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In vitro usage of various non-organic compounds to subdue acidogenic value and enhance the fermentation of alfalfa hay based diets by mixed rumen microbiota

M. Danesh Mesgaran[1]*, J. Amini[1] and M. Paktinat[2]

[1]Department of Animal Science, Faculty of Agriculture, Ferdowsi University of Mashhad, Mashhad, Iran, P. O. Box 91775-1163. Iran.
[2]Behta Taghzieh Sabz Co., Mashhad, Iran.

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Batch cultures of mixed rumen microorganisms were used in a randomized complete block design to study the effects of alfalfa hay-to-concentrate ratio and various non-organic buffering compounds on Acidogenic Value (AV), in vitro dry matter disappearance (IVDMD), medium pH, and AV: IVDMD ratio. Alfalfa hay was included in the experimental diets as: 80% (F80), 60% (F60), 40% (F40), and 20% (F20) on a dry matter (DM) basis. Buffering compounds were added to the experimental diets as: Sodium bicarbonate [SB; 5 or 10 mg.g^{-1} DM], magnesium oxide (MgO; 5 or 10 mg.g^{-1} DM), sodium bentonite (bentonite;10 or 20 mg.g^{-1} DM), Acid Buf® (5, 10 or 20 mg.g^{-1} DM), Acid Buf + SB in a 3:4 ratio (11 or 16.5 mg.g^{-1} DM), Acid Buf + SB + MgO in a 3:4:1 ratio (12.5 or 18.75 mg.g^{-1} DM), and Herod's Buffer (5, 10 or 20 mg.g^{-1} DM); keeping one group as control (no supplementation). After 24 h incubation, no significant differences observed in medium pH among SB, MgO, bentonite and the control, but Herod's Buffer, Acid Buf, Acid Buf + SB, and Acid Buf + SB + MgO kept it up (P≤ 0.05). The lowest AV and AV: IVDMD ratios were observed when SB was used in the cultures (P≤ 0.05). Herod's buffer IVDMD was the lowest (P≤ 0.05). The results indicated that the diet containing Acid Buf and SB had a relatively low AV and AV: IVDMD, and could maintain a relatively high rumen fluid pH compared with those of the others.

Key words: Acidogenic value, *in vitro* dry matter disappearance, buffers.

INTRODUCTION

The challenge 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; Krause and Oetzel, 2006). Increasing energy supply through increased use of concentrates or rapidly fermentable fiber can plunge the rumen into acidosis (Tajik and Nazifi, 2011). Failure to maintain a consistent rumen pH in high yielding dairy cows may result in metabolic disorders and reduced production performance. Subacute ruminal acidosis (SARA) is a common and economically important problem in well managed dairy herds (Enemark, 2008; Tajik and Nazifi, 2011). The classical view of acidosis in the rumen is that as grain increases,

*Corresponding author. E-mail: danesh@um.ac.ir. Tel: +98 511 8795618. Fax: +98 511 8796845.

so does the prevalence of starch-fermenting bacteria like *Streptococcus bovis* (Russell and Hino, 1985). Thus, there should be a high population ratio of lactate-consuming bacteria like *Megasphaera elsdenii* to prevent lactic acid acidosis (Russell et al., 1981). Subacute ruminal acidosis occurs during early and mid-lactation and has traditionally been characterized by low rumen pH, but lactic acid does not accumulate as in acute lactic acid acidosis (Khafipour et al., 2009). Detailed analysis of the rumen microbial populations showed substantial differences in the types of microorganisms which proliferated when SARA was induced by cereal or ground alfalfa-based diets (Khafipour et al., 2009). The real-time PCR data also indicated that severe grain-induced SARA was dominated by *Streptococcus bovis* and *Escherichia coli*, whereas mild grain-induced SARA was dominated by *Megasphaera elsdenii*. Although, Khafipour et al. (2009) showed that the amylolytic bacterium whose abundance most closely mirrored the severity of SARA was *S. bovis*, and other major amylolytic bacteria were less affected. *Megasphaera elsdenii* populations were synchronized with the *S. bovis* numbers, indicating that this bacterium was effectively eliminating rumen lactate (Khafipour et al., 2009). At 6 h after feeding, the severe grain-induced SARA group was dominated by *M. elsdenii* and *S. bovis*, while the mild group was dominated by *M. elsdenii*, *Succinivibrio dextrinisolvens*, *Prevotella bryantii*, and *Ruminococcus flavefaciens*. Therefore, providing high-yielding dairy cows adequate levels of dietary buffers could prevent SARA and the resulting depressions in fiber digestion, dry matter intake (DMI), and milk production (NRC, 2001; Danesh Mesgaran, 2005) as well as alterations in milk composition (NRC, 2001). Erdman (1988) suggested that forage type and content affect the response to dietary buffers. Alfalfa hay differs from corn silage in at least three aspects that may reduce the need for supplemental buffers. First, fiber is greater per unit for alfalfa such that in comparison of diets with equal forage to concentrate, alfalfa based diets offer more total fiber. Second, alfalfa has higher buffering capacity than corn silage. And finally, alfalfa offered as hay is not acidic (DePeters et al., 1984).

The likelihood of encountering problems with rumen acidity relates both to the amount of acid produced as feeds ferment and the ability of the feed to promote chewing and consequently production of salivary buffers (Wadhwa et al., 2001). Cows maintain rumen pH within a relatively narrow range, and only rarely succumb to clinical disorders, by adopting a wide range of behavioral, physiological, and metabolic adaptations to high levels of acid production in the rumen. These include reducing DMI, changing meal patterns, and altering rumination activity and absorption from the rumen (Ketelaars and Tolkamp, 1992). These adaptations make it difficult to study this area; concerns about feeding extreme diets to experimental animals compound the difficulties. Consequently, *in vitro* feed evaluation systems that are independent of animal effects are developed. Wadhwa et al. (2001) developed a simple *in vitro* technique for evaluating the production and neutralization of acid as feeds ferment. It is based on *in vitro* estimates of rumen acid load [acidogenic value, (AV)]. Rumen AV is based on measurements of dissolution of Ca from insoluble $CaCO_3$ powder added at the end of a 24 h *in vitro* fermentation to assess residual acidity. Therefore, AV represents the amount of alkali needed to neutralize the medium, owing to buffering effects of feed (Wadhwa et al., 2001). Rustomo et al. (2006) reported that fibre 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 (6.27, 11.71. 12.63 and 12.86 mg Ca g^{-1} feed DM, respectively).

The objective of this research was *in vitro* investigation of the effect of varying alfalfa hay-to-concentrate ratios and different mineral buffer including: sodium bicarbonate (SB), magnesium oxide (MgO), sodium bentonite, an absorbent aluminium phyllosilicate with chemical formula $(Na,Ca)_{0.33}(Al,Mg)_2Si_4O_{10}(OH)_2.(H_2O)_n$; Acid Buf, a commercial product of the skeletal remains of the seaweed *Lithothamnium calcareum* (CelticSea Minerals, Cork, Ireland); sodium bicarbonate, Acid Buf + SB in a 3:4 ratio, Acid Buf + SB + MgO in a 3:4:1 ratio, and a mixture of bentonite, monobasic potassium phosphate, magnesium carbonate, magnesium oxide, and sodium carbonate in a 5:22:22:35:16 ratio introduced by Herod et al. (1978) (Herod's Buffer) as non-organic buffers on AV, pH, and IVDMD using rumen mixed microbiota.

MATERIALS AND METHODS

To assess *in vitro* rumen acidogenic values of different rations including several non-organic buffering additives, batch cultures of mixed rumen microbiota were carried out.

Experimental diets and non-organic buffering compounds

Four diets composed of alfalfa hay and concentrate in proportions of 80:20 (F80 diet), 60:40 (F60), 40:60 (F40), and 20:80 (F20) (DM basis) were used as experimental diets. Alfalfa harvested in the afternoon at Late bud (June 26) and Early flower (July 18) from a second year alfalfa field seeded with cv. Ranger at the Research Farm of Ferdowsi University of Mashhad with semi arid climate condition (Mashhad, Iran; 36° 14′ 30.7″ N, 59° 41′ 6.6″ E). After air drying in the shade (*ca.* 10–15 days), two *samples* of alfalfa hay were mixed in *equal* proportions and used in this study [17.85% crude protein (CP), 46.7% Neutral detergent fiber (NDF), 2.4% ether extract (EE), 9.15% ash; and 30.1% non-fibrous carbohydrate (NFC)].

The basal diets Ingredient and nutrient compositions are presented in Table 1. The diets Ingredients were ground through a Wiley mill with a 1-mm sieve,oven-dried (48 h, 60°C), and then nitrogen content 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) was determined according to Van Soest et al. (1991). Samples were also analyzed for EE [(AOAC, 2002), ID 920.39] and ash by igniting the samples in muffle furnace at 525°C for 8 h. Non-fibrous

Table 1. Ingredient and chemical compositions of the rations used in the experiment.

Item	F20	F40	F60	F80
Ingredient (% of DM)				
Alfalfa Hay	20.00	40.00	60.00	80.00
Barley grain	49.65	37.24	24.82	12.41
Soybean meal	14.57	10.93	7.28	3.64
Wheat bran	14.46	10.84	7.23	3.61
Vitamin-mineral premix[1]	1.34	1.00	0.67	0.33
Chemical composition (mg g^{-1} of DM) of rations				
Crude protein	175.2	174.9	174.6	174.3
NDF	312.8	352.8	392.9	432.9
EE	31.0	28.2	25.5	22.7
Ash	53.8	63.1	72.4	81.7
NFC	427.2	380.9	334.6	288.3

[1]Each gram contains: 195 mg Ca, 90 mg P, 3 mg Fe, 2 mg Mn, 280 µg Cu, 100 µg Co, 100 µg, 500 IU vitamin A, 100 IU vitamin D, and 100 µg vitamin E.

carbohydrate (NFC) is calculated using the equation of NRC (2001); NFC = 100 – (NDF + CP + EE + Ash).

The oven-dried basal diets were supplemented with following buffering treatments: Control (no added buffering agent); 5, 10 $mg.g^{-1}$ of DM sodium bicarbonate (SB); 5, 10 $mg.g^{-1}$ of DM magnesium oixde [(MgO); Ghatran Shimi Co., Iran]; 5, 10, 20 $mg.g^{-1}$ of DM bentonite; 5, 10 $mg.g^{-1}$ of DM Acid Buf; 11, 16.5 $mg.g^{-1}$ of DM Acid Buf + sodium bicarbonate in a 3:4 ratio; 12.5, 18.75 $mg.g^{-1}$ of DM Acid Buf + sodium bicarbonate + magnesium oixde combined in a 3:4:1 ratio; 5, 10, 20 $mg.g^{-1}$ of DM Herod's buffer (a combination of bentonite, monobasic potassium phosphate, magnesium carbonate, magnesium oxide, and sodium carbonate in a 5:22:22:35:16 ratio) (Herod et al., 1987). Unless otherwise stated, all chemicals were of analytical reagent grade (≥99%; Merck, Germany).

In vitro incubation

The fermentation-incubation technique used in this experiment was adapted from Wadhwa et al. (2001). One-gram (DM) of each sample was placed into a culture vial, then incubated with 30 ml of buffered rumen liquor comprising 60% buffer and 40% rumen liquor. Buffer used was made up at 20% of the strength of that already described by Tilley and Terry (1963). Cysteine hydrochloride monohydrate (0.025% wt/vol) was added just prior to the incubations. A set of vials without feed sample was also incubated similarly which served as blank. Rumen fluid was collected, approximately 3 h after morning feeding, from four adult ruminally fistulated sheep (49.5 ± 2.5 kg, body weight) that were fed 0.6 kg of alfalfa hay and 0.4 kg of concentrate (24% corn grain, 20.4% barley grain, 27% soybean meal, 13.8% canola meal, 13.8% wheat bran, 0.3% calcium carbonate, 0.5% mineral and vitamin premix, and 0.2% salt). Ruminal fluid was strained through 4 layers of cheesecloth and stored in a pre-warmed thermos until it was transported to the laboratory. The incubations were carried out anaerobically, under oxygen-free CO_2 flush, in triplicate and in three independent sets (run) using 125 ml culture vials, fitted with rubber stoppers and one-way gas release valves, held in a shaking water bath at 39°C for 24 h, with the oscillation set at 30 rpm. An incubation period of 24 h was chosen as this is representative of average rumen retention times and preliminary studies revealed that only a small amount of further acid load accumulated after that time (Cruywagen et al., 2007).

Immediately after each incubation, the vials 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). Vial content was filtered (42 µm pore size) and a 2 ml sample of each filtrate vial was taken to analyze residual acidity (acidogenic value). The filtrated residual was oven dried (60°C for 48 h), weighted and used to calculate *in vitro* dry matter disappearance (IVDMD). Acidogenic value (AV) was determined by means of an adapted method from Wadhwa et al. (2001). Briefly, sample from each vial was transferred into a 2.5 ml centrifuge tube containing excess amount of $CaCO_3$ powder (50 mg). The mixture was shaken manually for 5 s and then centrifuged at 4000 ×*g* for 10 min. The supernatant Ca content was then immediately determined using a flame photometer (Model 410, Sherwood Scientific Ltd, Cambridge, UK). A measurement of dissolution of Ca from insoluble $CaCO_3$ powder makes it possible to assess residual acidity after fermentation of feeds.

Calculations and statistical analysis

The AV (mg Ca g^{-1} DM) of each sample was calculated as the product of Ca concentration (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 et al., 2011):

$$IVDMD\ (\%) = [(A-(B-C))/A] \times 100$$

Where: A = dry weight of sample; B = dry weight of residue after incubation, and C = dry weight of blank

Data were analyzed as a randomized complete block design using PROC MIXED of SAS (SAS Institute Inc., 2008). The model used was: $Y_{ijk} = \mu + T_i + D_j + R_k + \varepsilon_{ijk}$, where Y_{ijk} is the dependent variable, μ is the overall mean, T_i is the fixed effect of the non-organic buffering compounds, D_j is the fixed effect of the experimental diets, R_k is the random effect of run (incubation day), and e_{ijkl} is the random residual error, which is assumed to be normally distributed. Means were compared using the least square means method.

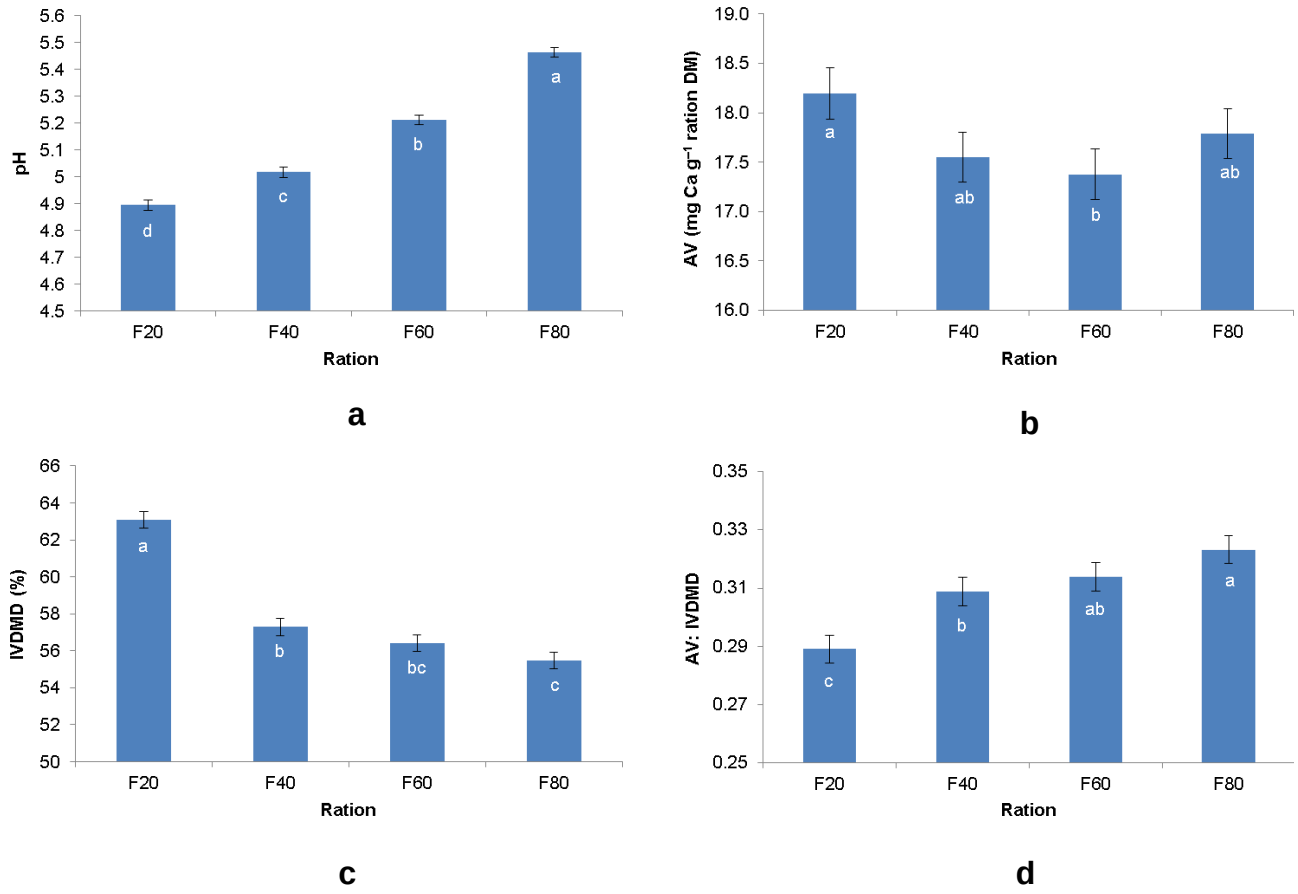

Figure 1. Effects of rations with different alfalfa hay ratios on the *in vitro* rumen fluid pH (**a**), apparent acidogenic value [AV; dissolved Ca (mg Ca g^{-1} ration DM)] (**b**), dry matter disappearance (IVDMD, %) (**c**), and AV: IVDMD ratio (**d**) after 24 h incubation.

Mean differences were considered significant at P< 0.05.

RESULTS AND DISCUSSION

The effects of the experimental diets on medium pH, AV, IVDMD, and AV: IVDMD are presented in Figure 1. In all rations, the medium pH decreased from 0 to 24 h incubation. Previous studies (Rezaii et al., 2010; Jahani et al., 2011) showed that the pH of incubation medium declined through the first 24 h and then stabilized. furthermore, Rustomo et al. (2006) found no difference in AVs between the 24 and 48 h incubation for all feed classes and concluded that there was little further fermentation after 24 h. Statistical evaluations revealed a significant (P≤ 0.01) downward trend in the *in vitro* rumen fluid pH among the rations evaluated, with the lowest pH (pH = 4.89) in F20 (Figure 1a). The substrate had a great impact on the fermentation end products (Russell 1998). High concentrate diets containing high amounts of nonstructural carbohydrates are quickly fermented by ruminal microbes, resulting in a greater decline in ruminal pH (Kalscheur et al., 1997). Extent of microbial fermentation is a key factor in ruminal digestibility. Another aspect to be taken into consideration is the medium pH, which determines the equilibrium of microbial population (Hungate, 1966), and is therefore a key factor in the development of fermentative activity (Fondevila and Pérez-Espés, 2008). Subacute ruminal acidosis is characterized by ruminal pH depression and microbial perturbation (Hook et al., 2011). In general, by transition from a hay-based to a grain-based diet, the availability of rapidly fermentable carbohydrate goes up, coinciding with an increase in bacterial growth rates and increase in volatile fatty acids (VFAs), which are responsible for the reduction in ruminal pH (Nocek, 1997). As the rumen pH drops, the proportion of lactate producers, such as *Streptococcus bovis*, increase and the result is a rise in lactate production. The lactate is metabolized by a growing lactate-utilizer population, represented by *M. elsdenii* and *Selenomonas ruminantium*, until the rumen pH drops below 5.6, when the proportion of lactate producers to lactate utilizers increases, lactic acid begins to accumulate, and density

Table 2. Effects of different buffering system on the *in vitro* rumen fluid pH, apparent acidogenic value [AV; dissolved Ca (mg Ca g^{-1} ration DM)], dry matter disappearance (IVDMD, %), and AV: IVDMD ratio after 24 h incubation.

Variable	Treatments[1]									
	Control	Sodium bicarbonate (mg.g^{-1})		MgO (mg.g^{-1})		Bentonite (mg.g^{-1})		Acid Buf (mg.g-^{1})		
		5	10	5	10	10	20	5	10	20
pH	5.02^{i}	5.07^{fghi}	5.08^{fghi}	5.07^{fghi}	5.11^{efghi}	5.04^{i}	5.06^{hi}	5.07^{ghi}	5.15^{defgh}	5.17^{cdef}
AV (mg Ca g^{-1} ration DM)	17.79^{cde}	15.25^{fg}	15.09^{g}	16.85^{ef}	16.78^{ef}	17.61^{de}	17.50^{de}	17.44^{de}	18.38^{cd}	17.48^{de}
IVDMD, %	57.6^{cde}	59.0^{abcde}	60.3^{abc}	58.1^{abcde}	59.4^{abcd}	59.7^{abcd}	60.2^{abc}	60.7^{a}	56.5^{ef}	57.4^{de}
AV: IVDMD	0.312^{cdefgh}	0.262^{j}	0.264^{ij}	0.298^{efgh}	0.282^{hij}	0.295^{fghi}	0.291^{fghij}	0.287^{ghij}	0.330^{bc}	0.307^{defgh}

Variable	Treatments[1]							S.E.M2	P- Value
	Acid Buf + SB (mg.g^{-1})		Acid Buf + SB + MgO (mg.g^{-1})		Herod's Buffer (mg.g^{-1})				
	11	16.5	12.5	18.75	5	10	20		
pH	5.17^{cdefg}	5.20^{bcde}	5.17^{cdef}	5.24^{bc}	5.24^{bcd}	5.28^{ab}	5.37a	0.0166	< 0.0001
AV (mg Ca g^{-1} ration DM)	17.48^{de}	18.37^{cd}	18.27^{cd}	19.53^{ab}	19.93^{a}	19.38^{abc}	18.28^{bcde}	0.1463	< 0.0001
IVDMD (%)	56.8^{ef}	59.4^{abc}	58.2^{bcde}	60.0^{ab}	53.1^{g}	56.4^{ef}	54.4^{fg}	0.3067	< 0.0001
AV: IVDMD	0.310^{cdefgh}	0.312^{cdefg}	0.316^{cdef}	0.327^{bcd}	0.381^{a}	0.348b	0.326^{bcde}	0.00313	<0.0001

[1]Control = no added dietary buffer; MgO = magnesium oxide; acid Buf + SB = a 3:4 mixture of acid Buf and sodium bicarbonate; acid Buf + SB + MgO = a 3:4:1; mixture of acid Buf, sodium bicarbonate, and magnesium oxide; Herod's buffer = a mixture of bentonite, monobasic potassium phosphate, magnesium carbonate, magnesium oxide, and sodium carbonate combined in a 5:22:22:35:16 ratio. [2] Standard error of mean. [a-j] Means in a row with unlike superscripts indicate significant differences (P< 0.05).

of fibrolytic bacteria decreases. This is the point where the animal is considered to have SARA (Hook et al., 2011).

The disappearance of DM after 24 h of *in vitro* incubation was significantly increased by concentrate ratio in diet and this was due to high amounts of carbohydrates fermentation and in consent with pH results. Acidogenic value was lower (P≤ 0.05) for F60 than that of F20 diet (17.38 and 18.67 mg Ca g^{-1} DM, respectively) and there was not any differences between these diets and those of the others (Figure 1c). In addition, a high AV was associated with a greater decrease in medium pH (Figure 1a, b). As prescribed by Rustomo et al. (2006) energy sources and fiber sources have highest and intermediate AV, respectively, whereas protein sources has lower AV. Diet containing 60% alfalfa hay F60 had enough concentration of available fiber and in consequence had the lowest expected AV value, as it was observed in this experiment. Additionally, as seen in the pH values, a significant (P≤ 0.01) upward trend was detected in the AV: IVDMD among the rations evaluated, with the lowest AV: IVDMD (AV: IVDMD = 0.289) in F20 (Figure 1d), which imply that the fermentation of high concentrate diets, containing high amounts of nonstructural carbohydrates, yielded lower acidogenicity for each unit of disappeared DM, compared with high fiber diets.

The effects of different buffering agents used on medium pH, AV, IVDMD, and AV: IVDMD are shown in Table 2. In present study several non-organic compounds with different buffering properties were evaluated alone or together. Main goal to use such these feed additive buffers was to prevent drastic fluctuation in rumen pH, to ensure optimum conditions for rumen microbial ecosystem. The results indicated that there were no significant differences in medium pH among SB, MgO, bentonite and control groups, but Herod's buffer, Acid Buf, Acid Buf + SB, Acid Buf + SB + MgO caused to reduce it significantly (P≤ 0.05). A diet containing 20 mg.g^{-1} of DM bentonite, had higher IVDMD, regardless of the concentration

of alfalfa hay in the rations. Addition of bentonite to the diets evaluated apparently resulted in a substrate that was more easily digested by the rumen microbiota. Using Herod's buffer maintained the highest medium pH, with an increase in AV: IVDMD ratio. However, it was not able to alter AV. When SB used as 5 or 10 $mg.g^{-1}$ DM, a considerable and more effectiveness of buffering function, which was expected to see, were observed. In addition, a combination of SB and Acid Buf showed the best buffering efficiency as observed in the lowest AV, and the highest IVDMD. This finding is in contrast with Russell and Chow (1993). They have suggested that sodium bicarbonate enhances water intake in lactating cows. Consequently, in this situation both rumen fluid dilution rate and the flow of undegraded starch from the rumen increase, with a reduction in the production of propionate which may cause a decline in the rumen acidosis. Already, an average of 3.6% increase in acid detergent fiber (ADF) digestion has been observed for each 0.1 unit increase in ruminal pH (Erdman, 1988). Thus, it seems that SB used in the present experiment alone or composed with Acid Buf might provide a better situation in which the rumen micrbiota activity optimized, due to the subacute ruminal acidosis (SARA), and even enhance feed digestibility. Krause and Oetzel (2006) suggested that the risk of developing SARA can be reduced by adopting a feeding regime, which balances ruminal buffering with the production of volatile fatty acids mainly from fermented carbohydrates.

The lowest both AV and AV: IVDMD were observed in the medium containing for SB ($P \leq 0.05$). In vitro dry matter disappearance of the Herod's buffer was lower compared with those of control; moreover, it was lower than those of the other treatments ($P \leq 0.05$). In F20 ration, Acid Buf used as 10 $mg.g^{-1}$ DM and AB + BC as 16.5 $mg.g^{-1}$ DM had the lowest AV: IVDMD (0.263 and 0.264, respectively) which indicated these buffering agents are more effective when a high concentrate portion used in ruminant feeds.

Conclusion

Decreasing forage in ration altered in vitro ruminal fermentation and led to higher AV and IVDMD, and lower pH. Ability of feed additive non-organic buffering compounds to maintain optimum conditions for rumen microbial ecosystem in different forage-containing diets was evaluated. Since there is no general agreement on the pH threshold that is definitive of SARA, rumen AV could be better index for studying the production and neutralization of acids during rumen fermentation of low forage rations. In F20 ration, although Herod buffer had highest pH, Acid Buf and AB + BC had the lowest AV: IVDMD which indicated these buffering agents are more effective when a high concentrate portion used in ruminant feeds. In general, best buffering efficiency (minimum AV, and highest IVDMD) obtained by SB with or without Acid Buf. Therefore, they would be beneficial in high concentrate rations. Furthermore, they can be recommended as a buffer of choice in further *in vivo* evaluations.

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Abbreviations: AV, acidogenic value; **DM,** dry matter, **IVDMD**, *in vitro* dry matter disappearance.

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