# Rumen $CO_2$ species equilibrium might influence performance and be a factor in the pathogenesis of subacute ruminal acidosis

**José A. Laporte-Uribe**[1]

GEA Farm Technologies GmbH, 59199 Boenen, Germany

**ABSTRACT:** This experiment was conducted to explore rumen carbon dioxide ($CO_2$) species equilibrium. Three lactating, fistulated cattle were consecutively exposed to three dietary treatments tailored to produce low rumen pH and increase the risk of subacute ruminal acidosis (SARA) by reducing physically effective neutral detergent fiber (Low $_{pe}$NDF), increasing rumen degradable starch (High RDS) or both (Combined). Under these conditions, high and varied rumen concentrations of the $CO_2$ associated to water or dissolved $CO_2$ ($dCO_2$) were found. The results suggest that the activity of $dCO_2$ and bicarbonate ($HCO_3^-$) represents an important component of the rumen environment. Rumen $CO_2$ holdup was associated with high $dCO_2$ and $HCO_3^-$ activity as well as changes in the viscosity and surface tension of the rumen fluid. All dietary treatments produced low rumen pH, <5.5 for >3 h/d, a condition associated with SARA, but clinical SARA was observed only during $CO_2$ holdup. This pilot study highlights the possible role of $CO_2$ holdup and rumen $CO_2$ species in cattle performance and nutritional diseases. In the future, better estimations of $CO_2$ species might help clarify these findings.

**Key words:** bicarbonate, $CO_2$ holdup, dissolved $CO_2$, nutritional diseases, rumen pH, subacute ruminal acidosis



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## INTRODUCTION

The pH of a solution is measured by a change in the electrical field of an operational cell electrode (i.e., hydrogen cell electrode plus a reference electrode), and a one-unit, 6 to 7, change in pH is equivalent to a 60 mV change in fluid conductivity (Covington et al., 1985). The average rumen pH is 6.1 and a low rumen pH, <5.6 or <5.8, for an extended period of time, >3 or >5 h/d, triggers clinical signs of subacute ruminal acidosis or SARA (Plaizier et al., 2008; Humer et al., 2018). However, it seems unlikely that this small decrease in fluid conductivity can cause this disease. Instead, SARA might be caused by the ions that modify the electrical field which causes the pH decline (fluid ionization). For instance, volatile fatty acid (VFA) concentrations have been closely associated with rumen pH decline and the onset of SARA (Zebeli et al., 2010; Aschenbach et al., 2011). In in vitro studies, an increase in VFAs seems to directly affect the rumen epithelium (Aschenbach et al., 2011; Penner et al., 2011). However, rumen VFAs, the main energy source for ruminants, are widely metabolized by the rumen epithelium and liver (Aschenbach et al., 2010). There is little in vivo evidence that VFAs can have negative effects on cattle other than a decrease in feed intake (Bradford et al., 2006;

[1]Corresponding author: joselaporte@hotmail.com
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Plaizier et al., 2008). Moreover, within the rumen pH range (5 to 7), VFA activity is low, stable and dominated by bases; the VFA equilibrium constant, p$K_a$, is ~4.8 (Dijkstra et al., 1993). Thus, VFAs are unlikely to be the main source of pH fluctuations and decline.

Another factor associated with VFA production and rumen pH fluctuations is carbon dioxide ($CO_2$) species (Turner and Hodgetts, 1955; Waghorn, 1991). The main liquid $CO_2$ species in the rumen are bicarbonate ($HCO_3^-$) and dissolved $CO_2$ ($dCO_2$). Carbonic acid ($H_2CO_3$) is found in only low concentrations (<1 mmol/100 mL) in water, and carbonates ($CO_3^{-2}$) are minimal within the rumen pH range (Adamczyk et al., 2009; Laporte-Uribe, 2016). However, our use and misuse of standards in biology made this claim difficult to understand. For instance, water is defined as $H_2O$, but this is the water vapor molecule (gas). In its liquid state, $H_2O$ is dissociated in chains of hydronium ($H_3O^+$) and hydroxide ($OH^-$) ions (Agmon et al., 2016). In fact, the dissociation of water or ionization depends on the amount and nature of other molecules in the solution and, namely, change in pH of a solution denotes water ionization product of its interaction with other molecules (Covington et al., 1985; Valsaraj, 1999). Therefore, the pH decline represents the increase in proton activity ($H^+$) or, more appropriately, the increase in $H_3O^+$ activity, as $H^+$ ions are not alone in solutions (Covington et al., 1985; Agmon et al., 2016). Similarly, due to the environmental conditions on earth, $CO_2$ is found mainly as a gas, but due to its solubility in water, $CO_2$ can also be found in the above ionic forms in the rumen liquor (Valsaraj, 1999; Laporte-Uribe, 2016). Moreover, dissolved $CO_2$ can be defined as the $CO_2$ molecule weakly linked to $H_3O^+$, so high $CO_2$ production during rumen fermentation will increase $H_3O^+$ activity due to $dCO_2$ formation and reduce the rumen pH.

Despite the importance of $dCO_2$ in determining rumen pH, only $HCO_3^-$ is routinely reported. Rumen $dCO_2$ has been thought to be low, stable, or absent in the fluid due to the effect of eructation on gas $CO_2$ release and the constant $CO_2$ solubility across diets (Kohn and Dunlap, 1998; Hille et al., 2016). However, it is essential to differentiate between $CO_2$ solubility (entrance into the solution) and volatility (exit from the solution), as the sources of rumen $CO_2$ species are within the liquid, and changes in volatility might lead to higher $dCO_2$ concentrations (Laporte-Uribe, 2016). Moreover, SARA diets usually contain high levels of rumen degradable starch (RDS), which increase $CO_2$ production and the viscosity, Vis, of the rumen fluid (Cheng and Hironaka, 1973; Cheng et al., 1976). SARA diets also have low proportions of physically effective neutral digestible fiber, $_{pe}$NDF (Zebeli et al., 2010), leading to small rumen particle sizes and a decline in the rumen surface tension, ST (Kluytmans et al., 2001). These changes in physicochemical properties promote lower $CO_2$ volatility, $CO_2$ holdup and high $dCO_2$ concentrations, which have direct nutritional and physiological implications (Laporte-Uribe, 2016).

Liquid $dCO_2$ is a biologically active molecule that can cross almost freely through the rumen epithelium (Gutknecht et al., 1977; Endeward et al., 2013), which explains the vast $CO_2$ transaction between the rumen and blood in ruminants (Whitelaw et al., 1972; Veenhuizen et al., 1988). Sustaining high rumen $dCO_2$ for longer periods of time due to $CO_2$ holdup might create an even larger gradient between the blood and rumen, which might increase $dCO_2$ diffusion, saturate cellular buffer systems and lead to respiratory and metabolic acidosis, pathologies that are closely associated with SARA (Huber, 1976; Gianesella et al., 2010).

Normally diets might not lead to high $dCO_2$ concentrations or $CO_2$ holdup, because the exchange of rumen $CO_2$ species between liquid and the gas cap is not impaired. However, if this equilibrium is broken, rumen $dCO_2$ will not effervescent from the liquid and $CO_2$ can not be eliminated through eructation (Laporte-Uribe, 2016). Nevertheless, it remains unclear whether SARA diets produce high $dCO_2$ concentrations or how rumen $CO_2$ holdup develops under those conditions. This experiment investigated rumen $CO_2$ species equilibrium and the nature of $CO_2$ holdup. Moreover, SARA diets were used to explore how changes in feed components affect the rumen physicochemical properties, $CO_2$ species and cattle performance, trusting that evidence might arise regarding the role of $CO_2$ species in health and nutrition.

## MATERIALS AND METHODS

### *Cattle and Performance*

Three lactating and fistulated cattle (Bar Diamond, Inc., Parma, ID) were placed in tied stalls. They had similar days in milk (~100 DIM), but they differed in their dry matter intake (DMI) and milk yield (MY). Three experimental diets were composed to create the SARA condition of a low rumen pH (Zebeli et al., 2010). The first was aimed at reducing the particle size of the diet (Low

$_{pe}$NDF), which was achieved by grinding and pelletizing part of the dried grass. The second diet was aimed at increasing the RDS by exchanging part of the corn-based concentrate for wheat flour (High RDS). The last diet was a combination of the other two diets (Combined).

The experiment was approved by the Animal Care and Ethics Committee of Wageningen UR Livestock Research (Dairy Campus, the Netherlands). Cattle had free access to water and were fed simultaneously the same experimental diets ad libitum during consecutive 2-wk periods (Figure 1). During the first week (introduction), the original diet was incrementally replaced by the experimental diet (daily increments of ~20 g/100 g DM), and this setup provided 2 d of steady-state conditions before the rumen environment was sampled or continuously monitored (experimental week). Between each run, a 2 d washout period was provided were cattle returned to the herd and fed a standard production diet (pretrial). The diets were automatically mixed daily by a mixing wagon (total mixed ration, TMR) and offered in three bouts (at 7:30, 9:00, and 16:00 h). The total feed offered and refused was recorded and sampled daily for particle size determinations (Penn State Particle Separator, PSPS). The particle size was monitored following PSPS guidelines using three sieves: upper, >19 mm; middle, 19 to 6 mm; and lower, <6 mm (Kononoff et al., 2003). Individual feed ingredients were sampled daily to determine dry matter, and weekly samples were pooled and analyzed for chemical composition and nutritional value. The feed analyses were based on the VEM system (Van Es, 1978), the Dutch protein evaluation system (Tamminga et al., 1994), and the Nordic feed evaluation system, NorFor (Nørgaard et al., 2011). Cattle were milked twice daily at 7 and 16 h, and samples were drawn for milk component analysis using mid-infrared spectrometry (Qlip, The Netherlands). The energy-corrected MY (ECM) was calculated as described by Aguerre et al. (2011).

### *Rumen Physicochemical Properties*

The rumen pH and temperature were continuously monitored (every 15 s) for 4 d (sampling period) using indwelling data-loggers (Dascor, Inc.) that were weighted to remain in the ventral sac. The location of each sensor was assessed daily. After placing the rumen pH sensors (1st-d), the rumen fluid was manually sampled for 3 d using a manual pump. The rumen samples were taken from two locations, dorsal (10 cm from the fistula) and ventral (30 cm from the fistula), at five consecutive times (0.5, 1, 2, 4, 6 h) postprandially (7:30 h). The rumen pH of spot samples was monitored with a temperature-corrected handheld system (Seven2Go ProS8, Mettler-Toledo).

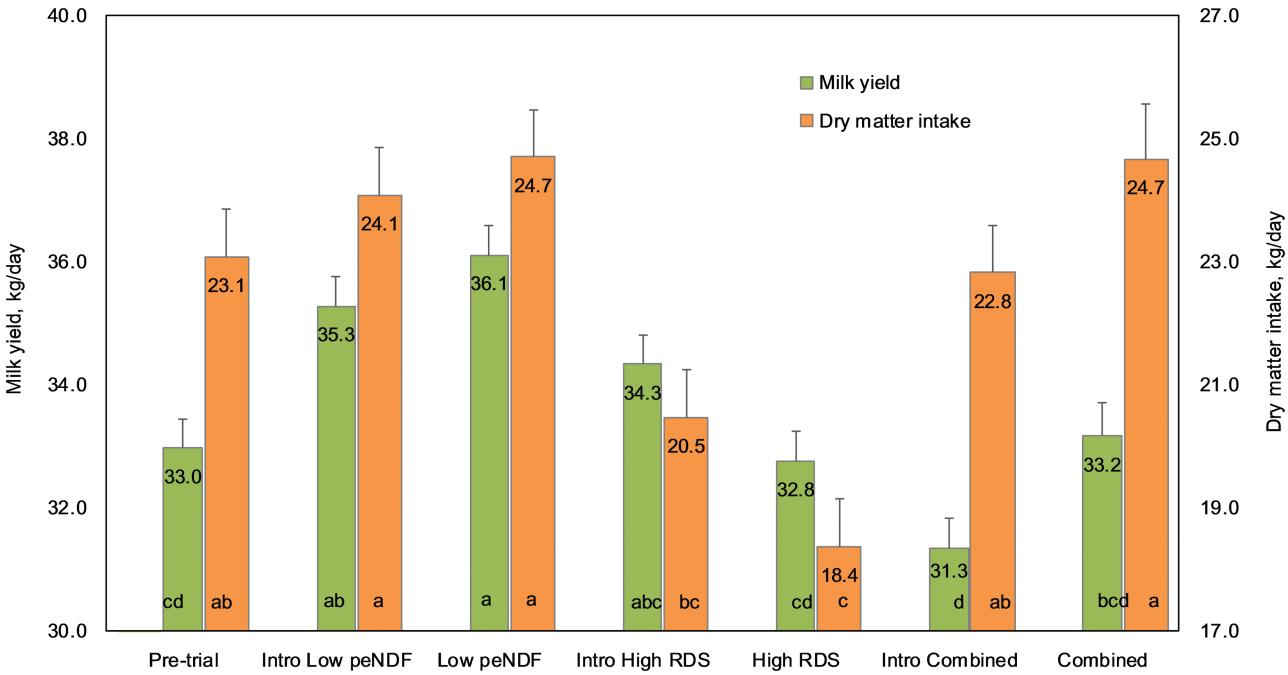


**Figure 1.** The cattle performance and the feeding sequence during the experiment. The bars represent the mean and standard error of the mean (SEM), for milk yield (MY), and dry matter intake (DMI). After the pre-trial period, three fistulated and lactating dairy cattle, ~100 DIM, were sequentially and simultaneously fed three diets: low physically effective neutral digestible fiber (Low $_{pe}$NDF), high rumen degradable starch (High RDS) and the combined (Combined) diet. The cattle were allowed a 1 week introduction (intro) to the diets before rumen indwelling sensors were deployed and rumen samples were collected. Means that do not share a letter are significantly different at the 95% confidence level ($P < 0.05$, Bonferroni).

Samples collected for total inorganic carbon (TIC) analysis were alkalinized by the addition of 1 ml sodium hydroxide (NaOH, 5 M) and quickly frozen (−20 °C). Rumen samples for Vis, ST, and VFA analyses were frozen (−20 °C) without further treatment. TIC was determined by gas chromatography (GC) at the Institute of Biochemical Engineering, University of Stuttgart (Buchholz et al., 2014). The GC method (Model 5890 II, Hewlett Packard, Germany) was also used for the VFA analysis at the Physiology Department of the Veterinary Physiology, Veterinary Medicine Hannover, Foundation (Geissler et al., 1976). The concentrations (mmol/L) of acetate, propionate, butyrate, and branched VFAs (iso-valerate and valerate) are reported. The iso-butyrate was below the detection limit in all samples (<0.04 mmol/L). Lactate (D-lactate, µmol/L) was analyzed in rumen samples at 2, 4, and 6 h postprandial as a reference for lactate accumulation. Vis (mPa·s) was analyzed in two temperature-controlled rheometers (MCR301 and MCR5.02, Anton Paar GmbH, Austria). ST (mN/m) was measured using a bubble pressure tensiometer (LAUDA TVT 2, LAUDA-Brinkmann, LP). All samples were analyzed at average rumen temperature (39.5 °C).

### *Calculations*

The laboratory TIC results were used to calculate the concentrations of $HCO_3^-$ and $dCO_2$ according to the following equations (Bjerrum plot equations):

$$dCO_2 = \frac{[H^+]^2}{[H^+]^2 + K_{a1}[H^+] + K_{a1} \cdot K_{a2}} \times TIC \quad (1)$$

$$HCO_3^- = \frac{K_{a1} \times [H^+]}{[H^+]^2 + K_{a1}[H^+] + K_{a1} \cdot K_{a2}} \times TIC \quad (2)$$

where $dCO_2$ is the dissolved carbon dioxide, mmol/L; $HCO_3^-$ is bicarbonate, mmol/L; TIC is the total inorganic carbon, mmol/L; $[H^+]$ is the hydrogen activity; $K_{a1}$ is the first dissociation constant, which is $4.45 \times 10^{-7}$ at 25 °C (Edsall, 1969); and $K_{a2}$ is the second dissociation constant, which is $4.69 \times 10^{-11}$ at 25 °C (Edsall, 1969).

The TIC analysis suggested that $HCO_3^-$ was preferentially retained in the rumen spot samples (see the Discussion). The following equations were used to test that hypothesis and calculate the $CO_2$ species (Henderson–Hasselbalch, HH):

$$K_{a1} = \frac{B^-[H^+]}{A} \quad (3)$$

$$-\log[H^+] = -\log(K_{a1}) + \log\left(\frac{B^-}{A}\right)$$

$$pH = pK_{a1} + \log\left(\frac{B^-}{A}\right) \quad (3a)$$

where $K_1$ is the first dissociation constant, which is $4.45 \times 10^{-7}$ at 25 °C (Edsall, 1969); $[H^+]$ is the hydrogen activity; $B^-$ is the conjugate base or dissociated molecule, for example, $HCO_3^-$; $A$ is the conjugate acid or undissociated molecule, for example, $dCO_2$; pH is the negative logarithm of $[H^+]$; $pK_{a1}$ is the negative logarithm of $K_{a1}$, and

$$dCO_2 + HCO_3^- = TIC \quad (4)$$

where $dCO_2$ is dissolved carbon dioxide, mmol/L; $HCO_3^-$ is bicarbonate, mmol/L; and TIC is the total inorganic carbon, mmol/L.

In short, the equilibrium (activity) between the acids and bases in solution is related to the compound's charge and concentration and the hydrogen activity, $H^+$, or more precisely hydronium, $H_3O^+$ (Dawes, 1972; Covington et al., 1985). Thus, by determining the base, in this case $HCO_3^-$, the acid ($dCO_2$ or $CO_2 + H_3O^+$) can be calculated if the pH and equilibrium constant ($K_{a1}$) for the solution are known (equation 3a). $H_2CO_3$ is not stable in aqueous solutions, and only a small proportion of $H_2CO_3$ can be found in water (<1 mmol/100 mL), so its role in rumen pH equilibrium can be disregarded (Laporte-Uribe, 2016).

### *Statistical Analysis*

The experimental design is not suitable to clearly define statistically the effect of diet on cattle performance (Bailey and Greenwood, 2018). However, the primary objective of this pilot experiment was to observe changes in the rumen physicochemical properties and $CO_2$ species. Thus, the factor dietary treatment (Treat), which combines period and diet, was introduced into the following general linear model (GLM):

$$Y_{ijkl} = \mu + D_i + A_j + ST_k + SS_l + e_{ijkl}$$

All variables were considered fixed factors, where µ is the overall mean, $D_i$ is the dietary treatment effect (Treat, $i$ = 1 to 3), $A$ is the cattle effect (Cows, $j$ = 1 to 3), ST = time after feeding effect (Time, $k$ = 0.5 to 6 h), SS = sampling site effect (Site, $l$ = 1 and 2), and $e_{ijkl}$ = residual error.

The model assumes that parameters were independent and normally distributed. All the results are presented as the mean and standard error of

the mean, SEM, unless stated otherwise. The factors Treat, Cows, and their interaction (Treat × Cows) were included in the GLM to analyze cattle performance. The model for rumen metabolites included two additional fixed factors, Site and Time, and all interactions. The rumen physicochemical properties (3 d) and cattle performance (4 d) were recorded throughout the experimental period, except for DMI on the last day for the Combined Treat, as the recorded fresh feed weight was unreliable, and therefore excluded (3 d). Two-way analysis of variance was used to describe the effect of treatments when measurements were bulked, that is, milk component proportions. The Bonferroni comparison method was applied in the post hoc analysis. In the tables, values with different letters differ significantly from one another ($P < 0.05$).

Box plots were used to describe the relationships among key metabolites, VFAs, pH, and $CO_2$ species. The central value of each boxplot is the median; the box ranges between the first ($Q_1$) and the third ($Q_3$) quartile; and the line ranges from the lower to the upper limit, $Q_n \pm 1.5 \times (Q_3 - Q_1)$. All variables are presented for three factors: Site, Time (h), and Treat. Due to similarities between the 0.5 h and 1 h samples, the former were excluded from the figures. A categorical analysis for continuous pH measurement was used and was based on the area under the curve (AUC), with the pH values categorized as follows: above 6.4, indicative of low fermentation; between 6.4 and 6.1, indicative of moderate fermentation; between 6.1 and 5.8, optimal; between 5.5 and 5.8, suboptimal; and below 5.5, acidic (Russell, 1987; De Veth and Kolver, 2001). The probability for each category was calculated every 15 min; the AUC values represented the mean probability (h/d ± SEM) of each category in a 24 h interval according to the GLM analysis. All the statistical analyses and figure construction were performed using Minitab 16 statistical software, Minitab, LLC, PA.

## RESULTS

### *Dietary Treatments and Cattle Performance*

The diets were tailored to produce similar MY and DMI. For instance, urea was added to both the High RDS and Combined diets to ensure that all treatments provided similar energy and crude protein (Table 1). However, feed intake varied among dietary treatments during the trial, which influenced the final composition (Table 1). For instance, when cattle were fed the High RDS diet, the resulting decline in feed intake reduced NEL and CP intake. Components did not differ between the Low $_{pe}$NDF diet and the Combined diet. Particle size varied among diets by design, but no difference within diets in fresh feed or refusal particle size was found (Table 1).

Figure 1 shows the sequence of events and the effect of dietary treatment on cattle performance before and during the experimental period. A difference among dietary treatments was already apparent in the introduction week (intro) for all periods (Figure 1). Differences became more apparent during the experimental week, suggesting that changes in cattle performance and the rumen physicochemical properties were closely associated with Treat, that is, the diet or the sequence in which diets were fed (Figure 1, Tables 2 and 3). Table 2 summarizes the performance of cattle in the three dietary treatments. The MY and lactose yield were higher when the cattle were fed the Low $_{pe}$NDF diet than when they were fed the other diets. In contrast, DMI and MY declined in all cattle under the High RDS Treat (Treat × Cow, NS). Similarly, although the fat and protein yields were low in all treatments when compared with literature values, the yields were lowest in the High RDS Treat (Table 2). The milk components proportion (fat, protein, and fat protein ratio) was similar among dietary treatments due to the differences in MY among periods. An effect of Treat on cattle performance was also observed when cattle were introduced to the Combined diet, but by the sampling period, DMI and MY had risen to values observed during the pretrial period (Figure 1).

### *Treatment Effects on Rumen pH*

The indwelling sensors showed that all of the dietary treatments produced acidic rumen environments (AUC < 5.5–3 h/d and AUC < 5.8–12 h/d, Table 2, Figure 2), those values resemble SARA conditions described in the literature. Moreover, differences in rumen pH were observed among treatments, among individual cows (Table 2) and throughout the day (Figure 2a). The lowest average rumen pH was recorded when the cattle were fed the Combined diet (Table 2). The highest pH and the largest fluctuations in pH were observed when the cattle were fed the High RDS diet (Figure 2a), and temperature also varied widely under this diet (Figure 2b). Cattle fed the Low $_{pe}$NDF diet exhibited the lowest pH fluctuations in the rumen (Figure 2a).

**Table 1.** Final mix of components, chemical composition, and particle size analysis of the three dietary treatments fed to fistulated dairy cattle on this experiment

| | Treat | | | | | |
|---|---|---|---|---|---|---|
| | Low $_{pe}$NDF | | High RDS | | Combined | |
| | $n$ = 4 | | $n$ = 4 | | $n$ = 4 | |
| | Mean | SEM | Mean | SEM | Mean | SEM |
| Item, g/100 g DM | | | | | | |
| Grass silage | 7.1 | 0.26 | 28.6 | 7.7 | 9.9 | 2.95 |
| Corn silage | 21.2 | 2.03 | 16.4 | 0.62 | 15.5 | 0.74 |
| Dried grass | 16.4 | 4.52 | 13.3 | 6.3 | 7.4 | 0.27 |
| Dried grass, pelleted | 15.2 | 5.39 | — | — | 27 | 0.89 |
| Concentrate | 34.4 | 0.82 | 25.1 | 4.55 | 10.9 | 0.36 |
| Wheat meal | — | — | 10.6 | 4.98 | 21.8 | 0.72 |
| Soybean meal | 5.3 | 0.4 | 5.2 | 0.69 | 6.3 | 0.21 |
| Premix minerals | 0.4 | 0.04 | 0.5 | 0.05 | 0.4 | 0.01 |
| Urea | — | — | 0.3 | 0.15 | 0.7 | 0.04 |
| Feed chemical components, g/kg DM | | | | | | |
| NEL, MJ/kg DM | 6.6 | 0.06 | 6.8 | 0.06 | 6.6 | 0.07 |
| DVE | 91.72 | 2.86 | 83.43 | 2.41 | 89.3 | 3.19 |
| OEB | 11.87 | 1.80 | 18.23 | 1.80 | 24.3 | 2.38 |
| CP | 160.7 | 5.22 | 155.2 | 5.22 | 173.8 | 6.91 |
| S + S | 237.8 | 13.25 | 246.1 | 13.25 | 278.1 | 17.53 |
| Sugar | 65.1 | 2.47 | 52.4 | 2.47 | 60.2 | 3.27 |
| Starch | 172.7 | 11.98 | 193.7 | 11.98 | 218.0 | 15.84 |
| NDF | 370.7 | 9.69 | 388.8 | 9.69 | 349.8 | 12.8 |
| $_{pe}$NDF | 209.1 | 20.50 | 302.8 | 20.50 | 156.4 | 27.12 |
| Penn State Particle separator, g/100 g | | | | | | |
| | Fresh feed, $n$ = 3 | | | | | |
| >19 mm | 11.3 | 6.25 | 55.8 | 6.25 | 25.7 | 6.25 |
| 19 to 6 mm | 47.2 | 4.55 | 8.6 | 4.55 | 28.4 | 4.55 |
| <6 mm | 41.5 | 3.95 | 35.6 | 3.95 | 45.9 | 3.95 |
| | Refusal, $n$ = 3 | | | | | |
| >19 mm | 10.4 | 6.25 | 70.9 | 6.25 | 22.3 | 6.25 |
| 19 to 6 mm | 50.4 | 4.55 | 5.9 | 4.55 | 26 | 4.55 |
| <6 mm | 39.2 | 3.95 | 23.2 | 3.95 | 51.8 | 3.95 |

Values represent the mean and the standard error of the mean (SEM) for each ingredient and component. The dietary treatments, Treat, were as follows: the low physically effective neutral detergent fiber, Low $_{pe}$NDF; high rumen degradable starch, High RDS; and the Combined. NEL, net energy for lactation; DVE, intestinal digestible protein; OEB, degraded protein balance; CP, crude protein; S + S, sugar plus starch; NDF, neutral detergent fiber; $_{pe}$NDF, physically effective NDF.

### *The Rumen Physicochemical Properties and $CO_2$ Species in Rumen Fluid*

The dietary treatments (Treat) had a significant effect on the rumen physicochemical properties (Table 3). For instance, all Treat produced very low A/P ratios, with the High RDS and Combined diets yielding the lowest values. Rumen propionate concentration was lower when cattle were fed the Low $_{pe}$NDF diet than when they were fed the other diets (Figure 3b), whereas the butyrate concentration was highest under the Low $_{pe}$NDF diet (Table 3). Propionate and acetate concentrations were highest when the cattle were fed the High RDS diet (Figure 3a, Table 3). All dietary treatments produced low lactate (Table 3). In general, rumen VFA activity was dominated by bases and dissociated VFAs, and only a small quantity of undissociated VFAs or acid was found (Table 3). High Vis was observed in the treatments that had higher levels of RDS (High RDS and Combined), with lower Vis under the Low $_{pe}$NDF diet (Figure 3e). In contrast, ST exhibited no apparent pattern: ST was lowest when the cattle were fed the Low $_{pe}$NDF diet and highest when they were fed the Combined diet; cattle fed the High RDS diet had intermediate ST values (Figure 3f). ST remained stable within each Treat except when cattle were fed the High RDS diet, in which postprandial ST tended to decline rapidly (Figure 3f, Table 3).

In general, high $dCO_2$ and $HCO_3^-$ activity in the ventral sac were found in all dietary treatments (Table 3, Figure 3c and d). The highest $dCO_2$

**Table 2.** Effect of the dietary treatments, Treat, on cattle performance, milk components, rumen pH and temperature measured continuously for 4 d

| | Treat | | | | | | Probability | | |
|---|---|---|---|---|---|---|---|---|---|
| | Low $_{pe}$NDF $n = 12$ | | High RDS $n = 12$ | | Combined $n = 9$ | | *P*-value | | |
| Item | Mean | SEM | Mean | SEM | Mean | SEM | Treat | Cow | Treat × Cow |
| Cattle performance, kg/d | | | | | | | | | |
| DMI | 24.7[a] | 0.85 | 18.4[b] | 0.85 | 24.7[a] | 0.98 | 0.000 | 0.017 | 0.676 |
| MY | 36.1[a] | 0.48 | 32.8[b] | 0.48 | 33.2[b] | 0.56 | 0.000 | 0.000 | 0.382 |
| ECM | 37.2[a] | 0.51 | 34.6[b] | 0.51 | 35.6[a] | 0.59 | 0.005 | 0.000 | 0.091 |
| Milk components, g/100 mL, $n = 3$ | | | | | | | | | |
| Fat | 3.90 | 0.278 | 3.95 | 0.278 | 4.04 | 0.278 | 0.794 | 0.031 | — |
| Protein | 3.40[a] | 0.144 | 3.55[ab] | 0.144 | 3.67[b] | 0.144 | 0.030 | 0.004 | — |
| Lactose | 4.50[ab] | 0.058 | 4.58[a] | 0.058 | 4.45[b] | 0.058 | 0.012 | 0.003 | — |
| Fat/protein ratio | 1.14 | 0.037 | 1.11 | 0.037 | 1.10 | 0.037 | 0.802 | 0.283 | — |
| Milk component yield, kg/d | | | | | | | | | |
| Fat | 1.37[a] | 0.019 | 1.27[b] | 0.019 | 1.33[ab] | 0.022 | 0.003 | 0.000 | 0.001 |
| Protein | 1.22[a] | 0.017 | 1.15[b] | 0.017 | 1.21[ab] | 0.020 | 0.033 | 0.000 | 0.720 |
| Lactose | 1.62[a] | 0.021 | 1.50[b] | 0.021 | 1.47[b] | 0.025 | 0.000 | 0.000 | 0.112 |
| Rumen continuous measurements | | | | | | | | | |
| Average pH | 5.78[a] | 0.001 | 5.91[b] | 0.001 | 5.65[c] | 0.001 | 0.000 | 0.000 | 0.000 |
| Average temperature, °C | 38.74 | 0.003 | 38.99 | 0.003 | 38.89 | 0.003 | 0.000 | 0.000 | 0.000 |
| AUC rumen pH, h/d | | | | | | | | | |
| <5.5 | 4.58[a] | 0.307 | 4.67[a] | 0.307 | 8.50[b] | 0.307 | 0.000 | 0.001 | 0.000 |
| 5.5–5.8 | 7.90[a] | 0.323 | 6.06[b] | 0.323 | 7.52[a] | 0.323 | 0.027 | 0.205 | 0.000 |
| 5.8–6.1 | 7.86[a] | 0.316 | 5.41[b] | 0.316 | 6.11[b] | 0.316 | 0.003 | 0.000 | 0.000 |
| 6.1–6.4 | 3.55[a] | 0.222 | 4.14[a] | 0.222 | 1.87[b] | 0.222 | 0.000 | 0.000 | 0.000 |
| >6.4 | 0.11[a] | 0.109 | 3.71[b] | 0.109 | 0.01[a] | 0.109 | 0.000 | 0.000 | 0.000 |

Values represent the mean and the standard error of the mean (SEM). The treatments, Treat, were low physically effective neutral detergent fiber, Low $_{pe}$NDF; high rumen degradable starch, High RDS; and the Combined. DMI, dry matter intake; MY, milk yield; ECM, energy-corrected MY. The area under the curve (AUC) for rumen pH was analyzed using a categorical analysis. The probability (*P* value) is given for the main factors and their interaction. All comparison were made at the 95% confidence level ($P < 0.05$), and means that do not share a letter are significantly different (Bonferroni).

concentrations in the rumen were observed when cattle were fed the Low $_{pe}$NDF diet, with intermediate concentrations observed under the High RDS diet; the lowest activity was found under the Combined Treat (Figure 3c, Table 3). The High RDS diet yielded the highest $HCO_3^-$ activity, followed by the Low $_{pe}$NDF and Combined diets (Figure 3c). Higher $dCO_2$ and lower $HCO_3^-$ activity were found postprandially in all treatments. In general, maximum $dCO_2$ and maximum $HCO_3^-$ concentrations were both close to 36 mmol/L (Figure 3c and d).

### *Manual Sampling and Total Inorganic Carbon Recovery*

There was a positive relationship between TIC and VFA concentrations (Figure 4a). Moreover, the negative relationship between pH and TIC and the almost disappearance of TIC below the 5.4 pH threshold (Figure 4b) suggested that $HCO_3^-$ was preferably retained during manual sampling. Assuming that most of the TIC was $HCO_3^-$ and using the HH equation (equation 3a), the $dCO_2$ concentrations and TIC (equation 4) were calculated. The results showed that $HCO_3^-$ values varied widely among treatments (Figure 5a) and that $dCO_2$ and TIC concentrations were larger than otherwise expected (Figure 5b, Table 3). For example, $dCO_2$ values ranged from 5 to 180 mmol/L and averaged approximately 60 mmol/L (Table 3). Greater $dCO_2$ concentrations were found when the cattle were fed the Low $_{pe}$NDF and High RDS diets; the Combined diet yielded the lowest $dCO_2$ concentrations at any given time (Figure 5b). As described above, the highest $HCO_3^-$ concentrations were observed when the cattle were fed the High RDS diet (Figure 5a).

## DISCUSSION

The values for $HCO_3^-$ obtained from the Bjerrum plot equation are similar to the values for the low-pH diets used in this experiment (Hille et al., 2016), suggesting that the rumen $dCO_2$ concentrations in dairy cattle potentially range from 5

**Table 3.** Descriptive analysis for the effect of the dietary treatments on the physicochemical properties of the rumen fluid of cattle

| | | | | VFAs, mmol/100 mL | | | | | | | VFA activity, mmol/100 mL | | | $CO_2$ species, mmol/L | | | | Calculated $CO_2$ species* | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | | | | | Br, | Lactate, | A/P | Total VFAs, | | | TIC, | | | Viscosity, | Surface tension, | TIC, | $CO_2$ activity, mmol/L | |
| Treat | Sites | Times, h | pH | Ac | Pr | Bu | mmol/L | µmol/L | ratio | mmol/L | Base | Acid | mmol/L | $HCO_3^-$ | $dCO_2$ | mPa·s | mN/m | mmol/L | $HCO_3^-$ | $dCO_2$ |
| Low peNDF | Dorsal | 0.5 | 6.04 | 59.7 | 28.5 | 11.5 | 0.45 | — | 2.09 | 121.2 | 94.1 | 5.9 | 23.3 | 8.1 | 15.3 | 2.53 | 68.5 | 72.0 | 23.3 | 48.6 |
| | | 1 | 5.94 | 58.7 | 29.0 | 11.7 | 0.77 | — | 2.03 | 121.1 | 91.9 | 8.1 | 23.2 | 7.9 | 15.3 | 2.06 | 67.5 | 82.1 | 23.2 | 58.8 |
| | | 2 | 6.04 | 57.4 | 29.8 | 12.3 | 0.73 | 25.9 | 1.94 | 119.0 | 93.1 | 6.9 | 31.0 | 11.4 | 19.6 | 1.83 | 66.6 | 107.6 | 31.0 | 76.6 |
| | | 4 | 5.88 | 58.2 | 29.4 | 11.6 | 0.78 | 7.6 | 1.99 | 115.6 | 90.7 | 9.3 | 23.9 | 7.3 | 16.6 | 2.02 | 66.8 | 102.1 | 23.9 | 78.2 |
| | | 6 | 5.79 | 58.4 | 29.6 | 11.7 | 0.49 | 6.6 | 1.98 | 124.7 | 90.0 | 10.0 | 19.1 | 4.7 | 14.4 | 2.37 | 66.7 | 84.7 | 19.1 | 65.6 |
| | | Mean | $5.94^{d}$ | $58.7^{a}$ | $29.3^{c}$ | $11.8^{a}$ | $0.47^{d}$ | | $2.01^{a}$ | $120.3^{bc}$ | $91.9^{c}$ | $8.1^{a}$ | $24.1^{bc}$ | $7.9^{bc}$ | $16.2^{ab}$ | $2.16^{c}$ | $67.2^{b}$ | $89.7^{a}$ | $24.1^{bc}$ | $65.6^{a}$ |
| | Ventral | 0.5 | 6.15 | 59.3 | 28.9 | 11.6 | 0.21 | — | 2.07 | 112.5 | 95.5 | 4.5 | 29.4 | 11.7 | 17.7 | 2.22 | 68.2 | 76.4 | 29.4 | 47.0 |
| | | 1 | 6.02 | 58.7 | 29.1 | 11.5 | 1.04 | — | 2.02 | 117.8 | 93.9 | 6.1 | 29.9 | 10.0 | 19.9 | 1.88 | 66.2 | 94.2 | 29.9 | 64.4 |
| | | 2 | 6.16 | 57.9 | 29.8 | 12.0 | 0.38 | 12.6 | 1.95 | 113.3 | 95.0 | 5.0 | 31.9 | 13.3 | 18.5 | 1.86 | 68.8 | 87.0 | 31.9 | 55.1 |
| | | 4 | 5.98 | 58.9 | 29.5 | 11.6 | 0.11 | 7.6 | 2.01 | 111.7 | 94.5 | 5.5 | 28.4 | 11.0 | 17.4 | 2.18 | 67.3 | 81.6 | 28.4 | 53.1 |
| | | 6 | 6.00 | 58.9 | 29.3 | 11.7 | 0.23 | 7.3 | 2.02 | 117.6 | 93.4 | 6.6 | 25.1 | 8.3 | 16.8 | 2.17 | 65.4 | 85.4 | 25.1 | 60.3 |
| | | Mean | $6.08^{bcd}$ | $58.7^{a}$ | $29.3^{c}$ | $11.7^{a}$ | $0.32^{d}$ | | $2.01^{a}$ | $114.6^{c}$ | $94.5^{ab}$ | $5.5^{bc}$ | $28.9^{ab}$ | $10.9^{bcd}$ | $18.1^{a}$ | $2.06^{c}$ | $67.2^{b}$ | $84.9^{a}$ | $28.9^{ab}$ | $56.0^{ab}$ |
| High RDS | Dorsal | 0.5 | 6.29 | 58.4 | 31.6 | 8.7 | 1.84 | — | 1.88 | 126.0 | 95.2 | 4.8 | 27.7 | 15.4 | 12.4 | 3.65 | 70.6 | 63.3 | 27.7 | 35.6 |
| | | 1 | 6.35 | 56.5 | 32.9 | 9.1 | 2.06 | — | 1.75 | 124.4 | 95.3 | 4.7 | 30.8 | 18.8 | 12.0 | 2.93 | 72.8 | 65.0 | 30.8 | 34.2 |
| | | 2 | 6.23 | 55.2 | 34.5 | 8.6 | 2.37 | 20.1 | 1.62 | 133.5 | 94.7 | 5.3 | 34.2 | 18.7 | 15.6 | 3.13 | 70.9 | 81.1 | 34.2 | 46.8 |
| | | 4 | 5.96 | 56.1 | 33.3 | 8.9 | 2.73 | 23.8 | 1.70 | 148.1 | 90.9 | 9.1 | 23.4 | 10.0 | 13.4 | 3.81 | 69.3 | 78.3 | 23.4 | 54.9 |
| | | 6 | 5.92 | 56.1 | 33.2 | 8.8 | 2.77 | 10.8 | 1.72 | 146.2 | 90.7 | 9.3 | 22.9 | 8.1 | 14.7 | 3.84 | 68.5 | 88.2 | 22.9 | 65.3 |
| | | Mean | $6.15^{abc}$ | $56.5^{b}$ | $33.1^{ab}$ | $8.8^{c}$ | $1.62^{ab}$ | | $1.73^{bc}$ | $135.6^{a}$ | $93.4^{bc}$ | $6.6^{ab}$ | $27.8^{b}$ | $14.2^{ab}$ | $13.6^{b}$ | $3.47^{b}$ | $70.4^{a}$ | $75.2^{ab}$ | $27.8^{b}$ | $47.4^{b}$ |
| | Ventral | 0.5 | 6.46 | 56.8 | 33.1 | 9.1 | 1.20 | — | 1.74 | 112.4 | 97.5 | 2.5 | 39.7 | 23.4 | 16.3 | 3.42 | 70.7 | 71.4 | 39.7 | 31.6 |
| | | 1 | 6.36 | 56.0 | 33.6 | 9.2 | 1.64 | — | 1.70 | 122.1 | 96.3 | 3.7 | 35.2 | 20.1 | 15.1 | 2.83 | 71.4 | 70.2 | 35.2 | 35.0 |
| | | 2 | 6.36 | 54.8 | 35.2 | 8.7 | 1.72 | 23.8 | 1.58 | 124.6 | 96.0 | 4.0 | 36.4 | 20.6 | 15.8 | 3.12 | 71.6 | 78.3 | 36.4 | 41.9 |
| | | 4 | 6.23 | 55.5 | 34.2 | 8.7 | 2.28 | 21.5 | 1.64 | 133.0 | 95.4 | 4.6 | 29.5 | 14.2 | 15.4 | 3.64 | 70.0 | 71.5 | 29.5 | 42.0 |
| | | 6 | 6.16 | 56.0 | 33.4 | 8.7 | 2.51 | 18.2 | 1.71 | 135.2 | 94.4 | 5.6 | 27.2 | 11.8 | 15.4 | 5.10 | 68.7 | 81.2 | 27.2 | 54.0 |
| | | Mean | $6.31^{a}$ | $55.8^{b}$ | $33.9^{a}$ | $8.9^{c}$ | $1.42^{ab}$ | | $1.67^{c}$ | $125.5^{ab}$ | $95.9^{a}$ | $4.1^{c}$ | $33.6^{a}$ | $18.0^{a}$ | $15.6^{ab}$ | $3.62^{b}$ | $70.5^{a}$ | $74.5^{ab}$ | $33.6^{a}$ | $40.9^{b}$ |
| Combined | Dorsal | 0.5 | 6.11 | 57.7 | 30.9 | 10.2 | 1.60 | — | 1.94 | 122.7 | 94.1 | 5.9 | 21.2 | 8.9 | 12.3 | 4.32 | 71.0 | 57.3 | 21.2 | 36.2 |
| | | 1 | 6.02 | 57.0 | 31.5 | 10.2 | 1.67 | — | 1.85 | 120.2 | 92.8 | 7.2 | 25.0 | 9.9 | 15.1 | 4.50 | 70.6 | 75.8 | 25.0 | 50.7 |
| | | 2 | 6.00 | 56.8 | 32.1 | 10.1 | 1.40 | 62.7 | 1.81 | 127.3 | 92.4 | 7.6 | 22.4 | 8.3 | 14.1 | 4.15 | 70.9 | 73.3 | 22.4 | 50.9 |
| | | 4 | 5.97 | 55.2 | 33.7 | 9.9 | 1.66 | 11.7 | 1.68 | 130.0 | 92.1 | 7.9 | 22.1 | 7.5 | 14.5 | 3.33 | 72.1 | 79.6 | 22.1 | 57.5 |
| | | 6 | 5.90 | 55.5 | 33.6 | 9.7 | 1.67 | 8.8 | 1.68 | 130.7 | 90.9 | 9.1 | 19.9 | 6.3 | 13.6 | 4.14 | 70.3 | 81.9 | 19.9 | 62.0 |
| | | Mean | $6.00^{cd}$ | $56.4^{b}$ | $32.3^{b}$ | $10.0^{b}$ | $1.21^{bc}$ | | $1.79^{b}$ | $126.2^{ab}$ | $92.5^{bc}$ | $7.5^{ab}$ | $22.1^{c}$ | $8.2^{cd}$ | $13.9^{b}$ | $4.09^{ab}$ | $71.0^{a}$ | $73.6^{ab}$ | $22.1^{c}$ | $51.5^{ab}$ |
| | Ventral | 0.5 | 6.31 | 58.3 | 30.9 | 10.1 | 0.96 | — | 1.96 | 109.5 | 96.8 | 3.2 | 28.9 | 14.0 | 15.0 | 4.89 | 70.7 | 62.2 | 28.9 | 33.3 |
| | | 1 | 6.26 | 57.3 | 31.7 | 10.2 | 0.98 | — | 1.87 | 105.5 | 96.5 | 3.5 | 28.4 | 13.3 | 15.1 | 4.22 | 71.4 | 62.4 | 28.4 | 34.0 |
| | | 2 | 6.21 | 56.6 | 32.3 | 10.1 | 1.18 | 41.2 | 1.79 | 118.8 | 95.9 | 4.1 | 28.6 | 12.6 | 15.9 | 4.64 | 71.9 | 68.7 | 28.6 | 40.1 |
| | | 4 | 6.15 | 55.7 | 34.0 | 9.5 | 0.95 | 26.2 | 1.69 | 119.1 | 94.8 | 5.2 | 24.3 | 9.8 | 14.5 | 4.14 | 72.5 | 69.2 | 24.3 | 44.9 |
| | | 6 | 6.17 | 54.9 | 34.4 | 9.4 | 1.66 | 24.8 | 1.65 | 121.7 | 95.7 | 4.3 | 26.8 | 10.9 | 15.9 | 4.22 | 71.0 | 67.9 | 26.8 | 41.2 |
| | | Mean | $6.22^{ab}$ | $56.6^{b}$ | $32.6^{b}$ | $9.9^{b}$ | $0.95^{c}$ | | $1.79^{b}$ | $114.9^{c}$ | $95.9^{a}$ | $4.1^{c}$ | $27.4^{b}$ | $12.1^{bc}$ | $15.3^{ab}$ | $4.42^{a}$ | $71.5^{a}$ | $66.1^{b}$ | $24.1^{bc}$ | $38.7^{b}$ |

**Table 3.** Continued

| Treat | Sites | Times, h | pH | VFAs, mmol/100 mL | | | | Br, mmol/L | Lactate, μmol/L | A/P ratio | Total VFAs, mmol/L | VFA activity, mmol/100 mL | | TIC, mmol/L | $CO_2$ species, mmol/L | | Viscosity, mPa·s | Surface tension, mN/m | Calculated $CO_2$ species* | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | | Ac | Pr | Bu | | | | | | Base | Acid | | $HCO_3^-$ | $dCO_2$ | | | TIC, mmol/L | $CO_2$ activity, mmol/L $HCO_3^-$ | $dCO_2$ |
| Treat, Site | | SEM, $n = 45$ | 0.041 | 0.29 | 0.29 | 0.11 | | 0.097 | — | 0.028 | 2.46 | 0.55 | 0.55 | 1.22 | 0.97 | 0.65 | 0.175 | 0.50 | 4.13 | 1.22 | 4.02 |
| Treat, Site, Time | | SEM, $n = 12$ | 0.061 | 0.64 | 0.65 | 0.24 | | 0.306 | 14.90 | 0.093 | 5.50 | 1.22 | 1.22 | 2.73 | 2.16 | 1.45 | 0.385 | 1.12 | 9.24 | 2.73 | 8.99 |
| | | Min | 5.39 | 49.3 | 21.1 | 7.1 | | 0 | 0 | 1.19 | 68.1 | 79.2 | 0.6 | 4.1 | 0.5 | 3.5 | 1.12 | 61 | 28.0 | 4.1 | 9.7 |
| | | Max | 7.01 | 64.7 | 41.4 | 13.5 | | 4.74 | 489.7 | 3.06 | 193.2 | 99.4 | 20.8 | 63.9 | 48.2 | 37.5 | 9.16 | 83.3 | 224.4 | 53.9 | 190.0 |

The dietary treatments, Treat; low physically effective neutral detergent fiber, Low$_{pe}$NDF; high in rumen degradable starch, High RDS; the Combined; the rumen dorsal and ventral sac, Sites; the postprandial sampling time, Time, h; the volatile fatty acids, VFAs; acetate, Ac; propionate, Pr; butyrate, Bu; the branched-VFAs, Br; the quotient between Ac and Pr, A/P ratio; total VFAs excluding lactate, VFA activity calculated with Ac, p$K'_a$ 4.76; Pr, p$K'_a$ 4.88; Bu, p$K'_a$ 4.82 at 25°C and equation 3a; bicarbonate, $HCO_3^-$; dissolved carbon dioxide, $dCO_2$; total inorganic carbon, TIC; $CO_2$ activity was calculated with equations 1 and 2, the sample pH, and the dissociation constants p$K_1$, $4.45 \times 10^{-7}$, and p$K_2$, $4.69 \times 10^{-11}$ at 25°C (Edsall, 1969).

*The calculated $CO_2$ species: $dCO_2$ and TIC, where calculated by using equations 3a and 4, the samples' pH and the p$K_1$, $4.45 \times 10^{-7}$ at 25°C (Edsall, 1969), assuming that $HCO_3^-$ was preferably retained during manual rumen sampling. Results are expressed as the mean and standard deviation of the mean, SEM, for the Treat, Site and Time interaction, plus the minimum, Min and maximum, Max, values for each variable and all treatments. The effects of Treat and Site were compared at the 95% confidence level ($P < 0.05$); means that do not share a letter are significantly different (Bonferroni).

to 36 mmol/L. These concentrations are lower than the values extrapolated from an experiment in sheep (Turner and Hodgetts, 1955); however, differences among species are expected (Laporte-Uribe, 2016). Nonetheless, $dCO_2$ concentrations in the range observed in this study might pose a significant risk to the cattle acid-base balance because the gradient of concentrations between the blood (5 mmol/L) and rumen (~15 mmol/L) remains sizeable (Turner and Hodgetts, 1955). The $dCO_2$ absorption into the rumen epithelium is passive (Ash and Dobson, 1963; Gutknecht et al., 1977; Endeward et al., 2013), and cattle seem to have a wider paracellular space and leakier rumen epithelium than do sheep (Laporte-Uribe, 2005). These differences might lead to a greater transepithelial $CO_2$ diffusion in cattle than in sheep, resulting in an apparently lower rumen $dCO_2$ concentrations. A high level of diffusion might also explain the high bidirectional $CO_2$ exchange between the blood and rumen (Ash and Dobson, 1963; Veenhuizen et al., 1988) and the fast postprandial appearance of rumen $CO_2$ in the blood (Whitelaw et al., 1972). Consequently, the greater exchange between compartments might have a greater impact on the acid-base balance in cattle than in sheep, especially if high rumen $dCO_2$ is sustained for extended periods of time.

### *Rumen pH and the Nature of $CO_2$ Holdup*

The activity of molecules in a solution defines the final pH; for example, the greater the ionization of molecules, the lower the pH, as the concentration of $H_3O^+$ increases (Dawes, 1972; Valsaraj, 1999). However, high VFAs alone do not explain low rumen pH; for example, the High RDS diet, which had the highest VFA concentration, resulted in the highest rumen pH. The rumen pH fluctuations were closely associated with $HCO_3^-$ and $dCO_2$ activities. Furthermore, VFA activity was dominated by bases (dissociated VFAs), and although higher VFA activity led to higher acid formation (undissociated VFAs) and lower rumen pH, the changes were small (~10 mmol/100 mL). Nevertheless, rumen pH is the quotient (balance) of ionic activities of all molecules, primarily $CO_2$ and VFA species, in the rumen fluid (acids and bases). Thus, high $dCO_2$ did not correspond to low pH unless $HCO_3^-$ was also low; similarly, an increase in VFAs did not correspond to low pH unless dissociated VFA levels were high.

Important contributors to the final ruminal pH were the changes in Vis and ST elicited by the diets. Rumen Vis increases when cattle are fed diets rich in RDS and pH is also low (Cheng and Hironaka,

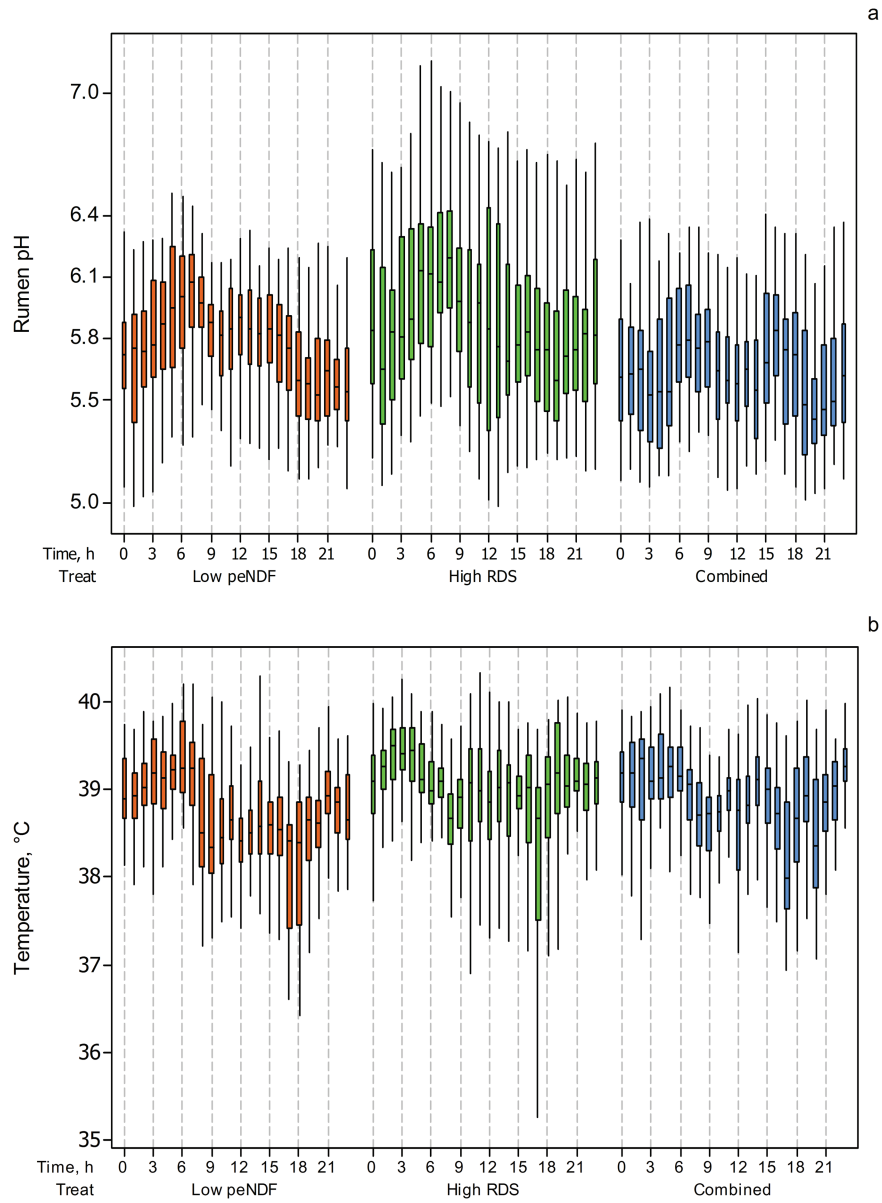


**Figure 2.** The rumen pH (a) and temperature (b), °C, measured every 15 s, from the ventral sac of each of three cows fed the Low $_{pe}$NDF, High RDS or Combined diet, Treat. The central value is the median for each dietary treatment every daytime hour, time (h) for 4 d ($n$ = 2,160), boxes range between quartiles (Q), $Q_1$ and $Q_3$, and the line ranges from the lower to the upper limit $Qn \pm 1.5 \times (Q_3 - Q_1)$.

1973; Cheng et al., 1976). ST has not been routinely monitored; however, earlier studies suggest large variation in ST among diets and a positive relationship of ST with rumen pH (Nichols et al., 1956; Blake et al., 1957). Both high Vis and low ST led to lower pH, which might indicate that Vis and ST influenced or were influenced by rumen $dCO_2$ concentration (Lubetkin, 2003; Islam and Carlson, 2012). In general, diets are regularly screened for $_{pe}$NDF and RDS contents, but the effects of these components on Vis and ST have not been considered. Vis and ST might influence $CO_2$ species equilibrium, leading to rumen pH fluctuations and explaining differences among and within diets (Dijkstra et al., 2012).

Notably, rumen $CO_2$ holdup was associated with high $HCO_3^-$ and $dCO_2$ activity, especially in the ventral sac. These results are important in the context of SARA pathogenesis: clinical signs of SARA are elicited by exposing cattle to diets high in RDS and low in $_{pe}$NDF (Krause and Oetzel, 2005; Dohme et al., 2008). In SARA studies, the cattle are "challenged" by restricting their intake for a day and then reintroducing them to SARA diets. These on-and-off feeding patterns produce large pH fluctuations and might trigger SARA in susceptible cattle (Gozho et al., 2005; Khafipour et al., 2009). Alternatively, SARA signs might be associated with the formation of a large $HCO_3^-$ pool ($CO_2$ holdup) during off-feed periods, as suggested by the observed

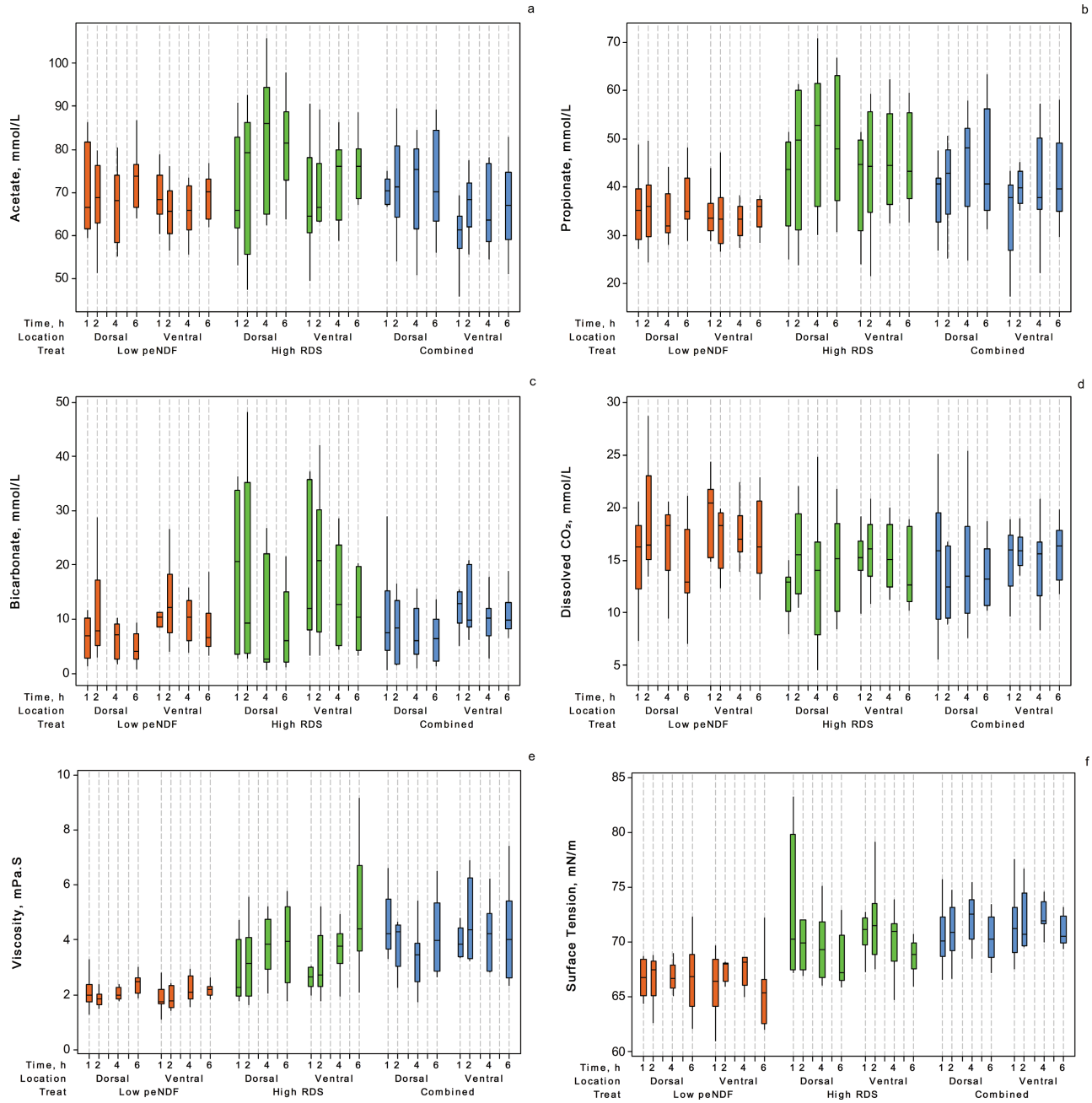


**Figure 3.** The concentrations, mmol/L, of acetate (a), propionate (b), bicarbonate (c), and dissolved $CO_2$ (d) and the viscosity (e), mPa·s, and surface tension (f), mN/m, in the rumen fluid when cattle were fed the Low $_{pe}$NDF, High RDS or Combined diet (Treat), and sampled from the dorsal or ventral sac (Site) at 1, 2, 4, and 6 h postprandially (Time, 7:30 h). Each bar represents the values for the three cattle on the 3-d measurement ($n$ = 12). The central value is the median, boxes range between quartiles (Q), $Q_1$ and $Q_3$, and the line ranges from the lower to the upper limit $Qn \pm 1.5 \times (Q_3 - Q_1)$.

high rumen pH, especially if the provided diets are known to reduce $CO_2$ volatility, for example, high RDS and lucerne diets (Nichols et al., 1956; Cheng et al., 1976). $CO_2$ holdup might lead to high $dCO_2$ when postprandial fermentation quickly transforms $HCO_3^-$ into $dCO_2$, which is detected as a decline in rumen pH (Waghorn, 1991; Laporte-Uribe, 2016). During bloat, a similar mechanism might be responsible for rapid $CO_2$ release, for example, 185 L of $CO_2$ during the first postprandial hour (Waghorn, 1991). Nevertheless, the differences among diets that produce stable foam during bloat (fast $CO_2$ release) and the diets that produce $CO_2$ holdup that leads to SARA might be related to changes in ST and Vis (Laporte-Uribe, 2016).

The highest pH and TIC were observed when cattle were fed the High RDS diet, suggesting that High $HCO_3^-$ levels, might lead to high $dCO_2$ concentration over long periods of time. Furthermore, postprandial rumen pH fluctuations were strongest under

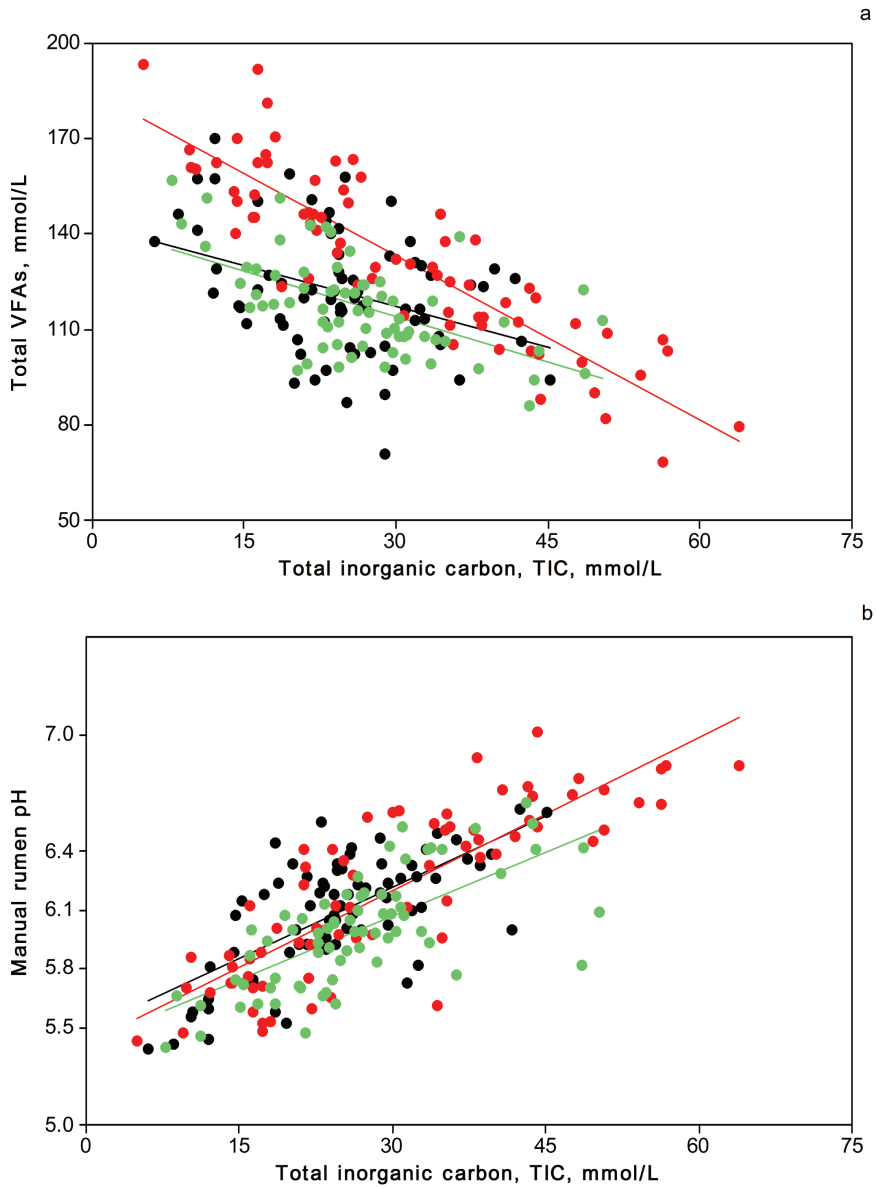


**Figure 4.** The relationship between the total inorganic carbon (TIC), mmol/L, the total volatile fatty acids (VFAs), mmol/L (a), and the pH of rumen samples manually drawn (b). Each dot and line represents values and linear relationship between variables for the three dietary treatments ($n$ = 90) independent of Time and Site: Low $_{pe}$NDF in green, High RDS in red and Combined in black.

the High RDS diet, and the cattle under this diet exhibited a rumen pH under 5.5 for more than 3 h, which is known to increase the risk of SARA (Plaizier et al., 2008). Under that threshold, $HCO_3^-$ no longer exists in solution, and all TIC is in the $dCO_2$ form (Hille et al., 2016; Laporte-Uribe, 2016). The high Vis in the High RDS diet might have reduced $CO_2$ fugacity and led to high and sustained $dCO_2$ concentrations, as the postprandial decline in ST seemed to indicate. SARA affects the blood acid-base status of cattle, that is, hypercapnia (Huber, 1976; Gianesella et al., 2010), and rumen $dCO_2$ quickly reaches the blood $CO_2$ pool (Whitelaw et al., 1972; Veenhuizen et al., 1988). The formation of $CO_2$ holdup ($HCO_3^-$ reservoir) might lead to considerable $dCO_2$ diffusion during periods of low pH ($dCO_2$ formation) and the onset of SARA, as observed when cattle were fed the High RDS diet (see below).

### *Cattle Performance Related to $CO_2$ Species Equilibrium*

The current dietary treatments, Treat, were tailored to lower the rumen pH and increase the risk of SARA by reducing $_{pe}$NDF and increasing RDS (Zebeli et al., 2010). Our experiment did not focus on a specific threshold for optimal $_{pe}$NDF and RDS proportions but rather on providing conditions under which high rumen $dCO_2$ might be found and the equilibrium of $CO_2$ species can be investigated (Laporte-Uribe, 2016). In our experiment, $dCO_2$ activity varied widely and was not

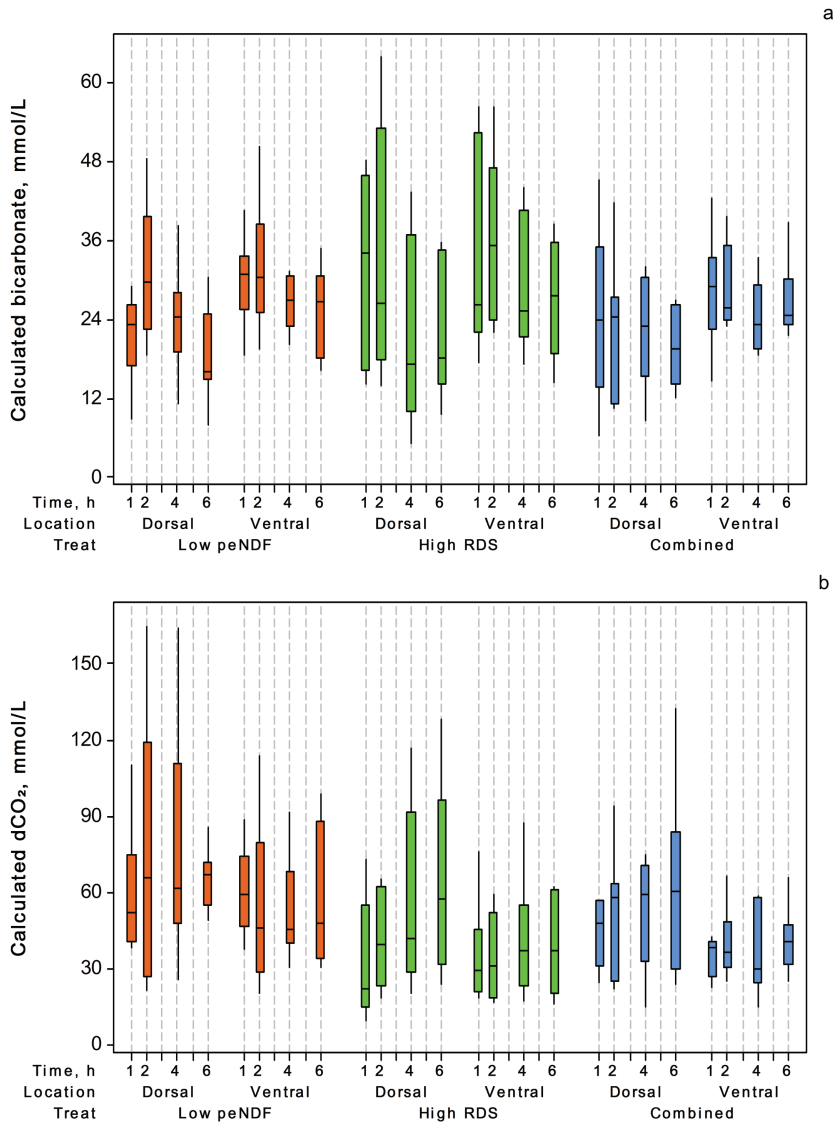


**Figure 5.** The calculated values of bicarbonate, mmol/L (a), and dissolved carbon dioxide ($dCO_2$), mmol/L (b) in the rumen fluid when cattle were fed the Low $_{pe}$NDF, High RDS or Combined diet (Treat), sampled from the dorsal or ventral sac (Site) at 1, 2, 4, and 6 h postprandially (Time, 7:30 h). Each bar represents the values for the three cows on the 3-d measurement ($n$ = 12). The central value is the median, boxes range between quartiles (Q), $Q_1$ and $Q_3$, and the line ranges from the lower to the upper limit, $Qn \pm 1.5 \times (Q_3 - Q_1)$.

constant, low or absent, in contrast to previous suggestions (Kohn and Dunlap, 1998; Hille et al., 2016). Nevertheless, all diets were balanced for energy and protein content, but different ingredients (sugar vs. starch) might have contributed to changes in DMI and MY; for example, the Low $_{pe}$NDF diet might have increased DMI, whereas the High RDS diet might have reduced it (Zebeli et al., 2010). Moreover, the experimental setup did not allow differentiation between the effect of diet on performance and the effect of the sequence in which the diets were provided (Bailey and Greenwood, 2018). Nonetheless, each diet and period combination (Treat) elicited significant changes in rumen physicochemical properties, and these changes provide insight into the potential role of $CO_2$ species on rumen function and cattle performance, as discussed below.

### *Effect of High $dCO_2$ on Energy Supply*

Because $dCO_2$ is a normal feature of the rumen environment, ruminants might have evolved mechanisms to compensate for high $dCO_2$ diffusion. For example, $dCO_2$ diffusion had a positive effect on protecting the epithelium from high rumen VFA concentrations, possibly through the increase in intracellular $HCO_3^-$ formation (Aschenbach et al., 2011; Rackwitz and Gäbel, 2018). High intracellular $HCO_3^-$ might increase VFA absorption, thereby improving energy metabolism and milk productivity (Ash and Dobson, 1963; Aschenbach

et al., 2010). For example, high propionate absorption is known to increase liver gluconeogenesis, MY and milk lactose yield (Aschenbach et al., 2010). Accordingly, when the cattle were fed the Low $_{pe}$NDF diet, rumen $dCO_2$ activity, MY and lactose yield were higher than when the cattle were fed the Combined diet at a similar DMI. Moreover, rumen VFAs and propionate concentrations were lower under the Low $_{pe}$NDF diet than under the Combined diet, suggesting that high $dCO_2$ might promote VFA absorption and thereby lead to greater lactose production and MY. However, the cellular mechanism is unclear, so further research is warranted.

### *Effect of $CO_2$ Holdup on SARA*

Unlike Treat with the $_{pe}$NDF diet, Treat with the High RDS diet lead to clinical SARA signs (Plaizier et al., 2008), including low MY, DMI, and low fat yield but this effect was not associated with rumen lactate accumulation (Table 3). The low DMI under the High RDS diet might have reduced the energy supply and led to MY decline (Humer et al., 2018). The High RDS Treat resulted in the highest rumen VFAs and propionate concentrations among the Treat; such high concentrations are also signs of SARA. This result also suggested low VFA absorption; otherwise, high VFA production should have led to high MY on this Treat. It is possible that $CO_2$ holdup (high $HCO_3^-$) created a negative gradient for VFA exchange, $K_m$ ~54 mmol/L (Aschenbach et al., 2009). Alternatively, the high rumen $HCO_3^-$ might signal impaired $HCO_3^-$ absorption, as both VFAs and $HCO_3^-$ are promoted by the $Na^+/H^+$ exchanger (NHE); that is, conditions that inhibit NHE activity reduce $HCO_3^-$ absorption (Gao and Oba, 2016; Caushi and Martens, 2018). NHE might play a central role in pH regulation, and its activity and expression might explain the susceptibility of cattle to SARA (Penner et al., 2009; Gao and Oba, 2016). Thus, the high VFAs and $HCO_3^-$ concentrations might be a sign of low NHE activity associated with SARA; that is, both VFAs and $HCO_3^-$ absorption were impaired. High $HCO_3^-$ ($CO_2$ holdup) might be the cause: after feeding, $HCO_3^-$ is rapidly transformed into $dCO_2$, which might sustain high $dCO_2$ concentrations, especially if volatility is impaired. As described above, an increase in $dCO_2$ absorption might challenge the intracellular buffer systems, increasing the risk of hypercapnia. In turn, cellular hypercapnia or concurrent hypoxemia might trigger cellular mechanisms that lead to NHE inactivation and clinical SARA signs.

### *Rumen pH Vs. High $dCO_2$ Concentrations*

An additive effect of high RDS and low $_{pe}$NDF was expected when cattle were offered the Combined diet. It generated the lowest average pH and the highest AUC <5.5 among the Treat. The highest rumen Vis and the highest proportion of small particles (<6 mm, ~46 g/100 g) were observed when the cattle were fed the Combined diet, which suggests that $CO_2$ fugacity might have been impaired. In contrast, under the Combined Treat, ST was high, and the $dCO_2$ and $HCO_3^-$ activities were the lowest among the dietary treatments. Furthermore, cattle should have been in greater risk of SARA due to the lowest rumen pH, however clinical SARA was not observed; that is, DMI and MY were similar to pre-trial values. It was assumed that the washout period during the introduction week would prevent any carryover effect from the previous Treat, but perhaps this was not the case. For instance, cattle recovering from SARA increase DMI, saliva secretion and upregulate mucin genes (Beauchemin et al., 2008; DeVries et al., 2009; Dionissopoulos et al., 2013). Saliva is a strong surfactant and has a lower ST than the rumen (~47 mN/m), but its addition to rumen samples increases ST (Blake et al., 1957; Van Horn, 1959). This effect might be caused by salivary mucin, which increases rumen $CO_2$ effervescence (Van Horn, 1959; Bartley and Yadava, 1961) and counteracts the negative effect of high Vis on $CO_2$ fugacity. Therefore, $dCO_2$ might have easily evolved, $CO_2$ holdup was not observed (low $HCO_3^-$), and rumen ST was high. The ST is a function of the $dCO_2$ in the solution (Lubetkin, 2003). Moreover, because both $HCO_3^-$ and $dCO_2$ were low, the quotient was low (rumen pH). Therefore, $CO_2$ holdup formation might be more important for the onset of clinical SARA than is rumen pH, as low rumen pH might not always coincide with high $dCO_2$ and $HCO_3^-$ concentrations. As in bloat, the large polymorphism of mucin genes (Clarke et al., 1974; Hoorens et al., 2011) might also explain the variation in susceptibly to SARA among cattle.

### *Remarks, Constraints, and Limitations*

SARA challenges use a limited number of cattle due to the ethical concern over feeding pathological diets (Maekawa et al., 2002; Krause and Oetzel, 2005). The consecutive SARA challenge in this work allowed for the preparation of the bulk diet, cattle feeding, and rumen sampling in a short period of time, although the same design has been used to observe the adaptation of

cattle to SARA conditions with a larger number of subjects (DeVries et al., 2008; Dohme et al., 2008). Furthermore, the effect of dietary treatment on cattle performance was significant, and all cattle reacted similarly to the diets (NS, Treat × Cows effect). Additionally, under the Combined Treat, the MY and DMI values at the end of the experiment were comparable to the pre-trial values. These results suggest that diet might have played a more important role in influencing performance than did any carryover effect due to the feeding sequence or the decline MY persistence during the trial, but this possibility was not tested.

The main limitation of this study was securing TIC recovery from manual sampling. The samples were pumped, alkalinized, and frozen to reduce $dCO_2$ losses (Buchholz et al., 2014). Theoretically, NaOH addition should inhibit the coalescence of bubbles (Craig et al., 1993), transform $dCO_2$ into $HCO_3^-$ (high pH) and increase TIC recovery, but in hindsight, NaOH addition might have salted out $dCO_2$ from the solution by increasing the ionic strength of the rumen fluid (Ho and Ilgen, 2017). Similarly, bubbling was observed during rumen sampling, which might have contributed to $dCO_2$ losses. In comparison, Hille et al. (2016) drew and snap-froze samples in liquid nitrogen, but $dCO_2$ was not found. Moreover, the TIC values in this study resembled the $HCO_3^-$ values described in sheep and cattle (Turner and Hodgetts, 1955; Hille et al., 2016). For instance, low rumen VFAs are associated with high $HCO_3^-$ due to low fermentation or high VFA absorption (Aschenbach et al., 2011). However, a constant or slightly high TIC might be observed during fermentation due to the high $dCO_2$ and low $HCO_3^-$ activities (Blombach and Takors, 2015). In the present study, TIC values were negatively related to VFA concentration and lowest at pH 5.4 (Dawes, 1972; Hille et al., 2016), suggesting that most of TIC recovered was $HCO_3^-$ and that $dCO_2$ was underestimated.

To test this hypothesis, $dCO_2$ values were calculated using the HH equation, assuming that the TIC was $HCO_3^-$. The calculated values agree with previous values described for both parameters (Turner and Hodgetts, 1955; Chou and Walker, 1964). For instance, the average $dCO_2$ concentrations are close to the theoretical maximum of ~60 mmol/L (Kohn and Dunlap, 1998) and to in vivo estimates of 41 to 60 mmol/L (Chou and Walker, 1964; Wang et al., 2019). The range is also consistent with previous extrapolations of 5 to 180 mmol/L (Laporte-Uribe, 2016). Therefore, rumen manual sampling might have preferably retained $HCO_3^-$ and led to underestimated $dCO_2$ concentrations. Nonetheless, if the actual $dCO_2$ concentrations were higher than those estimated, the above discussion remains relevant and increases the importance of evaluating the effect of rumen $CO_2$ species. Regardless, future experimental work might take advantage of the good $HCO_3^-$ recovery provided by manual sampling. The $dCO_2$ concentrations extrapolated from the HH equation might be accurate enough and simpler to evaluate than those obtained using other current methodologies (Hille et al., 2016; Wang et al., 2019).

Several important aspects remain to be elucidated. The sources of $CO_2$ species are within the fluid; thus, their concentrations depend on the volatility ($K_H$) or the $CO_2$ that can effervesce from the rumen liquor (Sander, 2015) and not on the $CO_2$ solubility or $CO_2$ diffusing into the fluid, which depends on the $_{pp}CO_2$ and Henry's constant ($H$), as previously described (Turner and Hodgetts, 1955; Hille et al., 2016). The function of $_{pp}CO_2$ is to control the exchange and release of $dCO_2$ from the fluid. For instance, the rapid decrease in rumen $_{pp}CO_2$ from ~65 kPa to ~1 kPa $CO_2$ (ambient) during sampling creates a large gradient for $CO_2$ to effervesce from the solution (Kohn and Dunlap, 1998). Similar to this experiment, such $dCO_2$ loss explains why spot sample pH is typically higher than the pH continuously measured with indwelling sensors, pH ~0.35 (Turner and Hodgetts, 1955; Duffield et al., 2004; Hille et al., 2016) and why fistulation reduces $dCO_2$ concentrations (Wang et al., 2019). Nevertheless, the equilibrium constants for $CO_2$ species ($pK_{a1}$ and $pK_{a2}$) depend on full TIC recovery, and $CO_2$ losses during sampling might lead to underestimations of these variables (Leung, 1961). Furthermore, this experiment shows that changes in ST and Vis might influence or are influenced by the rumen pH and $CO_2$ species equilibrium in SARA diets. But to date, $pK_{a1}$, $pK_{a2}$, $H$, and $K_H$ estimations under pathological conditions have not been performed. For this reason, no attempts were made to improve the $CO_2$ species and VFA calculations using established formulas, that is, correction for changes in temperature or the ionic strength of the rumen fluid (Stabenau and Heming, 1993; Hille et al., 2016). Consequently, a better understanding of the role of liquid $CO_2$ species on rumen fermentation and nutritional diseases can be gained by routinely including them in nutritional and physiological experiments. However, better methodologies to estimate rumen $CO_2$ species should be developed to corroborate the present findings.

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