtained were stored at −20 °C until total DNA isolation. The supernatant of rumen fluid was used to determine the rumen pH and short-chain fatty acid (SCFA) profile. The latter was done with a gas chromatograph (6890 series with flame ionization detector, Agilent Technologies, Inc., Palo Alto, CA, USA) equipped with a glass column with 5% Thermon 1000 and 0.5% H_3PO_4 on 80/100 mesh Chromosorb W (Wako Pure Chemical Ltd., Osaka, Japan).

2.4. DNA extraction and purification

Total DNA from the pellets was extracted using the same procedure as described before [18]. The extracted DNA was finally purified with Genomic Tip G100 (Qiagen GmbH, Hilden, Germany). The OD 260/280 reading of the purified DNA was >1.7. The DNA concentration was adjusted to 50 ng/μl, then the samples from four animals in each experimental data set were mixed in equal quantities to minimize animal-to-animal variation.

2.5. Statistical analyses

All data from animal feeding trail in each experiment were treated to determine the statistical significance with the GLM procedure in the SAS package [19]. If a significant difference was found in the factors compared, Tukey's multiple comparison was carried out [19]. The differences were considered significant if $P<0.05$. In the present experiments, there was no control in a strict sense because animals were exposed to the environmental changes in a stepwise manner. However, the main purpose of this study was to observe the relationship between the composition of rumen bacteria and environmental factors and we made the comparison of rumen bacterial diversity according to the environmental variables. Besides, our preliminary experiments suggested that the adaptation period of 2 weeks was sufficient to accommodate the transitional period and the establishment of the new steady-state (data not shown). Therefore, the experimental design with the stepwise changes of environmental variables was adapted in these experiments.

2.6. PCR, cloning and sequencing

The 16S rDNA libraries from the DNA samples were generated by PCR using a 27F and 1525R primer pair [20]. PCR was conducted with Takara ExTaq HS version (TAKARA BIO Inc., Shiga, Japan) and an iCycler thermal cycler (Bio-Rad, Hercules, CA, USA). To prevent PCR bias, the conditions were set as before [21], with the annealing temperature at 60 °C and 13 amplification cycles [18,21]. PCR products were cloned using a TOPO-TA cloning kit (Invitrogen Corp., Carlsbad, CA, USA). The colonies obtained were randomly picked up and the presence of inserts of expected size was confirmed by PCR using the M13 primers of the cloning vector kit. The resulting amplicons were purified with ExoSAP-IT kit (GE Healthcare Bio-Sciences Corp., NJ, USA) and used as templates for sequencing reaction with BigDye ver. 3.1 (Applied Biosystems, CA, USA). In the first experiment, M13 primers were used for sequencing. In the second and third experiments, 16S rDNA universal primer, 63F [22] was used. DNA sequence reading was carried out on an ABI PRISM 3730 Genetic Analyzer (Applied Biosystems). The length of sequences analyzed was over 580 bp.

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2.7. Sequence analysis

Sequence similarity search against database entries was done using on-line BLAST [23]. The presence of chimeric sequences in the libraries was detected by BLAST search and on-line analysis using CHIMERA_CHECK version 2.7 in RDP release 8.1. ClustalX ver 1.83 [24] was used for alignment and phylogenetic analysis using the neighbor-joining method. The operational taxonomy unit (OTU) and Shannon–Waver index were calculated with DOTUR program [25] by using a genetic distance level of 3%. The statistical significance of differences among the resulting nine 16S rDNA libraries was calculated with the web-LIBSHUFF program (http://libshuff.mib.uga.edu/) [26]. Genetic distance was calculated using the Jukes–Cantor formula in the DNADIST program of the PHYLIP 3.6 package (http://evolution.gs.washington.edu/phylip.html).

2.8. Quantification of bacteria

Quantification of three rumen bacteria, *Fibrobacter succinogenes*, *Ruminococcus flavefaciens*, and *Prevotella ruminicola* as well as of the uncultivated Cluster E group [27] in the rumen fluid was performed using real-time PCR as described before [28]. The known concentrations of plasmids containing target 16S rDNA sequence of the type strains and uncultivated bacterial group Cluster E were used as internal standards for quantification. Real-time PCR amplification reactions were performed with a Light-Cycler FastStart DNA Master SYBR Green I (Roche, Mannheim, Germany) on a LightCycler system (Roche).

2.9. Nucleotide sequence accession numbers

Nucleotide sequences generated in this work have been deposited in the GenBank database under the accession numbers AB269946–AB270154 (experiment 1), AB244030–AB244036, AB244102–AB244178, AB270155–AB270296 (experiment 2) and AB270297–AB270528(experiment 3).

3. Results

3.1. Body weight gain, feed intake and DM digestibility

Experiment 1: In this group of animals with the average body of 250 kg, which were maintained in the environment with the relative humidity of 60% throughout and under three temperature regimens (20, 28 and 33 °C), no significant differences in body weight gain and feed intake were detected (Fig. 1). At 28 °C, the body weight gain was slightly elevated in comparison with other temperatures. There was a tendency towards increased DM digestibility with the elevation of environmental temperatures, which became statistically significant between 20 and 33 °C (Fig. 1).

Experiment 2: In this experiment, the only variable which was different from those in Experiment 1 was the relative humidity, which was set at 80%. The combination of the elevated humidity and high temperatures had a profound effect on the body weight gain and feed intake, both decreasing rapidly with the rise of environmental temperatures (Fig. 2). The difference in feed intake was statistically significant between all three temperature settings. As for the body weight gain, there was no significant difference between 20 and 28 °C; however, the body weight gain at 33 °C was significantly decreased in comparison with the two other temperatures. Similarly to Experiment 1, the DM digestibility values were increasing with the rise in environmental temperatures but this increase was more substantial and the differences were statistically significant between each temperature set (Fig. 2).

Experiment 3: In this experiment, the environmental variables were the same as in the first experiment (temperatures set at 20, 28, and 33 °C and the relative humidity set at 60%) but the average body weight of heifers was 430 kg. Interestingly, despite that the environmental variables replicated that of the first experiment, the general trends followed experiment 2, with a significant decrease in body weight gain and feed intake and with a

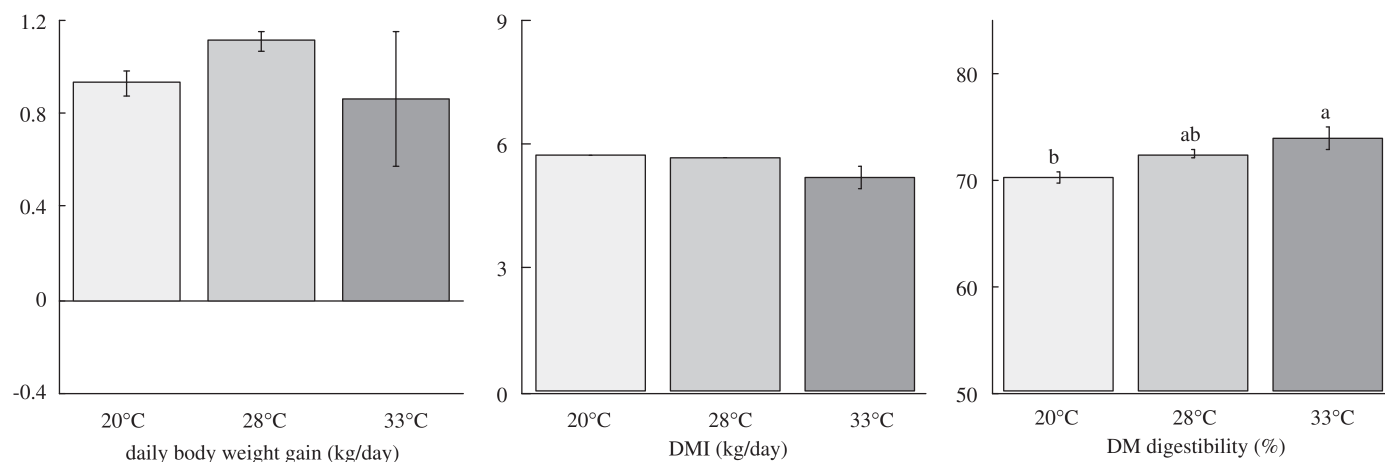

Fig. 1. Effect of heat and humidity on animal daily body weight gain, DMI, and DM digestibility in experiment 1 (animal body weight 250 kg, RH 60%). The significant differences are indicated by a–c above columns.

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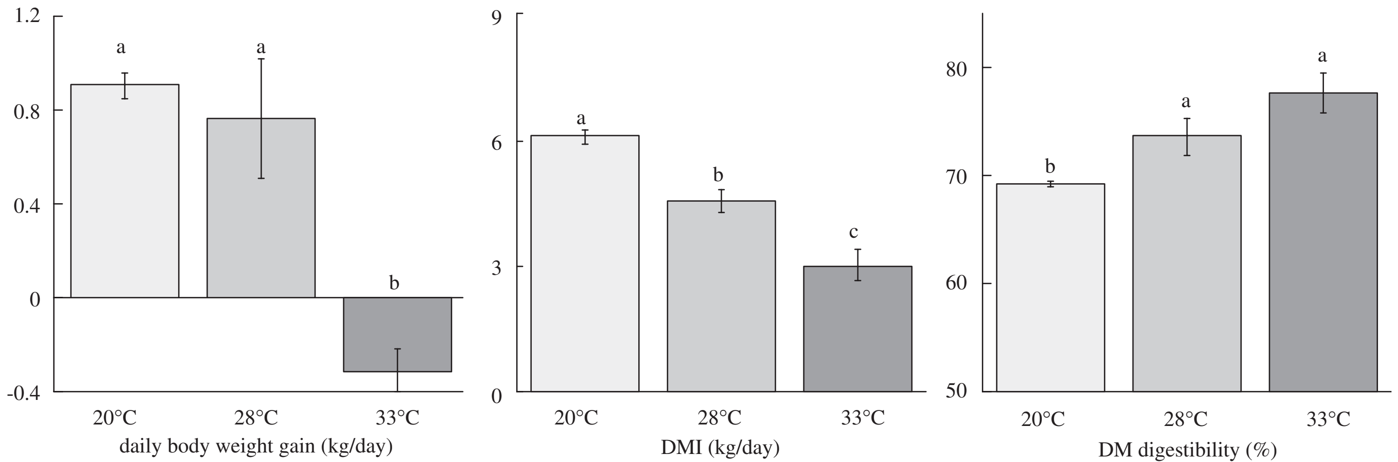

Fig. 2. Effect of heat and humidity on animal daily body weight gain, DMI, and DM digestibility in experiment 2 (animal body weight 250 kg, RH 80%). The significant differences are indicated by a–c above columns.

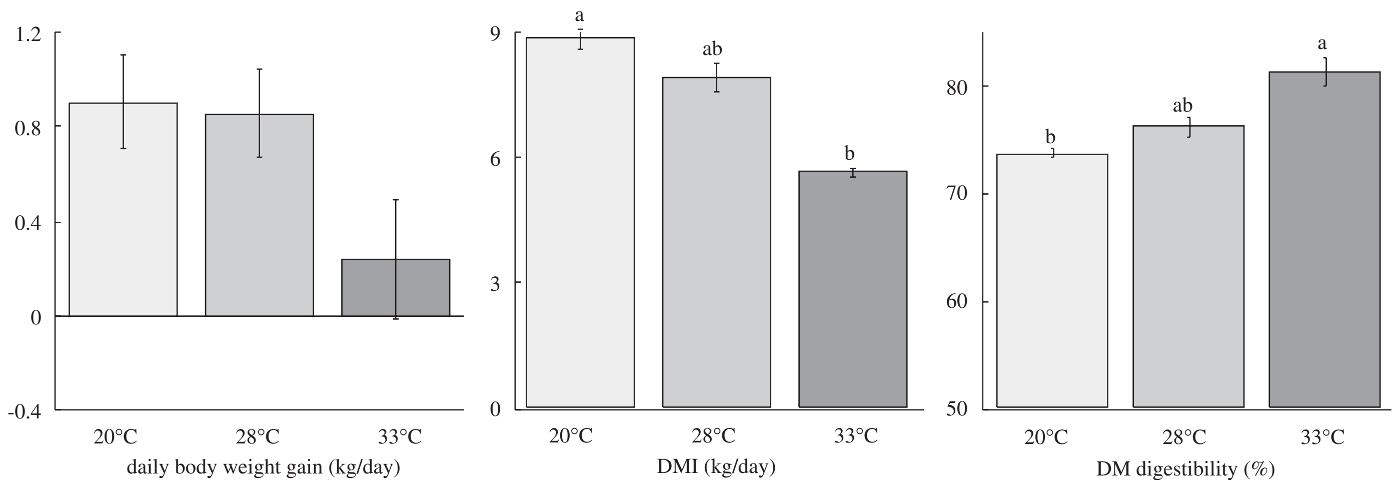

Fig. 3. Effect of heat and humidity on animal daily body weight gain, DMI, and DM digestibility in experiment 3 (animal body weight 430 kg, RH 60%). The significant differences are indicated by a–c above columns.

significant increase in DM digestibility values with the increase of temperature (Fig. 3).

3.2. Rumen fluid characteristics

In all three experiments, there was a tendency for decreasing of ruminal pH values with the increase of the environmental temperatures and it was statistically significant for the experiments 1 and 2 (Table 1). Total concentration of SCFA in the rumen was stable in experiment 1 but in experiments 2 and 3 the concentration dropped when the animals were kept at 33 °C. Only the proportion of propionic acid among other SCFAs was stable throughout the temperature shifts, while two other SCFAs, acetic and butyric acids, demonstrated the reverse tendencies, with the former decreasing and the latter increasing with the environmental temperatures rising (Table 1). Accordingly, the ratio of acetic acid to propionic acid was decreasing with the temperature increase (Table 1).

3.3. OTUs and diversity index in the libraries

In total, nine bacterial 16S rDNA libraries from the rumen content were constructed, which represented three temperature intervals in three experiments (Table 2). The result of analysis with DOTUR program is shown in Table 2. No differences were found in total number of OTUs and the Shannon index values suggesting that the environmental variables such as temperature and humidity as well as the weight of animals were not affecting general bacterial molecular diversity in the rumen of heifers.

3.4. Molecular bacterial diversity at different environments

The significance of differences between the ruminal 16S rDNA libraries was calculated with the LIBSHUFF program. Since this program is able to compare the differences only between two libraries, we applied the round robin approach in comparing the libraries, e.g.,

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20 vs. 28 °C, 20 vs. 33 °C and 28 vs. 33 °C. In the case of comparison of the three libraries, the critical *P*-value was 0.0085. The results of this analysis are shown in Table 3. In the first experiment (temperatures of 20, 28 and 33 °C, RH 60%, and BW 250 kg), no significant differences between the libraries were detected. When the relative humidity level was set to 80% (experiment 2), the composition of 16S rDNA sequences among the libraries demonstrated significant differences. The 16S rDNA library composition at 20 °C was significantly different than the libraries at 28 and 33 °C. No significant difference was detected between the libraries produced from the ruminal content of animals maintained at 28 and 33 °C (Table 3). In the case of larger animals (experiment 3), significant differences were found between the 16S rDNA library composition at 20 vs. 33 °C and 28 vs. 33 °C. But no significant difference was found between the 16S rDNA libraries produced from the rumen of animals maintained at 20 and 28 °C (Table 3).

Table 1
Rumen fluid characteristics

	20 °C	28 °C	33 °C	SEM
Experiment 1				
pH	7.1[a]	6.9[ab]	6.8[b]	0.1
Total VFA (mM)	77.8	77.2	77.5	4.8
Acetic acid (mol%)	71.6[a]	71.4[a]	67.8[b]	0.5
Propionic acid (mol%)	16.3	16.5	17.6	0.4
Butyric acid (mol%)	9.4	9.4	11.5	0.6
Acetic:propionic	4.4[a]	4.3[a]	3.9[b]	0.1
Experiment 2				
pH	7.3[a]	7.1[b]	6.9[c]	0.2
Total VFA (mM)	77.8	79.9	62.6	8.0
Acetic acid (mol%)	71.6[a]	67.7[b]	66.3[b]	2.7
Propionic acid (mol%)	16.1	15.2	16.1	1.7
Butyric acid (mol%)	7.1[b]	10.3[a]	10.5[a]	1.6
Acetic:propionic	4.5	4.5	4.1	0.6
Experiment 3				
pH	6.8	6.9	6.5	0.2
Total VFA (mM)	101	100	82.4	8.3
Acetic acid (mol%)	67.5[a]	66.8[ab]	62.1[b]	3.3
Propionic acid (mol%)	18.2	17.4	17.6	1.8
Butyric acid (mol%)	10.3[a]	11.4[ab]	14.4[b]	2.3
Acetic:propionic	3.7	3.8	3.5	0.5

[a–c]Statistically significant differences.

3.5. Transition of bacterial composition

The phylogenetic placement of 16S rDNA sequences was done on the basis of phylogenetic analysis using the neighbor-joining method and with the Tree View option of the BLAST search. The vast majority of sequences in our libraries belonged to the two phyla, *Bacteroidetes* and *Firmicutes*, with the minor inclusion of sequences related to the high G+C Gram-positive bacteria, *Spirochetes* and related bacteria, *Proteobacteria*, and others (Fig. 4). In all experiments, sequences belonging to the *Bacteroidetes* or *Firmicutes* were dominant with a proportion >80% of total rumen bacterial diversity. No obvious tendencies were revealed in this analysis, except that the older animals harbored a higher proportion of the *Bacteroidetes* in the rumen under the identical environmental conditions (experiments 1 and 3, Fig. 4).

3.6. Quantification of bacteria

No obvious trends in quantities of the two fibrolytic bacteria, *F. succinogenes* and *R. flavefaciens*, as well as of a more generalist bacterium, *P. ruminicola*, in relation to the environmental factors such as temperature and humidity could be demonstrated (Table 4). In the older animals, the quantities of the two fibrolytic bacteria tend to be more stable, while the quantities of *P. ruminicola* were decreasing with the increase of environmental temperatures. The only clear trend observed throughout was the behavior of the

Table 2
Effect of heat stress on rumen bacterial diversity

	Total no. of sequences	Shannon Index	No. of unique OTUs	No. of OTU with n_x sequences[b]								
				n_1	n_2	n_3	n_4	n_5	n_6	n_7	n_8	n_{10}
Experiment 1 (BW250 kg RH60%)												
20 °C	72	3.60	45	30	10	3	0	1	0	0	1	0
28 °C	62	3.67	45	35	6	1	3	0	0	0	0	0
33 °C	75	3.70	47	30	11	2	3	1	0	0	0	0
Experiment 2 (BW250 kg RH80%)												
20 °C	82	3.71	51	34	11	4	1	0	0	0	0	1
28 °C	78	3.92	57	44	7	4	2	0	0	0	0	0
33 °C	64	3.61	43	30	9	2	0	2	0	0	0	0
Experiment 3 (BW430 kg RH60%)												
20 °C	74	3.79	53	42	7	2	0	1	0	1	0	0
28 °C	78	3.66	48	33	9	2	0	3	1	0	0	0
33 °C	80	3.72	50	35	7	4	2	1	1	0	0	0

[a]OTUs and the corresponding Shannon indices were defined using a cutoff value of 97%.
[b]n_1, the number of singletons; n_2, the number of doubletons.

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Table 3
Significance of rumen bacterial composition difference under different environmental conditions and body weight

	P-value (*XY*)	*P*-value (*YX*)	Statistically different
Experiment 1 (BW250 kg RH60%)			
20 vs. 28 °C	0.208[a]	0.729	No
20 vs. 33 °C	0.676	0.715	No
28 vs. 33 °C	0.150	0.122	No
Experiment 2 (BW250 kg RH80%)			
20 vs. 28 °C	0.003	0.015	Yes
20 vs. 33 °C	0.002	0.001	Yes
28 vs. 33 °C	0.185	0.221	No
Experiment 3 (BW430 kg RH60%)			
20 vs. 28 °C	0.096	0.128	No
20 vs. 33 °C	0.002	0.001	Yes
28 vs. 33 °C	0.096	0.003	Yes

[a]In the LIBSHUFF program, the critical *P*-value is 0.0085, therefore, when the *P*-value of *XY* or *YX* is below 0.0085, two libraries are considered different.

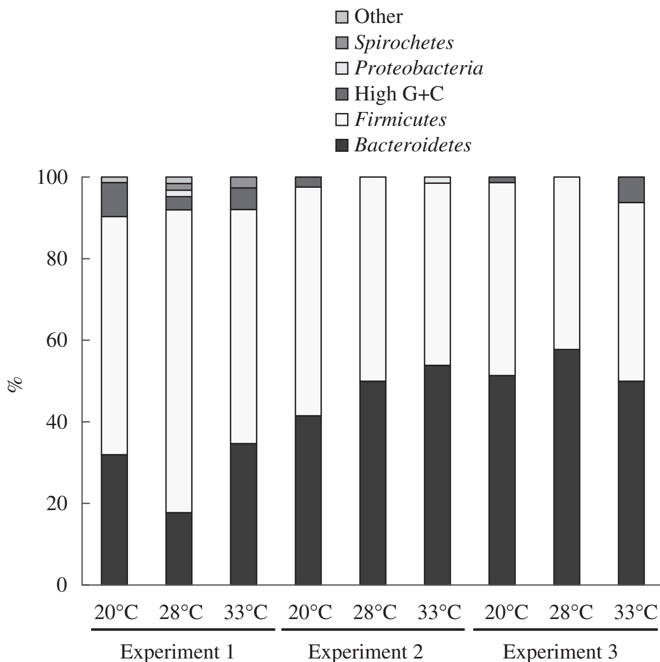

Fig. 4. Changes in proportion of major phylogenetic groups in the rumen in response to temperature shifts as determined by 16S rDNA clone library analyses.

uncultured Cluster E group of bacteria, quantities of which were falling with the increase in environmental temperatures in all three experiments (Table 4).

4. Discussions

Since the heat stress adversely affects the physiology, hormone balance and growth performance of cattle, there have been a number of studies reporting the response of animals to heat under conditions of various nutritional management, other environmental factors and genetic constituency [8–15]. No information, however, exists on how the heat stress affects the rumen microbiota. The ruminants rely on the microbial component of the rumen in order to utilize the nutrients of plant origin and the efficiency of this process directly affects the growth performance and feed conversion efficacy. The present study is the first attempt to answer the question on how the heat stress affects the composition of ruminal microbiota.

In our heifer feeding trials under different environmental temperatures, DM intake and body weight gain were decreased except for the first experiment, while digestibility was increased with the rising of temperature. The results of experiments 2 and 3 demonstrated the same tendency found in earlier studies [29–31]. It is well known that high environmental temperatures increase the DM digestibility, because the residence time of feeds is prolonged in the rumen by the inhibition of ruminal mobility from heat stress [32–34]. In the first experiment (RH 60% and body weight 250 kg), the temperature rise seems had no strong influence on the animal performance. At 28 °C, the daily weight gain was even increased in comparison with the moderate environmental temperature of 20 °C. Therefore, the Holstein heifers with the average body weight of 250 kg seems tolerate well the mildly elevated environmental temperatures such as 28 °C. The negative effect of high humidity (60% vs. 80%) on animal performance characteristics was more profound, especially at high temperatures (experiments 1 and 2, Figs. 1 and 2). Under these conditions, the rate of body heat dissipation is restricted and, therefore, in the heat stress trials it is important to take into consideration the humidity values as well. The larger sized animals appear to be more sensitive to elevated environmental temperatures (experiments 1 and 3, Figs. 1 and 3), probably because of less efficient body heat dissipation.

Except for the first experiment, temperature and humidity shifts had a profound effect on the ruminal molecular bacterial diversity (Table 3). In this regard, these results are in good agreement with animal performance

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Table 4
Quantification of three species of rumen bacteria by real-time PCR

	Experiment 1			Experiment 2			Experiment 3		
	20 °C	28 °C	33 °C	20 °C	28 °C	33 °C	20 °C	28°C	33 °C
Fibrobacter succinogenes	282	1259	545	6402	3070	1639	536	316	441
Ruminococcus flavefaciens	957	3780	1913	2756	912	4727	373	449	166
Prevotella ruminicola	24357	13407	28476	12260	17106	13598	4880	1814	532
Cluster-E	56655	40286	36058	17258	11731	9014	19170	6397	7068

Copy number/ng DNA derived from rumen fluid.

characteristics discussed earlier. Although the environmental parameters such as temperature do significantly affect the fermentation patterns and gas production in the systems such as the rumen-simulating fermentor (RUSITEC) [35], this is apparently not the case in the rumen of homeothermic animals such as cattle. This effect is indirect, through the changes in the physiology of animals in response to higher temperatures and humidity. Although the ruminal bacterial composition was altered in response to rising temperatures and humidity, the diversity indices such as Shannon index and the OTU numbers were very stable in all clone libraries. This suggests that the changed physiological parameters of animals in response to heat and humidity stresses are not selecting for a specific groups of bacteria thus diminishing the diversity indices in the rumen. Similarly to other rumen bacterial diversity studies, the vast majority of different library clones are represented by sequences related to the *Bacteroidetes* and *Firmicutes*. At the same time, the composition of this diversity is different, represented by different species/OTUs under different conditions. We monitored the quantity of several bacterial species in the rumen but no obvious trends were found for the two fibrolytic bacteria and *P. ruminicola*. Only the quantities of the uncultivated Cluster E group sequences were clearly decreasing in response to elevated temperatures in all experiments. In the previous study [28], the quantity of this group fluctuated in the presence or absence of rumen protozoa in Holstein cows. Therefore, this group seems responsive to the alteration of the rumen environment. In the absence of metabolic description of these bacteria, however, it is not possible to establish the functional significance of its dynamics.

The changes in the microbiota composition in response to heat stress at 33 °C are accompanied by a significant decrease in SCFA concentration in the rumen (Table 1). These microbial fermentation products are the primary energy and carbon sources for the host and the decreased growth performance of animals, therefore, is not only due to the higher maintenance energy expenditures at higher temperatures but also due to the diminished carbon and energy supply through the microbial fermentation. Another contributing factor to the impaired growth characteristics may be the changing proportion of different SCFAs in the rumen. In particular, the proportion of acetate was decreasing and the proportion of butyrate was increasing in response to the elevated environmental temperatures (Table 1). It is known that the absorption of acetate and propionate in the rumen is very efficient and close to a 100% while only 27% of butyrate could be accounted for in the portal vein because it is extensively metabolized in the ruminal epithelium of cattle [36].

In this study, we found that the environmental parameters such as temperature and humidity have a significant effect on bacterial composition of the rumen. These changes are accompanied by a substantial decrease in SCFAs concentration related to reduced DMI, which, in addition to the elevated energy expenditures, can be a contributing factor to the impaired animal growth performance at high temperature and humidity.

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