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In vitro Ruminal Acid Load and Methane Emission Responses to Supplemented Lactating Dairy Cow Diets with Inorganic Compounds Varying in Buffering Capacities

Research Article

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ABSTRACT

Using 24 hours *in vitro* cultures of rumen microorganisms, this study investigates the effect of buffering capacity of 2 inorganic compounds (M1=119.43 and M2=116.50 meq/L) on the *in vitro* rumen acidogenic value (AV), medium pH, dry matter disappearance (INVDMD) and methane emission of lactating dairy cow diets containing various forage to concentrate ratios as 40:60 ($FC_{40:60}$) and 30:70 ($FC_{30:70}$) in a completely randomized design. Inorganic compounds were included in the experimental diets at the rate of 0.0, 10 or 20 g/kg DM. Diet with higher amount of concentrate caused a decline in medium pH, an increase in both AV and IVDMD. The acidogenic value of $FC_{40:60}$ containing M2 at 20, M1 at 10 and 20 g/kg DM and FC 30:70 plus M1 and M2 at 20 g/kg DM was the lowest. The lowest level of CH_4 emission (mL/0.20 g DM) was observed in $FC_{30:70}$ plus M1 at the rate of 10 g/kg DM, while the highest level belongs to $FC_{40:60}$ plus M1 at 10 g/kg DM and $FC_{30:70}$ containing M1 at 20 and M2 at 10 g/kg DM. It has been concluded that the higher buffering capacity of a lactating diet might reduce the rumen acid load and increased IVDMD, while a diet with higher amount of concentrate causes to decline rumen methane emission.

KEY WORDS acidogenic value, buffering capacity, dairy cows, methane, rumen.

INTRODUCTION

The problem primarily met in dairy cow feeding is to provide an energetically high-density ration without jeopardizing ruminal ecosystem, animal welfare and production performances (Zebeli *et al.* 2008). Enhancing energy supply through increased use of concentrates or rapidly fermentable fiber can swallow the rumen into acidosis. Subacute ruminal acidosis (SARA) is a common and economically important problem in well managed dairy herds. Failure to maintain a consistent rumen pH in high yielding dairy cows may result in metabolic disorders and reduced production performance (Tajik and Nazifi, 2011).

The rumen pH will fall when organic acids that are produced during fermentation by rumen microbes accumulate and rumen buffering is not sufficient to prevent the increase in acidity (Plaizier *et al.* 2008). Chemical buffers in diets for ruminants to provide rumen pH in a range that is optimal for the activity of cellulose-degrading organisms (pH=6-7) are used. The need for buffering agents in dairy cattle diets depends on the secretion of salivary buffers, the buffering capacity of feed and feed acidogenic value. Wadhwa *et al.* (2001) have extended a simple laboratory based technique for evaluating ruminal acid load from feedstuffs based on the dissolution of Ca from $CaCO_3$. The acidogenic value (AV) of the feedstuffs different with the non-

fiber carbohydrate (NFC) content, protein and fiber concentrations. The highest AV was for starch rich feeds, forages were intermediate and protein sources had the lowest AV. Rustomo *et al.* (2006) reported that fiber sources had intermediate AV and protein sources had the lowest AV, wheat straw and alfalfa hay, as a fiber sources in ration, had lower AV than alfalfa pellet or corn silage. Additionally, the AV of feed ingredients were positively correlated to changes in rumen fluid pH after incubation, suggesting that high AV feeds were expected to increase the risk of rumen acidosis in dairy cows than low AV feeds.

Ruminal methanogenesis represents an alternative mechanism of reducing equivalent disposal for carbohydrate-fermenting bacteria, but interspecies hydrogen transfer is only exergonic at very low partial pressures of hydrogen (Wolin, 1975).

If the methanogens are inhibited, hydrogen accumulates, the hydrogenases are inhibited and the carbohydrate-fermenting bacteria utilize other mechanisms of reducing equivalent disposal (e.g. the dehydrogenases of propionate production) (Gottschalk, 1986).

Sauvant and Giger-Reverdin (2007) realize the relationship between methane production and proportion of concentrate in the diet to be curvilinear, with methane losses of 6-7% of gross energy (GE) being constant at 30-40% concentrate levels in the diet and then decreasing to 2-3% of GE with a concentrate proportion of 80-90%. The objective of the present experiment was to determine the effect of buffering capacity (BC) of various mixed inorganic compounds on *in vitro* rumen acidogenic value and methane emission from diets containing various forage to concentrate ratios.

MATERIALS AND METHODS

Diets, chemical composition and inorganic buffering compounds

The experimental diets were designed to provide two different forage to concentrate ratios as 40:60 ($FC_{40:60}$) and 30:70 ($FC_{30:70}$), respectively. Ration ingredients and nutrient compositions are presented in Table 1. Inorganic mixtures were made of different compositions of $NaHCO_3$, Na_2CO_3, $CaCO_3$, $MgCO_3$, MgO and bentonite Na as 500, 200, 90, 100, 100 and 10 g/kg (M1) or 300, 200, 200, 150, 100 and 50 g/kg (M2), respectively. This inorganic mixtures had the highest buffering capacity and obtained from Acros brand. A modification of the procedure of Evans and Ali (1967) was used to measure the buffering capacity. Approximately, 1 g DM sample of individual buffering compound or the mixtures of M1 and M2 was suspended in 100 mL distilled water and stirred continuously with a magnetic stir bar.

Titrations were performed by addition of HCl (83.3 mL/L) or NaOH (40 g/L) (Merck brand) in variable increments until pH was decreased to 4 or increased to 9. Buffering capacity and initial pH of the individual and the compositions used in the present experimental diets are shown in Tables 2 and 3, respectively.

Table 1 Ingredient and chemical compositions of the experimental diets

Diet (forage:concentrate)	$FC_{40:60}$	$FC_{30:70}$
Ingredient (% of DM)		
Alfalfa hay	19.72	13.89
Corn silage	19.72	13.89
Barley grain	18.33	21.11
Corn grain	16.67	20.00
Wheat bran	9.44	12.22
Soybean meal	12.22	13.89
Canola meal	3.33	2.78
Wheat straw	0.56	2.22
Chemical composition (mg/g of DM) of rations		
Crude protein	177.60	170.80
Neutral detergent fiber (NDF)	371.00	338.10
Acid detergent fiber (ADF)	208.40	171.40
Ash	52.90	49.50

Each composition (M1 or M2) were added to the diets of $FC_{40:60}$ and $FC_{30:70}$ at the rate of 0.0, 10 and 20 g/kg DM. Ingredients used in the diets were ground through a mill with a 1-mm sieve, then dried using air-forced oven (48 h, 65 ˚C). Nitrogen content of each ingredient was determined using Kjeldahl method (Kjeltec 2300 Autoanalyzer Foss Tecator AB, Hoganas, Sweden) and CP was calculated as N × 6.25.

Neutral detergent fiber (NDF) and acid detergent fiber (ADF) were determined according to Van Soest *et al.* (1991). Samples were also analyzed for ash by igniting the samples in muffle furnace at 525 ˚C for 8 h.

In vitro acidogenic value

In vitro technique used in this experiment adapted from Wadhwa *et al.* (2001). Appropriately, one-gram (DM) of each diet was placed into a 125-mL incubation bottle, then M1 or M2 was added as the experimental protocol, then bottles were held at 39 ˚C in a water-bath. The samples were incubated in a 3 run and quadruplicate with 30 mL of buffered rumen liquor comprising 60% buffer and 40% rumen liquor.

The buffer (5.880 g/L $NaHCO_3$; 5.580 g/L Na_2HPO_4; 0.282 g/L NaCl; 0.342 g/L KCl; 0.028 g/L $CaCl_2.2H_2O$ and 0.036 g/L $MgCl_2$) (Merck brand) was made up at 20% the strength of the Tilley and Terry (1963). Rumen fluid was collected from two rumen fistulated dairy cows fed corn silage, alfalfa hay and concentrates 25, 25 and 50%, DM, respectively; at 3 h after the morning feeding. Cysteine hydrochloride monohydrate (Merck brand) (0.025% wt/vol) was added just prior to the incubations.

Table 2 Buffering capacities and initial pH of the individual inorganic buffering inorganic compounds

Parameters	Buffering inorganic components					
	$NaHCO_3$	Na_2CO_3	$CaCO_3$	$MgCO_3$	MgO	Bentonite Na
Initial pH	8.31	11.18	8.97	10.45	10.56	9.58
Buffering capacity (meq/L)	114.93	171.13	152.56	171.86	184.00	1.96

The bottles were closed with gas release valves and shaken continuously. A set of bottles without feed sample was also incubated similarly which served as blank. After 24 of the incubation, the bottles were transferred to an ice bath to stop fermentation, and then opened to measure medium pH using a pH meter (Metrohm pH meter, model 691). Bottle contents were filtered and a 2 mL sample of the supernatant from each bottle was taken to analyze residual acidity (acidogenic value).

Table 3 Buffering capacities and initial pH of different composition of inorganic compounds

Parameters	Inorganic compounds*	
	M1	M2
Initial pH	9.67	9.33
Buffering capacity (meq/L)	119.43	116.50

* M1 and M2 were made of from $NaHCO_3$, Na_2CO_3, $CaCO_3$, $MgCO_3$, MgO and bentonite Na as 500, 200, 90, 100, 100 and 10 g/kg or 300, 200, 200, 150, 100 and 50 g/kg, respectively.

The filtrated residual was oven dried (75 ˚C for 48 h), weighted and used to calculate *in vitro* dry matter disappearance (IVDMD). The supernatant of each bottle was transferred into a 2 mL centrifuge tube containing excess amount of $CaCO_3$ powder (Merck brand) (50 mg). The mixture was shaken manually for 5 s and then centrifuged at 4000 × g for 10 min.

The supernatant Ca concentration was then immediately determined using an autoanalyzer (A15 Biosystem). A measurement of dissolution of Ca from insoluble $CaCO_3$ powder makes it possible to assess residual acidity after fermentation of feeds.

In vitro rumen methane emission

An *in vitro* incubations were carried out as proposed by Menke and Steingass (1988). Appropriately, 200 mg (DM basis) of each experimental diet was placed into a 125 mL incubation bottle, then inorganic mixtures of M1 or M2 was introduced as rate of 0.0, 10 or 20 g/kg DM. A set of bottles without feed sample was also incubated similarly which served as blank. The bottles were incubated with 30 mL of buffered rumen fluid (artificial saliva to the rumen liquid in ratio of 2:1) and held at 39 ˚C in a water-bath.

The artificial saliva was made up of 475 mL/L distilled water, 240 mL/L buffer solution (ammonium bicarbonate 4 g/L and sodium bicarbonate 35 g/L, Merck brand), 240 mL/L macromineral solution (5.7 g anhydrous Na_2HPO_4, 6.2 g anhydrous KH_2PO_4 and 0.6 g $MgSO_4.7H_2O$ per liter, Merck brand), 0.12 mL/L micro-mineral solution (13.2 g $CaCl_2.2H_2O$, 10.0 g $MnCl_2.4H_2O$, 1 g $CoCl_2.6H_2O$ and 8.0 g $FeCl_3.6H_2O$ per 100 mL, Merck brand), 1.22 mL/L Resazurin aqueous (Merck brand) (1 mg/1 mL). The medium was then reduced by addition of reducing agent (47.5 mL distilled water, 2 mL 1 N NaOH and 336 mg $Na_2S.9H_2O$, Merck brand) per liter of medium. Rumen fluid was collected as described previously.

The incubation was carried out in two set (run) and in triplicate. After 24 of incubation, the bottles were transferred to an ice bath to stop fermentation, then total gas was recorded by digital pressure indicator (model SEDPGB0015PG5) and methane emission was determined using a biological gas recorder (SR2-BIO).

Calculations and statistical analysis

The buffering capacity (BC, meq/L) was calculated by the following formula (Evans and Ali, 1967):

BC= [(milliliters of 1 N HCl) + (milliliters of 1 N NaOH)] × 10^3/30

In vitro acidogenic value (mg Ca/g DM) of each sample was calculated as the product of Ca concentration (mg/mL, from the analysis) and fluid volume (30 mL) divided by the sample weight (1 g). *In vitro* dry matter disappearance was calculated as follows (Jahani Azizabadi *et al*. 2011):

IVDMD (%)= [(A-(B-C)) / A] × 100

Where:
A: dry weight of sample.
B: dry weight of residue after incubation.
C: dry weight of blank.

Data were analyzed as a completely randomized design to compare the diets and buffering composition in each experimental diet with replications using Dunnett's test ($P<0.05$) procedure in SAS (2002).

RESULTS AND DISCUSSION

The effect of inorganic mixtures supplementation on the *in vitro* medium pH, AV and IVDMD after 24 hours incubation within the experimental diets containing 40:60 or 30:70 forage to concentrate ratios are shown in Table 4 and Table 5, respectively.

Table 4 *In vitro* ruminal medium pH, acidogenic value [AV, (mg Ca/g DM)] and dry matter disappearance (IVDMD, %) of a dairy cow diet containing different forage: concentrate ratio as 40:60 which supplemented with different composition of inorganic buffering compounds (M1 and M2), after 24 h incubation

Ration	Inorganic chemical compounds applied		Parameters		
	Inorganic compounds[1]	Concentration (g/kg DM ration)	pH	AV	IVDMD
$FC_{40:60}$[2]	-	0.0	5.50	6.60	55.50
$FC_{40:60}$	M1	10	5.59*	6.43	57.50
$FC_{40:60}$	M1	20	5.60*	6.93	64.50
$FC_{40:60}$	M2	10	5.54	7.59	54.25
$FC_{40:60}$	M2	20	5.63*	7.09	54.00
SEM	-	-	0.022	1.034	5.205
P-value	-	-	0.016	0.254	0.185

[1] M1 and M2 were made of from $NaHCO_3$, Na_2CO_3, $CaCO_3$, $MgCO_3$, MgO and bentonite Na as 500, 200, 90, 100, 100 and 10 g/kg or 300, 200, 200, 150, 100 and 50 g/kg, respectively.
[2] $FC_{40:60}$: a dairy cow diet containing 40% forage and 60% concentrate.
SEM: standard error of the means.
The means within the same column with at least one common letter, do not have significant difference (P>0.05).

Table 5 *In vitro* ruminal medium pH, acidogenic value [AV, (mg Ca/g DM)] and dry matter disappearance (IVDMD, %) of a dairy cow diet containing different forage: concentrate ratio as 30:70 which supplemented with different composition of inorganic buffering compounds (M1 and M2), after 24 h incubation

Ration	Inorganic chemical compounds applied		Parameter		
	inorganic compound[1]	Concentration (g/kg DM ration)	pH	AV	IVDMD
$FC_{30:70}$[2]	-	0.0	5.44	8.25	67.25
$FC_{30:70}$	M1	10	5.49	10.23	68.00
$FC_{30:70}$	M1	20	5.54*	7.09	69.00
$FC_{30:70}$	M2	10	5.52	8.58	66.00
$FC_{30:70}$	M2	20	5.54*	10.72	67.00
SEM	-	-	0.038	0.809	5.580
P-value	-	-	0.050	0.100	0.797

[1] M1 and M2 were made of from $NaHCO_3$, Na_2CO_3, $CaCO_3$, $MgCO_3$, MgO and bentonite Na as 500, 200, 90, 100, 100 and 10 g/kg or 300, 200, 200, 150, 100 and 50 g/kg, respectively.
[2] $FC_{30:70}$: a dairy cow diet containing 30% forage and 70% concentrate.
SEM: standard error of the means.
The means within the same column with at least one common letter, do not have significant difference (P>0.05).

Increased concentrate from 60 to 70%, due to increasing the level of rapidly fermentable carbohydrates caused a decrease in medium pH and a significant (P< 0.05) increase in AV. By increasing the amount of acid produced as a result of fermentation of carbohydrates, pH levels on the medium was reduced. High concentrate diets contain high amounts of non structural carbohydrates which are quickly fermented by ruminal microbes, resulting in a greater decline in ruminal pH (Kalscheur *et al.* 1997).

Results of previous studies have shown a decline in ruminal pH when more rapidly fermentable carbohydrates were included in the diet (Krause *et al.* 2002b). Danesh Mesgaran *et al.* (2009) reported that there is a positive correlation between non fibrous carbohydrates in diet and acidogenic value, so that by increasing the non fibrous carbohydrates in diet, the AV enhanced. As described by Rustomo *et al.* (2006), energy feeds and fiber sources have the highest and intermediate AV, respectively.

Results indicated that the adding of M1 at both rate and M2 at 20 mg/kg to $FC_{40:60}$ caused a significant (P<0.05) increase in medium pH. Inorganic buffers are capable in preventing pH reduction in the medium through neutralizing acids produced by bacterial activities.

The highest significant (P<0.05) level of medium pH was belonged to $FC_{30:70}$ plus M1 and M2 which were added at the rate of 20 g/kg DM. Inorganic buffers enhance ruminal environmental conditions by modulating acidity of the ruminal contents, preventing severe drops in pH (Le Ruyet and Tucker, 1992).

Tripathi *et al.* (2004) have also reported that $NaHCO_3$ supplementation caused a linear enhancement in ruminal fluid pH. In experiment of West *et al.* (1987), the addition of various buffers to the diet also resulted in a significant increase in rumen pH. Santra *et al.* (2003) reported that dietary buffers prevent the reduction of rumen pH when animal fed a high levels of concentrate. With regard to AV (Tables 4 and 5), none of the M1 and M2 supplementation had significant (P>0.05) effect compared with the non-supplemented diet, which might express to this cause that the inorganic mixtures could not significantly affected the buffering capacity and ultimately the acid load created in the medium.

Dietary buffers are widely used to improve the harmful effects of acidity in high concentrate diets (Coppock *et al.* 1986), but the response of buffer is variable and sometimes unpredictable.

Erdman (1988) have also reported that the buffering agents that possess a pK_a above the typical ruminal fluid pH will act as alkalinizing agents rather than simply as buffers to increase the resistance of the rumen to a change in pH. Present results indicated that the adding of the inorganic mixtures to the experimental diets, had no significant effect on IVDMD (Tables 4 and 5), which, probably indicated no effect of the inorganic mixtures on fermentation at the medium. Bodas *et al*. (2009) used sodium bicarbonate in the diet of lambs and did not obtain difference in dry matter digestibility. However, Mould and Qrskov (1983) reported that the addition of buffer due to maintenance of ruminal pH above the critical level might improve the DM digestibility.

The effect of inorganic mixtures supplementation on the *in vitro* total gas, CH_4 and CO_2 emission from diets containing different forage to concentrate ratios as 40:60 and 30:70 are shown in Table 6 and Table 7, respectively. Increased concentrate from 60 to 70%, caused a significant decrease in both total gas and CO_2 emission, which is probably due to the negative impact of rapid fermentation of carbohydrates on microorganisms. Rumen pH is one of the most critical determinants for rumen function as cellulolytic bacteria fail to grow below pH 6.0, while a slight increase in ruminal pH favors the activity of these bacteria (Santra *et al*. 2003). Within both the experimental diets, the supplementation with M1 and M2 alter total gas, methane and carbon dioxide produced in the medium significantly ($P<0.05$). The lowest levels of total gas were observed in $FC_{40:60}$ containing M1 at 20 and M2 at both 10 and 20 g/kg DM. Methane emission was significantly ($P<0.05$) higher when M1 was added to $FC_{40:60}$ at the rate of 10 g/kg DM and $FC_{30:70}$ at the rate of 20 g/kg DM compared with the non-supplemented diets.

Present results indicated that $FC_{40:60}$ containing M1 at 20 g/kg DM, M2 at 10 and 20 g/kg DM and $FC_{30:70}$ plus M1 at 10 g/kg DM had the lowest level CO_2 emission ($P<0.05$).

The pattern of the responses was influenced by the kind of inorganic composition and the concentration applied. Inorganic mixture M1 to M2, had the highest buffering capacity and by increasing the amount of M1 in the $FC_{40:60}$, decreased total gas, methane and carbon dioxide production, while, with the increasing the amount of M2 in the diet, increased total gas, methane and carbon dioxide production. But the results in the $FC_{30:70}$ against the results of the $FC_{40:60}$.

Table 6 *In vitro* total gas (mL/0.20 g DM), CH_4 (mL/0.20 g DM) and CO_2 (mL/0.20 g DM) of a dairy cow diet containing different forage: concentrate ratio as 40:60 which supplemented with different composition of inorganic buffering compounds (M1 and M2), after 24 h incubation

Ration	Inorganic chemical compounds applied		Parameter		
	Inorganic compound[1]	Concentration (g/kg DM ration)	Total gas	CH_4	CO_2
$FC_{40:60}$[2]	-	0.0	41.17	3.54	37.05
$FC_{40:60}$	M1	10	43.57*	3.84*	39.08*
$FC_{40:60}$	M1	20	38.77*	3.48	34.89*
$FC_{40:60}$	M2	10	36.42*	3.27	32.77*
$FC_{40:60}$	M2	20	37.67*	3.39	33.90*
SEM	-	-	0.360	0.061	0.327
P-value	-	-	0.0001	0.0008	0.0001

[1] M1 and M2 were made of from $NaHCO_3$, Na_2CO_3, $CaCO_3$, $MgCO_3$, MgO and bentonite Na as 500, 200, 90, 100, 100 and 10 g/kg or 300, 200, 200, 150, 100 and 50 g/kg, respectively.
[2] $FC_{40:60}$: a dairy cow diet containing 40% forage and 60% concentrate.
SEM: standard error of the means.
The means within the same column with at least one common letter, do not have significant difference ($P>0.05$).

Table 7 *In vitro* total gas (mL/0.20 g DM), CH_4 (mL/0.20 g DM) and CO_2 (mL/0.20 g DM) of a dairy cow diet containing different forage: concentrate ratio as 30:70 which supplemented with different composition of inorganic buffering compounds (M1 and M2), after 24 h incubation

Ration	Inorganic chemical compounds applied		Parameter		
	Inorganic compound[1]	Concentration (g/kg DM ration)	Total gas	CH_4	CO_2
$FC_{30:70}$[2]	-	0.0	39.17	3.52	35.44
$FC_{30:70}$	M1	10	36.67*	3.30*	33.00*
$FC_{30:70}$	M1	20	41.17*	3.70*	37.05
$FC_{30:70}$	M2	10	42.17*	4.21*	37.53*
$FC_{30:70}$	M2	20	38.17	3.43	34.35
SEM	-	-	0.465	0.041	0.421
P-value	-	-	0.0001	0.0001	0.0001

[1] M1 and M2 were made of from $NaHCO_3$, Na_2CO_3, $CaCO_3$, $MgCO_3$, MgO and bentonite Na as 500, 200, 90, 100, 100 and 10 g/kg or 300, 200, 200, 150, 100 and 50 g/kg, respectively.
[2] $FC_{30:70}$: a dairy cow diet containing 30% forage and 70% concentrate.
SEM: standard error of the means.
The means within the same column with at least one common letter, do not have significant difference ($P>0.05$).

It seems that increase the buffering capacity environment by adding the inorganic mixtures, as well as increasing its concentration with effects on pH, rumen bacterial fermentation performance is affected.

Dietary buffers by increasing the buffering capacity of the medium might produce a situation by which a huge decrease in pH may prevent and thereby causing an increase in methane levels in the culture medium. It was reported that dietary buffer would prevent depression in rumen pH and improve rumen ecology associated with high concentrate feeding (Santra *et al.* 2003). The amount of decrease in pH after an increase in the fermentation rate will depend on the buffering capacity of the rumen fluid (Counotte *et al.* 1979). Supplementation of minerals in the diet of animals is known to increase the number of total ruminal bacteria especially the cellulolytic bacteria which contributed to better cellulose digestibility (Koul *et al.* 1998). However, diets high in cereals consequently reduce ruminal pH and cellulolytic activity (Franzolin and Dehority, 1996). It has long been recognized that the addition of cereal grains to ruminant diets causes a decrease in methane and an increase in propionate production (Czerkawski, 1986), but the cause of this fermentation shift was not clear.

CONCLUSION

It has been concluded that there is a chance to increase the buffering capacity of inorganic composition when different amount of the inorganic chemical compounds were used compared with the sodium bicarbonate. Adding inorganic compound had the highest buffering capacity, reducing the amount of the acid load and increased IVDMD, although the effect was not significant compared to the control that might be due to lack of impact of the inorganic compound on the buffering capacity of the medium. Increasing the concentration of mixture M1 in the diet, causes was reduced in the total gas, CH_4 and CO_2. But with regard to mixture M2, the opposite was observed may be due to the effect of buffering capacity created by this mixtures on pH medium and microbial activity.

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