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Evaluation of Suitability of Buffer to Induce Acidosis on Rice (*Oriza sativa*) based Diet in RUSITEC

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ABSTRACT

Background: Rice feeding followed in Tamil Nadu for dairy cattle induces acidosis and the effect of acidosis studies through *in vitro* techniques is desirable to reduce the animal experiment. Since Mc Dougall Buffer neutralizes acidity produced on incubation with rice (*Oriza sativa*) in semi continuous culture, it is difficult to mimic acidosis in RUSITEC. Hence a study was conducted in RUSITEC with cooked rice incubated for 24 hours to evaluate the effect of diluting Mc Dougall buffer to establish rumen acidosis (pH < 5.8).

Methods: Two treatments were followed with undiluted Mc Dougall Buffer (MDB) and diluting Mc Dougall Buffer (MMDB) as per Mickdam *et al.* (2016) in RUSITEC experiments with six replicates for each treatment. This experiment was conducted with equivalent weight of test material (Cooked rice on DMB). The pH of artificial buffer was maintained at 7.4 during start of incubation for both buffers.

Result: The rumen fermentation pattern for establishment of onset of acidosis due to over feeding of cooked rice was evinced by the onset of acidosis (<5.8 pH) recorded at 3 hours of incubation in MMDB infusion and at 18 hours of incubation in MDB infusion. The concentration of NH_3-N declined after acidosis induction; however, it reached its minimum in MMDB at significant difference ($p<0.05$) than MDB at all incubation hours. Total gas production (Methane and Carbon dioxide) was reduced with MMDB incubation. The CH_4 proportion declined strongly ($p<0.01$) at all incubation hours in MMDB than MDB. The production of volatile fatty acid was reduced significantly ($p<0.05$) in MMDB at all incubation period compared with MDB. This was based on a significant decrease in the acetate production ($p<0.05$), an increase in the propionate and butyrate production ($p<0.05$). Acidosis challenge resulted in significantly ($p<0.01$) elevated lactate levels in MMDB at all incubation hours which remained high at 24 hours of incubation. Compared to MDB condition, the LPS concentration was largely elevated ($p<0.01$), by 36% on average till 12 hours of incubation, due to severe acidosis condition (pH 4.9) created by MMDB. The early occurrence of acidosis condition in MMDB, increased significantly ($p<0.01$) gram positive organisms in the rumen. The MMDB infusion showed a strong effect on degradation of DM, OM and N and it was decreased, similarly the degradation rate of DM and N was lowest. This study proves that the MMDB is the suitable acidosis inducing buffer for establishing the *in vitro* models to cooked rice.

Key words: Acidosis, Buffer, Rice, RUSITEC.

INTRODUCTION

Dairy farmers of southern India, especially Tamil Nadu, have been feeding their dairy cattle with cheap and abundantly available feed resources, which includes cooked rice. Alam *et al.* (2014) reported that in Bangladesh, most of the cases of ruminal acidosis resulted from accidental intake of large amounts of cooked rice or rice gruel in addition to feeding dairy animals with potato/bread/jackfruit residue or other easily digestible carbohydrates.

Consumption of excessive amounts of fermentable carbohydrates resulted in increased fermentation and volatile fatty acids production, resulting in moderately acidic ruminal fluid pH with values ranging from 5 to 5.5 (Nagaraja and Titgemeyer, 2007). The sub-acute ruminal acidosis was designated with rumen pH of 5.2 to 9 by Thorat *et al.* (2021). It is a well-established fact that feeding cereal grains or cereal flours or boiled cereal flour in excess to cattle leads to acidosis, ruminal bloat, loose dung, laminitis and reproductive problems (Gao and Oba, 2014 and Aschalew *et al.,* 2020). Earlier study on survey of cooked rice feeding and its consequence on ruminal acidosis indicated that the quantum of cooked rice influences the severity of ruminal acidosis (Murugeswari *et al.*, (2018).

The establishment of rumen acidosis by feeding cooked rice needs to be studied and find out the effect on animal productivity. The study requires the ruminal cannulated animals and also the repeated acidosis induction enhances the severity of acidosis and might therefore affect results of

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repeated experiments as per Dohme *et al.,* (2008). Therefore, the establishment of *in vitro* models is desirable to reduce the number of animal experiments and provide more standardized test conditions. Since Mc Dougall Buffer neutralizes acidity produced on incubation with rice (*Oriza sativa*) in semi continuous culture, it is difficult to mimic acidosis in RUSITEC. A recent study investigated changes of rumen bacteria in sub-acute ruminal acidosis conditions (pH<5.8) in the RUSITEC by reducing the concentrations of the mentioned salts and increasing the dietary concentrate proportion (Mickdam *et al.*, 2016). In this study, the effect of cooked rice feeding on rumen acidosis and its fermentation pattern needs to be conducted. Therefore, establishment of rumen acidosis through *in vitro* models is to be established. Thence, this study investigated changes of rumen pH in acidosis conditions (pH<5.8) on a cooked rice-based feeding regimen in the RUSITEC studies by reducing the concentrations of the $NaHCO_3$ and Na_2HPO_4 salts in the artificial saliva (Mickdam *et al.,* 2016) and its efficacy compared to McDougall's buffer (1948).

MATERIALS AND METHODS

Rice samples (500 g of each) were collected from dairy farmers adopting cooked rice-based feeding regimen for this trial. Rice samples were cooked with water in the ratio of 1:10, in boiling water bath to a gel like consistency which was similar to cooked rice fed by farmers (Bhattacharjee *et al.,* (2020).

Experimental design

The experiment was conducted with two different buffers MDB (McDougall buffer, 1948) and MMDB (Modified McDougall buffer as per Mickdam *et al.,* 2016), whose composition of $NaHCO_3$ (MDB - 116.50 mmol/l Vs. MMDB - 50 mmol/l) $Na_2HPO_4.2H_2O$ (MDB -26.30 mmol/l Vs. MMDB - 10 mmol/l), Nacl (MDB and MMDB - 8.04 mmol/l), Kcl (MDB and MMDB - 7.64 mmol/l), $CaCl_2.2H_2O$ (MDB and MMDB - 0.37 mmol/l) and $MgCl_2.6H_2O$ (MDB and MMDB - 0.63 mmol/l). Whose composition is presented in Table 1. Two treatments were followed with undiluted Mc Dougall Buffer (MDB) and diluting Mc Dougall Buffer (MMDB) as per Mickdam *et al.* (2016) in RUSITEC experiments with six replicates for each treatment. Sample size of cooked rice was eight grams on as such basis to equalize the requirement of RUSITEC analysis in both treatments.

RUSITEC experiment

For the *in vitro* induction of acidosis, six fermentation vessels of a RUSITEC system (Czerkawski and Breckenridge, 1977) were inoculated with rumen liquor was collected in slaughterhouse from six cows immediately prior to slaughter and brought to the laboratory by maintaining the temperature of rumen liquor at 39°C under anaerobic condition during the transit. Ruminal fluid was filtered through four layers of muslin cloth and stored in pre warmed thermos container at 39°C till its use. To begin the experiment the fermenters (800 ml capacity) were filled with 600 ml of strained rumen liquor and 100 ml of artificial saliva. Eighty grams of solid digesta (solid inoculum) and eight gram of test feed (cooked rice on DM basis) were taken in nylon bags and placed in feed container. The pore size of nylon bag was 100 microns. Artificial saliva was infused continuously into the fermenters at a flow rate of 326±19.2 ml/day. The solid inoculum bag was replaced by a feed bag after 24 hours. During the change of bags, the fermenters were flushed with carbon dioxide to maintain anaerobic condition. After removal, nylon bags were squeezed with pre-warmed buffer solution and the obtained fluid was given back into the fermentation vessel to ensure transfer of solid-phase-associated micro-organisms. This experiment was also conducted in two buffers (MDB Vs MMDB). The pH of artificial buffer was maintained at 7.4 during start of incubation for both buffers. Seven days of adaptation was followed by collection period.

Measurement of fermentation parameters

Starting at day one and in the end of collection period of 7 days at 3, 6, 9, 12, 18 and 24 hours, the pH was measured immediately before feeding, fermentation fluid was sampled directly from each vessel through a three-way valve using a syringe and it was immediately determined for pH using a pH-meter (Mickdam *et al.,* 2016). Effluent samples were collected during collection period and stored at -20°C for subsequent analysis of NH_3-N concentrations, SCFA production and lactate concentrations.

The ammonia nitrogen concentration was determined as per method described by Souza *et al.* (2013). Daily VFA production was calculated by multiplying volumes of effluents with concentrations determined by gas chromatography (Koch *et al.,* 2006). Lactic acid concentration of effluent was determined as per the procedure of Lee *et al.* (2011).

Gas volume was measured by water replacement method at the specified incubation hours. Gas samples for assessing the composition of the fermentation gas were stored in vacutainer tubes (Venosafe type VF-109 SP, Terumo, Eschborn, Germany). The total gas was partitioned as carbon dioxide and methane the percentages of CH_4 and CO_2 in the fermentation gas were analysed as described by Meibaum *et al.* (2012).

The fluid samples were frozen at -20°C for determination of Lipopolysacchrides (LPS) concentration. LPS was determined with a chromogenic kinetic Limulus Amebocyte Lysate (LAL) assay kit as per (Gozho *et al.,* (2006). Gram-positive organisms were identified and a count of them made from the ruminal effluent collected. The effluent samples were smeared on a glass slide followed by gram staining (Bartholomew and Mittwer, 1952). Bacterial count was made by direct microscopic count (Newbold *et al.,* 1998).

Loss in weight of nylon bag after 3, 6, 9, 12, 18 and 24 hours of incubation in RUSITEC after washing and drying was recorded to calculate dry matter disappearance. The rumen degradation kinetics of dry matter was calculated using the non linear model proposed by Mc Donald, (1981). Effective degradability was calculated using equation

described by Orskov and Mc Donald (1979). Samples of feed and the collected substrate residues were analysed for OM and nitrogen according to AOAC (2012).

Statistical analysis

Data were analysed with analysis of variance (ANOVA) and linear regression analysis using IBM® SPSS® Statistics version 20.0 for Windows® software as per the Snedecor and Cochran (1989). The critical difference between the groups was analysed by Duncan's multiple range test. Data are presented as means±SE.

RESULTS AND DISCUSSION

Rumen fermentation pattern for establishment of onset of acidosis due to over feeding of cooked rice in RUSITEC

The development of the pH at different incubation hours under MDB and MMDB is presented in Fig 1. The fall in pH with MMDB was steep and significantly ($p<0.01$) lower than MDB in all the incubation tested. The rumen fermentation pattern for establishment of onset of acidosis due to over feeding of cooked rice was evinced by the onset of acidosis (<5.8 pH) recorded at 3 hours of incubation in MMDB infusion and at 18 hours of incubation in MDB infusion. The reduction in the buffer capacity was more pronounced in MMDB infusion and therefore resulted in a lower pH (Mickdam *et al.*, 2016).

The concentration of NH_3-N at different incubation hours under MDB and MMDB is presented in Fig 2. The concentration of NH_3-N declined after acidosis induction; however, it reached its minimum in MMDB (0.68±0.001 mM) at significant difference ($p<0.05$) than MDB (2.14 mM±0.01) at all incubation hours Orton *et al.* (2020) Chiquette *et al.* (2012). The decline of ammonia nitrogen with time is because of its utilization for microbial protein production. A higher pH sensitivity of proteolytic bacteria might explain the longer duration of depressed NH_3-N concentrations in the present study.

The volume of total gas (ml) and methane (ml) at different incubation hours under MDB and MMDB due to over feeding of cooked rice are presented in Fig 3. The volume of fermentation gas 1615±79.48 ml in MDB and 1450±59.35 ml in MMDB at 24 hours of incubation was recorded. The gas production was decreased significantly ($p<0.01$) in MMDB. The CH_4 proportion declined strongly ($p<0.01$) at all incubation hours in (335±12.75 ml) MMDB than (303±10.55 ml) MDB. RUSITEC studies confirm that gas production, particularly methane production, is reduced in acidotic conditions (Mickdam *et al.,* 2016).

The cooked rice fermentation pattern in RUSITEC with MDB or MMDB infusion at 3, 6, 9, 12, 18 and 24 hours of incubation fermentation pattern for establishment of onset of acidosis due to over feeding of cooked rice in RUSITEC is presented in Table 1. The production of volatile fatty acid was reduced significantly ($p<0.05$) in MMDB (79.8±3.7 mM) than MDB (98.32±3.7 mM) at 24 hours of incubation.

Table 1: Cooked rice* fermentation pattern in RUSITEC with MDB or MMDB infusion at 3, 6, 9, 12, 18 and 24 hours of incubation (Mean**±SE).

Parameters	MDB						MMDB						p-value
	Incubation period (hours)												
	3	6	9	12	18	24	3	6	9	12	18	24	
VFA total (mmol/lit)	100.45±5.1^{a}	99.28±5.7^{a}	97.31±4.5^{a}	99.17±4.7^{a}	98.49±3.9^{a}	98.32±3.7^{a}	105.2±8.9^{b}	105.7±7.6^{b}	99.4±6.2^{b}	97.6±6.8^{b}	86.2±7.1^{b}	79.8±3.7^{b}	<0.05
Acetate (mmol/lit)	55.33±2.3^{b}	54.65±3.2^{a}	53.63±3.5^{a}	54.14±3.2^{a}	53.05±2.9^{a}	53.59±2.9^{b}	50.56±4.2^{b}	49.89±4.9^{b}	47.62±4.8^{b}	47.29± 4.5^{b}	40.53±3.6^{b}	37.27±3.3^{b}	<0.05
Propionate (mmol/lit)	42.68±1.9^{a}	42.22±1.9^{a}	40.22±1.7^{a}	41.81±2.1^{a}	42.79±2.0^{a}	42.18±2.2^{a}	51.14±1.9^{b}	52.48±1.4^{b}	49.41±1.8^{b}	48.63±1.1^{b}	42.03±0.9^{b}	39.34±0.9^{b}	<0.05
Butyrate (mmol/lit)	3.69±0.05^{a}	2.71±0.05^{a}	2.65±0.06^{a}	2.56±0.06^{a}	2.59±0.06^{a}	2.78±0.06^{a}	4.68±0.06^{b}	4.53±0.06^{b}	3.14±0.05^{b}	3.31±0.06^{b}	3.17±0.05^{b}	3.18±0.05^{b}	<0.05
Lactic acid (mmol/lit)	0.22±0.04^{a}	1.06±0.1^{a}	1.95±0.2^{a}	2.68±0.3^{a}	3.37±0.4^{a}	6.01±0.4^{a}	1.78±0.1^{b}	2.18±0.2^{b}	3.63±0.3^{b}	6.24±0.7^{b}	14.45±0.5^{b}	15.52±0.6^{b}	<0.01
Lipopolysacchrides ($\times 10^3$ EU/ml)	16.37±0.8^{a}	22.98±1.02^{a}	29.81±1.9^{a}	33.64±1.8^{a}	38.14±2.0^{a}	41.02±2.92^{a}	28.42±1.52^{b}	35.76±2.06^{b}	38.78±1.75^{b}	42.34±2.18^{b}	44.62±2.87^{b}	48.51±2.41^{b}	<0.01
G+ count (No. $\times 10^9$/ml)	0.07±0.01^{a}	0.35±0.01^{a}	0.92±0.1^{a}	1.45±0.1^{a}	1.98±0.1^{a}	2.31±0.1^{a}	1.04±0.1^{b}	1.89±0.1^{b}	2.63±0.1^{b}	3.56±0.1^{b}	4.87±0.1^{b}	5.42±0.1^{b}	<0.01

*On dry matter basis; **Mean of six sample. Numbers with different superscripts differ significantly within one row at different incubation hours.

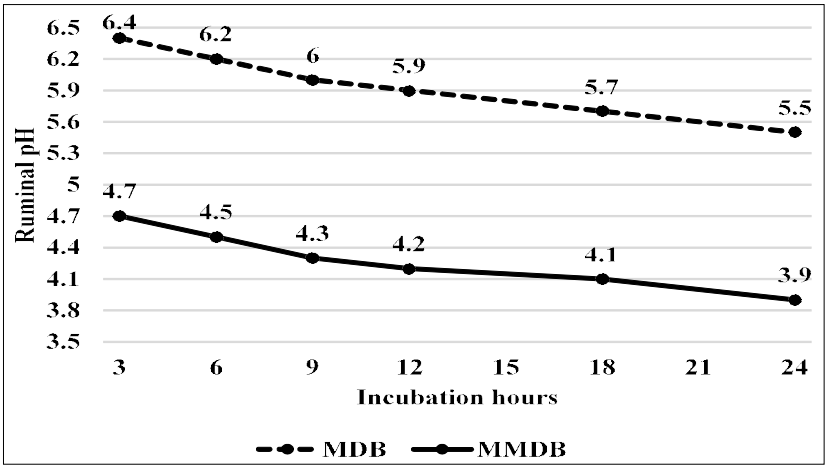


Fig 1: Development of the pH at different incubation hours under MDB and MMDB due to over feeding of cooked rice (P<0.01).

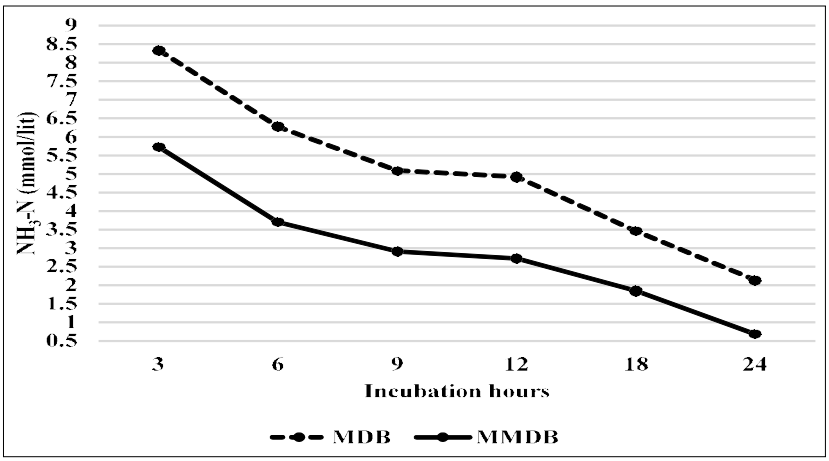


Fig 2: Concentration of NH_3-N (mmol/lit) at different incubation hours under MDB and MMDB due to over feeding of cooked rice (P<0.05).

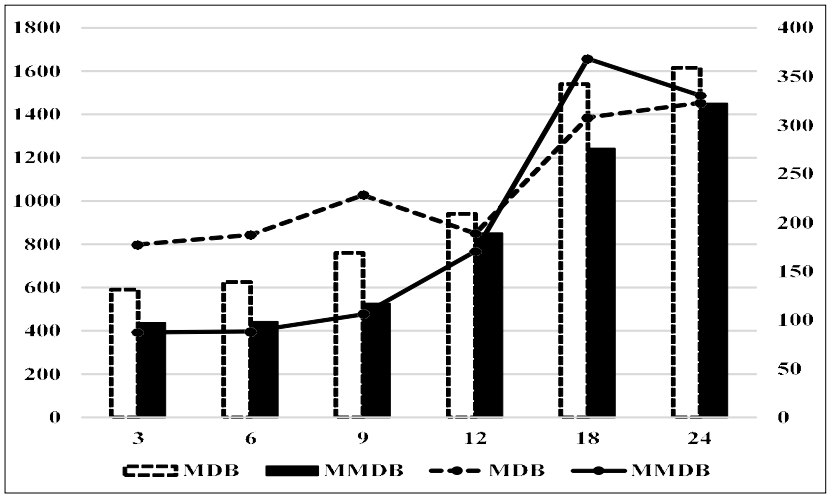


Fig 3: Volume of total gas (ml) and methane (ml) at different incubation hours under MDB and MMDB due to over feeding of cooked rice (P<0.01).

This was based on a significant decrease in the acetate production (53.59±2.9 mM - MDB Vs 37.27±3.3 mM - MMDB; P<0.05), an increase in the propionate and butyrate production (42.18±2.2 mM - MDB Vs 39.34±0.9 mM - MMDB and 2.78±0.06 mM - MDB Vs. 3.18±0.05 mM - MMDB; p<0.05) in MMDB at 24 hours incubation (Eger *et al*., 2016) probably due to the cooked rice alone fed. Acidosis challenge resulted in significantly (p<0.01) elevated lactate levels in MMDB at all incubation hours which remained high at 24 hours of incubation. This might be based on a proliferation of *Lactobacilli* and inhibition of fibrolytic and lactate-utilizing bacteria during prolonged time spans of low pH (pH<5.0) which was documented in MMDB infusion (Newbold and Wallace, 1988). Compared to MDB condition, the LPS concentration was largely elevated (p<0.01), by 36% on average till 12 hours of incubation, due to severe acidosis condition (pH 4.9) created by MMDB. The hypothesis that the free rumen ruminal lipopolysacchrides concentration increases following grain engorgement (Monteiro and Faciola, 2020) (Emmanuel *et al*., 2008), especially during sub-acute ruminal acidosis Jiang *et al*. (2022), (Gozho *et al*., 2007), has been clearly proven in this study wherein when cooked rice alone was fed in *RUSITEC,* the ruminal lipopolysaccharides (LPS) concentration was found to increase with increase in incubation hours up to 24 hours of incubation in both MDB and MMDB infusion. The increase in ruminal lipopolysaccharides (LPS) concentration was significantly (p<0.01) higher in MMDB (48.51±2.41×10^3 EU/ml) than MDB (41.02±2.92×10^3 EU/ml) infusion at 24 hours incubation. The gram-positive count was increased according to the acidotic condition develops with incubation period in both MDB and MMDB infusion and it was significantly (p<0.01) higher in MMDB (5.42±0.1×10^9/ml) infusion than MDB (2.31±0.1×10^9/ml) infusion at 24 hours incubation. The increased gram-positive organisms in the rumen because other species are very sensitive and require a specific pH and the proper substrate for their role and activity (Monteiro and Faciola, 2020) (Akbar and Kumari, 2005).

Nutrient degradability for establishment of onset of acidosis due to feeding of cooked rice in RUSITEC

The nutrient degradability under MDB and MMDB infusion in cooked rice (g) at 3, 6, 9, 12, 18 and 24 hours of incubation nutrient degradability for establishment of onset of acidosis due to over feeding of cooked rice with RUSITEC is presented in Table 2. The DM (68.5±3.7% - MDB Vs 60.5± 3.3% - MMDB), OM (65.7±3.2% - MDB Vs 58.4±3.1% - MMDB) and N (44.3±2.3% - MDB Vs 38.7±2.3% - MMDB) degradation were inhibited by MMDB, showing a reduction of 5-6% in DM and OM degradation and 4-6% in N degradation from that of MDB condition (p<0.01) at 24 hours incubation. The overall lowered fermentation is mirrored by a reduced digestibility of OM which may be mainly attributed to decreased fibre degradation (Mickdam *et al.,* 2016) in acidotic condition.

Degradability characteristics of nutrients for establishment of onset of acidosis due to feeding of cooked rice in RUSITEC

The degradability characteristics of nutrients under MDB and MMDB infusion in cooked rice (g) at 3, 6, 9, 12, 18 and 24 hours of incubation is presented in Table 3. The degradability

Table 2: Nutrient degradability under MDB and MMDB infusion in cooked rice* (g) at 3, 6, 9, 12, 18 and 24 hours of incubation (Mean**±SE).

Treatments	MDB	MMDB	MDB	MMDB	MDB	MMDB	p value
parameter	DM degradability (%)		OM degradability (%)		N degradability (%)		
Incubation hours 3	32.2±1.8^b	26.7±1.4^a	30.4±1.4^b	24.8±1.3^a	16.2±1.2^b	12.5±0.9^a	<0.01
6	39.6±2.1^b	34.5±1.9^a	37.3±1.8^b	32.7±1.4^a	24.6±1.1^b	19.4±1.1^a	<0.01
9	45.8±2.7^b	41.1±2.6^a	43.6±2.5^b	39.6±1.6^a	31.5±1.8^b	24.8±1.8^a	<0.01
12	51.7±2.5^b	46.5±2.3^a	48.6±2.4^b	43.7±2.2^a	34.7±1.9^b	29.1±1.9^a	<0.01
18	59.5±3.3^b	54.8±2.8^a	56.4±3.7^b	51.6±2.6^a	41.9±2.2^b	35.0±2.2^a	<0.01
24	68.5±3.7^b	60.5±3.3^a	65.7±3.2^b	58.4±3.1^a	44.3±2.3^b	38.7±2.3^a	<0.01

*On dry matter basis; **Mean of six sample. Numbers with different superscripts differ significantly within one row at different incubation hours.

Table 3: Degradability characteristics of nutrients under MDB and MMDB infusion in cooked rice* (g) at 3, 6, 9, 12, 18 and 24 hours of incubation (Mean**±SE).

Treatments	MDB	MMDB	MDB	MMDB	p-value
degradability characteristics	DM		N		
Nutrient degradation rate/ hour	0.07±0.01^b	0.06±0.01^a	0.10±0.01^b	0.08±0.01^a	<0.01
Soluble degradable fractions (%)	19.3±0.8^b	17.6±0.7^a	5.4±0.2^b	3.7±0.2^a	<0.01
Insoluble degradable fractions (%)	59.3±2.9^b	55.9±3.1^a	44.3±2.2^b	40.9±2.1^a	<0.01
Undegradable fractions (%)	29.4±1.6^b	26.5±1.8^a	51.3±2.5^b	55.47±2.6^a	<0.01
Effective degradability of nutrients (%)	52.5±2.8^b	47.8±2.2^a	33.2±1.9^b	28.1±1.7^a	<0.01

*On dry matter basis; **Mean of six sample. Numbers with different superscripts differ significantly within one row at different incubation hours.

characteristic of nutrients for establishment of onset of acidosis due to over feeding of cooked rice in RUSITEC is presented in Table 3. The degradation rate of DM and N was significantly ($p<0.01$) highest in MDB (0.07 and 0.10) than MMDB (0.06 and 0.08). The degradability characteristic of nutrients evinced the effective degradability of DM and N was significantly ($p<0.01$) lowest in MMDB (47.8% and 28.1%) than in MDB (52.5% and 33.2%). Cumulative effect of better pH and synchronized release of nitrogen to degradable organic matter led to conducive environment for microbial proliferation which in turn lead to above mentioned desirable effect on degradability in MDB infusion due to the buffering capacity of the saliva.

CONCLUSION

The acidosis was developed in RUSITEC by using cooked rice at 3rd hour of incubation with diluted buffer of McDougall infusion when compared to normal McDougall buffer infusion at 18 hours of incubation. It indicates that the rumen acidosis could be established using the RUSITEC system when the diluted McDougal buffer infused as artificial saliva. Therefore, the RUSITEC provides stable conditions for testing cooked rice feeding, influencing rumen acidosis with dilution of McDougal buffer.

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