Increasing Buffering Capacity Enhances Rumen Fermentation Characteristics and Alters Rumen Microbiota Composition of High-Concentrate Fed Hanwoo Steers

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Abstract

This study determined the buffering capacity of buffer agents and its effect on *in vitro* and *in vivo* rumen fermentation characteristics and bacterial composition of a high-concentrate fed Hanwoo steers. Both $BC_{0.9\%}$ and $BC_{0.5\%}$ had significantly highest buffering capacity, pH, and ammonia-nitrogen (NH_3-N) than $BC_{0.3\%}$ and CON at 24 h incubation. Individual and total volatile fatty acids (VFA) were significantly lowest in CON. Phylum Bacteroidetes dominated all treatments but a higher abundance of Firmicutes in $BC_{0.5\%}$ than others. *Ruminoccocus bromii* and *Succiniclasticum ruminis* were dominant in $BC_{0.5\%}$ and *Bacteroides massiliensis* in $BC_{0.3\%}$. The normalized data of relative abundance of observed OTUs' representative families have grouped the CON with $BC_{0.3\%}$ in the same cluster, whereas $BC_{0.5\%}$ and $BC_{0.9\%}$ were clustered separately which indicates the effect of varying buffering capacity of buffer agents. Principal coordinate analysis (PCoA) on unweighted UniFrac distances revealed close similarity of bacterial community structures within and between treatments and control, in which $BC_{0.9\%}$ and $BC_{0.3\%}$ groups showed dispersed community distribution. Overall, the increasing buffering capacity enhances rumen fermentation parameters and affects rumen microbiome by altering bacterial community through distinct structure between high and low buffering capacity, thus an important factor to prevent ruminal acidosis during a high-concentrate diet.

Introduction

Energy and essential nutrients are obtained by ruminants through a complicated symbiotic relationship with the rumen microbiome [1] and bacterial community alterations can affect the productivity and health of the host animal [2]. A high forage diet is usually switched to a high concentrate diet to improve the productivity of the ruminants; however, it alters the rumen ecosystem due to high non-structural carbohydrates level [1]. Meanwhile, the core ruminal bacterial community is dominated by the phyla Bacteroidetes, Firmicutes, and Proteobacteria regardless of diet composition [3]. However, a high-concentrate diet induces death and cell lysis [4], thus decrease in abundance of Firmicutes in the rumen [3]. It also enhances the growth of lactic acid utilizers like *Megasphaera elsdenii*, *Selenomonas ruminantium*, and *Veillonella parvula* resulting to a drastic reduction of fiber-degrading bacteria such as *Fibrobacter succinogenes* and *Ruminococcus* spp [1]. Feeding of highly fermentable diets is the current practices in high producing beef to increase growth rates, but it causes microbial disturbances resulting to digestive disorders such as ruminal acidosis [5]. The rapid fermentation of non-structural carbohydrates resulted in the accumulation of volatile fatty acid and lactic acid in the rumen causing a drastic decrease in pH [6]. Hence, the use of buffer could be useful to resist changes in rumen pH whenever cattle are being fed with high concentrate, low forage, fermented and fine-chopped forage [7]. Compounds that increase the buffering capacity of ruminal fluid help maintain a more stable ruminal pH and direct neutralization of VFA especially during a diet or fermentation-related acid challenge [5, 6]. Rumen buffering could avert the sudden decrease in pH, thus could enhance rumen microbial growth, activity and diversity, microbial protein synthesis, and fermentation end product [10]. Buffering capacity (BC) is then referred to as the

number of moles of H^+ that should be added to a 1L solution to decrease pH by 1 unit [11]. Weak acids and bases are known to provide better buffering in comparison to strong acids and bases because of the equilibrium establishment between the acid and the conjugate base [12].

Various studies have reported that adding a buffer solution, such as sodium bicarbonate ($NaHCO_3$) with magnesium oxide (MgO) increased dry matter intake when corn silage was the sole or major source of forage in the diet [13]. $NaHCO_3$ is commonly used in preventing ruminal acidosis because it provides a natural buffer; however, its high solubility limits the buffering activity against acidic conditions [14]. Le Ruyet and Tucker [15] proved that $NaHCO_3$ had high BC in an *in vitro* study. It contained 26% more actively buffering the CO_3 portion of the molecule that is important to neutralize the acid. MgO, on the other hand, appears to work efficiently in combination with $NaHCO_3$[14]. Shaver et al. [16] stated that supplementing $NaHCO_3$ and MgO in a 3:1 ratio is the recommended level of dietary buffer for the best response. The efficiency and mechanisms of buffer responsible for alleviating chronic acidosis are variable and often inconsistent [9]. Research on different level of buffering capacity in enhancing rumen fermentation parameters and microbiome during high concentrate diet has not yet been investigated. In the present work, ruminal metataxonomic and fermentation characteristics analyses were conducted using rumen fluid samples to evaluate the effect of different buffering capacities on *in vitro* and *in vivo* trials in high-concentrate fed Hanwoo steers.

Methods

Animal care and ethics approval

Animals used in this experiment and all experimental protocols were reviewed and approved by the Sunchon National University Animal Research Ethics Committee (SCNU IACUC, approval number: SCNU IACUC-2018-01). All experiments were performed in accordance with the guidelines and regulation set by the governing body, and carried out in compliance with the Animal Research Reporting In Vivo Experiments (ARRIVE) guidelines.

Animals, rumen fluid collection and in vitro **rumen fermentation**

Three ruminally cannulated Hanwoo steers (500 ± 47 kg body weight; 20 mos. of age) were used to provide ruminal fluid for *in vitro* rumen fermentation. The animals were fed twice daily with concentrate feed and kleingrass. Ruminal contents were collected before morning feeding. Samples were squeezed and strained through four layers of surgical gauze and pooled in an amber bottle with an oxygen-free headspace, which was subsequently capped after collection. Collected samples were immediately transported to the laboratory while being maintaining at a temperature of 39°C [49].

Seventy milliliters of rumen fluid were dispensed into serum bottles containing each treatment and 2.5 g dry matter of ground corn grain served as substrate, mixed, and flushed with CO_2 [50]. Samples were in

triplicate and incubated at 39°C for 3, 6, 12, and 24 h while shaking horizontally at 100 rpm, as described by Hattori and Matsui [51]. The buffer used in treatments is composed of calcium carbonate, magnesium oxide, sodium carbonate, and calcified seaweed (Rupromin Balance™, Rotterdam, Netherlands). Treatments consisted of CON (negative control, no buffer added), $BC_{0.3\%}$ (low buffering capacity, 0.3% buffer), $BC_{0.5\%}$ (medium buffering capacity, 0.5% buffer), and $BC_{0.9\%}$ (high buffering capacity, 0.9% buffer). The buffer and the concentrate given to experimental animals were supplied by Purina® Cargill, Korea. The ingredients and chemical composition of the experimental concentrate offered are presented in Supplementary Table S3. Treatments were initially tested for determining their neutralizing (NC) and buffering capacity (BC) through titration using 2N acetic acid from its initial pH to 6.50, and 5.50, respectively (Supplementary Table S4). The buffering agents used in every treatment are in powdered form.

Analyses of in vitro **rumen fermentation parameters and buffering capacity**

Ruminal fermentation parameters were monitored at the end of each incubation time period. Total gas production was measured from each serum bottle after the incubation time using a pressure meter (Laurel Electronics, Inc., Costa Mesa, Calif., USA). Consequently, a needle channel connected to the machine was extended into the sealed fermentation bottle for measuring positive pressure created by the gas build up inside the bottle. A gas flow regulator was then opened to allow gas flow inside a syringe barrel and the plunger was subsequently pulled gradually until the pressure reading on the machine display was zero. The volume of gas trapped inside the barrel was recorded as the total gas produced [49,52].

The pH value was determined using a pH meter (Metler Toledo, Germany) after uncapping each serum bottle. Samples of fermenta were also collected into two 1.5 ml microcentrifuge tubes and stored at -80°C prior to ammonia-nitrogen and VFA analyses. Frozen samples were thawed at room temperature; after which, they were centrifuged for 10 min at 13,000 rpm at 4°C using a Micro 17TR centrifuge (Hanil Science Industrial, Korea). The resulting supernatant was used for ammonia-nitrogen and VFA concentration analyses. Ammonia-nitrogen concentration was measured according to the colorimetric method developed by Chaney and Marbach [53] using a Libra S22 spectrophotometer (Biochrom Ltd., CB40FJ, England) at an absorbance of 630 nm. NH_3-N is the vital source of nitrogen for microbial protein synthesis in the rumen [54]. Analysis of volatile fatty acid concentration was done using high-performance liquid chromatography (Agilent Technologies 1200 series, Tokyo, Japan) with a UV detector set at 210 nm and 220 nm. Samples were isocratically eluted with 0.0085N H_2SO_4 at a flow rate of 0.6 mL/min and a column temperature of 35°C.

Ruminal fluid pH was recorded following 1 min of equilibration. Buffering capacity, defined as the resistance to change in pH from pH 7 to 5, was determined by titrating a 30 ml aliquot of ruminal fluid with continuous stirring from its initial pH to pH 5 with 1N HCl and titrating an additional 30 ml aliquot from its initial pH to a pH of 7 with 1N NaOH. If the initial pH was higher than 7, only the volume of acid

required to reduce the pH from 7 to 5 was recorded. Buffering capacity was converted to milliequivalents per liter as follows: BC = [(milliliters of 1N HCl) + (milliliters of 1N NaOH)] × $10^3/30$ [15].

Analysis of rumen fermentation characteristics in Hanwoo steers

In vivo experiment was conducted using four Hanwoo steers (765 ± 60 kg body weight; 24 mos. of age) in a 4 × 4 Latin square design to assess the effects of treatments on rumen fermentation characteristics and ruminal bacterial composition and diversity of the experimental animals for four months. The feeding trial was conducted with 4 treatments comprised of CON which served as the negative control, $BC_{0.3\%}$, $BC_{0.5\%}$, and $BC_{0.9\%}$.

The Hanwoo steers were fed daily of 2:8 forage and concentrate ratio in 2 equal portions at 0900 and 1600 h. Animals in all treatments received the same vaccinations, medications, and were under the same management programs unless otherwise stated. Steers were confined in free-stall barns and had free access to water and exercise lots.

Rumen fluid samples were collected before morning feeding using an oral stomach tube on the 30th day right before transitioning to the next feeding trial for the analysis of ruminal fermentation parameters. These parameters were all evaluated using the same protocol as used in the *in vitro* experiment. However, rumen pH change in every experimental period of about 30 days was monitored using eCow (hathor.ecow.co.uk). It was done basically to monitor the occurrence of acidosis through a pH value of <5.8 for several hours a day.

16S rRNA amplicon sequencing and metataxonomic analyses

Samples obtained from each treatment were sent to Macrogen, Korea for DNA extraction, 16S rRNA sequencing and microbiome analysis. In brief, DNA was extracted using DNeasy Power Soil Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. The extracted DNA was quantified using Quant-IT PicoGreen (Invitrogen). The sequencing libraries were prepared according to the Illumina 16S Metagenomic Sequencing Library protocols to amplify the V3 and V4 region. The input gDNA was PCR amplified with 1 × reaction buffer, 1 nM of dNTP mix, 500 nM each of the universal F/R PCR primer, and 2.5 U of Herculase II fusion DNA polymerase (Agilent Technologies, Santa Clara, CA). The cycle condition for 1st PCR was 3 min at 95°C for heat activation, and 25 cycles of 30 sec at 95°C, 30 sec at 55°C and 30 sec at 72°C, followed by a 5-min final extension at 72°C. The universal primer pair with Illumina adapter overhang sequences used for the first amplification was V3-F (5’-TCG TCG GCA GCG TCA GAT GTG TAT AAG AGA CAG CCT ACG GGN GGC WGC AG-3’) and V4-R (5’- GTC TCG TGG GCT CGG AGA TGT GTA TAA GAG ACA GGA CTA CHV GGG TAT CTA ATC C-3’). The 1st PCR product was purified with AMPure beads (Agencourt Bioscience, Beverly, MA). Following purification, the 2 uL of 1st PCR product was PCR amplified for final library construction containing the index using NexteraXT Indexed Primer. The cycle condition for 2nd PCR was the same as the 1st PCR condition except for 10 cycles. The PCR product was

purified with AMPure beads. The final purified product is then quantified using qPCR according to the qPCR Quantification Protocol Guide (KAPA Library Quantification kits for Illumina Sequencing platforms) and qualified using the TapeStation D1000 ScreenTape (Agilent Technologies, Waldbronn, Germany).

Sequencing was done using the Illumina Miseq (Illumina Inc., San Diego, CA, USA) platform. The raw data files (fastq) containing the sequenced paired-end (PE) reads were obtained using the bcls2fastq package (Illumina Inc., San Diego, CA, USA) from the base call binary data produced by real-time analysis. The PE raw reads were filtered from adapter sequences using Scythe (v0.994) [55] and Sickle [56] programs then assembled using Fast Length Adjustment of Short Reads (FLASH 1.2.11) [57]. Assembled reads were quality filtered and trimmed for short and extra-long reads, and duplicate reads were removed, then clustered at 100% identity using CD-HIT-OTU [58]. Chimeric reads were identified and the initial clusters were recruited to primary clusters. Then, noise filtering was done and the remaining non-chimeric clusters were binned to operational taxonomic units (OTU) following a greedy algorithm with a cut-off value of 97% species level identity using CD-HIT-OTU [58]. Representative sequences from the clustered OTU were taxonomically assigned using Quantitative Insights Into Microbial Ecology (QIIME Version 1) [18] from the NCBI 16S rRNA database, and the taxonomy composition was generated using QIIME-UCLUST [59]. The produced bacterial taxonomy and composition data were used to generate a biological information matrix (BIOM) [60] in Mothur [61]. The generated BIOM file were used to visualize the alpha and beta diversity indices, and the bacterial composition using programs utilized by Metagenomics Core Microbiome Exploration Tool (MetaCOMET) [17].

Statistical analysis

Data analysis was performed using Statistical Analysis Systems (SAS) version 9.1 (SAS Institute Inc., Cary, NC). The data of rumen fermentation, alpha diversity indices and relative abundance of individual taxa of rumen microbiota were statistically evaluated using Proc general linear model (GLM) for a completely randomized design. All treatments in the *in vitro* experiment were conducted in triplicate and Duncan's Multiple Range Test (DMRT) was used to identify differences between specific treatments. The linear effects of different buffering capacity concentrations were analyzed using orthogonal polynomial coefficients to describe the functional relationships among the control and treatment groups. A $P < 0.05$ was considered indicative of significant differences.

Results

Effect of different buffering capacities on in vitro rumen fermentation parameters

The buffering capacity of $BC_{0.9\%}$ and $BC_{0.5\%}$ were significantly greatest ($P < 0.05$) after 24 h incubation compared to $BC_{0.3\%}$ and CON (Table 1). Both $BC_{0.9\%}$ and $BC_{0.5\%}$ exhibited significantly highest ($P < 0.05$) buffering capacity value of 106.00 meq/L, hence had a similar effect on *in vitro* after 24 h. The ruminal pH obtained from $BC_{0.9\%}$ and $BC_{0.5\%}$ showed similar effects and were consistently higher ($P < 0.05$) than the other treatments throughout the incubation period. In gas production, $BC_{0.9\%}$, $BC_{0.5\%}$, and $BC_{0.3\%}$ had

significantly higher ($P < 0.05$) gas produced than CON and showed similar effects at 3 and 12 h incubation. Ruminal NH_3-N concentration was significantly higher ($P < 0.05$) in $BC_{0.9\%}$ and $BC_{0.5\%}$, thus, it also had a similar effect on this parameter. However, at 6 and 12 h, no effect observed on treatments except that $BC_{0.5\%}$ tended to increase ($P = 0.073$) NH_3-N concentration followed by $BC_{0.9\%}$ and the rest treatments.

Significantly higher concentrations ($P < 0.05$) of acetate were observed in $BC_{0.9\%}$ at 12 h; however, $BC_{0.5\%}$ and $BC_{0.3\%}$ obtained the highest value ($P < 0.05$) after 24 h (Table 2). Propionate and butyrate concentrations were both highest ($P < 0.05$) in $BC_{0.3\%}$ and $BC_{0.9\%}$ at 6 h. Subsequently, distinct effects of $BC_{0.3\%}$, $BC_{0.5\%}$, and $BC_{0.9\%}$ were observed at 24 h which had significantly higher ($P < 0.05$) propionate concentrations than CON. A similar pattern was noticeable with butyrate at 12 h such that $BC_{0.3\%}$, $BC_{0.5\%}$, and $BC_{0.9\%}$ obtained the highest concentration ($P < 0.05$) compared with CON. During this period, a similar effect can be seen between the 3 treatments; however, no significant effect was observed after 24 h. Total volatile fatty acid contents were greater ($P < 0.05$) in $BC_{0.3\%}$, $BC_{0.5\%}$ and $BC_{0.9\%}$ at 12 h but had a slight change after 24 h. At this time point, treatments $BC_{0.3\%}$ and $BC_{0.5\%}$ were highest ($P < 0.05$) compared to $BC_{0.9\%}$ and CON. Furthermore, there were no treatment effects on acetate to propionate ratio after 24 h incubation. Consequently, increasing the concentration of buffering capacity showed linear effects ($P < 0.05$) on pH, total gas production, NH_3-N, and at some certain time point of individual VFA.

Table 1

Effect of different buffering capacity concentrations on *in vitro* rumen fermentation parameters at 3, 6, 12 and 24 h. [e] CON (no buffer added); $BC_{0.3\%}$ (0.3% buffer); $BC_{0.5\%}$ (0.5% buffer); $BC_{0.9\%}$ (0.9% buffer). SEM = standard error of the mean. [a-d] Means with different superscripts in a row differ significantly ($P < 0.05$); [x,y,z] Means within a row indicate linear effect among CON, $BC_{0.3\%}$, and $BC_{0.5\%}$ ($P < 0.05$).

Parameters	Time (h)	Treatment [e]				SEM	*P*-value	
		CON	**$BC_{0.3\%}$**	**$BC_{0.5\%}$**	**$BC_{0.9\%}$**		**All**	**Linear**
Buffering capacity (meq/L)	3	$76.44^{d,z}$	$83.89^{c,y}$	$87.56^{b,x}$	90.11^{a}	0.398	<0.001	<0.001
	6	$76.45^{c,z}$	$84.17^{b,y}$	$92.89^{a,x}$	92.89^{a}	0.246	<0.001	<0.001
	12	$85.33^{c,y}$	$87.22^{c,y}$	$96.67^{b,x}$	99.22^{a}	0.469	<0.001	<0.001
	24	$100.22^{c,z}$	$102.56^{b,y}$	$106.00^{a,x}$	106.00^{a}	0.462	<0.001	<0.001
pH	3	6.00^{c}	6.03^{bc}	6.08^{ab}	6.13^{a}	0.013	0.012	0.052
	6	$5.68^{c,z}$	$5.80^{b,y}$	$5.91^{a,x}$	5.93^{a}	0.014	<0.001	<0.001
	12	$5.42^{c,z}$	$5.54^{b,y}$	$5.60^{a,x}$	5.62^{a}	0.014	<0.001	<0.001
	24	$5.14^{b,y}$	$5.15^{b,y}$	$5.22^{a,x}$	5.24^{a}	0.009	0.001	0.003
Total gas (mL)	3	$74.67^{b,y}$	$82.00^{a,x}$	$81.67^{a,x}$	82.00^{a}	1.287	0.016	0.004
	6	122.33	124.33	123.00	123.67	1.353	0.777	0.488
	12	$169.00^{b,y}$	$187.00^{a,x}$	$179.00^{a,x}$	182.67^{a}	2.492	0.007	0.010
	24	251.67	269.00	256.00	256.67	3.877	0.078	0.195
NH_3-N (mg/dL)	3	$11.26^{c,y}$	$12.24^{b,x}$	$13.05^{a,x}$	13.39^{a}	0.205	0.001	0.003
	6	13.35	13.60	16.09	14.74	0.569	0.178	0.095
	12	13.90^{y}	14.95^{xy}	16.23^{x}	15.84	0.482	0.073	0.010
	24	19.90^{c}	20.39^{bc}	21.35^{ab}	22.43^{a}	0.378	0.012	0.052

Table 2

Volatile fatty acid production during *in vitro* rumen fermentation incubated at 3, 6, 12, and 24 h. [e] CON (no buffer added); $BC_{0.3\%}$ (0.3% buffer); $BC_{0.5\%}$ (0.5% buffer); $BC_{0.9\%}$ (0.9% buffer). SEM = standard error of the mean. [a-c] Means with different superscripts in a row differ significantly ($P < 0.05$); [x,y] Means within a row indicate linear effect among CON, $BC_{0.3\%}$, and $BC_{0.5\%}$ ($P < 0.05$)

Parameters	Time (h)	Treatment [e]				SEM	*P*-value	
		CON	**$BC_{0.3\%}$**	**$BC_{0.5\%}$**	**$BC_{0.9\%}$**		**All**	**Linear**
Acetate (mmol/L)	3	100.13	94.81	93.41	90.36	2.505	0.265	0.237
	6	100.03	100.31	99.79	100.39	0.592	0.901	0.775
	12	102.52^{b}	104.34^{b}	103.36^{b}	107.76^{a}	0.291	0.001	0.412
	24	$103.71^{c,y}$	$112.05^{ab,xy}$	$117.27^{a,x}$	108.47^{bc}	2.182	0.018	0.009
Propionate (mmol/L)	3	27.44	26.51	24.02	25.59	1.395	0.598	0.280
	6	27.81^{b}	28.67^{a}	27.66^{b}	28.37^{a}	0.085	0.001	0.421
	12	32.19	32.65	30.59	33.85	0.756	0.227	0.350
	24	$35.24^{b,y}$	$40.32^{a,xy}$	$42.31^{a,x}$	38.09^{ab}	1.203	0.045	0.020
Butyrate (mmol/L)	3	17.60	20.15	19.60	19.42	0.501	0.088	0.098
	6	$22.23^{a,x}$	$22.78^{a,x}$	$20.95^{b,y}$	22.81^{a}	0.276	0.009	0.006
	12	$27.46^{b,y}$	$40.52^{a,x}$	$39.69^{a,x}$	40.08^{a}	1.660	0.007	<0.001
	24	53.85	54.58	56.43	54.94	0.783	0.327	0.125
Total VFA (mmol/L)	3	145.16	141.47	137.02	135.37	3.381	0.430	0.285
	6	150.07	151.76	148.40	151.57	0.803	0.077	0.196
	12	$162.18^{b,y}$	$177.51^{a,x}$	$173.63^{a,x}$	181.69^{a}	2.424	0.005	0.005
	24	$192.80^{c,y}$	$206.95^{ab,xy}$	$216.01^{a,x}$	201.51^{bc}	3.447	0.013	0.008
A:P ratio	3	3.74	3.57	3.89	3.53	0.134	0.618	0.670
	6	3.60^{ab}	3.50^{c}	3.61^{a}	3.54^{bc}	0.018	0.014	0.719
	12	3.19	3.20	3.41	3.18	0.084	0.465	0.266
	24	2.94	2.78	2.78	2.85	0.069	0.434	0.178

Effect of different buffering capacities on rumen fermentation characteristics in Hanwoo steers

The effect of different buffering capacity concentrations on rumen fermentation characteristics of Hanwoo steers in four treatments are presented in Supplementary Table S1. Average pH had no significant effects among CON and treatments. However, $BC_{0.3\%}$, $BC_{0.5\%}$, and $BC_{0.9\%}$ had significantly higher ($P < 0.05$) buffering capacity value than CON, and showed linearly significant effect ($P < 0.05$). Ammonia-nitrogen, acetate to propionate ratio, individual and total VFA concentrations of rumen fluid from steers under all treatments were not significant and showed similar effects after the *in vivo* experiment.

Bacterial diversity of the rumen contents of Hanwoo steers

The boxplot representation of alpha diversity indices is shown in Fig. 1. Alpha diversity indices are composite indices that reflect abundance and consistency. Chao1 which reflect the OTU abundance in the samples showed that $BC_{0.9\%}$ was the highest among treatments followed by $BC_{0.5\%}$ and the rest of the treatments (Fig. 1a). Shannon index which reflects the diversity of the OTU in samples presented $BC_{0.9\%}$ as the most diverse among treatments and $BC_{0.3\%}$ being the least (Fig. 1b). Moreover, Figure 1c showed the boxplot of OTUs of observed species from the samples. The number of OTUs in $BC_{0.9\%}$ was higher followed by $BC_{0.5\%}$ and the rest of the treatments. The diversity index is used to analyze the temporal and spatial changes in species composition which reflects whether bacterial communities between groups have differences. Our results showed that the rumen bacterial composition of $BC_{0.5\%}$ and $BC_{0.9\%}$ had overall higher alpha diversity than other treatment groups, although no significant difference was observed after statistical analysis.

Effect of treatments on bacterial community composition of Hanwoo steers rumen contents

Bacterial taxonomic compositions at the phylum, genera, and species level are shown in Fig. 2. Results at the phylum level revealed that 15 bacterial phyla were identified in the rumen digesta samples of Hanwoo steers (Fig. 2a). The majority of the sequences obtained from all treatments belonged to Bacteroidetes followed by Firmicutes. It was noticeable that $BC_{0.3\%}$ had the highest abundance of Bacteroidetes (71.90%) and lowest Firmicutes (22.13%). On the contrary, $BC_{0.9\%}$ had the lowest abundance of Bacteroidetes (54.19%) among treatments; however, $BC_{0.5\%}$ had the highest Firmicutes (33.84%) relative abundance. Furthermore, Proteobacteria was dominant upon incorporating $BC_{0.9\%}$ in the diet resulting in declining its abundance from that of $BC_{0.3\%}$ and $BC_{0.5\%}$. Spirochaetes increased its abundance when animals received supplementation of $BC_{0.9\%}$, whereas there was a sudden decrease once the steers received $BC_{0.5\%}$ in their diet. At the genus level (Fig. 2b), *Prevotella* was the predominant among genera in all treatments followed by *Bacteroides* (10.36%) and *Ruminococcus* (8.93%) in $BC_{0.3\%}$ and $BC_{0.5\%}$, respectively. Furthermore, *Succiniclasticum* was dominant among genera after supplementing $BC_{0.5\%}$; however, a decreasing abundance of *Paludibacter* was noticeable as $BC_{0.5\%}$ is incorporated into the diet, which had a reverse effect as did CON. Species-level analyses revealed that *Prevotella ruminicola* predominated the treatments CON, $BC_{0.3\%}$, $BC_{0.5\%}$ and $BC_{0.9\%}$ with the relative abundance of 24.85%,

32.16%, 26.73%, and 23.17%, respectively (Fig. 2c). The comparison of single species analyzed through statistical analysis showed a significant effect of the treatments only in the case of *Prevotella brevis.* This species was more abundant (P = 0.015) in the CON and as steers received a diet supplemented with $BC_{0.9\%}$, $BC_{0.5\%}$ and $BC_{0.3\%}$ its abundance decreased. Owing to the $BC_{0.5\%}$ supplemented in the diet, a decreasing abundance of *Paludibacter propionicigenes* was observed; however, it increased in CON. Incorporation of $BC_{0.5\%}$ increased the microbial population of *Ruminococcus bromii* and *Succiniclasticum ruminis.* Moreover, the smaller percentage of $BC_{0.3\%}$ resulted in a higher abundance of *Bacteroides massiliensis* which led to a sudden decrease in its population as the concentration of treatments increases. Supplementing buffers of different buffering capacity concentration may affect the rumen microbiota through the relative abundance of bacterial species.

The core, shared and unique bacterial community of observed species of the rumen microbiome after treatment of buffer agents with varying level of buffering capacity is presented in Fig. 3a as Venn diagram. A total of 211 (59.6%) observed species can be found across all the samples (core), 79 (22.32%) for shared by 2 or 3 samples, and 64 (18.08%) are specific and are distributed to the four samples. The comparison of the bacterial communities by principal coordinate analysis (PCoA) is presented in Fig. 3b. The PCoA plots showed close similarity within and between treatments and control, whereas those under $BC_{0.9\%}$ and $BC_{0.3\%}$ groups showed dispersed distribution of bacterial communities. The PCoA plot showed dissimilarity of bacterial community and revealed a distinct structure between high buffering capacity and low buffering capacity.

The normalized data presented in Fig. 4 shows the clustering based on the similarity of relative abundance between representative families of OTUs (row), and treatments (column). The analysis divided the representative families into two major clusters distinguishing families which represents low relative abundance on all treatments (upper cluster in red), and families that have varying relative abundance between treatments (lower cluster, colored from peach to blue). On the cluster presenting varying abundance between treatments, two sub-clusters were also distinguishable; (1) families which represent variation from very low (red) to average (peach) abundance, and (2) cluster representing families which have average to high (blue) abundance. The cluster in the bottom of the heatmap (labelled) contains the families that represent the above average relative abundance. On this cluster, family Prevotellaceae had branched out because it presents the highest abundance with very small variations between treatments (p = 0.092). Family Ruminicoccaceae were also found in all treatments, but varying relative abundance was observed, with $BC_{0.5\%}$ presenting the highest. Families Acidaminococcaceae and Lachnospiraceae were significantly highest (P < 0.05) in $BC_{0.3\%}$ and $BC_{0.5\%}$, respectively. Also, the Unclassified Clostridiales had significantly highest (P < 0.05) relative abundance in $BC_{0.5\%}$. A certain unclassified family under order Bacteroidales also showed major abundance especially in $BC_{0.3\%}$, while families Vibrionaceae and Spirochaetaceae were highest in $BC_{0.9\%}$. Meanwhile, the normalized data of relative abundance of representative families of observed OTUs have grouped the control sample together with $BC_{0.3\%}$ in a single cluster, while $BC_{0.5\%}$ and $BC_{0.9\%}$ are on their own cluster, which could indicate the effect of varying buffering capacity of buffer agents.

Monitoring of acidosis

The changes in the 24 h mean ruminal pH monitored for 30 d is presented in Supplementary Table S2. During this period of the feeding challenges, mean pH values were >5.8. Minimum pH was lowest in CON, whereas it was highest in $BC_{0.5\%}$. Additionally, $BC_{0.9\%}$ had a low minimum pH value second to that of CON. It was noticeable that $BC_{0.3\%}$ and $BC_{0.5\%}$ had higher minimum and mean pH values compared to $BC_{0.9\%}$ and CON. Obtained results indicated that the duration of time where pH was <5.8 and 5.8 < 6.0 was longer in CON followed by $BC_{0.9\%}$ and $BC_{0.3\%}$. Meanwhile, $BC_{0.3\%}$ also exhibited good results in the duration of time where pH was approximately 6.0 and above; however, $BC_{0.5\%}$ had even better effects and did not show any signs of acidosis in the rumen. Based on the data gathered, $BC_{0.5\%}$ stabilized the pH of rumen preventing it from becoming acidotic.

Discussion

Currently, one of the major health issues in dairy farming is the sudden decline of ruminal pH which causes a reduction of feed intake, problems with digestion, and production losses. Cattle health mainly suffers and additional costs in management increase due to its prevalence. Sodium bicarbonate is widely used for the prevention of rumen acidosis because it serves as a natural buffer in the rumen. Despite its buffering ability, it only functions for a short period of time and because of the high solubility, it is rapidly used by the ruminants. Most studies have suggested that magnesium oxide act either as a neutralizer or buffer in rumen or intestine [22]. It also increases starch digestion in the intestine of animals fed with a high-concentrate diet. This may result in an increase of pH in the small intestines allowing starch-digesting enzymes to become more active [16]. Mao et al. [23] reported that supplementation of the bicarbonate group had higher pH, total gas production, and total VFA concentration although ammonia-nitrogen concentrations remained unaltered. Addition of combined buffers in high concentrate rations altered rumen pH, liquid turnover, and patterns of rumen fermentation [24]. Consequently, commercial buffer agent (CBA) is developed as a buffer premix and considered as more powerful alternative to sodium bicarbonate. This premix is a mixture of various raw materials, differing in acid-binding capacity and solubility that contained live yeast, which promoted the conversion of lactate to propionate; thus, improving rumen conditions. Research data have shown its efficiency in maintaining the stability of ruminal pH, thus preventing the stimulation of subacute ruminal acidosis (Provimi™, Rotterdam, Netherlands). Meanwhile, the results of the present study are in accordance with their experimental output.

The result of the present study showed that $BC_{0.9\%}$, as well as the $BC_{0.5\%}$, had similar effects on rumen content. Both treatments had significant effects on pH, buffering capacity, and ammonia-nitrogen concentration relative to that of the negative control. An increase in ruminal pH upon supplementation of sodium bicarbonate is a result of dissociation of sodium (Na^+) and bicarbonate (HCO_3^-) [11]. Meanwhile, the results on gas production were supported by the claims of Rauch et al. [25] and Kang and Wanapat [10],

who stated that supplementation with sodium bicarbonate enhanced gas production. The increase in gas production might be caused by the dissociation of sodium bicarbonate resulting to increase gas volume because of CO_2 liberation [25]. Also, it might be due to the conversion of some bicarbonate to carbonic acid which soon released as carbon dioxide [11]. Moreover, obtained data from the present study is in accordance with the results of Le Ruyet and Tucker [15] on the temporal effects of ruminal buffers in terms of buffering capacity and pH of ruminal fluid from cows fed a high concentration diet. Buffering compounds increased the ruminal fluid buffering value index and were beneficial in preventing postprandial increases in ruminal fluid hydrogen ion concentration. Shaver et al. [16] also stated that magnesium oxide and sodium bicarbonate were the best rumen buffers, which increased the acetate: propionate ratio and prevented declines in pH. The effect of buffers on VFA in this study was the same as the data obtained by Kang and Wanapat [10] wherein supplementation with buffering agents increased the total VFA. High ruminal VFA concentration is caused by increased carbohydrate fermentation in the rumen [26]. Although the present study did not show a significant effect on molar concentration of VFA, the noticeable increasing numerical values were observed in buffer-supplemented treatments.

Subsequently, the metagenomic survey of bacterial community composition was identified in the rumen digesta samples of Hanwoo steers. Obtained results at the phylum level were in accordance with the data gathered by Nagata et al. [27] wherein the relative abundance of Bacteroidetes was higher during the high-concentrate period of the experimental animals. Additionally, Zhao et al. [28] stated that the microbial community of beef cattle was dominated by Bacteroidetes and Firmicutes at the phylum level regardless of group. An increase in the phylum Bacteroidetes resulted in increased *Prevotella* and repressed Firmicutes, which was attributed to decreasing Ruminococcaceae. Dodd et al. [29] and Naas et al. [30] indicated that the Bacteroidetes in the rumen represented another numerically dominating phylum that was not associated with cellulose degradation, rather its saccharolytic status is based on limited case studies of noncellulolytic *Prevotella* rumen isolates. Because of the ability of *Prevotella* to use a variety of substrates, it tends to dominate in the rumen under a range of diets [31]. In the present study, *Prevotella ruminicola* appeared to be the predominant species among all treatments. This species constitutes one of the most numerous groups recovered from the rumen and plays important roles in the utilization of polysaccharides of plant origin [32–34] and the metabolism of peptides and proteins [35–39]. Moreover, the low-relative abundance of *Ruminococcus* (8.93%) in this study was in contrast with the findings obtained by Klieve et al. [40], who used a high-grain diet (75% barley) for the animals, although this genus was identified and largely comprised the cellulolytic bacteria. High propionate concentration of $BC_{0.5\%}$ might be caused by the high relative abundance of *Succiniclasticum ruminis*. This result is in accordance with the study of Van Gylswyk [41], who stated that this species specializes in fermenting and converting succinate to propionate, which is an important precursor of glucose in ruminants. Ueki et al. [42] described *Bacteroides massiliensis* as a producer of acetate, propionate, and succinate which can explain the increase in molar concentrations of VFA on *in vivo* study. The abundance of *Paludibacter propionicigenes* might be due to its description as a sugars utilizer and a producer of acetate and propionate, an end product of fermentation [43].

Acidosis was defined as impaired ruminal health accompanied by a reversible ruminal pH depression [40,44–47]. Ruminal microbes convert carbohydrates to short-chain fatty acids at a rate that exceeds the rumen's absorptive, buffering, and outflow capacity causing a rapid decrease in ruminal pH [48]. Data gathered in this experiment agreed with the results obtained by Tucker et al. [8] that the addition of a buffer, especially sodium bicarbonate, was effective in reducing ruminal fluid acidity and retards the drop in pH that normally occurs from 6 to 12 h post-feeding. Also, Zamarreño et al. [9] stated that the use of sodium bicarbonate and magnesium oxide or even mixed antacids were recommended for satisfactory results. They concluded that the increase in buffering capacity and increase in acid consuming capacity contributed to the correction of animal acidosis.

In conclusion, increasing buffering capacity enhances rumen fermentation and alters rumen microbiome which is an important factor contributed positively to the correction of animal acidosis during a high-concentrate diet.

Declarations

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Author contributions

Conceptualization: S.S.L., C.D.J., T.G.K., J.S.L., K.K.C.; Supervision: S.S.L.; Experiment: S.C.R., C.D.J.; Data curation: S.C.R., C.D.J.; Formal analysis: S.C.R., C.D.J., S.S.L.; Methodology: S.C.R., C.D.J., S.S.L.; Software: S.H.Kim, A.R.S., Y.I.C., Sung Sill Lee (S.S.L.); Validation: S.S.L.; Investigation: S.C.R., C.D.J., S.S.L.; Writing – original draft: S.S.L., C.D.J., S.C.R., T.G.K., J.S.L.; Writing – review and editing: S.C.R., L.L.M., S.H.Kang, S.S.L. All authors have read and agreed to the published version of the manuscript.

Competing interests

The authors declare no competing interests.

Additional information

Supplementary information is available for this paper at….

Correspondence and requests for materials should be addressed to S.S.L.

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Figures

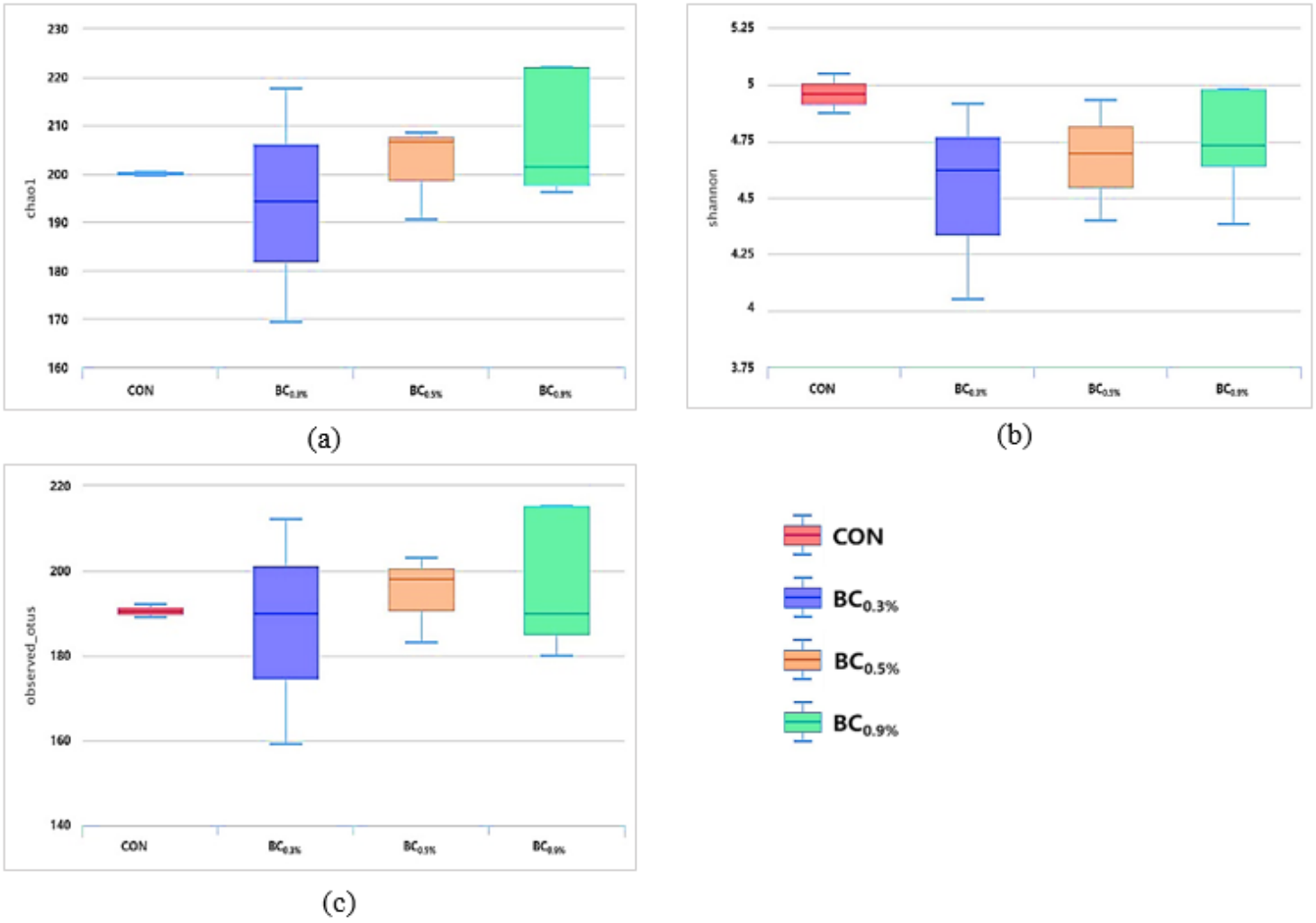
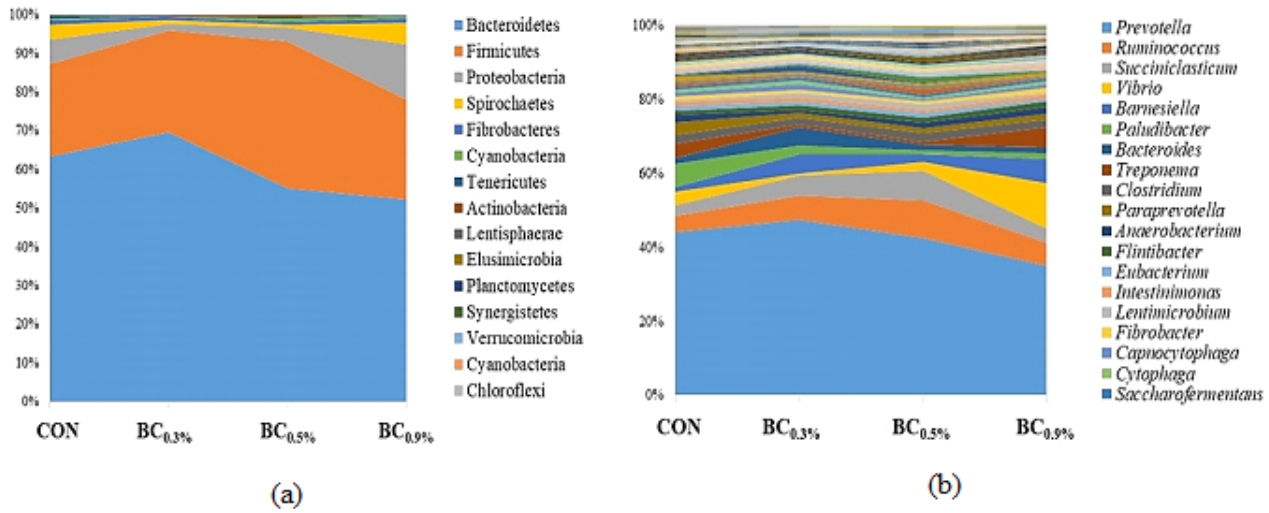
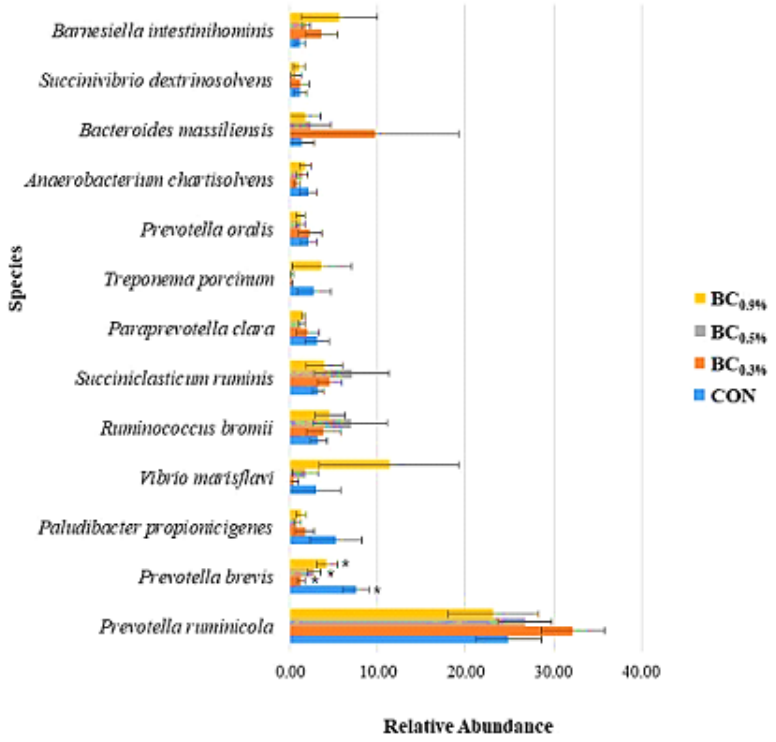


Figure 1

Boxplot representation of alpha diversity indices: (a) chao1, (b) Shannon, and (c) observed OTUs, between treatment groups. Alpha-diversity metrics visualization were done in MetaCOMET 17 and computed using QIIME 18. CON (no buffer added); BC0.3% (0.3% buffer); BC0.5% (0.5% buffer); BC0.9% (0.9% buffer).



(a) (b)



(c)

Figure 2

Relative abundance of the observed (a) phyla, (b) genera, and (c) species from the four different treatments. Relative abundance was computed using QIIME 18. CON (no buffer added); BC0.3% (0.3% buffer); BC0.5% (0.5% buffer); BC0.9% (0.9% buffer); asterisk (*): represents significant differences ($P < 0.05$).

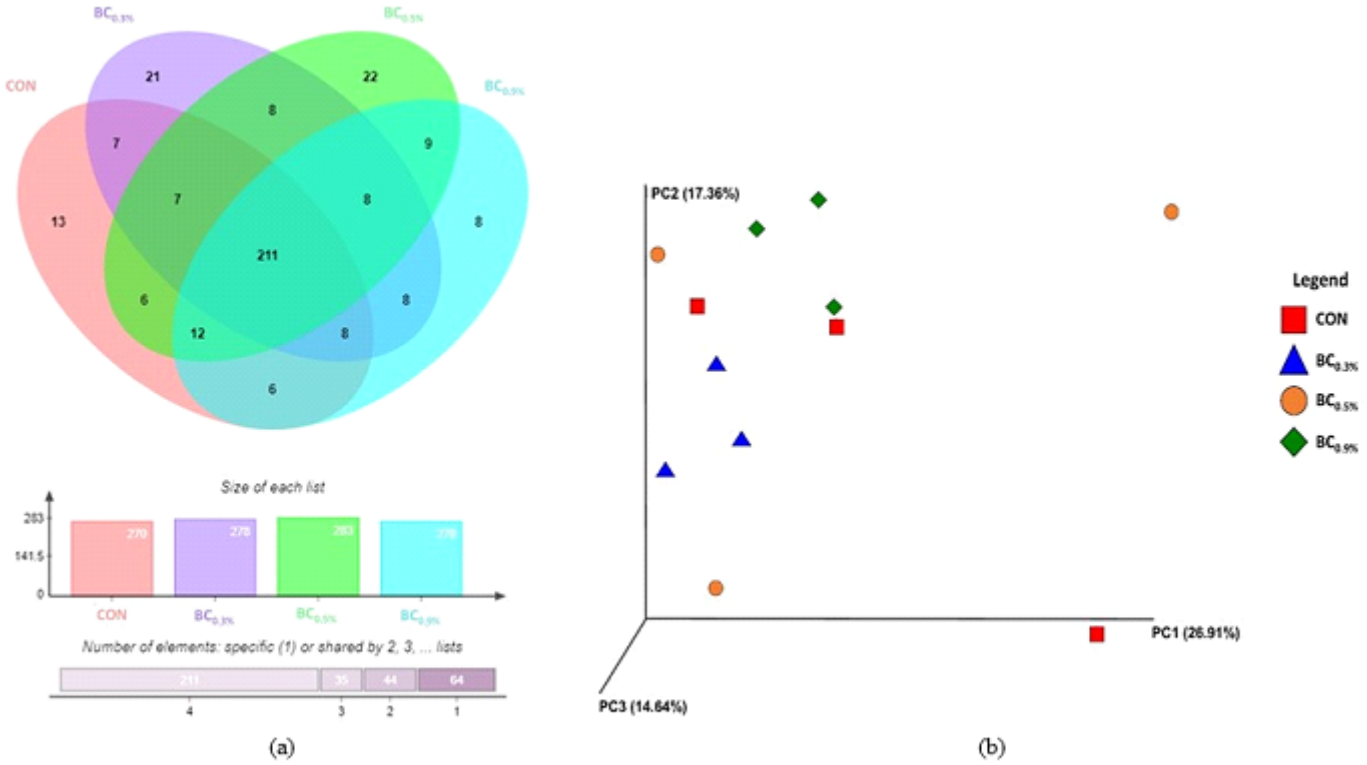


Figure 3

(a) Membership-based representation of unique, shared and core bacterial community of rumen microbiome after treatment supplementation with varying level of buffering capacity, and the total size of observed species per treatment. Venn diagram was generated in MetaCOMET 17 using jvenn 19. (b) Principal Coordinate Analysis (PCoA) of all samples using Bray-Curtis distance derived from the subset of identified OTUs. PCoA plot was generated using EMPeror 21. CON (no buffer added); BC0.3% (0.3% buffer); BC0.5% (0.5% buffer); BC0.9% (0.9% buffer).

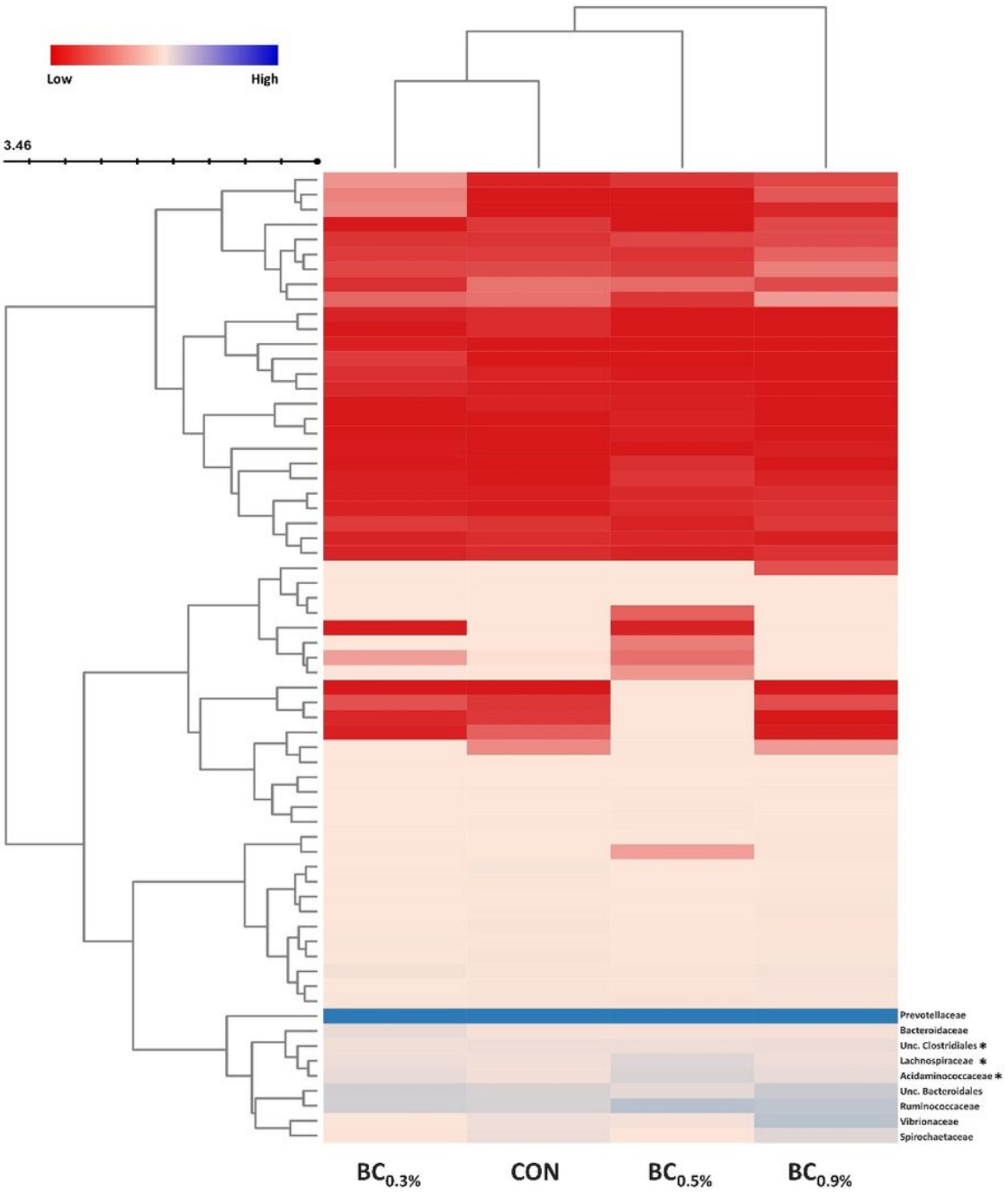


Figure 4

Heatmap presentation of relative abundance of representative families of observed OTU's. Treatments (columns) and families (rows) are clustered using Bray-Curtis dissimilarity test and Ward linkage. Normalized relative abundance are plotted from low (red), mid (peach), and high (blue). Heatmap clustering was generated in MetaCOMET 17 utilizing the lnCHlib application 20. CON (no buffer added); BC0.3% (0.3% buffer); BC0.5% (0.5% buffer); BC0.9% (0.9% buffer); asterisk (*): represents significant differences ($P < 0.05$).

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