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

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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;

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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, pK_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).

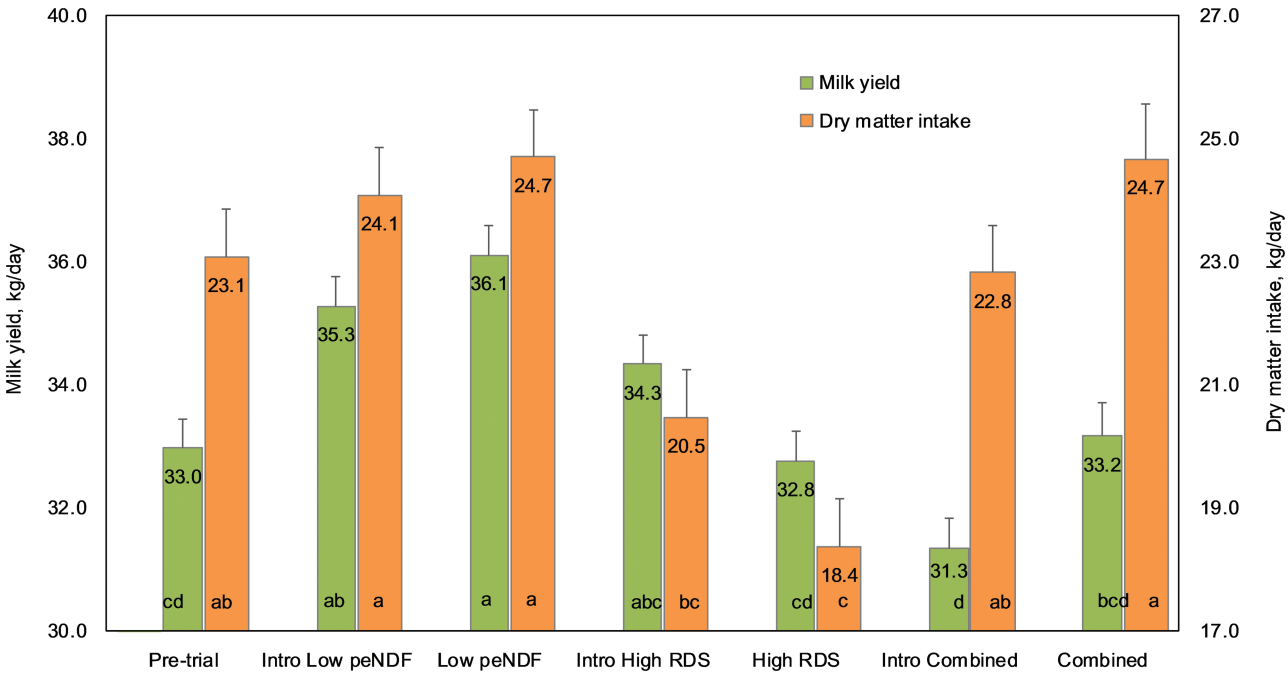

Figure 1. The cattle performance and the feeding sequence during the experiment. The bars represent the mean and standard error of the mean (SEM), for milk yield (MY), and dry matter intake (DMI). After the pre-trial period, three fistulated and lactating dairy cattle, ~100 DIM, were sequentially and 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 4.45×10^{-7} at 25 °C (Edsall, 1969); and K_{a2} is the second dissociation constant, which is 4.69×10^{-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 4.45×10^{-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

																		Calculated CO_2 species*		
				VFAs, mmol/100 mL			Br,	Lactate,	A/P	Total VFAs,	VFA activity, mmol/100 mL		TIC,	CO_2 species, mmol/L		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, pK_a 4.76; Pr, pK_a 4.88; Bu, pK_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 pK_1, 4.45×10^{-7}, and pK_2, 4.69×10^{-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 pK_1, 4.45×10^{-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,

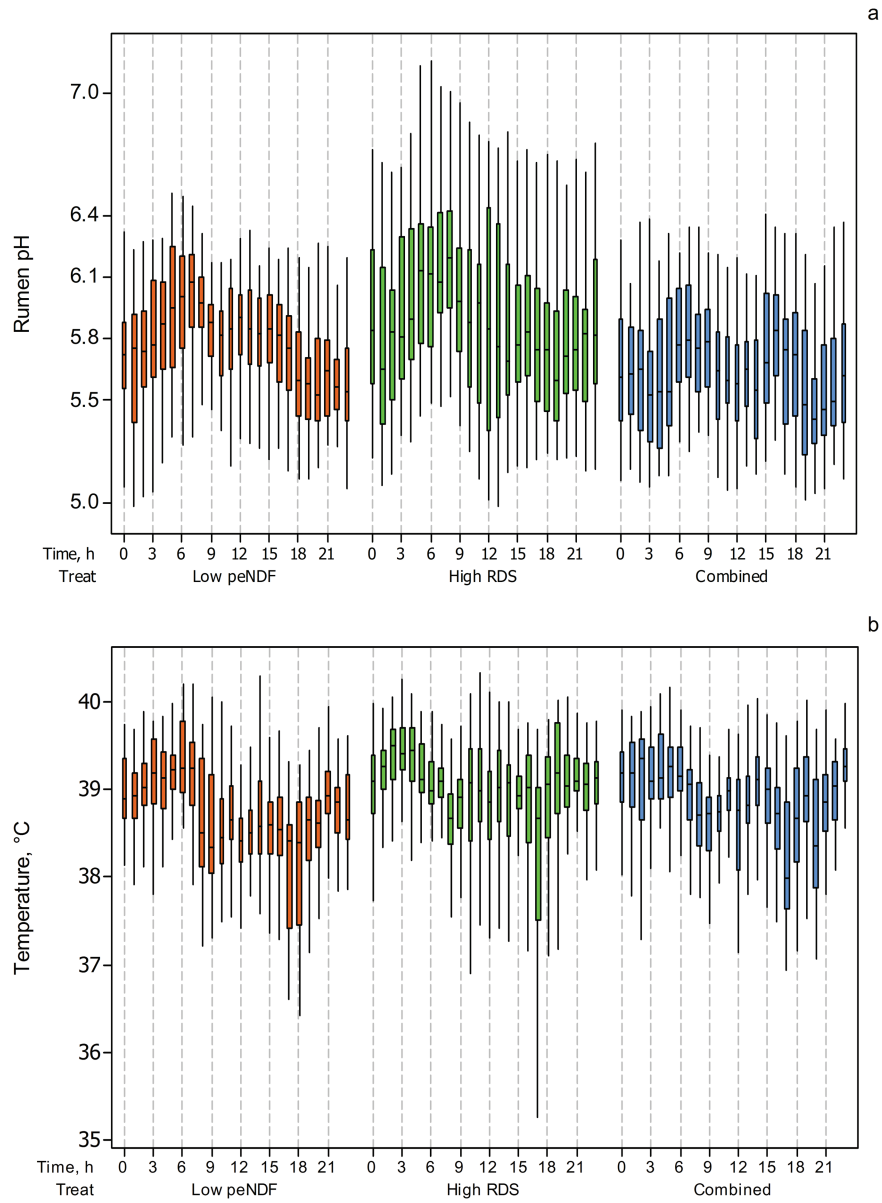

Figure 2. The rumen pH (a) and temperature (b), °C, measured every 15 s, from the ventral sac of each of three cows fed the Low $_{pe}$NDF, High RDS or Combined diet, Treat. The central value is the median for each dietary treatment every daytime hour, time (h) for 4 d (n = 2,160), boxes range between 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

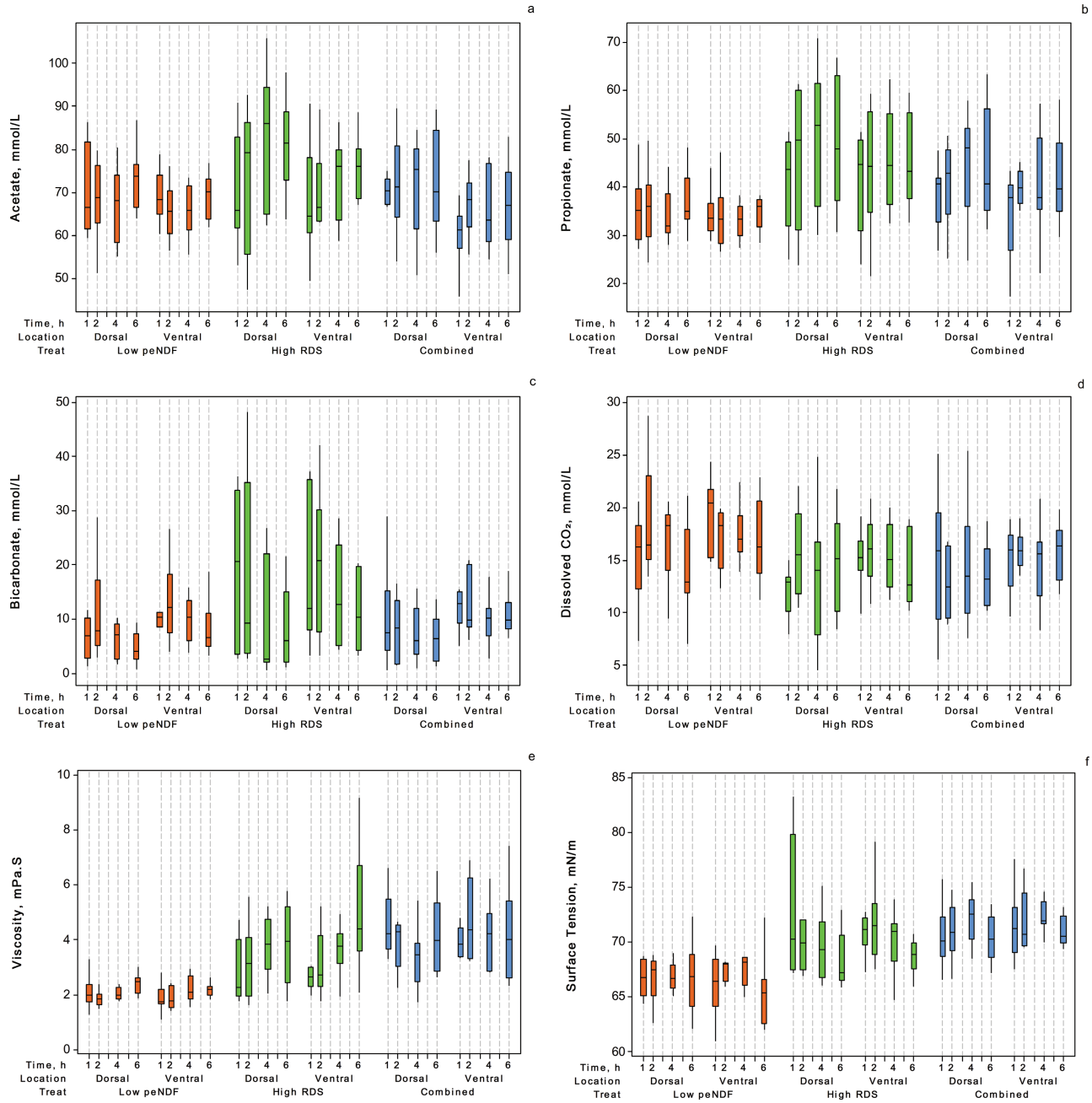

Figure 3. The concentrations, mmol/L, of acetate (a), propionate (b), bicarbonate (c), and dissolved CO_2 (d) and the viscosity (e), mPa·s, and surface tension (f), mN/m, in the rumen fluid when cattle were fed the Low $_{pe}$NDF, High RDS or Combined diet (Treat), and sampled from the dorsal or ventral 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

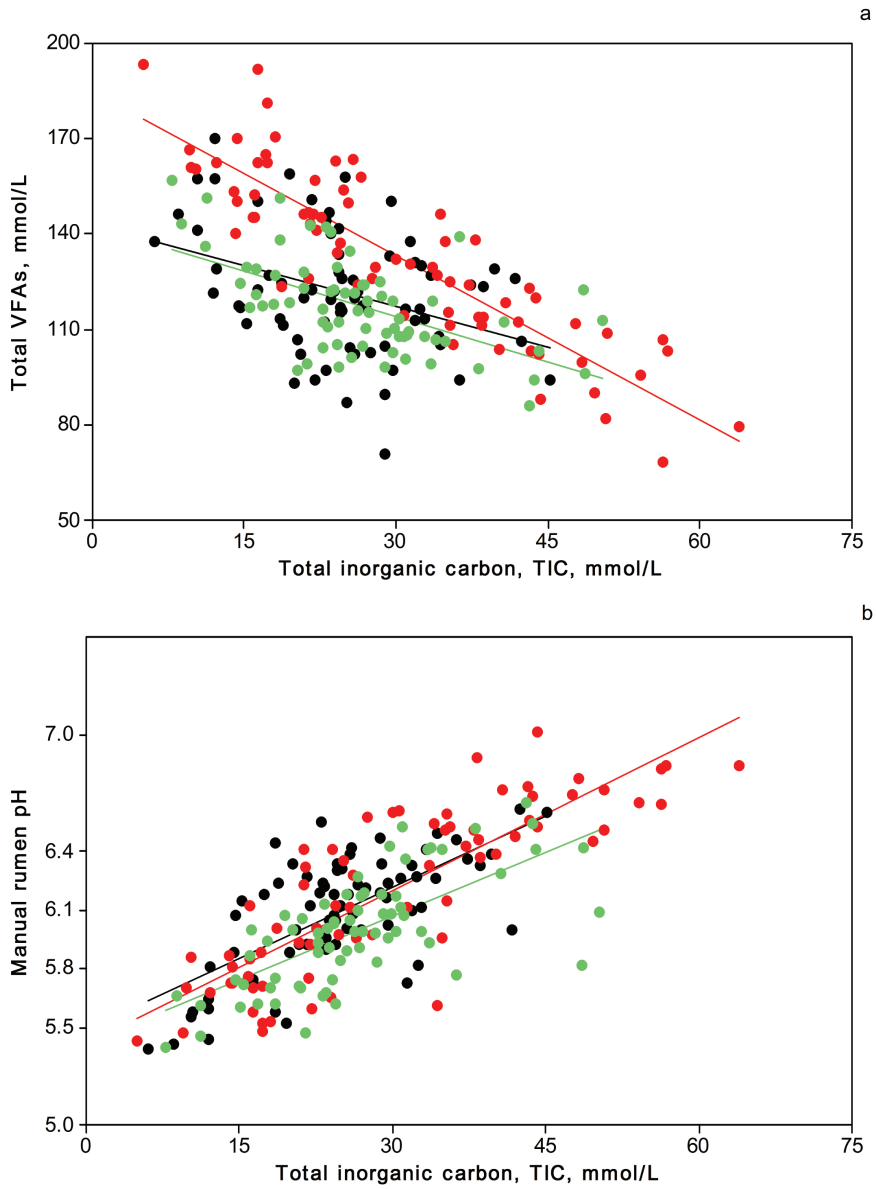

Figure 4. The relationship between the total inorganic carbon (TIC), mmol/L, the total volatile fatty acids (VFAs), mmol/L (a), and the pH of rumen samples manually drawn (b). Each dot and line represents values and linear relationship between variables for the three dietary treatments (n = 90) independent 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

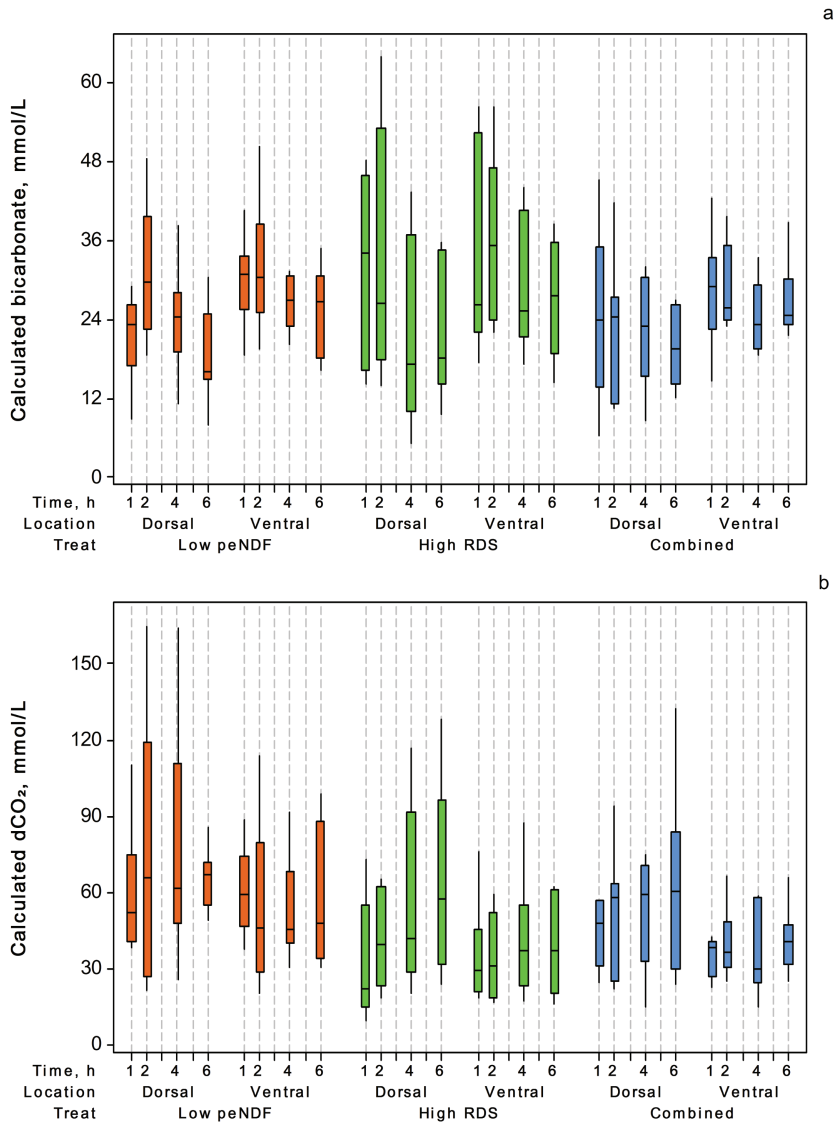

Figure 5. The calculated values of bicarbonate, mmol/L (a), and dissolved carbon dioxide (dCO_2), mmol/L (b) in the rumen fluid when cattle were fed the Low $_{pe}$NDF, High RDS or Combined diet (Treat), sampled from the dorsal or ventral sac (Site) at 1, 2, 4, and 6 h postprandially (Time, 7:30 h). 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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