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Original article

Evaluation of fresh rumen fluid challenge on rumen eco-system of buffalo calves under harsh environmental conditions

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ABSTRACT

Environmental factors as temperature and humidity are the most influential factor within any ecosystem with no exclusion. Ruminal ecosystem is greatly variated according to the physiological, nutritional and the microclimatic conditions. Temperature and humidity influences were evaluated against the efficiency of fresh rumen fluid (FRF) challenge in buffalo calves on some ruminal performance parameters and microbial community. Twenty male buffalo calves of 5 months old age were selected and divided into four groups, 5 calves for each. Buffalo calves G1; G2; and G3 were challenged with 1 L; 500 mL; and 300mL FRF; respectively. The 1stgroup challenged with 1 L FRF showed the highest improvement represented in a highly significant increases ($P < 0.01$) in the log Total bacterial count (log TBC); Log Lactobacillus count; Log Ruminococcus count; log Total protozoal count (log TPC); Rumen pH; protozoal motility and VFAs at 1st week of challenge. Ambient temperature revealed a significant intermediate positive (+0.634) correlations with log TPC and relative humidity revealed highly significant strong positive (+0.927) correlation with log Lactobacillus count. Significant intermediate positive (+0.698); a highly significant strong positive

(+0.711) correlations between log Ruminococcus count with lactate and log TPC; respectively. Ruminal pH showed a highly significant strong positive (+0.771) correlation with log TPC of sampled ruminal fluid from challenged animals.

1. Introduction

Ruminant animals are known to be least tolerant to the impact of heat stress because of the fact that ruminal fermentation produces excessive heat, which needs to be efficiently dissipated in order to maintain homeostasis (Kiyoshi et al., 2007). Heifers are more tolerant to impact of the heat stress than the mature cattle, because of lower metabolic heat production rate than that in cows. As well as, heifers have a greater body surface area allowing more efficient dissipation of the body heat (West, 2003). Rumen pH and short chain fatty acids as acetic, propionic, and butyric acid, and total fatty acids as well as, microbial population that underlie energy and protein supply in cows were affected by the great variation in temperature and relative humidity (Gianesella et al., 2012). Fresh ruminal fluid is considered to be the best available ruminotoric; as it contains a high population of microorganisms responsible for digestion of nutritive substances; these organisms as viable ruminal bacteria (10^9-10^{11}/mL), ruminal protozoa (10^5-10^6/mL) and anaerobic fungi (10^3-10^5 zoospores/mL) as well as many useful fermentation factors such as volatile fatty acids, microbial protein,minerals, vitamins and buffers (Carlos and Angela, 2011). The biological byproduct results from slaughtering of animals (cattle; buffaloes; sheep, and goat) in abattoirs are not recycled and usually workers get rid of it;as it constitute a great source of environmental pollution.The major objectives of this study was to run an epidemiological clinical trialto measure the influence of harsh environmental conditions (high ambient temperature, and high relative humidity) against the efficiency of fresh rumen fluid (FRF) challenge in buffalo calves onrumen physiological function, performance and microbial population.

2. Materials and methods

The present clinical trials were conducted on male buffalo calves in research animal farm, Collage of Veterinary Medicine, Suez Canal University, Ismailia, Egypt.

2.1. Fresh rumen fluid inoculum

Rumen fluid was collected from Abo-Kalifa Abattoir, Ismailia, Egypt into thermos insulated drink container, pre-warmed 39 $^{\circ}$C, flushed with $CO_2$and sealed before being transported to the laboratory.

2.2. Animals and treatments

A total number of 20 male buffalo calves of 5 months old age with average live weight of 110kg were selected and divided into four groups, 5 calves for each. Calves of 1st group (G1) were challenged with 1000 mL fresh rumen fluid (FRF); 2nd group (G2) were challenged with 500 mL FRF; and the 3rd group (G3) were challenged with 300 mL FRF and the fourth group (G4) act as a control healthy group. Calves were challenged with FRF for 7 consecutive days during which they were all kept under the same environmental conditions in open half shelter system.

Buffalo calves were fed at the entire duration of the clinical trial on a concentrate and roughage diet mixture. Concentrate diets consisted of 10% dried beet pulp; 30% ground yellow corn; 15% sunflower seed meal; 15% DDGS (Distiller's dried grains with soluble); 26.5% wheat bran; 1.5% ground lime stone; 0.5% vitamin premix; 0.9% sodium chloride and 0.6% mineral premix; and these concentrate diets were provided to the calves at a rate of 2.5 - 3kg per day per calf divided into three feeding times daily. Roughage were provided to the animals in the form of wheat straw at a rate of 2 kg per calf and tap water was provided ad libitum. Regular recording of the environmental temperature and relative humidity using Clock &Hygro-Thermometer, (Boeco Germany, model: BOE 325); Indoor/Outdoor-MIN/MAX Thermometer, (Boeco Germany, model: BOE 325) from the initial point of the experiment (Zero time), then on a daily basis during challenge periods (7 consecutive days), and at sampling times.

2.3. Sampling and measurements

2.3.1. Samples

A total number of 80 ruminal fluid samples were obtained from all animals (four samples per each calf) during the experiment at zero time before challenge, 1 week, 2 week and 3 week post challenge. Ruminal fluid samples (250mL) were collected from each calf using stomach tube with a conical flask connected to its free end, while a vacuum pump was connected to the side tube of the flask. Samples were transferred in thermos insulated drink container, pre-warmed at 39°Cand flushed with CO_2 within half hour from collection to the research laboratory.

2.3.2. Ruminal fluid analysis

Each sample was divided into three portions. 50mL was used for estimation of ruminal fluid pH and evaluation of protozoal activity; rumen fluid pH was measured immediately after collection of samples using a calibrated electronic digital portable pH meter; Hanna instruments, Italy (Bramley et al., 2008). The activity and population density of the protozoa was judged by placing one drop of rumen fluid on a gently warmed glass slide; covered with a cover slip and examined under 4× lens magnification, the motility was described using the following scale: highly motile and abundant (+ + +), motile and moderate number (+ +), sluggish and low number (+), no or sporadic alive protozoa (±) and dead protozoa (-) (Fouda, 1995).

100 mL was strained through 4 layers of cheesecloth, then centrifuged for 15 minutes at 4000rpm, clear supernatant fluid was poured off into a clean plastic tube and was kept frozen at -20°C until used for the biochemical analysis of lactate, ammonia and glucose levels that were estimated calorimetrically using reagent test kits supplied by Ben company – Italy and Diamond company - Egypt, respectively (Young, 2001). About 2 mL aliquot of collected ruminal fluid was placed into a glass bottle contain 1mL of sulfuric acid (0.1M) and kept frozen at -20°C for subsequent analysis of volatile fatty acids (VFAs) by gas chromatography(Abo-Donia et al., 2011). The last 100 mL was kept to conduct the microscopical count of ruminal protozoa and microbiological culture of rumen bacteria.

2.3.3. Rumen protozoal count in rumen content

1.0 mL of ruminal fluid was strained and diluted 5 times with saline lugol's iodine mixture solution (15mL saline solution and 5mLlugol's solution) to fix and stain the protozoal cells. The mixture was shaken thoroughly and exactly 0.1mL was poured on a dry clean slide and spread under a cover slip of 1100 mm² area (22 × 50mm). Counting was carried out using a low power lens. Thirty fields were counted in each slide. The field area of the lens was 1mm², and the average protozoal counts in 30 fields represents 0.2 mL of the original sample, therefore, to get the protozoal count per mL of ruminal contents, the obtained value was multiplied by 50, thus the total protozoal count (TPC)/mL of ruminal fluid was calculated according to the following equation (Wang et al., 2009): TPC = (Total protozoal counts in 30 fields / 30) × 1100 × 50.

2.4. Bacteriological examination of ruminal fluid

2.4.1. Preparation of samples

All ruminal fluid samples were prepared according to (APHA, 2001).Tenfold decimal serial dilution up to 10^{-8} was prepared to cover the expected range of colonial growth using tubes containing 9mL sterile anaerobic dilution solution (ADS). The composition of ADS (Chandrasekharaiah et al., 2004); was as follows: (I) Mineral solution I- 0.3% dipotassium hydrogen phosphate, 15.0mL; (II) Mineral solution II- 0.3% potassium hydrogen phosphate + 0.6% ammonium sulfate + 0.6% sodium chloride + 0.6% magnesium sulfate + 0.06% calcium chloride, 15.0 mL; cysteine hydrochloride, 0.05%; sodium carbonate, 0.3%; resazurin, 0.001%; and distilled water, to make up to 100mL. The ADS was sterilized by autoclaving at 121 °C and 1.5 pressure for 20 minutes, after cooling; the diluent was displaced into tubes; each tube containing 9mL to be ready for serial dilution. The tubes were flushed with carbon dioxide to enhance the anaerobic conditions.

2.4.2. Total Anaerobic Bacterial Count (TABC)

Total Anaerobic micro-organisms count in ruminal fluid was carried out using Pour Plate Method (Cruickshank et al., 1980) with Standard Plate Count Agar (SPCA) that were treated with anti-fungal growth agents; Cyclo-heximide 0.5mL /100mL agar and Amphotricin-B 1.5mL/100mL agar (Jens Christian Pedersen, 1992; Mahdy et al.,

2010). Inoculated plates were placed in anaerobic jar using Carbon dioxide gas producing kits andincubated at 37°C for 72h. Counting the colonies and calculations were carried out according to Zelver et al. (1999); Herigstad et al. (2001).

2.4.3. Total Ruminococcus count

Total Ruminococcus species count in ruminal fluid was conducted using Pour Plate Method (Cruickshank et al., 1980) with Rumen Glucose Cellobiose Agar (RGCA). RGCA Medium used for the isolation of the cellulolytic bacteria was as follows: clarified rumen fluid, 20.0%; glucose, 0.0248g; cellobiose, 0.0248g; ammonium sulfate, 0.1g; agar, 2.0g /100mL; mineral solutions I and II, 15% each; haemin, 50mg; vitamin K1, 0.1mg; L-cysteine hydrochloride, 0.5mg; resazurin, 0.1mg/100 mL; distilled water, to make up to 100mL (Chandrasekharaiah et al., 2004). Inoculated plates were placed in anaerobic jar using Carbon dioxide gas producing kits and incubated at 37°C for 72 h. Counting the colonies and calculations were carried out according to Zelver et al. (1999); Herigstad et al. (2001).

2.4.4. Total Lactobacillus count

Total lactobacillus species count in ruminal fluid samples was counted by Pour Plate Method (Cruickshank et al., 1980) using lactobacillus selective medium (MRS) agar (Pfaller et al., 1999; Anwar, 2014). Inoculated plates were placed in anaerobic jar using Carbon dioxide gas producing kits and incubated at 37°C for 72 h. Counting the colonies and calculations were carried out according to Zelver et al. (1999); Herigstad et al. (2001).

2.5. Statistical analysis

The obtained data were analyzed statistically using factorial experiments of Analysis of Variance (ANOVA) and the results are considered significant at probability level of 0.05 ($P \leq 0.05$). Statistical analysis was run through SPSS (version, 20) for windows Levesque, (2007). The mean values, standard error (SE) were calculated by using Microsoft Excel program. For protozoal and bacterial count a logarithmic transformation were done before analysis. The correlation co-efficient was calculated to compare the influence of each measured parameter mean values on each other Fulekar, (2009).

3. Results and discussion

Biochemical analysis of ruminal fluid revealed that lactate and glucose levels showed highly significant differences ($P<0.01$) between G3; G2; and G1; consequently, ammonia and VFAs levels showed highly significant differences ($P<0.01$) in G2 with a non-significant difference between G1 and G3; Table 1. Ruminal fluid pH declared a significant decline ($P\leq0.05$) in G3, G2 and G1 compared to control (G4); non-significant difference between G1 and G2 in pH; Table 2. Examination of Table 3; revealed high significant differences ($P<0.01$) in log Lactobacillus count (G1; G3; and G2); log Ruminococcus count (G3; G2 and G1); log TBC (G2; G3 and G1), and log TPC (G2; G3 and G1) compared to control (G4). Biochemical analysis of ruminal fluid of the 1st group revealed a highly significant ($P<0.01$) increase of lactate, glucose and VFAs at 1st week, followed by a continuous decline until the end of 3rd week except lactate that showed a non-significant decline on 2nd and 3rd weeks. Ammonia levels showed a highly significant decline ($P<0.01$) at 1st week, then increased gradually at the 2nd and 3rd weeks; Table 1.

The 1st group showed a highly significant increases ($P<0.01$) in the log Total bacterial count (TBC) at 1st week followed by a highly significant decrease and increase ($P<0.01$) at 2nd and 3rd weeks, respectively; Log Lactobacillus count revealed a highly significant gradual increase ($P<0.01$) until the end of 2nd week then a highly significant decline ($P<0.01$) was noticed at 3rd week; Log Ruminococcus count showed a highly significant wave of increase; decrease and increase ($P<0.01$) at 1st; 2nd; and 3rd week, while log Total protozoal count (TPC) revealed a highly significant increase ($P<0.01$) at 1st week followed by continuous highly significant decline ($P<0.01$) at 2nd and 3rd weeks; Table 3. Biochemical analysis of rumen fluid of the 2nd group revealed a highly significant decrease ($P<0.01$) in ammonia level at 1st and 2nd weeks then increased at 3rd week; a highly significant decline ($P<0.01$) in glucose level at 1st week then gradual increase at the 2nd and 3rd weeks; a highly significant ($P<0.01$) increase of VFAs at 1st week followed by continuous decline until the end of 3rd week, while lactate level showed a non-significant change at 1st week, then highly significant increase at 2nd week followed by nonsignificant decline at 3rd week; Table 1.

The 2nd group showed a non-significant change in the log TBC at 1st week, followed by a highly significant gradual decline (P<0.01) at 2nd and 3rd weeks; Log Lactobacillus count revealed a highly significant gradual increase (P<0.01) until the end of 2nd week then a highly significant decline (P<0.01) was noticed at 3rd week; Log Ruminococcus count revealed a non-significant change at 1st week, followed by a highly significant gradual increase (P<0.01) until the end of 3rd weeks, while log TPC revealed a highly significant increase (P<0.01) at 1st week followed by highly significant decrease and increase (P<0.01) at 2nd and 3rd weeks; respectively, Table 3. Biochemical analysis of rumen fluid of the 3rd group revealed a highly significant (P<0.01) increase of lactate and VFAs at 1st week followed by gradual decline until the end of 3rd week; a highly significant decline (P<0.01) in glucose level until the end of 2nd week followed by a highly significant increase (P<0.01) at the 3rd week, while ammonia level showed wavy pattern of highly significant increase; decreases; and increase (P<0.01) in order between the sampling times; Table 1.

The 3rd group showed a non-significant change in the log TBC at 1st week, followed by a highly significant increase and decrease (P<0.01) at 2nd and 3rd weeks respectively; Log Lactobacillus count showed cyclic highly significant (P<0.01) decrease; increase and decrease at 1st, 2nd and 3rd week respectively; Log Ruminococcus count revealed a highly significant increase (P<0.01)at 1st week, followed by a highly significant gradual decrease until the end of 3rd weeks, while log TPC revealed a highly significant increase (P<0.01) at 1st week followed by highly significant decrease at 2nd and non-significant increase at 3rd weeks; Table 3. All groups revealed a significant improvement (P≤0.05) in ruminal pH at 1st and 3rd weeks only interrupted with a significant decline (P≤0.05) in the other sampling time. On the other hand; protozoal motility revealed a significant improvement (P≤0.05) that was clear in 1st week in all challenged groups followed by instant decline in activity; Table 2.

Table 1
Biochemical analysis of ruminal fluid sampled from challenged and control buffalo calves.

Treatment		Analyte			
		Lactate (mg/dL)	Ammonia (umol/L)	Glucose (mg/dL)	VFAs
Groups	Group (1)	$1.42^{c} \pm 0.12$	$1027.5^{c} \pm 109.8$	$4.25^{d} \pm 0.54$	$5.93^{b} \pm 0.08$
	Group (2)	$1.83^{b} \pm 0.45$	$1289.9^{b} \pm 23.8$	$6.14^{c} \pm 0.88$	$5.45^{c} \pm 0.8$
	Group (3)	$2.24^{a} \pm 0.38$	$1021.6^{c} \pm 153.1$	$7.22^{b} \pm 1.05$	$5.87^{b} \pm 0.07$
	Group (4)	$1.35^{c} \pm 0.18$	$1444.2^{a} \pm 15.9$	$8.73^{a} \pm 0.16$	$6.21^{a} \pm 0.07$
Group * Time					
Group (1)	Zero time	$0.81^{fgh} \pm 0.06$	$1031.06^{h} \pm 12.08$	$3.6^{f} \pm 0.29$	$6.2^{c} \pm 0.07$
	1st week	$1.62^{de} \pm 0.10$	$550.73^{k} \pm 2.91$	$7.26^{c} \pm 0.03$	$7.3^{a} \pm 0.05$
	2nd week	$1.86^{d} \pm 0.03$	$955.46^{i} \pm 2.96$	$3.26^{fg} \pm 0.09$	$5.2^{def} \pm 0.09$
	3rd week	$1.37^{def} \pm 0.10$	$1572.83^{a} \pm 3.71$	$2.86^{g} \pm 0.18$	$5.1^{def} \pm 0.12$
Group (2)	Zero time	$0.23^{h} \pm 0.02$	$1288.2^{e} \pm 3.05$	$6.63^{d} \pm 0.09$	$5.4^{de} \pm 0.07$
	1st week	$0.46^{gh} \pm 0.01$	$1261.03^{f} \pm 3.33$	$2.73^{g} \pm 0.12$	$6.4^{bc} \pm 0.07$
	2nd week	$3.39^{b} \pm 0.09$	$1196.7^{g} \pm 2.0$	$4.63^{e} \pm 0.14$	$5^{ef} \pm 0.05$
	3rd week	$3.21^{bc} \pm 0.06$	$1413.9^{c} \pm 3.51$	$10.56^{a} \pm 0.12$	$4.9^{f} \pm 0.07$
Group (3)	Zero time	$0.66^{gh} \pm 0.08$	$896.96^{j} \pm 5.67$	$10.33^{a} \pm 0.37$	$4.3^{g} \pm 0.14$
	1st week	$4.02^{a} \pm 0.10$	$1348.46^{d} \pm 4.33$	$6.3^{d} \pm 0.12$	$7.5^{a} \pm 0.05$
	2nd week	$2.69^{c} \pm 0.09$	$254.2^{l} \pm 2.89$	$1.93^{h} \pm 0.15$	$6.6^{bc} \pm 0.07$
	3rd week	$1.57^{de} \pm 0.16$	$1586.73^{a} \pm 3.93$	$10.3^{a} \pm 0.12$	$5.1^{def} \pm 0.07$
Group (4)	Zero time	$1.41^{def} \pm 0.37$	$1420.5^{c} \pm 2.89$	$8.8^{b} \pm 0.3$	$5.5^{d} \pm 0.13$
	1st week	$1.35^{def} \pm 0.41$	$1417.93^{c} \pm 6.96$	$8.36^{b} \pm 0.09$	$6.4^{bc} \pm 0.08$
	2nd week	$1.59^{de} \pm 0.56$	$1404.6^{c} \pm 9.17$	$8.8^{b} \pm 0.57$	$6.7^{b} \pm 0.19$
	3rd week	$1.04^{efg} \pm 0.07$	$1533.63^{b} \pm 5.93$	$8.96^{b} \pm 0.17$	$6.3^{bc} \pm 0.32$

Means carrying different superscripts in the same column are significantly different at (P ≤ 0.05) or highly significantly different at P < 0.01). Means carrying the same superscripts in the same column are non-significantly different at (P >0.05).

Table 2
pH and protozoal activity of ruminal fluid sampled from challenged and control buffalo calves.

Treatment		Parameters	
		Ruminal fluid pH	Protozoal activity
Groups	Group (1)	$6.65^{c} \pm 0.11$	++
	Group (2)	$6.67^{c} \pm 0.10$	++
	Group (3)	$6.79^{b} \pm 0.12$	+
	Group (4)	$7.01^{a} \pm 0.10$	++
Group * Time			
Group (1)	Zero time	$6.30^{f} \pm 0.05$	±
	1st week	$7.20^{ab} \pm 0.06$	+++
	2nd week	$6.47^{ef} \pm 0.09$	++
	3rd week	$6.63^{d} \pm 0.03$	++
Group (2)	Zero time	$6.50^{ef} \pm 0.06$	±
	1st week	$7.13^{b} \pm 0.03$	++
	2nd week	$6.30^{f} \pm 0.05$	++
	3rd week	$6.73^{cd} \pm 0.03$	+
Group (3)	Zero time	$6.63^{de} \pm 0.09$	±
	1st week	$7.37^{a} \pm 0.08$	++
	2nd week	$6.30^{f} \pm 0.05$	++
	3rd week	$6.87^{c} \pm 0.03$	+
Group (4)	Zero time	$6.93^{c} \pm 0.13$	++
	1st week	$7.30^{ab} \pm 0.05$	+++
	2nd week	$6.63^{de} \pm 0.09$	++
	3rd week	$7.17^{ab} \pm 0.03$	++

Means carrying different superscripts in the same column are significantly different at ($P \leq 0.05$) or highly significantly different at ($P < 0.01$). Means carrying the same superscripts in the same column are non-significantly different at ($P > 0.05$).

Table 3
Log protozoal and bacterial count of ruminal fluid sampled from challenged and control buffalo calves.

Treatment		Log count			
		Log Lactobacillus count /ml	Log Ruminococcus count /ml	Log TBC /ml	Log TPC /ml
Groups	**Group (1)**	$5.6816^{b} \pm 0.51$	$5.8031^{d} \pm 0.37$	$6.5979^{d} \pm 0.578$	$5.0980^{c} \pm 0.39$
	Group (2)	$5.4834^{d} \pm 0.29$	$6.0006^{c} \pm 0.34$	$7.2847^{a} \pm 0.460$	$5.4262^{b} \pm 0.19$
	Group (3)	$5.6502^{c} \pm 0.46$	$6.1074^{b} \pm 0.33$	$7.1497^{b} \pm 0.686$	$5.4174^{b} \pm 0.17$
	Group (4)	$5.8155^{a} \pm 0.39$	$6.1453^{a} \pm 0.10$	$6.9497^{c} \pm 0.219$	$6.0677^{a} \pm 0.10$
Group * Time					
Group (1)	**Zero time**	$4.4419^{k} \pm 0.02$	$5.2299^{l} \pm 0.02$	$6.5601^{b} \pm 0.01$	$4.1313^{f} \pm 0.01$
	1st week	$5.6197^{f} \pm 0.01$	$6.7922^{b} \pm 0.01$	$7.8172^{c} \pm 0.01$	$5.9647^{b} \pm 0.01$
	2nd week	$6.8303^{b} \pm 0.00$	$5.3977^{k} \pm 0.01$	$5.1454^{k} \pm 0.02$	$5.2122^{cd} \pm 0.02$
	3rd week	$5.8346^{e} \pm 0.02$	$5.7924^{i} \pm 0.004$	$6.8691^{e} \pm 0.01$	$5.0840^{e} \pm 0.01$
Group (2)	**Zero time**	$5.2548^{h} \pm 0.01$	$5.5797^{j} \pm 0.01$	$8.0184^{b} \pm 0.04$	$5.08870^{e} \pm 0.03$
	1st week	$5.5681^{fg} \pm 0.01$	$5.5678^{j} \pm 0.03$	$8.0294^{b} \pm 0.02$	$5.6451^{c} \pm 0.01$
	2nd week	$6.2129^{d} \pm 0.01$	$5.9224^{h} \pm 0.01$	$6.8129^{ef} \pm 0.04$	$5.1414^{de} \pm 0.02$
	3rd week	$4.8976^{j} \pm 0.01$	$6.9327^{a} \pm 0.01$	$6.2784^{i} \pm 0.01$	$5.8296^{b} \pm 0.04$

Group (3)	**Zero time**	$5.5184^{g} \pm 0.01$	$5.5678^{j} \pm 0.01$	$6.8957^{e} \pm 0.01$	$5.2560^{de} \pm 0.01$
	1st week	$5.2197^{h} \pm 0.03$	$6.7269^{c} \pm 0.01$	$6.8367^{ef} \pm 0.01$	$5.8925^{b} \pm 0.01$
	2nd week	$6.9428^{a} \pm 0.003$	$6.5714^{d} \pm 0.02$	$8.9912^{a} \pm 0.02$	$5.2005^{de} \pm 0.02$
	3rd week	$4.9201^{j} \pm 0.02$	$5.5636^{j} \pm 0.02$	$5.8750^{j} \pm 0.03$	$5.3208^{d} \pm 0.01$
Group (4)	**Zero time**	$5.2206^{h} \pm 0.02$	$6.4034^{e} \pm 0.01$	$6.7757^{f} \pm 0.002$	$6.2437^{a} \pm 0.01$
	1st week	$6.4760^{c} \pm 0.02$	$6.1454^{f} \pm 0.02$	$7.5681^{d} \pm 0.007$	$6.2170^{a} \pm 0.03$
	2nd week	$6.4666^{c} \pm 0.02$	$6.0170^{j} \pm 0.004$	$6.6857^{g} \pm 0.06$	$5.8843^{b} \pm 0.02$
	3rd week	$5.0989^{i} \pm 0.04$	$6.0156^{j} \pm 0.003$	$6.7694^{f} \pm 0.05$	$5.9257^{b} \pm 0.02$

Means carrying different superscripts in the same column are significantly different at ($P \leq 0.05$) or highly significantly different at ($P < 0.01$). Means carrying the same superscripts in the same column are non-significantly different at ($P > 0.05$).

Recorded ambient temperature revealed a significant intermediate positive (+0.634) correlations with log TPC. On the other hand; relative humidity revealed highly significant strong positive (+0.927) correlation with log Lactobacillus count; Table 4. Log Ruminococcus count revealed a significant intermediate positive (+0.698); a highly significant strong positive (+0.711) correlations with lactate and log TPC; respectively. Ruminal pH showed a highly significant strong positive (+0.771) correlation with log TPC of sampled ruminal fluid from challenged animals; Table 4.

Table 4

Log bacterial and protozoal counts correlations with recorded temperature and relative humidity (Above diagonal), Log bacterial and protozoal counts correlations with Biochemical analysis of ruminal fluid (Below Diagonal).

r	Log LC	Log RC	Log TBC	Log TPC	Temp	RH%			
Log LC	**1**	0.078^{NS}	0.197^{NS}	0.104^{NS}	0.108^{NS}	0.729^{**}			
Log RC	0.078^{NS}	**1**	0.305^{NS}	0.711^{**}	-0.388^{NS}	0.233^{NS}			
Log TBC	0.197^{NS}	0.305^{NS}	**1**	0.126^{NS}	-0.315^{NS}	0.240^{NS}			
Log TPC	0.104^{NS}	0.711**	0.126^{NS}	**1**	0.634^{*}	0.258^{NS}			
Lactate	0.220^{NS}	0.698*	-0.158^{NS}	0.407^{NS}	**1**	0.396^{NS}			
Ammonia	-0.530^{NS}	-0.252^{NS}	-0.505^{NS}	0.019^{NS}	-0.008^{NS}	**1**			
Glucose	-0.538^{NS}	0.222^{NS}	-0.313^{NS}	0.370^{NS}	0.049^{NS}	0.286^{NS}	**1**		
VFAs	-0.005^{NS}	0.460^{NS}	0.467^{NS}	0.299^{NS}	0.233^{NS}	-0.381^{NS}	-0.356^{NS}	**1**	
pH	-0.306^{NS}	0.416^{NS}	0.052^{NS}	0.771**	0.126^{NS}	0.219^{NS}	0.297^{NS}	0.535^{NS}	**1**

**. Correlation is significant ($P < 0.01$). *. Correlation is significant ($P < 0.05$). ^{NS}. Correlation is non-significant ($P > 0.05$).

The shifting in bacterial population in fresh rumen fluid samples may have been due to poor access of bacteria to nutrients as a result of higher motility and rate of passage of digesta throughout the gut (Romero-Pérez et al., 2011). The competitive exclusion have established that microorganisms with similar physiological needs should not coexist in ecosystems where the environment and flow of nutrients fluctuate greatly (Kudva et al., 1998).

The activity of microbial populations in the rumen can be affected by environmental factors, such as antimicrobial compounds in plants, e.g., tannins (Molan et al., 2001; smith et al., 2003), and temperature, e.g., cold conditions (von Keyserlingk and Mathison, 1993). Temperature has long been considered the most influential factor within any ecosystem (Bhakooand Herbert, 1979), the rumen microbial ecosystem is not an exception. However, when ruminants are subject to cold temperatures, the rumen environment remains thermally stable (Goel et al., 2005). Thermoregulation of the animal maintains the temperature of the internal organ systems, extremities (feet, tail, ears, etc.) are usually affected by ambient temperature first, and only in extreme situations are the organ systems affected (Fuller et al., 2005). In our study; the influence of temperature was reflected on microbial population by strong significant correlation with ruminal protozoa, and the humidity influenced by strong highly significant correlation with bacterial population (Ruminococcus count). The other correlations predominate a nonsignificant weak positive or negative.

The improvement of ruminal fluid pH; protozoal motility and protozoal count of all animals within the 1st week post-challenge; followed by retraction by the end of 2nd week; this may be attributed to the increase in log lactobacillus count at the same time. The improvement of log TPC in the 1st group was attributed to the

synchronized increase in log TBC; Which in-turn increase the protozoal predation of bacteria as they digest and utilize the engulfed bacteria to satisfy their needs for amino acids (Belzecki et al., 2013). This improvement in both parameters was lost by the end of the 2nd week of the challenge; and this may be attributed to the increase of log lactobacillus count, which in-turn caused a reduction of ruminal pH that was determinaned to both anaerobic ruminal protozoa and bacteria.

Increased ruminal ammonia concentration of all animals at the 3rd week of challenge may be attributed to excessive degradation of feed protein and lysis and breakdown of microbial protein (recycling) within the rumen (Karnati, 2006). Meanwhile, the increased SCFA's concentration in ruminal fluid among buffalo calves challenged with 1 liter FRF in the 1st week was attributed to fermentation of carbohydrates by rumen microbial community by the corresponding enzymes (Wang et al., 2012) and this synchronized with increase of log TBC in 1st week of the 1st group.

4. Conclusion

The study revealed a good and promising response as well as transient improvement of the rumen functional status after challenging buffalo calves with fresh rumen fluid. 1L of FRF showed the best results, but the improvement was lost after only one week of challenge; suggesting that the FRF challenge is recommended to last for a duration longer than that used in the experiment as well as using FRF of good quality.

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