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In vitro Effect of the Inorganic Buffers in the Diets of Holstein Dairy Cow Varying in Forage: Concentrate Ratios on the Rumen Acid Load and Methane Emission

Research Article

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ABSTRACT

The present research was conducted aimed at using the approach involving the *in vitro* tests to evaluate effect by various inorganic buffers used in the diets of Holstein dairy cow on the dietary buffering capacity, ruminal acidogenecity value (AV), methane emission and assess the relationship between them. The buffers were sodium bicarbonate (SB), sodium sesquicarbonate (SSc), a commercial buffer [BEHINA®, (BH)], and potassium carbonate (PC). Basal diets were low forage [30% forage and 70% concentrate, ($FC_{30:70}$)], mid forage [35% forage and 65% concentrate, ($FC_{35:65}$)], and high forage [40% forage and 60% concentrate, ($FC_{40:60}$)]. The buffers were added to the diets in the concentrations of 0.0, 8.0 and 12.0 g/kg dry matter (DM). *In vitro* pH, AV, and methane emission of the experimental diets were determined using the gas production technique. Results showed that buffering capacity was significantly the highest for the PC, followed by BH, SB, and SSc (143.3, 138.3, 136.6, and 135, respectively). Analysis of the acid load revealed that adding 8 g/kg of DM of SB in the $FC_{40:60}$ diet led to the lowest AV (9.6 mg Ca g^{-1} DM). In addition, adding 12 g/kg of DM of BH in the $FC_{30:70}$ and 8 g/kg of DM of SB in the $FC_{35:65}$ diet caused the lowest (5.27) and highest (5.43) pH compared to the other treatments, respectively. The $FC_{40:60}$ diet containing 8 g/kg DM of PC had the lowest level of methane emission (1.01 mL/0.20 g DM). Our findings demonstrated that the rumen acid load and methane emission may alter when the dietary buffering capacities are changed using the inorganic buffers.

KEY WORDS acidogenecity value, buffer, buffering capacity, *in vitro*, methane, pH.

INTRODUCTION

The common practice of feeding high grain diets for the dairy cows can result in the reversible rumen pH depressions (Plaizier *et al.* 2018). Therefore, the dairy cows fed for maximum milk production are at the risk of experiencing the ruminal acidosis, as reported by Krause and Oetzel (2006). In high-producing dairy cows, ruminal pH fluctuates during the day as the processes of eating, rumination, ruminal digestion, and volatile fatty acids (VFA) absorption occur. Subacute ruminal acidosis (SARA) is the most important nutritional disease and represents a significant concern as it can negatively impact the dairy industry by decreasing dry matter intake, milk production, profitability, and increasing culling rate and death loss (McCann *et al.* 2016). SARA occurs when the ruminal pH decreases into a zone that is suboptimal for the ruminal function (pH from 5.2 to 6.0; Plaizier *et al.* 2008). The length of time per day when the ruminal pH is below 5.6 (Keunen *et al.* 2002) or below 5.8 (Krause *et al.* 2002; Krause and Combs, 2003) is

a highly important determinant of the rumen acidosis than the mean daily ruminal pH. Krause and Oetzel (2006) suggested that three main factors cause the occurrence of SARA in the milk-producing cows: residual use of the carbohydrates capable of being fermented fast, insufficient adjustment of the rumen to a diet with high fermenting capacity, and insufficient performance of making buffer in the rumen due to the lack of enough fiber or physically fibers in the diets. Feed additives can be used in the role of a complementary diet to the levelheaded diets, and they can decrease the probability of SARA as they are consisted of the antibiotics, solutions and neutralizing materials, yeasts, supplements containing live, naturally-occurring microorganisms, enzymes, and maybe essential oils (Khorrami *et al.* 2015; Malekkhahi *et al.* 2015). Feed additives have been found to affect the reticulum and rumen through various ways (Plaizier *et al.* 2018). Animal to animal difference as a reaction to the above materials has been shown that no rumen modifier can stop the SARA in all the cows in any condition (Golder *et al.* 2014a). Maybe, there is a need for using various rumen modifiers with respect to the diet constituents. It has been strongly proposed that the inclusion of inorganic buffers may help the milk producers to reduce the acidosis risk in the lactating dairy cows (Plaizier *et al.* 2018). Buffers are generally added to the diets of the milk-producing cows to stabilize the ruminal pH (Golder, 2014). Sodium bicarbonate and magnesium oxide are generally applied as the ruminal buffers (Staples and Lough, 1989; Hu and Murphy, 2005; Golder, 2014). Besides act of making buffer, sodium bicarbonate may stabilize the level of acid in the rumen by elevating the water use and decreasing the digestion in the rumen (Russell and Chow, 1993). Erdman (1988) in a study found that inclusion of a mixture of MgO and $NaHCO_3$ to the diets of dairy cows elevated the milkfat by 0.3-0.4%. Moreover, Golder *et al.* (2014b) demonstrated that inclusion of the above mixture to their diets decreased the variation in the diet use throughout a carbohydrate challenge experiment. Results of a previous experiment showed that using the other buffers added to the dairy cows̀ diets, such as potassium carbonate, potassium bicarbonate, and sodium sesquicarbonate had some benefit for the animals (Erdman, 1988; Golder, 2014; Lean *et al.* 2014). Alfonso-Avila *et al.* (2017) reported that using the potassium carbonate (K_2CO_3) as a buffer influenced the rumen environment. It has been well demonstrated that the digestibility and availability of the nutrients in the rumen depends on the pH value. The ingestion of the feed containing both acidic and basic substances, the microbial fermentation of the carbohydrates and proteins to volatile fatty acids, and the end products of the digestion and metabolism all influence the concentration of H^+ (Chalupa and Kronfeld, 1983).

High buffer value index (BVI) of a feed means that it can manage the H^+ concentration in the rumen and that the pH of the rumen will remain too high. Thus, the role of buffering capacity (BC) of the diet in the pH of rumen fluid, as well as the overall effect of feed fermentation and digestion are among the important factors influencing the acid-base neutrality in the ruminants.

Recently, the role of rumen acid load in different feeding strategies has been determined by the ruminal acidogenecity value (AV) (Rustomo *et al.* 2006a). This approach has been applied to prioritize the diets regarding the amount of acid aggregated in the rumen over the process of breaking down of the substances by the organisms. According to which, the total acid load of a medium is measured using the dissolution of Ca from $CaCO_3$ added to the media at the end of an *in vitro* fermentation. However, the effect of the BC of the feed on the AV value of the diets has not been evaluated yet. Danesh Mesgaran *et al.* (2011) reported that overall subsequent milk yield of the animals was higher in presence of high AV compared to the other group (41.9 and 37.8 kg), although milk fat percentage of the low AV group was higher than the other groups (3.7 and 3.5%). They developed and evaluated the technique in various diets of the ruminants.

Biomethanation in the rumen offers another approach for decreasing the similar release of the bacteria capable of fermenting the carbohydrates, but transition of hydrogen between the species occurs only with negative change in free energy at very low partial pressures of hydrogen (Wolin, 1975). If the methane-producing microorganisms are held back, then the hydrogen is aggregated, the enzymes catalyzing the reversible oxidation of molecular hydrogen are held back, and the bacteria capable of fermenting the carbohydrates use another approaches to reduce the similar release (e.g., by sufficient generation of the enzymes catalyzing the removal of hydrogen atoms) (Gottschalk, 1986).

Sauvant and Giger-Reverdin (2007) found a curved-line association between the methane generation and generation of concentrate in the diet, while the reduction of methane was fixed by 6-7% of whole energy at concentrate amounts of 30-40% in the diet followed by a decrease to 2-3% of whole energy at concentrate amounts of 80-90%. It has been well demonstrated that the inorganic buffering compounds that increase the BC of the ruminal fluid enable the cow to maintain a more stable ruminal pH while facing with a diet-related or a fermentation-related acid challenge. However, the relationship between the BC and ruminal acid load in the dairy cows̀ diet with methane emission has not been investigated. Therefore, the present study was carried out to investigate the *in vitro* role of some inorganic buffers used in various diets of lactating dairy cows (with different forage: concentrate ratios) regarding the ruminal AV and

methane emission, and evaluating the relationship between them.

MATERIALS AND METHODS

Inorganic buffers and experimental diets

The inorganic buffers were sodium bicarbonate (SB), sodium sesquicarbonate (SSc), potassium carbonate (PC), and a commercial buffer (BEHINA®) [containing sodium, calcium, and magnesium at the concentrations of 28, 21, and 35 g/kg, respectively, JAVANEH KHORASAN CO., IRAN]. Treatments were developed at three various forage-concentrate proportions including the low forage (30% forage and 70% concentrate, $FC_{30:70}$), mid forage (35% forage and 65% concentrate, $FC_{35:65}$), and high forage (40% forage and 60% concentrate, $FC_{40:60}$). Table 1 shows the ingredients and chemical composition of the diets. The buffers were added to the diets at the concentrations of 0.0, 8.0, and 12.0 g/kg DM of the diets.

Feed ingredients were milled and were passed through a 1-mm sieve (Retsch Muhle mill, Retch EPP 15X20, Germany) and then, were applied for chemical experiment and *in vitro* techniques. Composition of the material remaining after water removal was specified in every specimen by a fan-equipped oven at 95 ˚C, over a day. Amount of nitrogen was specified by the Kjeldahl approach (Kjeltec 2300 Autoanalyzer, Foss Tecator AB, Hoganas, Sweden) and crude protein was measured as follows: N × 6.25. The neutral detergent fiber was determined in accordance with the study by Van Soest *et al.* (1991). Acid detergent fiber [(AOAC, 2000), ID 973.18] was also determined. The amounts of ether extract [(AOAC, 2000), ID 920.39], and ash [(AOAC, 2000), ID 942.05] were also determined in the specimens.

Buffering capacity (BC) and *in vitro* ruminal acidogenecity value (AV)

The BC of the inorganic compounds added to the diets was measured according to some changes in the method proposed by Evans and Ali (1967). Each sample (1 g DM) was added into 100 ml of double distilled water and was stirred gently (2 min). The initial pH and all further pH measurements were recorded following a 3-min equilibration period after addition of the acid or base (Metrohm pH meter, model 691). Titrations were performed by addition to one 30-ml aliquot of initial solution ranging between its primary pH to pH 4 using 1 N HCI [83.3 mL/L, Merck brand] and by titrating a separate, 30 mL fraction ranging between its primary pH to pH 9 using 1N sodium hydroxide [40 g/L, Merck brand] through various increasing concentrations (0.1 to 10 mL, depending on the buffer and stage of titration). BC was calculated as the sum of milliequivalents of H^+ required to titrate the solution with a pH between 4 to 9. Samples with an initial pH higher than 9 were titrated to pH 9 using the HCl, titration then was continued to calculate the milliequivalents of H^+ required to reduce pH from 9 to 4. As, BC is measured only between the pH of 4 to 9, it does not account for total acid-neutralizing capacity of the buffers raising the initial pH above 9; the milliequivalents of H^+ required to reduce the initial pH to 9 are not included in the BC calculation. In addition, the amount of acid needed for reducing fluid to pH 9 during titration is typically very small because of the low H^+ concentration at high pH. Hence, this omission should only result in a minor error in the calculation of BC.

$$BC = [(\text{mL of 1N HCl}) + (\text{mL of 1N NaOH})] \times 10^3 / 30$$

Standard pH (STPH) of 6 and standard BC (STBC) of 50 meq/L were assumed as a base point (BVI=100) to calculate the BVI of the samples; these values were selected as typical and normal according to the previous studies (Aslam *et al.* 1991; Hogue *et al.* 1991). The BVI was calculated from sample's pH (SAPH) and sample's BC (SABC; milliequivalents per liter) by the following formula:

$$BVI = ((((\text{antilog}_{10}) ((-STPH-(\text{antilog}_{10} (-SAPH))) / (\text{antilog}_{10} (-STPH) + ((SABC-STBC)/STBC))\times 10) + 100$$

This formula was modified slightly from that presented previously (Aslam *et al.* 1991; Hogue *et al.* 1991) so that, a factor of 10 was included to increase the sensitivity of BVI to pH and BC alterations and to enhance the evaluation of differences between the buffer samples. Although, pH values were inserted in the formula, these values were converted to H^+ concentration during calculation of BVI hence; the resulting index should be more accurate as an assessment of changes in the acidity of the buffer compared to when the logarithmic pH was used in the calculations (Murphy, 1982).

The ruminal AV was assessed through the incubation occurring *in vitro* in a buffered rumen liquor for 24 h. The AV for each treatment was specified by the approach proposed by Tilley and Terry (1963) that has been changed by Wadhwa *et al.* (2001). The novelty of the present study was measuring the breaking down of calcium from calcium carbonate powder incapable of being dissolved to evaluate the excess level of acid after the process of breaking down of the substances in a feed by the organisms. The incubation was adopted for 24 h to indicate the mean rumen retention times (Lopez-Guisa and Satter, 1991; Nelson and Satter, 1992) and primary investigations have shown that just a low volume of excess acid is aggregated after the RRT (Wadhwa *et al.* 2001).

Table 1 Ingredients and chemical composition of the experimental diets (%DM basis)

Ingredients	Experimental diets (forage:concentrate)		
	$FC_{30:70}$	$FC_{35:65}$	$FC_{40:60}$
Corn silage	13.7	15.9	17.4
Alfalfa hay	17.5	20.8	23.2
Barley grain	19.7	18.4	17.4
Corn grain	18.8	17.7	16.7
Soybean meal	12.9	11.5	10.3
Wheat bran	10.5	9.5	9.1
Sugar beet pulp without molasses	4.2	3.5	3.2
Mineral-vitamin mix[1]	1.2	1.2	1.2
Salt (NaCl)	0.25	0.25	0.25
Sodium bentonite	0.65	0.65	0.65
Calcium carbonate	0.6	0.6	0.6
Chemical composition % dry matter (DM)			
Crude protein	15.23	14.81	14.47
Neutral detergent fiber (NDF)	33.37	34.24	35.06
Acid detergent fiber (ADF)	21.43	22.28	23.05
Ether Extract	8.15	7.51	7.2
Ash	5.83	6.13	6.36
Net energy for lactation (NE_L) (Mcal/kg)*	1.68	1.65	1.63

$FC_{40:60}$: a dairy cow diet containing 40% forage and 60% concentrate; $FC_{35:65}$: a dairy cow diet containing 35% forage and 65% concentrate and $FC_{30:70}$: a dairy cow diet containing 30% forage and 70% concentrate.
Mix supplied (on a concentrate DM basis): vitamin A: 34350 IU; vitamin D_3: 6870 IU; vitamin E: 46 mg; Zn: 229 mg; Mn: 126 mg; Fe: 69 mg; Cu: 33 mg; I: 2.6 mg; Co: 0.8 mg and Se: 0.46 mg.
* Net energy lactation, NRC (2001).

One g of every specimen was weighed and incubated in triplicate, in 30 mL of the buffered rumen liquor comprising 60% of buffer and 40% of rumen liquor obtained from the Holstein lactating dairy cow. The buffer was made up at 20% of the strength of the Tilley-Terry's (1963) buffer (diluted with distilled water). Cysteine hydrochloride monohydrate (0.025% wt/vol) was added just prior to the incubations. The rumen fluid was collected 3 h after the morning feeding, from three fistulated lactating Holstein dairy cows fed (g/kg, DM basis) by a diet consisting of 50% lucerne-containing forage, 20% wheat straw, 15% barley grain, 14% soybean meal, and 1% mineral-vitamin premix (Ca: 195000 mg/kg; P: 90000 mg/kg; Na: 55000 mg/kg; Mg: 20000 mg/kg; vitamin A: 500000 IU; vitamin D_3: 100000 IU, and vitamin E: 100 IU) [17% of crude protein (CP), 34% of neutral detergent fiber (NDF), 1.6 Mcal/kg of NE_L, 24.2 kg/d of DMI, and 36 kg/d of milk production]. Rumen fluid was filtered through four layers of cheesecloth before mixing with buffer, and was kept at 39 ˚C. The incubations were carried out in 100-Ml bottles held in a water bath at 39 ˚C. Bottles were shut by the gas relief windows and were rattled constantly. Samples (2 mL) were withdrawn from each bottle after 24 h and were transferred to 8-mL centrifuge tubes containing 50 mg (excess) of $CaCO_3$ powder (catalog no. C6763; Sigma Chemical Co., St. Louis, MO). The mixture was shaken manually for 5 s and then, was centrifuged at 4000 × *g* for 10 min and then, Ca content in the supernatant was determined using the atomic absorption spectroscopy (AAS, PG Instruments, England).

All the blanks were included using the same procedure.

The AV was measured through multiplying the calcium concentration of the product (obtained by the experiment) into amount of the fluid (30 mL) divided by the weight of the sample (1 g; Wadhwa *et al.* 2001).

AV= [Ca concentration × amount of the fluid (30 mL)] / sample weight (1 g)

Primary AV was measured after modifying the broken down calcium before adding $CaCO_3$ to exclude the role of calcium in the diet. The pH was calculated pre (0.0 h) and post-incubations (24 h) using a Fisher Accumet Digital pH/Ion meter (Model 425).

In vitro methane release from the rumen

The incubations were performed *in vitro* according to the study by Menke and Steingass (1988). To do this, 200 mg (based on dry matter) of every treatment was put in a 125 mL incubation bottle, and inorganic buffers were added at the concentrations of 0.0, 8.0, and 12.0 g/kg DM. A series of bottles containing no diet were also incubated similar to preparation of the blank. The bottles were incubated in 30 mL of the buffered rumen fluid (non-natural extracellular fluid secreted by the mouth with the rumen liquid at a ratio of 2:1) and were kept at 39 ˚C in a water-bath. The non-natural extracellular fluid secreted by the mouth was consisted of 475 mL/L of distilled water, 240 mL/L of buffer mixture, 240 mL/L of the macromineral solution, 0.12

mL/L of the micromineral solution, and 1.22 mL/L of Resazurin aqueous solution (Merck Company). Then, the medium was reduced by addition of reducing agent per liter of medium. Rumen fluid was obtained as explained before. The incubation was done in three sets (run) in triplicate. After the incubation for a day, the bottles were taken to an ice bath for inhibiting the process of breaking down of the substances by the organisms and the total amount of gas was registered using a digital pressure indicator (model SEDPGB0015PG5) and the release of CH_4 was specified by a biological gas recorder (SR2-BIO).

Statistical analysis

Data obtained from assessing the BC and BVI parameters were statistically analyzed using a completely randomized design, generalized linear model (GLM) procedure in the SAS (2002). The model used for analysis the data of pH, BC and ruminal methane emission was as follows:

$$Y_{ij}= \mu + A_i + e_{ij}$$

Where:
Y_{ij}: dependent variable.
μ: overall mean for the variable.
A_i: effect of the treatment i.
e_{ij}: random error associated with the observation i.

Data obtained for the BC, BVI, pH and AV (Tables 3 and 4) were analyzed as a completely randomized design using the statistical model:

$$Y_{ijk}= \mu + R_i + C_j + RC_{ij} + e_{ijk}$$

Where:
Y_{ijk}: dependent variable.
μ: overall mean.
R_i: main effect of diet (main plot).
C_j: main effect of buffer concentration (treatment).
RC_{ij}: interaction between diet and buffer concentration.
e_{ijk}: experimental error.

The significance criterion between the treatments was reported as the differences more than two times of the standard error of the means ($P<0.05$) (Danesh Mesgaran and Stern, 2005). Differences between treatments in BC, BVI, pH, AV and CH_4 were analysed using the GLM procedure of SAS (SAS, 2002), with Duncan's multiple range test used for the comparison of means at $P < 0.05$. Three dimensional graph was created using the SigmaPlot software through fitting data of all the treatments with respect to paraboloid equation ($Z=Z_0+ax+by+cx^2+dy^2$).

RESULTS AND DISCUSSION

Table 2 shows the BC and BVI of the inorganic compounds. BC was significantly the highest for the PC, followed by BEHINA®, SB, and SSc (143.3, 138.3, 136.6 and 135 meq/L, respectively). The highest BVI, indicating the changes in H^+ amount and BC was related to the PC and then, BEHINA®, SB, and SSc. Dietary buffers are able to elevate the pH and BC of the rumen the reactions of which are useful for the host animal. As, the BVI can account for the alterations in both of these measures thus, it is believed to provide a more complete evaluation of the diet-induced changes in the ruminal acid-base status, which should be useful in the evaluation of the dietary buffers (Tucker *et al.* 1992).

Sodium sesquicarbonate (SSc) is a combination of SB and Na_2CO_3 with a pK at 6.2 (for HCO_3^-) and another at 10.3 (for CO_3^{-2}). At the ruminal pH, the CO_3^{-2} portion of SSc would be a base, not a buffering compound, unless it is combined with H^+ to yield HCO_3^-. Then, it would be an active buffer at pH of 6.2. Compared to the BC alone, BVI partially considers the compounds elevating the pH level. At pH 7, both $NaHCO_3$ and SSc should be primarily consisted of HCO_3^- hence, BC should be similar.

Tucker *et al.* (1992) reported 16% lower BC for the SSc. The $NaHCO_3$ in the SSc contains 2 mol of Na/mol of CO_3; $NaHCO_3$ contains only 1 mol of Na and 1 mol of H/mol of CO_3. Therefore, $NaHCO_3$ contains 26% more CO_3 per unit weight than the Na_2CO_3, as one of the primary components of the SSc. Presumably, all the CO_3^{-2} in the SSc should be converted into HCO_3^-; during the reduction of ruminal fluid pH to 7, but $NaHCO_3$ would provide more HCO_3^- than the SSc during the reduction of pH from 7 to 5 (thus, leading to more BC).

BVI of the media culture was the highest for K_2CO_3, followed by BEHINA® buffer, $NaHCO_3$, and SSc, as H^+ was not significantly different between the $NaHCO_3$ and SSc, the increased BVI for $NaHCO_3$ is attributed solely to its higher BC. The BVI only considers the buffers increasing the pH BC of the ruminal fluid conversely, BVI is lowered as a result of decrease in either of these variables.

A high BVI of ruminal fluid may result from the buffering compounds increasing the ruminal fluid pH. Increasing the ruminal fluid pH to 6.3 or above may increase the rate of ruminal fiber digestion (Erdman, 1988).

An increase in the BC of ruminal fluid enhances the ability of the rumen to maintain a stable pH while facing with an acid challenge thus, increasing the ruminal fluid pH above 6.3 is desirable to maximize the fiber digestion (Tucker *et al.* 1992), alter the VFA patterns and ruminal function (Tucker *et al.* 1992).

Table 2 Initial pH, buffering capacity (meq/L) and buffering value index of the inorganic buffers

Parameter	Treatments				SEM	P-value
	SB	SSc	BH	PC		
Initial pH	8.37	8.38	10.74	10.66	-	-
Buffering capacity	136.6^{b}	135^{b}	138.3^{b}	143.3^{a}	1.47	0.019
Buffer value index	118.3^{b}	117.9^{b}	118.6^{ab}	119.6^{a}	0.306	0.023

SB: sodium bicarbonate; SSc: sodium sesquicarbonate; BH: BEHINA (a commercial buffer containing: sodium 280, calcium 21, and magnesium 35 g/kg produced by JAVANEH KHORASAN CO., Iran) and PC: potassium carbonate.
The means within the same row with at least one common letter, do not have significant difference (P>0.05).
SEM: standard error of the means.

Table 3 Buffering capacity (meq/L) and buffering value index of the treatments that are different forage: concentrate ratio supplemented by the different inorganic buffers

Parameters	Buffer concentration (g/kg)	Treatments															SEM	P-value[1]		
		$FC_{40:60}$					$FC_{35:65}$					$FC_{30:70}$								
		NB	SB	SSc	BH	PC	NB	SB	SSc	BH	PC	NB	SB	SSc	BH	PC		1	2	3
Buffering capacity	0.0	39.7	-	-	-	-	40.5	-	-	-	-	38.7	-	-	-	-	0.60	*	*	*
	8.0	-	49.8	48.3	46	44.3	-	46.5	46.7	41	43.8	-	43	40	39.2	43.7				
	12.0	-	44.3	49.8	48.2	44.2	-	45.8	45.3	45.5	45.8	-	44.2	48.5	43.3	43.8				
Buffer value index	0.0	83.7	-	-	-	-	88.7	-	-	-	-	89.4	-	-	-	-	0.69	NS	*	*
	8.0	-	97.2	98.4	97.4	97.4	-	96.9	97.3	96.4	97.1	-	98.7	96	96.5	97.2				
	12.0	-	97.5	99	99.1	98.1	-	98.9	99	98.4	98.8	-	97.9	99.7	98.2	97.5				

[1] 1: diet effect; 2: buffer concentration effect and 3: diet × buffer concentration effect.
$FC_{40:60}$: a dairy cow diet containing 40% forage and 60% concentrate; $FC_{35:65}$: a dairy cow diet containing 35% forage and 65% concentrate and $FC_{30:70}$: a dairy cow diet containing 30% forage and 70% concentrate.
NB: non buffered; SB: sodium bicarbonate; SSc: sodium sesquicarbonate; BH: BEHINA (a commercial buffer containing: sodium 280, calcium 21 and magnesium 35 g/kg produced by JAVANEH KHORASAN CO., Iran) and PC: potassium carbonate.
* (P<0.05).
SEM: standard error of the means.
NS: non significant.
The treatments were significant when the difference between the treatments was twice more than the SEM.

Table 4 Final medium pH and ruminal acidogenecity value (mg Ca g^{-1} DM) of the experimental diets varying in forage: concentrate ratio supplemented by the different inorganic buffers

Parameters	Buffer concentration (g/kg)	Treatments															SEM	P-value[1]		
		$FC_{40:60}$					$FC_{35:65}$					$FC_{30:70}$								
		NB	SB	SSc	BH	PC	NB	SB	SSc	BH	PC	NB	SB	SSc	BH	PC		1	2	3
pH	0.0	5.39	-	-	-	-	5.37	-	-	-	-	5.32	-	-	-	-	0.008	*	*	*
	8.0	-	5.41	5.39	5.38	5.41	-	5.43	5.34	5.39	5.38	-	5.31	5.34	5.31	5.39				
	12.0	-	5.37	5.39	5.37	5.36	-	5.37	5.41	5.38	5.39	-	5.32	5.29	5.27	5.31				
Acidogenecity value	0.0	11.9	-	-	-	-	11.2	-	-	-	-	12.3	-	-	-	-	0.19	*	*	*
	8.0	-	9.6	11.1	11.3	11.8	-	12	12.7	12.1	13	-	13.2	13.3	12.9	11				
	12.0	-	11.7	11.2	12.4	12.9	-	11.5	11.4	12	11.8	-	12.6	13.8	14.4	12.3				

[1] 1: diet effect; 2: buffer concentration effect and 3: diet × buffer concentration effect.
$FC_{40:60}$: a dairy cow diet containing 40% forage and 60% concentrate; $FC_{35:65}$: a dairy cow diet containing 35% forage and 65% concentrate and $FC_{30:70}$: a dairy cow diet containing 30% forage and 70% concentrate.
NB: non buffered; SB: sodium bicarbonate; SSc: sodium sesquicarbonate; BH: BEHINA (a commercial buffer containing: sodium 280, calcium 21 and magnesium 35 g/kg produced by JAVANEH KHORASAN CO., Iran) and PC: potassium carbonate.
* (P<0.05).
SEM: standard error of the means.
NS: non significant.
The treatments were significant when the difference between the treatments was twice more than the SEM.

BC of the ruminal fluid is important primarily through inhibiting a pH drop but, BC is not useful alone unless it is challenged by the acid. Because, BVI appraises both pH and BC of a compound's ability to withstand an acid challenge, it is a useful indicator regarding the influence of the dietary buffers on the acid-base status or stability of the ruminal fluid, or both. Tucker *et al.* (1992) reported the largest increase in the ruminal fluid pH for SSc, followed by $NaHCO_3$ and multielement buffer although, the H^+ concentration was the same in the fluid obtained from the rumen for the SSc and $NaHCO_3$. Because, BC was the highesting ability than either H^+ or BC alone. Given that, the STPH and STBC selected for inclusion in the BVI equation can influence the ranking of the dietary buffers, Erdman (1988) suggested that the STPH of 6 and STBC of 50 meq/L can be utilized for the future studies. Because, dietary pH and BC both can modulate effect of the diet buffers on the acid-base status of the ruminal fluid (Erdman, 1988), evaluating the influence of the dietary BVI upon ruminal fluid BVI may yield a more precise method for predicting the types of diets in which, supplemented dietary buffers will be most effective.

The BVI increases as BC H^+ decreases or BC increases; each of these responses typically would be beneficial for the lactating dairy cows. Conversely, an increase in the H^+ concentration or a reduction in the BC would lower the BVI.

Consolidating the effects of the dietary buffers on the ruminal fluid H^+ and BC into a single, numeric value will allow the evaluation of the total effects of dietary buffers on the rumen more completely (Tucker *et al.* 1992). Rustomo *et al.* (2006b) reported that the effects of dietary treatments on the ruminal pH were likely related to the differences in the feed fermentability reflected by the differences in the estimated total acid load produced from feed fermentation.

Both BC and BVI of the experimental diets were significantly influenced by the concentration of the used inorganic buffers (Table 3). BC was higher in the high forage diet. Adding 8.0 g/kg of DM of SB in the $FC_{40:60}$ diet led to the highest BC (49.8 meq/L). Forages required more acid and base during the titrations than the concentrates. Also, BC tended to increase with the increase in the ratio of forages in the diets indicating that the forage: concentrate ratio was an important factor influencing the BC that confirms the results of the previous studies (Levic *et al.* 2005) reporting that the cereals (concentrate) had the lowest BC. As expected, the BC was increased by increasing the buffer concentration. Higher BVI of the inorganic compounds, included to the experimental diets caused a positive significant reflect in the BVI of the buffer and buffer concentration. Thus, BVI has a direct association with the BC but has an inverse correlation with the H^+ concentration (acidity).

Jasaitis *et al.* (1987) in a study investigated the pH and BC of various feed components. Feed components have an effect on the acid-base condition of the rumen through their pH, BC, and induction of salivating process (Le Ruyet *et al.* 1992). Tucker *et al.* (1992) found that the BVI can be applied to assess the pH and buffering capacity of both diet and fluid obtained from rumen. If the total BVI in the diet estimates the condition of the fluid obtained from rumen, this measure can be employed to determine the beneficial time for using the supplemented dietary buffers.

Le Ruyet and Tucker (1992) reported that the sodium bicarbonate and sodium sesquicarbonate had a complete activity during the first 12 h of incubation; a plateau was observed in the activity of the multielement buffer and magnesium oxide for a day. In comparison with the multielement buffer and magnesium oxide, sodium bicarbonate and sodium sesquicarbonate have more advantages in avoiding the increments in the H^+ amount of the ruminal fluid shortly after receiving the diet; the multielement buffer and magnesium oxide contribute to stabilization of the acid-base condition in the rumen due to their slower secretion amounts, but the efficiency may be decreased through passaging out of the rumen. Tucker *et al.* (1992) in a study reported that the effect of dietary BC on the BC of ruminal fluid has not been delineated clearly. In the present study, the effect of different inorganic buffers on the AV and pH at different levels was investigated using different ratios. Table 4 shows the values of final medium pH and ruminal AV. Analysis of the H^+ concentration in the culture media revealed that $FC_{40:60}$ diet containing 8.0 g/kg of DM of SB had the lowest AV (9.6 mg Ca g^{-1} DM). The diets had a significant effect on both variables ($P<0.05$). Buffer supplementation increased ($P<0.05$) the final medium pH and ruminal AV. Buffer concentration had also significant ($P<0.05$) effect on the final medium pH. Possibly, the decreased yield of rumen acid was due to the diet's fermentation potential and the buffering capacities used in the present study. In addition, generally, high inclusion of the buffers in the diets increased the final medium pH and decreased the rumen AV (Table 4). These results are consistent with the higher buffering capacities of the used inorganic buffers and experimental diets. It has been previously demonstrated that the rumen acid depression is closely associated with the increased BC of the diet and the buffer (Jafarpour Boroujeni *et al.* 2016).

Clearly, the organic compounds used in the present study were able to modify the acid load of the diets. As expected, the final medium pH was reduced by reducing the forage to concentrate ratio (5.38 and 5.32 for $FC_{40:60}$ and $FC_{30:70}$ diets, respectively). A decrease has been reported in the ruminal pH following adding more carbohydrates capable of being fermented fast to the diet (Krause *et al.* 2002; Danesh Mesgaran *et al.* 2013). Danesh Mesgaran *et al.* (2009) found that the non-fibrous carbohydrates in the diet are positively correlated with the AV so that, the acidogenecity value was increased with an increase in the concentration of the non-fibrous carbohydrates in the diet. Rustomo *et al.* (2006a) reported that the energy feeds and fiber sources had the largest and medium aAVs, respectively.

Inorganic buffers can stop the pH decrease in the medium by making the acids neutral generated from the activation of the rumen microbiota. They improve the condition of the rumen by modifying the acid level of the components in the rumen, and avoiding high declines in the pH level (Le Ruyet and Tucker, 1992). The levels of pH in the medium were reduced by elevating the acid level generated from the process of fermenting of the carbohydrates. The use of the diets containing great concentrations of the non-structural carbohydrates capable of being fermented fast by the microbes in the rumen has been shown to result in a higher decrease in the pH (Kalscheur *et al.* 1997). Tripathi *et al.* (2004) found that adding the sodium bicarbonate resulted in a linear improvement in the pH of the ruminal fluid. West *et al.* (1987) showed that inclusion of different buffers to

the diet led to a remarkable elevation in the pH of the rumen. Santra *et al*. (2003) indicated that dietary buffers inhibited the rumen pH decline after applying high amounts of concentrate. Regarding the AV (Table 4), all the inorganic buffers ($P<0.05$) significantly influenced on the studied variables compared to the diet not supplemented by the treatments, attributing to the fact that the inorganic buffers have remarkably influenced the BC and consequently, the acid level of the medium. Rustomo *et al.* (2006a) concluded that the increase in the feed AV and the depression of ruminal pH could be predicted by the rumen acid load estimated from the feed fermentation. Rustomo *et al.* (2006b) originally hypothesized that increasing the concentrate AV would increase the ruminal pH below a suboptimal pH and that the animals would adjust their feed intake to avoid the excessive rumen acid load.

Erdman (1988) found that the buffering materials having a pK above the normal pH of the ruminal fluid will serve as the alkalinizing materials rather than the buffers for elevating the tolerance of the rumen against the pH changes. Our findings revealed that inclusion of the inorganic buffers to the treatments significantly influenced on the final pH medium (Table 4), possibly indicating the impact of the inorganic buffers on the process of fermenting in the medium. Furthermore, the effects of increasing the concentrate AV on the ruminal pH depression would depend on the forage proportion in a diet.

In addition, it has been reported that increasing the rumen AV and the correlation between the rumen acid load and ruminal pH was stronger than the correlation between the rumen acid load and the intake of starch, NFC, NDF, ADF, or CP.

Table 5 *In vitro* total gas (mL/0.20 g DM) and CH_4 (mmol/0.20 g DM) in a dairy cow's diet with 40% forage and 60% concentrate, supplemented by the different inorganic buffers, after 24 h of incubation

Parameters	Buffer concentration (g/kg)	Treatments[1]					SEM	P-value
		$FC_{40:60}$						
		NB	SB	SSc	BH	PC		
Total gas	0.0	67.1	-	-	-	-	1.71	*
	8.0	-	62.4	57.9	62.7	49.7		
	12.0	-	62.6	58.3	65.6	62		
CH_4	0.0	1.27	-	-	-	-	0.048	NS
	8.0	-	1.46	1.24	1.45	1.01		
	12.0	-	1.18	1.19	1.39	1.32		

$FC_{40:60}$: a dairy cow diet containing 40% forage and 60% concentrate.
NB: non buffered; SB: sodium bicarbonate; SSc: sodium sesquicarbonate; BH: BEHINA (a commercial buffer containing: sodium 280, calcium 21 and magnesium 35 g/kg produced by JAVANEH KHORASAN CO., Iran) and PC: potassium carbonate.
* ($P<0.05$).
SEM: standard error of the means.
NS: non significant.
The treatments were significant when the difference between the treatments was twice more than the SEM.

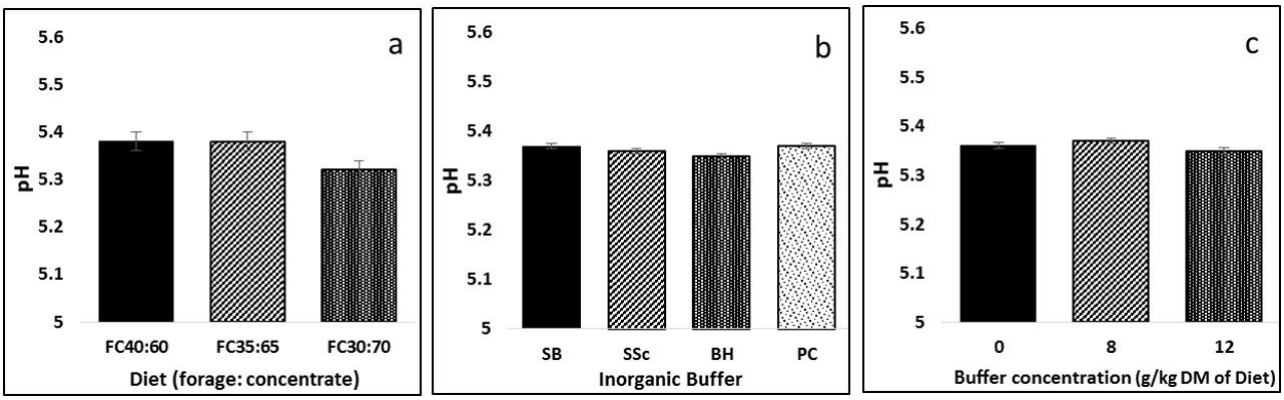

Figure 1 *In vitro* effect of the experimental **a**: diets; **b**: inorganic buffers and **c**: buffer concentration on the final pH medium
SB: sodium bicarbonate; SSc: sodium sesquicarbonate; BH: BEHINA® [a commercial buffer containing: sodium 280, calcium 21, and magnesium 35 g/kg produced by JAVANEH KHORASAN CO., Iran] and PC: potassium carbonate
⊕$FC_{40:60}$: a dairy cow diet containing 40% forage and 60% concentrate; $FC_{35:65}$: a dairy cow diet containing 35% forage and 65% concentrate and $FC_{30:70}$: a dairy cow diet containing 30% forage and 70% concentrate

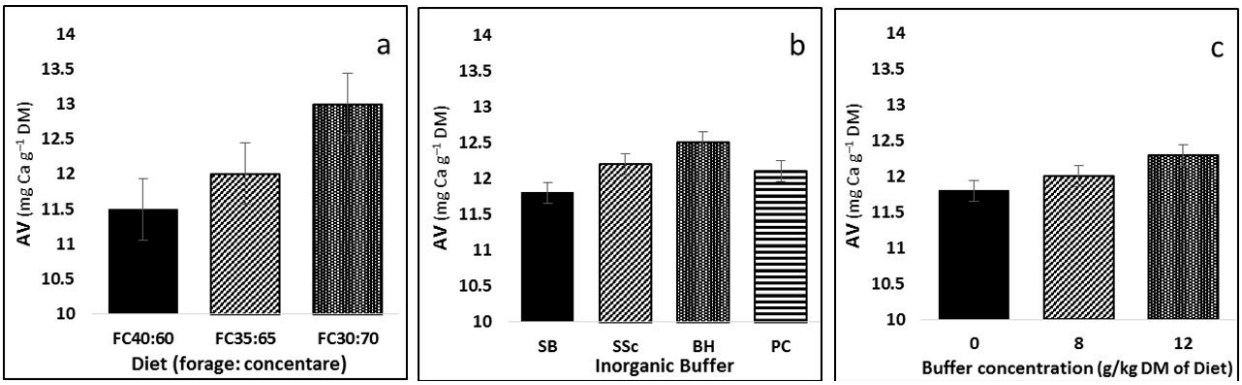

Figure 2 *In vitro* effect of the experimental **a**: diets; **b**: inorganic buffers and **c**: buffer concentration on the acidogenecity value, AV (mg Ca g^{-1} DM)

SB: sodium bicarbonate; SSc: sodium sesquicarbonate; BH: BEHINA® [a commercial buffer containing: sodium 280, calcium 21, and magnesium 35 g/kg produced by JAVANEH KHORASAN CO., Iran] and PC: potassium carbonate.

$^{\oplus}FC_{40:60}$: a dairy cow diet containing 40% forage and 60% concentrate; $FC_{35:65}$: a dairy cow diet containing 35% forage and 65% concentrate and $FC_{30:70}$: a dairy cow diet containing 30% forage and 70% concentrate

Table 5 shows the results regarding the impact of inorganic buffers on the total gas and methane release from the $FC_{40:60}$ diet in the *in vitro* experiments. The treatment buffers significantly influenced on both variables ($P<0.05$). The supplementation decreased the total gas produced in the medium. The smallest amounts of total gas were related to the $FC_{40:60}$ diet containing the PC at the concentration of 8.0 g/kg DM (49.7 mL/0.2 g DM). Methane emission (CH_4) had a remarkably ($P<0.05$) lower release after addition of the PC to the $FC_{40:60}$ diet at the concentration of 8.0 g/kg DM (1.01 mmol/0.20 g DM) compared to the diet not supplemented with the treatments. Increased BC of the rumen following addition of the inorganic buffers, and also elevated buffer amount caused a positive effect on the fermentation performance of the rumens microbiota. Dietary buffers might establish a condition by which a high decline in pH may be prevented by elevating the BC of the medium and also influencing on the methane levels in the culture medium. It has been found that the dietary buffer inhibits the rumen pH decline and enhances the rumen environment accompanied with using the high levels of concentrate (Santra *et al.* 2003). Therefore, pH decline after an elevation in the process of fermenting will rely on the BC of the ruminal fluid (Counotte *et al.* 1979). Adding the buffers in the diet of the ruminants has been shown to elevate the count of total microbiota in the rumen specifically the cellulolytic bacteria involving in better digestion of the cellulose (Koul *et al.* 1998). Although, diets having the cereals would diminish the ruminal pH and cellulolytic action (Franzolin and Dehority, 1996). It has been found that adding the cereals to the ruminants diets results in the reduction of methane and increased generation of the propionate (Czerkawski, 1986), but there is no clear cause for this change in the process of fermenting.

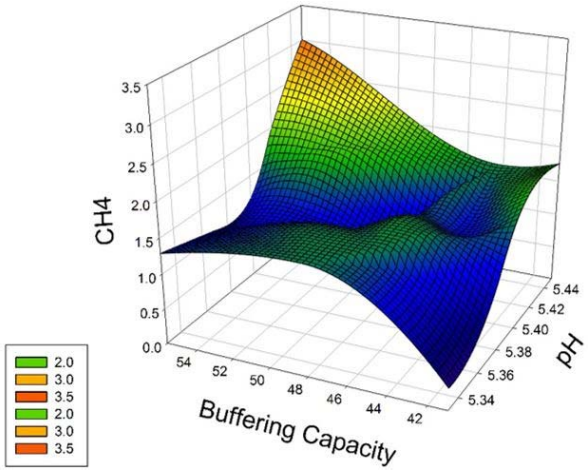

Figure 3 Response surfaces of the CH_4 emission and buffering capacity with pH

Figures 1 and 2 show the results regarding the effects of experimental parameters on the final pH medium and AV. It was concluded that the reactions were affected by the type of inorganic buffer, the buffer amount, and forage: concentrate ratio applied. Elevating the amount of concentrate from 60 ($FC_{40:60}$) to 70% ($FC_{30:70}$) led to a remarkable ($P<0.05$) increase in the AV, attributing to the negative effect of fast process of fermenting of the carbohydrates on the microorganisms to produce H^+ and decrease the pH of the medium. Although, BH was the least effective among the studied buffers in reducing the H^+ concentration (12.5 mg Ca g^{-1} DM) in Figure 2, the amount added to the media (0.0, 8.0, and 12.0 g/kg) was identical to that of the SB, SSc and PC; as demonstrated by Staples *et al.* (1988), addition of 3% of multielement buffer (combined buffer like BH) to the diet of the lactating dairy cows resulted in the ruminal fluid H^+ concentration similar to that observed for supple

menting the diet with 1% of $NaHCO_3$ (SB). Assuming that commercial combined buffers such as BEHINA® may contain clay, Sulzberger *et al.* (2016) reported that according to production and physiological parameters, clay may be an alternative buffer in diets for dairy cows. Ruminal pH is among the most influential factors for rumen function as the cellulolytic bacteria cannot grow below pH of 6.0, while a low increment in the ruminal pH is beneficial for their activity (Santra *et al*. 2003).

In this study, the response surface figure was also developed for CH_4 emission, ruminal BC, and pH (Figure 3). Response surface as a tool was used for investigating the effect of an independent variable (CH_4, BC, and pH) on the rumens fermentation pattern. Based on the results, both BC and pH influenced on the CH_4 emission from the rumen (Figure 3). The lowest ruminal CH_4 emission was found to be accompanied with the highest BC and lowest pH. This finding showed an optimal rumens fermentation condition regarding the CH_4 emission, BC, and pH.

CONCLUSION

It has been concluded that the addition of the inorganic buffers increased the diet BC and rumen pH, while decreased the AV. Also, buffer supplementation reduced the ruminal CH_4 emission. It was concluded that the response of the inorganic buffers to the increase in the diet BC and decrease in the acid load provides an alternative to the sodium bicarbonate as a means to optimize the rumen fermentation. This also results in greater improvement in CH_4 emission from the rumen. However, the *in vitro* effect of adding exogenous buffers in the dairy cows diets depends on the type of diets and buffer concentration. According to the results of this study when the amount of concentrate increasing from 60 ($FC_{40:60}$) to 70% ($FC_{30:70}$), the AV increased significantly ($P<0.05$), that might be due to the negative effect of fast process of fermenting of the carbohydrates on the microorganisms to produce H^+ and decrease the pH of the medium. By examining the results of this research, it may be concluded that the *in vitro* effect of the inorganic buffers was less pronounced on both pH and AV of the rumen in the $FC_{40:60}$ diets than the $FC_{30:70}$ diets and also demonstrated that the rumen acid load and methane emission may alter when the dietary buffering capacities are changed using the inorganic buffers. However, it needs to evaluate the buffers used in this study in *in vivo* experiments.

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