INTERPRETIVE SUMMARY: Effects of calcium and buffer sources on lactational performance, ruminal fermentation, nutrient digestibility, and metabolism of dairy cows. By Martins et al., page 000. The objective of the study was to investigate aragonite (a calcium carbonate) as rumen buffer, compared with sodium bicarbonate, and as a calcium source, compared with limestone, in lactating dairy cows. Results showed that treatments had no effect on rumen pH, but both aragonite and sodium bicarbonate increased blood pH 6 h after feeding. Aragonite also increased overall blood ionized calcium concentration. Aragonite decreased dry matter intake while not affecting milk production, which increased feed efficiency of the cows. Both aragonite and sodium bicarbonate increased milk fat concentration compared with the control.

RUNNING HEAD: RUMEN BUFFER AND CALCIUM SOURCES

Effects of calcium and buffer sources on lactational performance, ruminal fermentation, nutrient digestibility, and metabolism of dairy cows

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ABSTRACT

The objective was to investigate the effect of calcium and rumen buffer sources on lactational performance, ruminal fermentation, enteric gas emissions, apparent total-tract digestibility of nutrients, and blood variables of lactating dairy cows. A replicated 3 × 3 Latin Square design experiment was conducted with 9 primi- and 9 multiparous mid-lactation Holstein cows. Cows were fed the same basal diet, except for the inclusion (as % DMI) of the following minerals: 1) CON: 0.80% limestone and 0.55% NaCl; 3) BICARB: 0.80% limestone and 0.80% $NaHCO_3$; and 3) ARAG: 0.80% aragonite and 0.55% NaCl. Compared with CON, DMI was decreased by ARAG and tended to be decreased by BICARB. Treatments did not affect milk yield, ECM, or yields of milk components. Compared with CON, milk fat, milk TS, and MUN concentrations were increased by both BICARB and ARAG. Milk protein concentration was slightly decreased (i.e., 2%) by ARAG, compared with both CON and BICARB. Feed efficiency was increased by BICARB and ARAG, compared with CON. Treatments did not affect ruminal fermentation variables, except BICARB and ARAG increased or tended to increase butyrate molar proportion, compared with CON. Additionally, ruminal NH_3 concentration was greater for ARAG than BICARB and tended to be greater for ARAG than CON. Enteric gas emission and apparent total-tract digestibility of nutrients were not affected by treatments in the current study. Blood pH was increased at 6 h after feeding by ARAG and BICARB, compared with CON. Blood ionized Ca concentration was greater for ARAG than BICARB, but both were not different from CON. Treatments did not affect blood haptoglobin, β-hydroxybutyrate, and urea nitrogen concentrations. Overall, rumen buffering capacity of ARAG appears to be similar to that of $NaHCO_3$, which was supported by increased milk fat and blood pH, compared with CON. Additionally, ARAG appears to increase Ca availability for absorption compared with BICARB

(i.e., diet supplemented with $CaCO_3$ and $NaHCO_3$). The mechanism by which ARAG affects the acid-base status and Ca metabolism in dairy cows remain to be investigated.

Keywords: calcium, dietary cation-anion difference, milk production, rumen buffer

INTRODUCTION

Volatile fatty acids are the main source of energy for ruminants (Bergman, 1990) and their production occurs upon fermentation of OM (i.e., feedstuffs, primarily carbohydrates) in the rumen (Allen, 1997). With increasing milk yield (**MY**) of dairy cows in the last 60 years (Cole and Spurlock, 2017; Brito et al., 2021), DMI has also increased, and diets have been formulated to enhance ruminal fermentation and VFA production, providing greater energy availability for lactation. Volatile fatty acid accumulation and changes in VFA profile may lead to decreased ruminal pH (Dijkstra et al., 2012) and greater risk of sub-acute rumen acidosis in high producing dairy cattle (Plaizier et al., 2009). Absorption of VFA in the rumen, flow of VFA to the intestine, and neutralization of acids by salivary bicarbonate and phosphate buffers are the main physiological mechanisms of regulating rumen pH (Allen, 1997; Aschenbach et al., 2009, 2011). Additionally, the supplementation of neutralizing agents (i.e., rumen buffers) such as $NaHCO_3$ and MgO (Bach et al., 2018, 2023) may play a role in preventing excessive decrease in ruminal pH of dairy cows, leading to better animal performance and health.

Limestone (i.e., $CaCO_3$) is commonly used as a Ca source in ruminant diets. Although $CaCO_3$ has a carbonate group, the potential of $CaCO_3$ to promote rumen buffering is limited because of its low solubility at pH > 5.5 (Rogers et al., 1982; Wohlt et al., 1987). Aragonite is an important $CaCO_3$ polymorph present in naturally occurring carbonate sediments, and it is formed by biological and physical processes such as precipitation from marine environments (Wood et al., 2023). Unpublished data from an in vitro experiment (D. E. Wasson and A. N. Hristov, The Pennsylvania State University) demonstrated that ruminal pH at ≥ 6 h of incubation was similar between aragonite and $NaHCO_3$, both supplemented at up to 3% (DM basis) to a fermentation substrate mimicking a typical lactating diet containing 60:40 forage-to-concentrate ratio (average pH: 5.28 and 5.32 ± 0.006 for aragonite and $NaHCO_3$, respectively). Particle size and surface

area are important factors when considering the reactivity of $CaCO_3$ sources and their capacity to neutralize fermentation acids (Jasaitis et al., 1987). In this sense, the positive effect of aragonite on in vitro ruminal pH could be potentially explained by its smaller particle size (100 versus …… μm for aragonite and limestone, respectively) and greater surface area than limestone (Figure 1). Additionally, increased surface area could potentially contribute to increased availability of Ca for absorption.

We hypothesized that diet supplementation of aragonite would promote a similar buffering capacity as supplementation of $NaHCO_3$ (i.e., a standard rumen buffer used in the dairy industry). Consequently, rumen pH, milk fat content, digestibility of nutrients, and lactational performance of the cows would be enhanced by both aragonite and $NaHCO_3$, compared with a control diet supplemented with limestone. Additionally, we hypothesized that dietary supplementation of aragonite would increase blood ionized Ca (**iCa**) concentration in dairy cows, compared with diets supplemented with limestone. Thus, the objective of this study was to investigate the effect of Ca and rumen buffer sources on the lactational performance, ruminal fermentation, enteric gas emission, apparent total-tract digestibility of nutrients, and blood variables of mid-lactation dairy cows.

MATERIAL AND METHODS

Animals involved in this experiment were cared for according to the guidelines of The Pennsylvania State University Institutional Animal Care and Use Committee.

Animals, Experimental Design, and Treatments

The experiment was conducted in the tie-stall barn at The Pennsylvania State University's Dairy Teaching and Research Center from July to October 2022. Eighteen Holstein cows (9 primiparous and 9 multiparous) averaging (± SD) 121 ± 98 DIM, 629 ± 76 kg BW, and 43 ± 8 kg/d MY, at the beginning of the study, were used in a replicated 3 × 3 Latin square design

experiment. Cows were grouped into 6 Latin squares based on lactation number, DIM, and MY. The experiment consisted of 3 periods of 28 days each, with 18-d for adaptation to the diets (i.e., adaptation periods) and 10-d for data and samples collection (i.e., data and sample collection periods). Treatment allocation was balanced for carry-over effects, and cows within square were assigned to 1 of 3 treatments, as follows: (1) basal diet containing 0.80% limestone and 0.55% NaCl – **CON**; (2) basal diet containing 0.80% limestone and 0.80% $NaHCO_3$ – **BICARB**; and (3) basal diet containing 0.80% aragonite (Rumen Cal +, Ag Source, LLC) and 0.55% NaCl – **ARAG**. The variation among treatments consisted of Ca (i.e., limestone versus aragonite) and buffer (i.e., $NaHCO_3$ versus aragonite) sources. The basal diet was formulated to meet NE_L and MP requirements (NRC 2001) of a multiparous cow weighing 650 kg BW, producing 44 kg/d milk with 3.8% milk fat and 3.2% milk true protein, and 27 kg/d DMI. Diets were balanced to provide equal amounts of absorbable Ca, P, and Na (g/d), according to NRC (2001). Forages and concentrate feeds were mixed in a stationary mixer (Rissler Electra-Mix, model 1052, I. H. Rissler Mfg., LLC) once daily, at approximately 0600 h. Treatments were weighed daily, mixed with the basal diet for at least 3 minutes, and delivered as TMR to the cows once-a-day, at approximately 0700 h, in a mobile forage blender (Rissler, model 1050, I. H. Rissler Mfg., LLC). Feeding was ad libitum targeting 10% refusals and cows had free access to drinking water.

Sampling and Measurements

Diet and Feed Ingredients. Weights of the offered TMR and orts were recorded daily, and daily TMR intake was measured during the entire experiment. Samples of forages and concentrate feeds were collected once weekly and offered TMR and orts samples were collected twice weekly. Forage and TMR samples were immediately dried for 72 h at 55°C in a forced-air oven and ground in a Wiley Mill (Thomas Scientific) through a 1-mm sieve. Offered TMR and

orts samples were composited by week, on an equal DM basis, and forage and concentrate feeds were composited for the entire experiment. Weight of TMR offered and orts and DM content of the weekly composited TMR and orts were used to calculate DMI of the cows. Composite samples of the feed ingredients were submitted to Cumberland Valley Analytical Services for wet chemistry analysis of CP (method 990.03; AOAC International, 2000), amylase-treated NDF (**aNDF**; Van Soest et al., 1991), ether extract (**EE**; method 2003.05; AOAC International, 2006), ADF (method 973.18; AOAC International, 2000), ash (method 942.05; AOAC International, 2000), minerals (Ca, P, and Na; method 985.01; AOAC International, 2000), and estimated NFC (NRC, 2001). Samples were also analyzed for starch according to Hall (2009). Composite TMR samples were analyzed for indigestible NDF (**iNDF**) as described in Huhtanen et al. (1994) and modified by Lee et al. (2012). Nutrient composition of the diets (i.e., CP, aNDF, ADF, EE, starch, ash, Ca, P, and Na) was reconstituted from the analyzed composition of individual feed ingredients and their inclusion rates in the diets (Table 1). Estimated RDP, RUP, NE_L, and MP concentrations and balances were calculated using NRC (2001) considering average DMI, MY, milk composition, and BW of cows within treatment throughout the experiment. Intake of nutrients was calculated based on nutrient composition of the offered TMR and DMI of individual cows during data and sample collection periods, without correction for composition of the orts.

Milk Production, Milk Composition, BW and BCS. Milk production was automatically recorded (DeLaval milk meter, MM27BC) daily at each milking (a.m. and p.m.) throughout the experiment. Milk samples were collected from 4 consecutive milkings (a.m. and p.m.) on d 8 and 9 during the data and sample collection periods. Milk samples were placed into 50 mL tubes containing bromo-2-nitropropane-1,3-diol and submitted to Dairy One (Dairy One Cooperative

Inc.) for analysis of milk fat, milk true protein, lactose, other solids, TS, and MUN by infrared spectroscopy (MilkoScan 4000, Foss), and SCC by flow cytometry (Fossomatic models 5000 or FC; Foss Electric A/S). Separate, unpreserved milk samples were also collected as described above and stored frozen at -20°C until further analysis. These samples were thawed, composited per cow and period, and analyzed for milk fatty acids (**FA**) profile as described in Rico and Harvatine (2013). Milk composition data were weighted for the corresponding 10-d averaged a.m. and p.m. MY, and total yields of milk fat, milk true protein, lactose, and TS were calculated from averaged MY and weighted milk composition. Energy-corrected milk yield was calculated as follows: ECM, kg/d = kg of milk x [(38.3 × % milk fat × 10 + 24.2 × % (milk true protein ÷ 0.93) × 10 + 16.54 × % lactose × 10 + 20.7) ÷ 3,140] (Sjaunja et al., 1990), where the 0.93 factor was used to convert milk true protein into milk CP according to NRC (2001).

Ruminal Fermentation. Samples of ruminal fluid were collected from all cows using the ororuminal tubing technique (Lage et al., 2020). Samples were collected at 0 and 4 h relative to feeding on d 9 and 10 during the data and sample collection periods, respectively. Approximately 200 mL of the initially sampled ruminal fluid were discarded to avoid possible saliva contamination. Whole ruminal contents were filtered through 2-layers of cheesecloth and the filtered fluid samples were analyzed immediately for pH (59000-60 pH Tester, Cole-Parmer Instrument Company). Aliquots of filtered rumen fluid were processed and later analyzed for VFA (Yang and Varga, 1989) and NH_3 (Chaney and Marbach, 1962) concentrations.

Fecal and Urine Sampling. Spot fecal samples (approximately 300 g/cow) were collected from the rectum of the cows during 3 consecutive days at intervals staggered in time to cover a 24-h period sampling. Samples were collected at 0500, 1100, 1700, and 2300 h on d 7; at 0800, 1400, and 2000 h on d 8; and at 0200 h on d 9 during the data and samples collection periods.

Fecal samples were oven-dried at 55°C for 72 h and ground using a Wiley Mill (Thomas Scientific) through a 1-mm sieve. Ground fecal samples were composited per cow and period and analyzed for CP (N × 6.25) using the Costech ECS 4010 C/N/S elemental analyzer (Costech Analytical Technologies Inc.), and aNDF and ADF using an Ankom 200 fiber analyzer (Ankom Technology Corp.). Total-tract apparent digestibility of nutrients was estimated using indigestible iNDF as an internal marker. Briefly, composited fecal samples were incubated for 12 d in the rumen of a lactating rumen-cannulated cow following Huhtanen et al. (1994) recommendations, except 25-µm pore size filter bags (Ankom Technology Corp.) were used (Lee et al., 2012).

Urine samples (approximately 300 mL/cow) were collected by perineal stimulation at the same time points as for fecal samples and added to 2M H_2SO_4 in the ratio of 60 mL of acid per 1,000 mL of urine to reach a pH < 3.0. Acidified samples were diluted 1:10 with distilled water and stored at -20ºC for further analyses. Urine samples were composited on an equal volume basis per cow and period and analyzed for urea N (**UUN**; Urea nitrogen kit 580; Stanbio Laboratory Inc.), uric acid (Uric acid kit 1045; Stanbio Laboratory Inc.), creatinine (Creatinine kit 420; Stanbio Laboratory Inc.), and allantoin (Chen et al., 1992). Composite urine samples were freeze-dried (HarvestRight Home Freeze Dryer) and analyzed for N using a Costech ECS 4010 C/N/S elemental analyzer (Costech Analytical Technologies Inc.). Daily urinary volume was estimated based on urinary creatinine concentration, assuming a creatinine excretion rate of 29 mg/kg of BW (based on unpublished total urine collection data from Hristov et al., 2011). Estimated daily urine output was used to calculate daily excretions of urine N, UUN, and purine derivatives (**PD**; allantoin and uric acid). Total excreta N was calculated as the sum of excreted urine and fecal N. Unaccounted N was calculated as follows: Unaccounted N, g/d = [(N intake –

(total excreta N + milk N)]. Milk N secretion was calculated as: Milk N, g/d = [(Milk true protein ÷ 6.38) + MUN].

Enteric Gas Emissions. Enteric gas (CH_4, CO_2, and H_2) emissions were measured using the GreenFeed system (C-Lock Inc.). Two GreenFeed units were maintained and calibrated following the manufacturer's recommendations (https://globalresearchalliance.org/wp-content/uploads/2018/08/GreenFeeds-SOP-_final.pdf; accessed on October 24, 2023). Cows were fitted with a unique radio-frequency identification ear tag for recognition by the GreenFeed system. Gas measurements were taken 8 times over 3 consecutive days at intervals staggered in time to cover a 24-h period sampling. GreenFeed units were pushed in front of the cows at 0100, 1000, and 1900 h on d 4, 0400, 1300, and 2200 h on d 5, and 0700 and 1600 h on d 6 of the data and samples collection periods. Cows were attracted by a pelletized bait feed (Stocker Grower 14, Purina Animal Nutrition LLC). Individual breath samples were collected during 5 min sampling events, followed by 2-min intervals for background air collection between cows. Gas samples were collected following the sequence of cows in the tie-stall barn, and the sequence of sampling was maintained throughout the entire experiment. Average DMI, MY, ECM yield, and CH_4 production were used to calculate CH_4 yield (i.e., g CH_4 ÷ kg of DMI) and intensity (i.e., g CH_4 ÷ kg of MY and ECM). Enteric gas emissions data were averaged per cow and period.

Blood Sampling. Blood samples were collected from the tail vein or artery of the cows using a 20-gauge × 2.54 mm needle into 9 mL vacutainer tubes containing spray-dried sodium heparin (BD Vacutainer) at the same timepoints as for fecal and urine samples. Samples were centrifuged at 1,500 × g at 4°C for 15 min for plasma collection. Plasma samples were composited per cow and period and stored frozen at -20°C until analyzed for haptoglobin (PHASE Haptoglobin Assay, Tridelta Development Ltd.), BHB (Autokit 3-HB Microliter

Procedure; Wako Diagnostics), and urea N (**BUN**; Urea nitrogen kit 580; Stanbio Laboratory Inc.). Separate aliquots of blood samples were also collected as described above at 0400, 1000, 1600, and 2200 h and analyzed, within 30 min of collection, for pH, bicarbonate (**HCO_3^-**), Na, K, iCa, and glucose concentrations using an iSTAT hand-held biochemical analyzer (VetScan iSTAT, CG8+ cartridge, Abbott Point of Care).

Statistical Analysis

Statistical analyses were performed using SAS (release 9.4, SAS Institute Inc.). Two cows were removed from the experiment after being diagnosed with severe mastitis during the 1st week of adaption in period 2. Data collected from these cows during period 1 were included in the statistical analysis. These cows were replaced with similar cows considering lactation number, DIM, and MY during the 2nd week of adaptation in period 2. All data were tested for normality using the UNIVARIATE procedure and processed for outlier identification based on an absolute studentized residual value ≥ 3 using PROC REG. Log-transformed data were analyzed when the W statistic of the Shapiro-Wilk test was less than 0.05 (i.e., SCC data). Statistical analyses were completed using the MIXED procedure. Dry matter intake, MY, and feed efficiency data from the last 10-d of each experimental period were analyzed as repeated measures. Statistical models included the fixed effects of period, treatment, day, and treatment × day interaction. Day was the repeated term, AR(1) was the covariance structure, and the effect of cow within period × treatment × square was the subject. The fixed effects of parity and treatment × parity interaction were tested and removed from the final models, if non-significant ($P > 0.10$). Square and cow within square were random effects. Milk composition data, ECM, ECM feed efficiency, enteric gas emissions, nutrient intake, total-tract apparent digestibility of nutrients, milk FA profile of milk fat, urinary excretions, and blood concentrations of haptoglobin, BHB,

and BUN data were analyzed as described above without the repeated term. Ruminal fermentation and blood variables data collected with the iSTAT biochemical analyzer were analyzed as repeated measures with the fixed effects of treatment, time, and treatment × time interaction. Time was the repeated term, AR(1) was the covariance structure, and cow within period × treatment × square was the subject. The fixed effects of parity and treatment × parity interaction were also tested and removed from the final models, if non-significant ($P > 0.10$). Square and cow within square were random effects. Means were separated by pairwise *t*-test (diff option of PROC MIXED). Statistical differences were considered significant at $P \leq 0.05$, and tendency was declared at $0.05 < P \leq 0.10$. Data are presented as least squares means.

RESULTS AND DISCUSSION

Lactational Performance

Dry matter intake was decreased ($P < 0.01$) by ARAG and tended to be decreased ($P = 0.09$) by BICARB, compared with CON (Table 2). Milk yield was not affected by treatment in the current study. As a result of the decrease in DMI, feed efficiency was increased ($P \leq 0.05$) by both BICARB and ARAG, compared with CON. Milk fat ($P < 0.01$) and TS ($P \leq 0.04$) concentrations were also increased by BICARB and ARAG, compared with CON. Milk true protein concentration was slightly decreased (-2%, $P \leq 0.06$) by ARAG, compared with BICARB and CON. Treatments did not affect milk components yield, ECM, and ECM feed efficiency. Concentration of MUN was increased ($P = 0.02$) by BICARB and ARAG, compared with CON.

Readers should be aware that the majority of references used in the discussion are relatively dated because of a lack of recent studies investigating $CaCO_3$ as a Ca source and rumen buffer. In fact, studies evaluating $CaCO_3$ and $NaHCO_3$ as rumen buffer sources in dairy cattle date from the early 1960's. Emery et al. (1964) reported limited effects of $CaCO_3$ or

$NaHCO_3$ supplementation on the overall lactational performance in cows producing ≤ 20 kg/d MY, despite an increased milk fat and ruminal acetate concentration by $NaHCO_3$. Feeding 1.4% $CaCO_3$ alone or in combination with 1.2% $NaHCO_3$ to high starch diets (e.g., 43% starch and 40:60 forage-to-concentrate ratio), Rogers et al. (1985) reported a decreased DMI by $CaCO_3$ and $CaCO_3$ + $NaHCO_3$, and a tendency for decreased MY by $CaCO_3$ + $NaHCO_3$, compared with a basal diet containing 0.5% $CaCO_3$ (29.7 versus 31.1 kg/d MY, respectively). When $CaCO_3$, NaCl, or $NaHCO_3$ were supplemented at 2.0% DM to diets designed to cause milk fat depression (25:75 forage-to-concentrate ratio), Rogers et al. (1982) reported decreased DMI and increased feed efficiency by $CaCO_3$, compared with NaCl, $NaHCO_3$ and a non-supplemented basal diet. Milk fat was increased by $NaHCO_3$ but not affected by $CaCO_3$, compared with control (Rogers et al., 1982). Overall, data did not support the efficacy of $CaCO_3$ as rumen buffer and a strategy to prevent milk fat depression in dairy cows fed high concentrate diets. Thus, limestone was used in CON and BICARB diets as a Ca source, assuming that its effects on rumen pH and lactational performance would be minimal. For $NaHCO_3$, on the other hand, a meta-analysis by Hu and Murphy (2005) concluded that the supplementation at 0.7 to 1% of DM in corn silage-based diets enhanced DMI by 1.2 kg/d, milk fat concentration by 2.7 g/kg, without affecting milk production, and milk protein yield or concentration. These results agree with data from the present study, except for the decreased DMI by the rumen buffers.

Studies evaluating aragonite as a source of $CaCO_3$ are scarce. Clark Jr. et al. (1986) did not report treatments effects of diets containing different $CaCO_3$ sources (i.e., calcite flour, aragonite, and albacar) and Ca concentrations (i.e., 0.6 versus 0.9% DM). In contrast, cows fed calcite flour and aragonite had higher DMI and MY during week 5 to 10 in an 18-wk experiment in the study by Wohlt et al. (1986). Finkelstein et al. (1993) used aragonite as a reference

standard at 1.6% inclusion (DM basis) in diets containing 0.9% Ca to evaluate the effects of ocean quahog and surf clam shells as Ca supplements in lactating dairy cow diets. In that study, treatments did not affect MY, milk components, or BW, even though DMI was numerically lower in cows fed aragonite. When compared with $CaCO_3$ supplemented diets, decreased DMI by NaCl and $NaHCO_3$ has been explained by an increased water intake, rumen fluid dilution rate, and passage rate as a result of greater Na intake in cows fed the latter diets (Rogers et al., 1982). In the current study, diets were balanced to provide equal amounts of absorbable Na, and differences in water intake and rumen fluid dilution rate among treatments were not expected. Therefore, $CaCO_3$ source was the only variable to be considered when evaluating the effects of ARAG versus CON diets. The reason for the decrease in DMI by ARAG relative to CON is not clear and could not be explained by or associated with differences in digestibility of nutrients, as presented later in the manuscript. The current study also suggests that both BICARB and ARAG maintained a more adequate rumen environment, compared with CON, resulting in greater milk fat concentration in the former treatments. It should be noted, however, that our study was not able to determine whether this effect was a consequence of increased buffering capacity and decreased production of biohydrogenation intermediates in the rumen, increased milk FA precursors (see following section), or decreased DMI and consequently fermentable OM in the rumen of BICARB and ARAG cows.

Ruminal Fermentation and Enteric Gas Emission

Ruminal pH was not affected by treatments in the current study (Table 3; Figure 2). Ruminal NH_3 concentration was increased ($P < 0.01$) by ARAG, compared with BICARB, and tended ($P = 0.06$) to be increased by ARAG, compared with CON. Treatments did not affect total VFA concentration and VFA profile, except for an increased ($P < 0.01$) or a tendency for

increased (P = 0.09) butyrate concentration in ARAG and BICARB, respectively, compared with CON. It is noted that both ARAG and BICARB numerically increased A:P by 0.20 units, compared with CON (P = 0.11). Enteric gas emission metrics were not affected by treatments in the current study (Table 4).

Calcium carbonate was ineffective in altering ruminal pH, fluid dilution rate, molar percentages of acetate and propionate, and synthesis of milk fat in the study by Rogers et al. (1982). According to these authors, previous research demonstrating a positive effect of $CaCO_3$ sources on rumen pH used samples obtained from stomach tubing or slaughter, and results might not be comparable to a sample taken directly from a rumen-fistulated animal. Our results align with the Rogers et al. (1982) study since treatments, including BICARB, did not affect ruminal pH and ruminal fluid samples were collected using the stomach tubing technique. It is important to note that $CaCO_3$ rapidly decreases in reactivity when pH is increased above 5.5 (Rogers et al., 1982), despite its high acid consumption capacity (Erdman, 1988). Similarly, ruminal solubility of aragonite should not be expected to be greater than the solubility of $CaCO_3$ since both have similar chemical composition. However, an in vitro study conducted in our laboratory (unpublished data by D. E. Wasson and A. N. Hristov, The Pennsylvania State University) provided evidence for similar ruminal pH between aragonite and $NaHCO_3$ (average pH: 5.28 and 5.32 ± 0.006, respectively) at ≥ 6 h of incubation when supplemented at up to 3% of DM, which may indicate a greater rumen solubility of aragonite, possibly related to its smaller and more uniform particle size (100 µm), compared with a conventional $CaCO_3$ source used in the study (…….. µm). Overall, coarser particles are negatively associated with solubility and reactivity of $CaCO_3$ sources (Jasaitis et al., 1987). Reaction times (i.e., $T_{50, min}$; time for 50% of the sample to react at a specific pH) for calcite flour, aragonite, and albacar samples were 300,

1,500, and 3.3 min at pH 6, and 10.8, 40.0, and 0.6 min at pH 3, respectively, in the study by Wohlt et al. (1986). These $CaCO_3$ sources had different origins (quarry, ocean precipitate, and chemically precipitated), and their particle size ranged from 10-70, 50-1190, and 0.35-6 μm for calcite flour, aragonite, and albacar, respectively (Wohlt et al., 1986).

Erdman (1988) summarized 82 experiments and concluded that the use of $NaHCO_3$, MgO, $KHCO_3$, and other buffering agents were effective in increasing rumen pH, A:P, and milk fat concentration in dairy cows fed low forage (i.e., < 30% forage) and corn silage-based diets. Erdman (1988), however, did not investigate the effects of $CaCO_3$ sources. Even though $NaHCO_3$ was used as a positive control for rumen buffering, treatments did not affect rumen pH in the current study. It is noted, however, that studies have reported inconsistent effects of $NaHCO_3$ supplementation on rumen pH of dairy cows. When evaluating $NaHCO_3$ and $CaCO_3$ (alone or in combination) on ruminal fermentation in a high-starch diet, Rogers et al. (1985) described a decreased ruminal pH, and increased total VFA concentration by $NaHCO_3$, compared with $CaCO_3$ alone, which might be related to an increased feed intake of cows in the former treatment. The supplementation of $CaCO_3$ increased total VFA and lactic acid, compared with $NaHCO_3$ and control (Emery et al., 1964), while only $NaHCO_3$ decreased the molar proportion of propionate by 35% and tended to increase molar proportion of butyrate in the experiment by Rogers et al. (1982). More recently, Bach et al. (2018) concluded that $NaHCO_3$ prevents the decline in milk production, but not the decrease in DMI, and the proportion of time that rumen pH was < 5.8 when cows were challenged with 3 kg/d of barley. Similarly, Bach et al. (2023) demonstrated that cows supplemented with $NaHCO_3$ had lower rumen pH and increased proportion of time with pH < 5.8 than cows fed control or MgO at 44:56 and/or 36:64 forage-to-concentrate ratio diets.

Despite the inconsistent effect of $NaHCO_3$ on rumen pH described in the literature, the meta-analysis by Hu and Murphy (2005) supported 0.13 units increase in ruminal pH and decreased molar proportion of propionate in cows fed corn silage-based diets supplemented with $NaHCO_3$. The mechanisms by which treatments affected the diurnal pattern of ruminal fermentation, VFA production and absorption, and rates of digestion and passage were not evaluated in the current study. Nevertheless, based on the sustained milk fat content, increased butyrate molar proportion, and numerically increased A:P, it can be speculated that both BICARB and ARAG treatments promoted an enhanced rumen environment, compared with CON. These results also align with the increased *iso*-FA and decreased odd-chain FA concentrations in milk fat by BICARB and/or ARAG, indicating an increased fibrolytic activity, compared with CON (please find discussion below). Considering all results, it is plausible to propose that rumen buffers decreased pH fluctuations and proportion of time with low pH (e.g., < 5.8 or 5.5) in this study, but differences could not be detected in rumen fluid samples collected at 0 and 4 h relative to feeding, when rumen samples were collected using the ororuminal tubing technique.

Fatty Acid Profile of Milk Fat

Fatty acid profile of milk fat data are presented in Table 5. Milk concentrations of C4:0 and C6:0 were increased ($P \leq 0.03$) by BICARB, compared with CON. Similarly, C18:0 was increased ($P = 0.01$) by BICARB, compared with CON, and tended ($P = 0.06$) to be increase by BICARB, compared with ARAG. De novo, mixed, and preformed FA were not affected by treatments in the current study. Branched-chain FA were increased ($P < 0.01$) by BICAR, compared with CON, and tended ($P = 0.06$) to be increased by BICARB, compared with ARAG. Odd-chain FA was decreased ($P \leq 0.03$) by BICARB and ARAG, compared with CON.

BICARB increased ($P < 0.01$) *iso*-FA, compared with CON. It is noted that C4:0 response in milk fat aligned with the tendency for increased molar proportion of butyrate in the rumen of BICARB cows. Additionally, treatment differences in *iso*-FA and OCFA are indicative of enhanced ruminal environment for fibrolytic bacteria by both BICARB and ARAG, compared with CON. Vlaeminck et al. (2006) reported that fibrolytic and amylolytic bacteria are generally enriched with *iso*-FA and OCFA (and *anteiso*-FA), respectively, corroborating with the data presented in the current study (i.e., increased *iso*-FA and decreased OCFA concentrations in milk fat, and increased butyrate molar proportion and numerically decreased A:P in ruminal fluid samples).

Digestibility and Urinary Excretions

Treatments did not affect intake of nutrients during the fecal sampling week, except for a tendency for decreased ($P = 0.07$) aNDF and a decreased ($P = 0.05$) ADF intakes by BICARB and ARAG, compared with CON (Table 6). Apparent total-tract digestibility of nutrients was not affected by treatments in the current study. Additionally, treatments did not affect dietary N intake and urine and fecal outputs (Table 7). Treatments also did not affect daily N excretion or secretion variables expressed in g/d or as % of N intake.

Russell and Chow (1993) proposed an alternative mode of action for $NaHCO_3$, attributing its effects to increased water intake, fluid dilution rate, and flow of digesta and starch, limiting propionate production and enhancing rumen pH. These changes in the rumen kinetics could affect starch digestibility; however, potential effects of rumen buffers on decreasing ruminal starch digestibility would be offset by limestone supplementation for the BICARB diet through enhanced intestinal pH and α-amylase activity (Wheeler and Noller, 1976; Wheeler, 1980). Earlier studies reported that the apparent benefits from $CaCO_3$ supplementation on the

digestibility of nutrients occurred in the lower gastrointestinal tract through promotion of a more desirable pH for pancreatic α-amylase activity (i.e., pH = 6.9) in beef and dairy cattle. For instance, Wheeler and Noller (1976) and Wheeler (1980) confirmed their hypothesis that supplementation of $CaCO_3$ at 2.1% DM to diets containing 34 to 35% starch would improve starch digestibility and decrease fecal pH. Rogers et al., (1982) reported increased starch digestibility by both $NaHCO_3$ and $CaCO_3$ in dairy cows fed low forage and high starch diets (25:75 forage-to-concentrate ratio and 52% starch). On the contrary, supplementation of $NaHCO_3$ and $CaCO_3$ did not affect digestibility of nutrients in cows fed high starch diets in the study by Rogers et al. (1985). Overall, data from the current study do not indicate that the increased feed efficiency observed for BICARB and ARAG could be related to increased total-tract digestibility of nutrients and efficiency of N utilization.

Blood Acid-Base Balance and Metabolites

A treatment × time interaction was observed for blood pH in the current study ($P = 0.04$; Table 8), where BICARB tended ($P \leq 0.09$) to increase blood pH at 0 and 6 h relative to feeding, and ARAG increased ($P = 0.01$) it at 6 h relative to feeding, compared with CON (Figure 3a). Base excess (**BEecf**) was increased ($P \leq 0.03$) by BICARB, compared with CON and ARAG. Blood HCO_3 concentration was increased ($P \leq 0.02$) by BICARB, compared with CON and ARAG, and tended ($P = 0.07$) to be increased by ARAG, compared with CON. Blood concentration of Na was decreased ($P = 0.03$) by BICARB, compared with CON, and blood concentration of iCa was increased ($P = 0.02$) by ARAG, compared with BICARB. Glucose concentration was decreased ($P \leq 0.04$) by ARAG, compared with CON and BICARB, whereas haptoglobin, BHB, and BUN concentrations were not affected by treatment in the current study.

The supplementation of different $CaCO_3$ sources (calcite flour, calcite, and albacar) did not affect true absorption of Ca in the study by Wohlt et al. (1986), and Ca retention was increased by feeding 0.9 compared with 0.6% Ca across all $CaCO_3$ sources. Supplementation with $NaHCO_3$ increased blood pH, pCO_2 and HCO_3 more than $CaCO_3$, compared with a non-supplemented basal diet, in the study by Rogers et al. (1985). Erdman (1988) reported that very few studies have shown statistically significant responses in blood pH, pCO_2, or HCO_3 to dietary addition of $NaHCO_3$, $KHCO_3$ or MgO. Nevertheless, the general trend is toward increases in blood pH, HCO_3 and pCO_2 with added dietary $NaHCO_3$ (Erdman, 1988) in studies where significant effects were observed. Data from the current study showed that both BICARB and ARAG were able to increase blood pH in a timely manner, compared with CON, even though DCAD of ARAG was lower than that of BICARB (167 versus 260 mEq/kg DM, respectively). Blood pH and HCO_3 are positively associated with DCAD (quadratic response; $R^2 = 0.83$ and 0.88, respectively; Hu and Murphy, 2004), and the fact that ARAG increased blood pH in the current study indicates that there is a potential mechanism by which aragonite supplementation affects the acid-base status of dairy cows. The physiological control of the acid-base balance to maintain electroneutrality (Goff, 2018) may indicate some of this mechanism. Considering that aragonite increases Ca availability for absorption, the increased blood concentration of Ca^{+2} must be offset by a decrease in H^+ and increase in OH^- ions, thus increasing blood pH. This should be investigated in future research.

Although diets were formulated to provide equal amounts of absorbable Na, BICARB cows tended to have decreased blood Na concentration, which may be explained by the fact that NaCl was added to CON and ARAG diets only. It is noted that ARAG increased overall iCa concentration in blood compared with BICARB, but not with CON cows. When considering the

effects of treatments over time (treatment × time interaction, $P = 0.27$), ARAG increased ($P < 0.001$) iCa concentration at 12 h relative to feeding, compared with both CON and BICARB (Figure 3b). Acid-base balance and Ca concentrations in blood are strictly regulated physiological mechanism. Our data support the hypothesis that the increased surface area of ARAG may have a potential to enhance Ca absorption in dairy cattle, but the DCAD effect on blood iCa concentration should not be excluded. Feeding lower DCAD diets prepartum is known to increase blood iCa in the postpartum period in dairy cows (Santos et al., 2019; Glosson et al., 2020). Freitag et al. (2021) also demonstrated that feeding low DCAD (i.e., -335 and -289 versus 150 and 152 mEq/kg of DM) to neutered male sheep was associated with lower blood pH, higher urinary Ca excretion, higher iCa in blood, higher serum Ca concentrations, and increased apparent Ca digestibility. It should be noted, however, that the implications of slightly varying positive DCAD diets on the acid-base and Ca metabolism of lactating dairy cows have not been investigated. Although it can be assumed that feeding relatively lower DCAD diets during the lactation period would enhance Ca excretion in urine and iCa concentration in blood, the magnitude of these changes is expected to be small when comparing with the physiological effects reported for negative DCAD diets fed to cows during the prepartum period.

CONCLUSIONS

Dietary supplementation of BICARB and ARAG increased feed efficiency and milk fat content while not affecting MY or ECM in lactating dairy cows. Differences in lactational performance could not be explained by ruminal fermentation variables or total-tract digestibility of nutrients, except for an increase, or tendency to increase, butyrate molar proportion by BICARB and ARAG, relative to the CON. These results suggest a potential enhancement of ruminal environment and fibrolytic activity by BICARB and ARAG, aligning with the increased *iso*-FA and decreased OCFA concentrations observed in the milk fat of cows in those treatments.

Postprandial blood pH was increased by both BICARB and ARAG, whereas ARAG increased overall blood iCa concentration compared with BICARB. The mechanism by which ARAG affects the acid-base status and Ca metabolism in dairy cows remain to be investigated.

NOTES

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REFERENCES

Allen, M. S. 1997. Relationship Between Fermentation Acid Production in the Rumen and the Requirement for Physically Effective Fiber. J. Dairy Sci. J Dairy Sci 80:1447–1462. https://doi.org/10.3168/jds.S0022-0302(97)76074-0.

AOAC International. 2000. Official Methods of Analysis. 17th ed. AOAC International, Arlington, VA.

AOAC International. 2006. Official Methods of Analysis. 18th ed. AOAC International, Arlington, VA.

Aschenbach, J. R., S. Bilk, G. Tadesse, F. Stumpff, and G. Gäbel. 2009. Bicarbonate-dependent and bicarbonate-independent mechanisms contribute to nondiffusive uptake of acetate in the ruminal epithelium of sheep. Am. J. Physiol. Gastrointest. Liver. Physiol. 296: G1098–G1107 https://doi.org/10.1152/ajpgi.90442.2008.

Aschenbach, J. R., G. B. Penner, F. Stumpff, and G. Gäbel. 2011. RUMINANT NUTRITION SYMPOSIUM: Role of fermentation acid absorption in the regulation of ruminal pH. J. Anim. Sci. J. Anim. Sci. 2011. 89:1092–1107. https://doi.org/10.2527/jas.2010-3301.

Bach, A., I. Guasch, G. Elcoso, J. Duclos, and H. Khelil-Arfa. 2018. Modulation of rumen pH by sodium bicarbonate and a blend of different sources of magnesium oxide in lactating dairy cows submitted to a concentrate challenge. J. Dairy Sci. 101:9777–9788. https://doi.org/10.3168/jds.2017-14353.

Bach, A., M. Baudon, G. Elcoso, J. Viejo, and A. Courillon. 2023. Effects on rumen pH and feed intake of a dietary concentrate challenge in cows fed rations containing pH modulators with different neutralizing capacity. J. Dairy Sci. 106:4580–4598. https://doi.org/10.3168/jds.2022-22734.

Bergman, E. N., 1990. Energy contributions of volatile fatty acids from the gastrointestinal tract in various species. Physiol. Rev. 70:567-590. https://doi.org/10.1152/physrev.1990.70.2.567.

Brito, L. F., N. Bedere, F. Douhard, H. R. Oliveira, M. Arnal, F. Peñagaricano, A. P. Schinckel, C. F. Baes, and F. Miglior. 2021. Review: Genetic selection of high-yielding dairy cattle toward sustainable farming systems in a rapidly changing world. Animal. 15:100292. https://doi.org/10.1016/j.animal.2021.100292.

Chaney, A.L., and E. P. Marbach. 1962. Modified reagents for determination of urea and ammonia. Clin. Chem. 8:130–132. https://doi.org/10.1093/clinchem/8.2.130.

Chen, X.B., Y.K. Chen, M.F. Franklin, E.R. Orskov, and W.J. Shand. 1992. The effect of feed intake and body weight on purine derivative excretion and microbial protein supply in sheep. J. Anim. Sci. 70:1534–1542. https://doi.org/10.2527/1992.7051534x.

Clark Jr., W. D., J. E. Wohlt, R. L. Gilbreath, and P. K. Zajac. 1986. Phytate Phosphorus Intake and Disappearance in the Gastrointestinal Tract of High Producing Dairy Cows. J. Dairy Sci. 69:3151-3155. https://doi.org/10.3168/jds.S0022-0302(86)80780-9.

Cole, J. B., and M. Spurlock. 2017. Improving production efficiency through genetic selection. Large Dairy Herd Management, 3rd ed. https://doi.org/10.3168/ldhm.0523.

Dijkstra, J., J. L. Ellis, E. Kebreab, A. B. Strathe, S. López, J. France, and A. Bannink. 2012. Ruminal pH regulation and nutritional consequences of low pH. Anim. Feed Sci. Technol. 172:22-33. https://doi.org/10.1016/j.anifeedsci.2011.12.005.

Emery, R. S., L. D. Brown, and J. W. Thomas. 1964. Effect of sodium and calcium carbonates on milk production and composition of milk, blood, and rumen contents of cows fed grain ad libitum with restricted roughage. J. Dairy Sci. 47:1325-1329. https://doi.org/10.3168/jds.S0022-0302(64)88913-X.

Erdman, R. A., 1988. Dietary Buffering Requirements of the Lactating Dairy Cow: A Review. J Dairy Sci. 71 :3246—3266. https://doi.org/10.3168/jds.S0022-0302(88)79930-0.

Finkelstein, A. D., J. E. Wohlt, and S. M. Emanuele. 1993. Composition and Nutritive Value of Ground Sea Clam Shells as Calcium Supplements for Lactating Holstein Cows. J Dairy Sci. 76:582-589. https://doi.org/10.3168/jds.S0022-0302(93)77378-6.

Freitag, J. R. B., M. R. Wilkens, A. S. Muscher-Banse, K. Gerstner, N. Schnepel, P. R. Torgenson, and A. Liesengang. 2021. Effects of diets differing in dietary cation-anion difference and calcium concentration on calcium homeostasis in neutered male sheep. J. Dairy Sci. 104:11537–11552. https://doi.org/10.3168/jds.2021-20334.

Glosson, K. M., X. Zhang, S. S. Bascom, A. D. Rowson, Z. Wang, and J. K. Drackley. Negative dietary cation-anion difference and amount of calcium in prepartum diets: Effects on

milk production, blood calcium, and health. 2020. J. Dairy Sci. 103:7039–7054 https://doi.org/10.3168/jds.2019-18068.

Goff, J. P. 2018. Invited review: Mineral absorption mechanisms, mineral interactions that affect acid–base and antioxidant status, and diet considerations to improve mineral status. J. Dairy Sci. 101:2763–2813. https://doi.org/10.3168/jds.2017-13112.

Hall, M. B. 2009. Determination of starch, including maltooligosaccharides, in animal feeds: Comparison of methods and a method recommended for AOAC collaborative study. J. AOAC Int. 92:42–49. https://doi.org/10.1093/jaoac/92.1.42.

Hristov, A. N., C. Lee, T. W. Cassidy, M. Long, B. Corl, and R. Forster. 2011. Effects of lauric and myristic acids on ruminal fermentation, production, and milk fatty acid composition in lactating dairy cows. J. Dairy Sci. 94:382–395. http://doi.org/10.3168/jds.2010-3508.

Hu, W., and M. R. Murphy. 2004. Dietary Cation-Anion Difference Effects on Performance and Acid-Base Status of Lactating Dairy Cows: A Meta-Analysis. J. Dairy Sci. 87:2222–2229. https://doi.org/10.3168/jds.2006-515.

Hu, W., and M. R. Murphy. 2005. Statistical evaluation of early- and mid-lactation dairy cow responses to dietary sodium bicarbonate addition. Anim. Feed Sci. Technol. 119:43-54. https://doi.org/10.1016/j.anifeedsci.2004.12.005.

Huhtanen, P., K. Kaustell, and S. Jaakkola. 1994. The use of internal markers to predict total digestibility and duodenal flow of nutrients in cattle given six different diets. Anim. Feed Sci. Technol. 48:211–227. https://doi.org/10.1016/0377-8401(94)90173-2.

Jasaitis D. K., J. E. Wohlt, and J. L. Evans. 1987. Influence of Feed Ion Content on Buffering Capacity of Ruminant Feedstuffs In Vitro. J. Dairy. Sci. 70:1391-1403. https://doi.org/10.3168/jds.S0022-0302(87)80161-3.

Lage, C. F. A., S. E. Räisänen, A. Melgar, K. Nedelkov, X. Chen, J. Oh, M. E. Fetter, N. Indugu, J. S. Bender, B. Vecchiarelli, M. L. Hennessy, D. Pitta, and A. N. Hristov. 2020. Comparison of Two Sampling Techniques for Evaluating Ruminal Fermentation and Microbiota in the Planktonic Phase of Rumen Digesta in Dairy Cows. Front. Microbiol. 11:618032. https://doi.org/10.3389/fmicb.2020.618032.

Lee, C., A. N. Hristov, T. W. Cassidy, K. S. Heyler, H. Lapierre, G. A. Varga, M. J. De Veth, R. A. Patton, and C. Parys. 2012. Rumen- protected lysine, methionine, and histidine increase milk protein yield in dairy cows fed a metabolizable protein-deficient diet. J. Dairy Sci. 95:6042–6056. https://doi.org/10.3168/jds.2012-5581.

NRC. 2001. Nutrient Requirements of Dairy Cattle. 7th rev. ed. Natl. Acad. Press.

Plaizier, J. C., D. O. Krause, G. N. Gozho, and B. W. McBride. 2009. Subacute ruminal acidosis in dairy cows: The physiological causes, incidence and consequences. Vet. J. 176:21-31. https://doi.org/10.1016/j.tvjl.2007.12.016.

Rico, D. E., and K. J. Harvatine. 2013. Induction of and recovery from milk fat depression occurs progressively in dairy cows switched between diets that differ in fiber and oil concentration. J. Dairy Sci. 96:6621–6630. https://doi.org/10.3168/jds.2013-6820.

Rogers, J. A., C. L. Davis, and J. H. Clark. 1982. Alteration of Rumen Fermentation, Milk Fat Synthesis, and Nutrient Utilization with Mineral Salts in Dairy Cows. J. Dairy Sci. 65:577-586. https://doi.org/10.3168/jds.S0022-0302(82)82235-2.

Rogers, J. A., L. D. Muller, C. L. Davis, W. Chalupa, D. S. Kronfeld, L. F. Karcher, and K. R. Cummings. 1985. Response of Dairy Cows to Sodium Bicarbonate and Limestone in Early Lactation. J. Dairy Sci. 68:646-660. https://doi.org/10.3168/jds.S0022-0302(85)80871-7.

Russell, J. B., and J. M. Chow. 1993. Another Theory for the Action of Ruminal Buffer Salts: Decreased Starch Fermentation and Propionate Production. J Dairy Sci .76:826-830. https://doi.org/10.3168/jds.S0022-0302(93)77407-X.

Santos, J. E. P., I. J. Lean, H. Golder, and E. Block. 2019. Meta-analysis of the effects of prepartum dietary cation-anion difference on performance and health of dairy cows. J. Dairy Sci. 102:2134–2154. https://doi.org/10.3168/jds.2018-14628.

Sjaunja, L. O., L. Baevre, L. Junkkarinen, J. Pedersen, and J. Setälä. 1990. A Nordic proposal for an energy corrected milk (ECM) formula. Pages 156–157 in 27th Session of the International Commission for Breeding and Productivity of Milk Animals, Paris, France. Wageningen Academic Publishers.

Van Soest, P. J., J. B. Robertson, and B. A. Lewis. 1991. Methods for dietary fiber, neutral detergent fiber, and nonstarch polysaccharides in relation to animal nutrition. J. Dairy Sci. 74:3583–3597. https://doi.org/10.3168/jds.S0022-0302(91)78551-2.

Vlaeminck, B., V. Fievez, A. R. J. Cabrita, A. J. M. Fonseca, and R. J. Dewhurst. 2006. Factors affecting odd- and branched-chain fatty acids in milk: A review. Anim. Feed Sci. Technol. 131:389–417. https://doi.org/10.1016/j.anifeedsci.2006.06.017.

Wheeler, W. E., and C. H. Noller. 1976. Limestone Buffers in Complete Mixed Rations for Dairy Cattle. J. Dairy Sci. 59:1788-1793. https://doi.org/10.3168/jds.S0022-0302(76)84438-4.

Wheeler, W. E. 1980. Effect of Limestone Buffers on Digestibility of Complete Diets and on Performance by Dairy Cows. J. Dairy Sci. 63:1848-1854. https://doi.org/10.3168/jds.S0022-0302(80)83150-X.

Wohlt, J. E., D. E. Ritter, and J. L. Evans. 1986. Calcium Sources for Milk Production in Holstein Cows Via Changes in Dry Matter Intake, Mineral Utilization, and Mineral Source Buffering Potential. J. Dairy Sci. 69:2815-2824. https://doi.org/10.3168/jds.S0022-0302(86)80735-4.

Wohlt, J. E., D. K. Jasaitis, and J. L. Evans. 1987. Use of Acid and Base Titrations to Evaluate the Buffering Capacity of Ruminant Feedstuffs In Vitro. J. Dairy Sci. J Dairy Sci 70:1465-1470. https://doi.org/10.3168/jds.S0022-0302(87)80170-4.

Wood, M., C. T. Hayes, and A. Paytan. 2023. Global Quaternary Carbonate Burial: Proxy- and Model-Based Reconstructions and Persisting Uncertainties. Annu. Rev. Mar. Sci. 2023. 15:277–302. https://doi.org/10.1146/annurev-marine-031122-031137.

Yang, C.M.J., and G.A. Varga. 1989. Effect of Three Concentrate Feeding Frequencies on Rumen Protozoa, Rumen Digesta Kinetics, and Milk Yield in Dairy Cows. J. Dairy Sci. 72:950–957. https://doi.org/10.3168/jds.S0022-0302(89)79188-8.

Table 1. Ingredient and chemical composition of the diets fed to dairy cows during the experiment

Item	Treatment[1]		
	CON	BICARB	ARAG
Ingredient, % of DM or as indicated			
Corn silage[2]	35.5	35.2	35.2
Alfalfa haylage[3]	12.4	12.3	12.3
Grass hay[4]	4.3	4.2	4.2
Corn grain, ground	16.9	17.5	17.6
Canola meal	9.5	9.3	9.4
Heat-treated soybean meal[5]	4.8	4.7	4.7
Whole cottonseed	5.9	5.8	5.8
Roasted soybeans	4.8	4.7	4.7
Molasses[6]	3.3	3.2	3.3
Mineral and vitamin premix[7]	1.4	1.4	1.4
Limestone ($CaCO_3$)	0.80	0.80	-
$NaHCO_3$	-	0.80	-
Aragonite	-	-	0.80
NaCl	0.55	-	0.55
Composition, % of DM			
CP[8]	16.6	16.5	16.6
RDP[9]	10.2	10.1	10.2
RUP[9]	6.4	6.4	6.3
aNDF[8]	28.6	28.4	28.4
ADF[8]	19.9	19.7	19.7
Starch[8]	27.7	28.1	28.1
Ether extract[8]	5.4	5.4	5.4
NFC[8]	44.1	44.3	44.4
NE_L, Mcal/kg DM[9]	1.64	1.64	1.65
NE_L balance, Mcal/d[9]	1.3	0.0	0.3
MP supply, g/d[9]	2,531	2,525	2,491
MP balance, g/d[9]	-14	-37	3
Absorbable Ca, g/d[9]	91	90	94
Absorbable P, g/d[9]	65	64	64
Absorbable Na, g/d[9]	73	73	71
DCAD, mEq/kg DM[8]	168	260	167

[1]Treatments were: CON = basal diet supplemented with 0.80% limestone and 0.55% NaCl; BICARB = basal diet supplemented with 0.80% limestone and 0.80% $NaHCO_3$; ARAG = basal diet supplemented with 0.80% aragonite (Rumen Cal +, Ag Source, LLC) and 0.55% NaCl.

[2]Corn silage was 49.7% DM and contained (% of DM): 6.4 CP and 33.5 aNDF.

[3]Alfalfa haylage was 36.0% DM and contained (% of DM): 20.3 CP and 41.2 aNDF.

[4]Grass hay was 87.4% DM and contained (% of DM): 9.9 CP and 65.1 aNDF.

[5]Heat-treated soybean meal (SoyPLUS, Landus Cooperative).

[6]Liquid molasses from Westway Feed Products.

[7]Premix (Cargill, Inc.) contained (% of DM or as indicated): 19.2 CP, 12.7 ADF, 25.0 NDF, 0.39 Ca, 0.54 P, 4.69 Mg, 8.22 Cl, 0.71 K, 5.42 Na, 0.28 S, 8.77 mg/kg Co, 602.9 mg/kg Cu, 38.6 mg/kg I, 114.9 mg/kg Fe, 1,619.3 mg/kg Mn, 12.4 mg/kg Se, 2,145.9 mg/kg Zn, 223,000 IU/kg vitamin A, 81,300 IU/kg vitamin D, and 1395 IU/kg vitamin E.

[8]Values calculated using the nutrient analysis of the individual feed ingredients (Cumberland Valley Analytical Services Inc.) and their dietary inclusion rates.

[9]Estimated based on NRC (2001) using actual DMI, milk yield, milk composition, and BW of the cows throughout the experiment.

Table 2. Lactational performance of dairy cows fed diets supplemented with different sources of calcium and rumen buffer

Item	Treatment[1]			SEM[2]	*P*-value[3]
	CON	BICARB	ARAG		
DMI, kg/d	23.0^{ax}	22.6^{aby}	22.3^{b}	0.83	0.02
Milk yield, kg/d	37.9	38.4	37.6	2.05	0.46
Feed efficiency, kg/kg	1.64^{b}	1.71^{a}	1.70^{a}	0.091	0.03
Milk fat, %	3.32^{b}	3.58^{a}	3.58^{a}	0.116	0.003
Milk fat yield, kg/d	1.28	1.34	1.32	0.050	0.38
Milk true protein, %	3.09^{a}	3.08^{abx}	3.03^{by}	0.063	0.05
Milk true protein yield, kg/d	1.17	1.19	1.14	0.039	0.38
Milk lactose, %	4.93	4.92	4.91	0.041	0.25
Milk lactose yield, kg/d	1.87	1.89	1.85	0.109	0.68
TS, %	12.3^{b}	12.6^{a}	12.5^{a}	0.139	0.02
TS yield, kg/d	4.75	4.79	4.66	0.170	0.50
ECM yield, kg/d	34.1	35.2	34.2	1.38	0.56
ECM feed efficiency, kg/kg	1.46	1.53	1.53	0.084	0.19
MUN, mg/dL	8.17^{b}	8.96^{a}	8.92^{a}	0.487	0.03
SCC ($\times 10^3$ cells/mL)[4]	4.00 (153.0)	4.29 (162.7)	3.96 (85.9)	0.312 (60.9)	0.33

^{a,b}Means with different superscript letters differ at $P \leq 0.05$ separated by pairwise *t*-test.

^{x,y}Means with different superscript letters differ at $0.05 < P \leq 0.10$ separated by pairwise *t*-test.

[1]Treatments were: CON = basal diet supplemented with 0.80% limestone and 0.55% NaCl; BICARB = basal diet supplemented with 0.80% limestone and 0.80% $NaHCO_3$; ARAG = basal diet supplemented with 0.80% aragonite (Rumen Cal +, Ag Source, LLC) and 0.55% NaCl.

[2]Largest SEM published in table; n = 536 to 540 for DMI, milk yield, and feed efficiency; n = 53 to 54 for all other variables (n represents number of observations used in the statistical analysis).

[3]Main effect of treatment. Day effect: $P \leq 0.001$ for all variables. Parity effect: $P \geq 0.08$ for all variables. Treatment × day interaction: $P > 0.43$ for all variables. Treatment × parity interaction: $P = 0.06$ for DMI; for all other variables, $P \geq 0.28$.

[4]Statistical analysis was performed on log-transformed data. Actual data ($\times 10^3$ cells/mL) are given in parentheses.

Table 3. Ruminal fermentation of dairy cows fed diets supplemented with different sources of calcium and rumen buffer

Item	Treatment[1]			SEM[2]	*P*-value[3]
	CON	BICARB	ARAG		
pH	6.79	6.72	6.68	0.046	0.36
NH_3, m*M*	3.94^{aby}	3.30^{b}	4.82^{ax}	0.436	0.006
Total VFA, m*M*	99.6	103.4	105.7	2.86	0.30
VFA as % of total					
Acetate	58.9	59.6	58.8	0.82	0.20
Propionate	23.9	22.5	22.6	1.09	0.11
Isobutyrate	0.70	0.74	0.69	0.037	0.28
Butyrate	13.2^{bz}	13.9^{aby}	14.6^{ax}	0.61	0.005
Isovalerate	1.46	1.42	1.46	0.074	0.82
Valerate	1.73	1.59	1.72	0.198	0.46
Acetate:Propionate	2.53	2.75	2.72	0.161	0.11

[a,b]Means with different superscript letters differ at $P \leq 0.05$ separated by pairwise *t*-test.

[x,y,z]Means with different superscript letters differ at $0.05 < P \leq 0.10$ separated by pairwise *t*-test.

[1]Treatments were: CON = basal diet supplemented with 0.80% limestone and 0.55% NaCl; BICARB = basal diet supplemented with 0.80% limestone and 0.80% $NaHCO_3$; ARAG = basal diet supplemented with 0.80% aragonite (Rumen Cal +, Ag Source, LLC) and 0.55% NaCl.

[2]Largest SEM published in table; n = 103 to 108 for all variables (n represents number of observations used in the statistical analysis).

[3]Main effect of treatment. Time effect: $P \leq 0.03$ for all variables. Parity effect: $P \geq 0.08$ for all variables. Treatment × time interaction: $P > 0.18$ for all variables. Treatment × parity interaction: $P = 0.09$ for NH_3; for all other variables, $P \geq 0.13$.

Table 4. Enteric methane emissions of dairy cows fed diets supplemented with different sources of calcium and rumen buffer

Item	Treatment[1]			SEM[2]	*P*-value[3]
	CON	BICARB	ARAG		
CH_4, g/d	443	444	447	24.2	0.98
CH_4 per DMI, g/kg	19.6	19.8	20.1	0.84	0.84
CH_4 per milk yield, g/kg	11.9	12.1	12.6	1.02	0.54
CH_4 per ECM, g/kg	13.2	13.1	13.8	1.10	0.55
CO_2, g/d	13,036	13,131	12,819	241	0.60
H_2, g/d	1.62	1.67	1.51	0.166	0.37

[1]Treatments were: CON = basal diet supplemented with 0.80% limestone and 0.55% NaCl; BICARB = basal diet supplemented with 0.80% limestone and 0.80% $NaHCO_3$; ARAG = basal diet supplemented with 0.80% aragonite (Rumen Cal +, Ag Source, LLC) and 0.55% NaCl.

[2]Largest SEM published in table; n = 51 to 53 for all variables (n represents number of observations used in the statistical analysis).

[3]Main effect of treatment. Parity effect: $P > 0.05$ for all variables. Treatment × parity interaction: $P = 0.09$ for CH_4 per DMI; for all other variables, $P \geq 0.20$.

Table 5. Fatty acid composition of milk fat (g/100 g of fatty acids) in dairy cows fed diets supplemented with different sources of calcium and rumen buffer

Item	Treatment[1]			SEM[2]	*P*-value[3]
	CON	BICARB	ARAG		
C4:0	3.79^{b}	4.15^{a}	3.95^{ab}	0.126	0.03
C6:0	1.89^{y}	2.07^{x}	1.98^{xy}	0.101	0.09
C8:0	1.01	1.10	1.05	0.064	0.17
C10:0	2.32	2.45	2.37	0.153	0.52
C12:0	2.74	2.69	2.73	0.150	0.90
C14:0	9.93	9.91	9.75	0.189	0.68
cis-9 C14:1	0.86^{a}	0.71^{by}	0.81^{abx}	0.061	0.04
C15:0	1.03^{a}	0.90^{b}	0.93^{b}	0.044	0.02
C16:0	26.4	25.4	26.0	0.97	0.42
cis-9 C16:1	0.03	0.02	0.03	0.004	0.17
C17:0	0.52	0.50	0.50	0.028	0.24
C18:0	11.5^{b}	12.7^{ax}	11.8^{aby}	0.62	0.04
trans-4 C18:1	0.020^{b}	0.024^{a}	0.026^{a}	0.001	<0.01
trans-5 C18:1	0.018^{b}	0.022^{a}	0.025^{a}	0.002	0.08
trans-6,8 C18:1	0.44	0.43	0.45	0.039	0.90
trans-9 C18:1	0.35	0.34	0.35	0.016	0.61
trans-10 C18:1	1.75	1.25	1.58	0.437	0.22
trans-11 C18:1	1.24	1.16	1.18	0.061	0.55
trans-12 C18:1	0.66	0.66	0.70	0.024	0.15
cis-9 C18:1	21.2	20.9	21.4	0.69	0.83
cis-11 C18:1	0.99^{a}	0.88^{b}	0.93^{ab}	0.066	0.10
cis-9, *cis*-12 C18:2	0.06	0.06	0.06	0.003	0.76
C20:0	0.16^{ab}	0.15^{b}	0.17^{a}	0.007	0.10
cis-9, *trans*-11 CLA	0.120^{b}	0.129^{a}	0.126^{ab}	0.005	0.10
Total *trans* FA	4.96	4.44	4.88	0.486	0.42
Σ *De novo*[4]	22.4	23.0	22.6	0.83	0.59
Σ Mixed[5]	27.0	25.9	26.5	0.98	0.40
Σ Preformed[6]	44.4	45.2	45.0	1.55	0.80
Σ OBCFA[7]	2.85	2.84	2.71	0.092	0.22
BCFA	1.07^{b}	1.15^{a}	1.10^{b}	0.031	0.02
OCFA	1.71^{a}	1.54^{b}	1.57^{b}	0.065	0.03
iso-FA	0.69^{b}	0.75^{a}	0.72^{ab}	0.023	0.01
anteiso-FA	0.38	0.39	0.38	0.011	0.17

^{a,b}Means with different superscript letters differ at $P \leq 0.05$ separated by pairwise *t*-test.

^{x,y}Means with different superscript letters differ at $0.05 < P \leq 0.10$ separated by pairwise *t*-test.

[1]Treatments were: CON = basal diet supplemented with 0.80% limestone and 0.55% NaCl; BICARB = basal diet supplemented with 0.80% limestone and 0.80% $NaHCO_3$; ARAG = basal diet supplemented with 0.80% aragonite (Rumen Cal +, Ag Source, LLC) and 0.55% NaCl.

[2]Largest SEM published in table; n = 51 to 54 for all variables (n represents number of observations used in the statistical analysis).

[3]Main effect of treatment. Parity effect: $P \leq 0.06$ for *trans*-9, C18:1, *trans*-12, C18:1, and C20:0; for all other variables, $P \geq 0.12$. Treatment × parity interaction: $P = 0.02$ for C17:0; for all other variables, $P \geq 0.18$.

[4]Σ De novo = sum of C4:0; C6:0; C8:0; C10:0; C12:0; C14:0; and cis 9, C14:1.

[5]Σ Mixed = sum of C16:0; cis 9, C16:1, and C17:0.

[6]Σ Preformed = sum of ≥ 18 C.

[7]Σ OBCFA = sum of identified odd- and branched-chain fatty acids (OBCFA), branched-chain fatty acids (BCFA; *iso* C14:0 to *iso* C17:0, *anteiso* C15:0 to *anteiso* C17:0), odd-chain fatty acids (OCFA; C11:0 to C17:0), *iso* branched-chain fatty acids (*iso*-FA; *iso* C14:0 to *iso* C17:0), and *anteiso* branched-chain fatty acids (*anteiso*-FA; *anteiso* C15:0 to *anteiso* C17:0).

Table 6. Intake and apparent total-tract digestibility of nutrients in dairy cows fed diets supplemented with different sources of calcium and rumen buffer

Item	Treatment[1]			SEM[2]	*P*-value[3]
	CON	BICARB	ARAG		
Intake, kg/d[4]					
DM	21.3	21.0	20.6	0.79	0.17
OM	20.0	19.8	19.5	0.75	0.17
CP	3.53	3.46	3.42	0.131	0.17
aNDF[5]	6.08^{x}	5.95^{y}	5.86^{y}	0.225	0.07
ADF	4.23^{a}	4.13^{b}	4.06^{b}	0.156	0.05
Starch	5.88	5.95	5.86	0.223	0.56
Digestibility, % of intake					
DM	63.8	64.2	63.8	0.57	0.61
OM	65.1	65.4	65.0	0.59	0.65
CP	69.2	69.8	68.5	0.67	0.17
aNDF[5]	34.3	33.4	33.4	0.73	0.34
ADF	40.5	39.4	40.2	0.95	0.45
Starch	98.9	98.8	98.7	0.08	0.18

[a,b]Means with different superscript letters differ at $P \leq 0.05$ separated by pairwise *t*-test.

[x,y]Means with different superscript letters differ at $0.05 < P \leq 0.10$ separated by pairwise *t*-test.

[1]Treatments were: CON = basal diet supplemented with 0.80% limestone and 0.55% NaCl; BICARB = basal diet supplemented with 0.80% limestone and 0.80% $NaHCO_3$; ARAG = basal diet supplemented with 0.80% aragonite (Rumen Cal +, Ag Source, LLC) and 0.55% NaCl.

[2]Largest SEM published in table; n = 52 to 54 for all variables (n represents number of observations used in the statistical analysis).

[3]Main effect of treatment. Parity effect: $P \geq 0.13$ for all variables. Treatment × parity interaction: $P \leq 0.10$ for all variables, except $P \geq 0.21$ for DM, OM, and ADF digestibility.

[4]Average intake during the last 10-d of each experimental period (i.e., data collection period).

[5]Amylase-treated NDF.

Table 7. Nitrogen secretion and excretion of dairy cows fed diets supplemented with different sources of calcium and rumen buffer

Item	Treatment[1]			SEM[2]	*P*-value[3]
	CON	BICARB	ARAG		
N intake, g/d	564	553	548	20.9	0.16
Urine output, kg/d	23.5	23.1	23.7	2.03	0.98
Fecal output, kg/d DM	7.80	7.59	7.52	0.388	0.23
N excretion or secretion, g/d					
Urine N	183	173	190	13.2	0.44
UUN[4]	176	168	186	15.7	0.42
Fecal N	177	170	175	9.2	0.46
Total excreta N	360	345	370	20.8	0.18
Milk N[5]	188	191	184	7.5	0.45
Unaccounted N[6]	29.2	28.3	18.6	12.19	0.79
As % of N intake					
Urine N	33.1	32.2	35.1	2.51	0.50
UUN[4]	31.9	31.2	34.3	2.57	0.61
Fecal N	30.8	30.2	31.5	0.67	0.17
Total excreta N	66.0	62.0	63.2	1.93	0.33
Milk N[5]	33.3	34.9	33.6	1.45	0.12
Unaccounted N[6]	5.2	4.0	2.6	2.17	0.66
Urinary PD[7] excretion, mmol/d					
Allantoin	834	875	927	103.6	0.70
Uric acid	143	159	148	14.9	0.50
Total PD	913	889	1,020	87.0	0.42

[a,b]Means with different superscript letters differ at $P \leq 0.05$ separated by pairwise *t*-test.

[x,y]Means with different superscript letters differ at $0.05 < P \leq 0.10$ separated by pairwise *t*-test.

[1]Treatments were: CON = basal diet supplemented with 0.80% limestone and 0.55% NaCl; BICARB = basal diet supplemented with 0.80% limestone and 0.80% $NaHCO_3$; ARAG = basal diet supplemented with 0.80% aragonite (Rumen Cal +, Ag Source, LLC) and 0.55% NaCl.

[2]Largest SEM published in table; n = 48 to 54 for all variables (n represents number of observations used in the statistical analysis).

[3]Main effect of treatment. Parity effect: $P \geq 0.11$ for all variables. Treatment × parity interaction: $P \leq 0.06$ for N intake, unaccounted N (g/d and %), and fecal N (%); $P \geq 0.13$ for all other variables.

[4]UUN = urinary urea nitrogen.

[5]Milk N, g/d = [(Milk true protein, g/d ÷ 6.38) + (MUN, g/d ÷ 0.50)].

[6]Unaccounted N = [N intake – (Urinary N + Fecal N + Milk N)].

[7]PD = purine derivatives.

Table 8. Blood variables of dairy cows fed diets supplemented with different sources of calcium and rumen buffer

Item[1]	Treatment[2]			SEM[3]	*P*-value[4]
	CON	BICARB	ARAG		
pH	7.44	7.44	7.44	0.006	0.84
BEecf, mmol/L	3.60^{b}	4.51^{a}	2.99^{b}	0.366	<0.01
HCO_3, mmol/L	27.7^{bx}	28.7^{a}	27.1^{by}	0.32	<0.001
Na, mmol/L	137^{x}	136^{y}	137^{xy}	0.36	0.07
K, mmol/L	4.08	4.09	4.14	0.046	0.49
iCa, mmol/L	1.23^{ab}	1.22^{b}	1.24^{a}	0.009	0.07
Glucose, mg/dL	67.7^{a}	67.0^{a}	65.6^{b}	0.96	0.01
Hct % PCV	24.3^{b}	25.0^{a}	24.6^{ab}	0.35	0.05
Hb, g/dL	8.27^{b}	8.50^{a}	8.36^{ab}	0.120	0.06
Haptoglobin, mg/mL	1.75	1.53	1.32	0.141	0.16
BHB, mmol/L	0.54	0.57	0.63	0.051	0.17
BUN, mg/dL	25.9	32.9	39.0	18.87	0.88

[a,b]Means with different superscript letters differ at $P \leq 0.05$ separated by pairwise *t*-test.

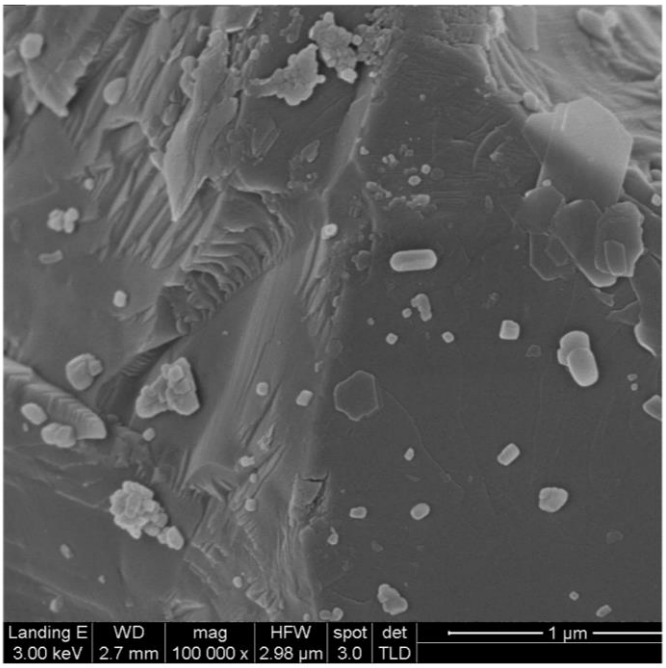
[x,y]Means with different superscript letters differ at $0.05 < P \leq 0.10$ separated by pairwise *t*-test.

[1]BEecf = base excess in the extracellular fluid compartment; HCO_3 = bicarbonate; Na = sodium; K = potassium; iCa = ionized calcium; Hct = hematocrit; Hb = hemoglobin; BHB = β-hydroxybutyrate; BUN = blood urea nitrogen.

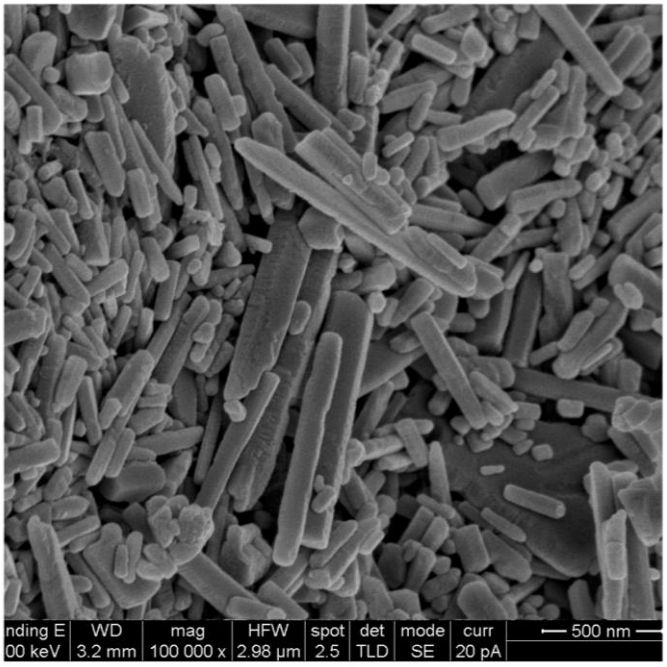
[2]Treatments were: CON = basal diet supplemented with 0.80% limestone and 0.55% NaCl; BICARB = basal diet supplemented with 0.80% limestone and 0.80% $NaHCO_3$; ARAG = basal diet supplemented with 0.80% aragonite (Rumen Cal +, Ag Source, LLC) and 0.55% NaCl.

[3]Largest SEM published in table; n = 206 to 210 for all variables, except n = 52 to 54 for Haptoglobin, BHB, and BUN (n represents number of observations used in the statistical analysis).

[4]Main effect of treatment. Parity effect: $P \geq 0.15$ for all variables. Treatment × time interaction: $P = 0.04$ for pH; for all other variables, $P \geq 0.12$. Treatment × parity interaction: $P \geq 0.11$ for all variables.



A



B

Figure 1. Microphotographs of limestone (**A**) and aragonite (**B**) particles amplified at 1×10^5 (courtesy of Ag Source, LLC). Scale bar = 0.5 and 1 µm for aragonite and limestone, respectively.

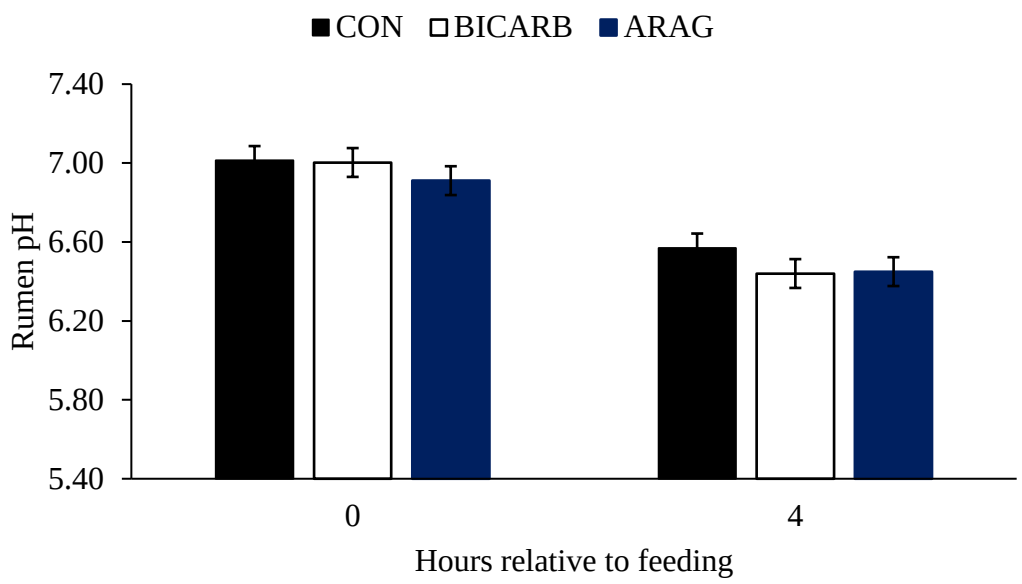


Figure 2. Rumen pH in dairy cows fed diets supplemented with different sources of calcium and rumen buffer. Treatments were: CON = basal diet supplemented with 0.80% limestone and 0.55% NaCl; BICARB = basal diet supplemented with 0.80% limestone and 0.80% $NaHCO_3$; ARAG = basal diet supplemented with 0.80% aragonite (Rumen Cal +, Ag Source, LLC) and 0.55% NaCl. Largest SEM = 0.073; n = 108 (n represents number of observations used in the statistical analysis). Treatment effect: $P = 0.36$; Treatment × time interaction effect: $P = 0.73$.

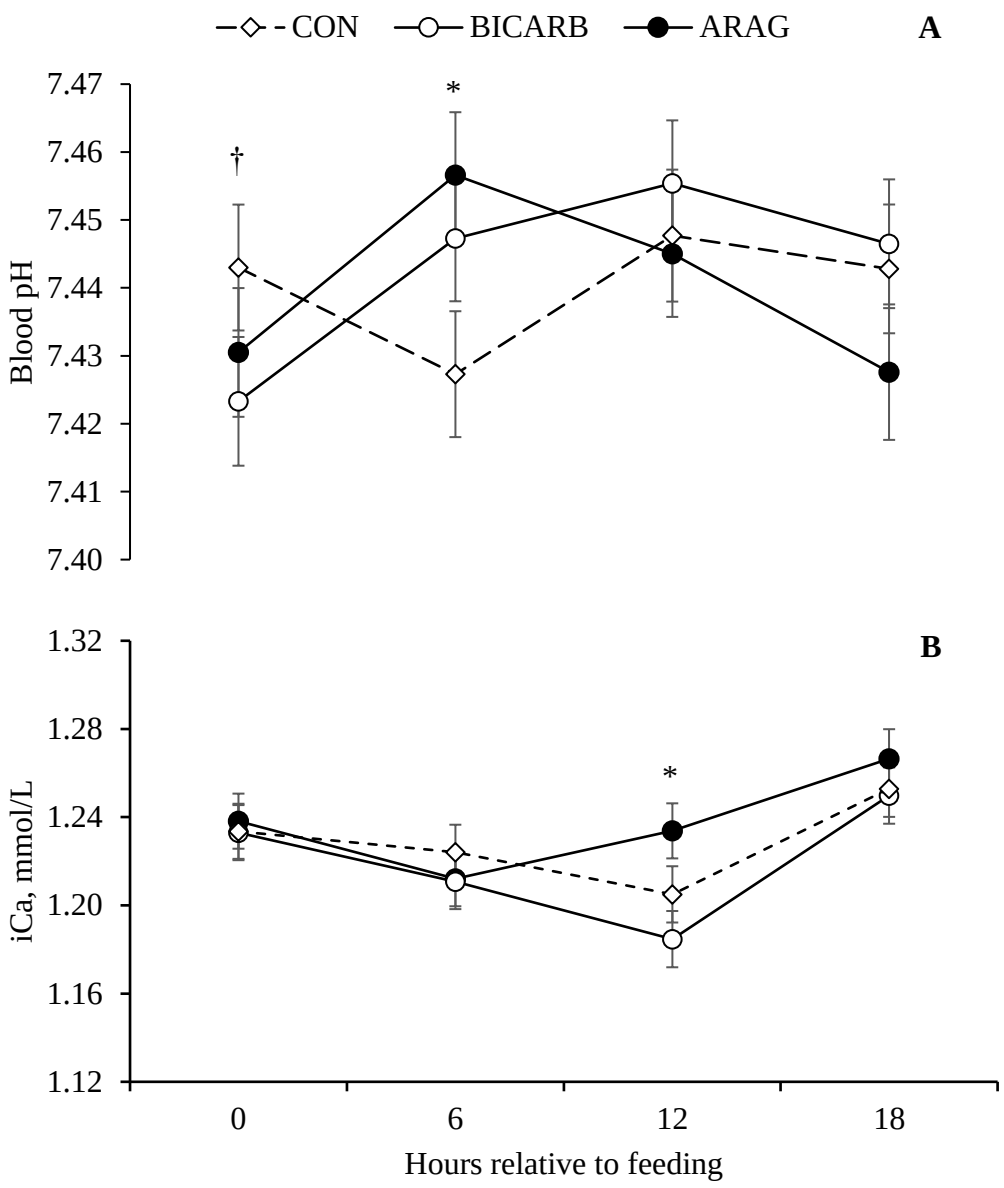


Figure 3. Blood pH (**A**) and ionized Calcium (iCa; **B**) concentration in dairy cows fed diets supplemented with different sources of calcium and rumen buffer. Treatments were: CON = basal diet supplemented with 0.80% limestone and 0.55% NaCl; BICARB = basal diet supplemented with 0.80% limestone and 0.80% $NaHCO_3$; ARAG = basal diet supplemented with 0.80% aragonite (Rumen Cal +, Ag Source, LLC) and 0.55% NaCl. Asterisk (*) represents statistical significance at $P \leq 0.05$. Cross (†) represents tendency at $0.05 < P \geq 0.10$. Largest SEM = 0.009 for (**A**) and 0.013 for (**B**); n = 207 and 209 for [A and B, respectively (n represents number of observations used in the statistical analysis)]. Treatment × time interaction effect: P = 0.04 for (**A**) and P = 0.27 for (**B**).