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Research Article

Ruminal CO2 Holdup Monitoring, Acidosis Might Be Caused by CO2 Poisoning

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The ruminal buffering system is composed of bicarbonate (HCO_3^-) and dissolved CO_2 (dCO_2). While low pH indicates high dCO_2 formation, the pH scale is a ratio between acids and bases in a solution, i.e., HCO_3^- and dCO_2, and fails to provide individual component concentrations. For instance, modern feeding practices can reduce CO_2 gas fugacity from the ruminal fluid or "CO_2 holdup". Under those conditions, not only can dCO_2 reach critical concentrations, but the buffering system might favour HCO_3^- formation, resulting in normal ruminal pH values, the quotient, regardless of the harmful dCO_2 accumulation. Consequently, subacute ruminal acidosis (SARA), traditionally associated with low or variable pH, might be triggered by CO_2 holdup. This observational study aimed to continuously monitor ruminal dCO_2 and characterised CO_2 holdup within the ruminal fluid, targeting the specific infrared signal of dCO_2 with an attenuated total reflectance infrared (ATR-IR) spectrometer. Three lactating dairy cattle were longitudinally exposed to diets designed to elevate both ruminal dCO_2 and SARA risk. Indwelling pH sensors and ruminal fluid samples served as references for dCO_2 analysis, while a categorical analysis detected CO_2 holdup from the output of the ATR-IR sensor. Milk yield, milk components, and feed intake supported the known positive role of high dCO_2 in rumen function. However, SARA was associated with ruminal CO_2 holdup, suggesting that prolonged exposure to critical dCO_2 concentrations during extended postprandial periods might trigger SARA. Continuous dCO_2 monitoring with the proposed methodology and analysis may offer a valuable tool for optimising rumen function and preventing SARA risk.

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Introduction

Arrhenius' theory suggests that the pH scale signals water (H_2O) ionisation into hydronium (H_3O^+) and hydroxides (HO^-) and their equilibrium. Therefore, the ruminal pH scale indirectly measures the effect of bicarbonate (HCO_3^-) buffering and dCO_2 formation on H_3O^+ activity[1][2]. For example, the ruminal CO_2 buffer system works by reducing H_2O dissociation. Ruminal H_3O^+ and HCO_3^- are combined to produce dCO_2, which is the main liquid form of CO_2 in the fluid, i.e., carbonic acid is short-lived in H_2O, <1%[1]. This step is catalysed by carbonic anhydrase[3]. The ruminal fluid is buffered, and H_2O dissociation is reduced when dCO_2 dissociates into H_2O and CO_2 gas, with the CO_2 gas subsequently evolving into the ruminal gas cap to be released via eructation (Diagram 2).

Traditionally, the ruminal short-chain fatty acid (SCFA) concentrations are blamed for pH variations; however, the primary driver of these fluctuations is the CO_2 buffer system[4]. SCFA concentrations are seemingly constant[5], are dominated by the bases, not the acid forms of SCFA, pK_a ~4.7, even lactic acid that is commonly associated with ruminal acidosis is mainly found as lactate, the base, pK_a ~3.8[6]. As bases, SCFA play only a buffering role when the pH is below 5.4 and HCO_3^- is depleted[4][7]. Moreover, the threshold for ruminal acidosis, pH 5.5[8], the rumen fluid

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equilibrium or pKa' 6.1[7] and the pH scale range, 5 to 7[9], coincide with the CO_2 species equilibrium described by the Bjerrum equations[10]. In fact, the relationship between SCFA formation and the pH scale decline may simply be a consequence of dCO_2 released during fermentation[11][12]. Therefore, increased SCFA production leads to greater dCO_2 formation, with a concomitant ruminal pH decline.

Another aspect explaining the spurious relationship between pH, SCFA, and dCO_2 is the capnophilic nature of ruminal bacteria[13][14]. Ruminal succinate- and lactate-producing bacteria thrive in high dCO_2 environments[15][16]. Under these conditions, ruminal propionate production also increases, as succinate and lactate are the main precursors[17]. Consequently, a rumen environment rich in dCO_2 enhances propionate production, manifested as a low acetate to propionate ratio (A/P ratio) and low pH[18][19]. Moreover, high ruminal dCO_2 stimulates lipopolysaccharide (LPS) formation in *Streptococcus bovis* [20][21], a major factor described in the pathogenesis of ruminal acidosis[22]. Similarly, elevated lactate during ruminal acidosis may result from bacteria favouring the acrylate pathway, which does not involve decarboxylation reactions that can be limited by high ruminal dCO_2[15][23][24]. Therefore, the common clinical signs of ruminal acidosis may be a consequence of high dCO_2 concentrations.

The risk of subacute ruminal acidosis (SARA) is attributed to prolonged exposure to low ruminal pH[8][25][26], which might indicate high dCO_2 formation (Henderson-Hasselbalch equation, Eq. 2). However, the individual risk of SARA is associated with variable pH bouts[27], suggesting also an increased HCO_3^- formation. The ruminal HCO_3^- and dCO_2 concentrations are influenced by factors such as the diet (SCFA formation), metabolism (H_3O^+ buffering), and physicochemical characteristics of the rumen liquor[7][1][2]. For instance, modern feeding practices may reduce ruminal CO_2 effervescence because the small particles and highly fermentable materials present in modern diets make the ruminal fluid less ideal[1]. Under those conditions, CO_2 fugacity deviates from Henry's law, and ruminal dCO_2 accumulates beyond the ideal equilibrium with the gas cap, or CO_2 holdup[1]. Therefore, the variable pH bouts observed during the onset of SARA may be consequential to CO_2 holdup, as it might also increase HCO_3^- formation.

Ruminal dCO_2 and CO_2 blood pools rapidly equate[28][29] due to the positive ruminal gradient with the blood, ~60 vs. 2.5 mM[4][30] and the preferential use of ruminal CO_2 for SCFA uptake[31][32]. Consequently, the proposed timescale and risk for the onset of SARA might involve prolonged exposure of the ruminal epithelium to critical dCO_2 concentrations, and SARA signs might be caused by CO_2 poisoning.

Ruminal dCO_2 concentrations are partially characterised, and CO_2 holdup has never been observed *in situ*[2][33][34][35]. This observational study aimed to use a wired attenuated total reflectance infrared spectrometer (ATR-IR) that detected the distinctive IR signal of dCO_2 at 4.27 µm within the ruminal liquor[36]. The more reliable ATR-IR technique and output evaluation might help us to confirm the following hypotheses: (first) to confirm the ruminal dCO_2 range, (second) to unveil the relationship between CO_2 species and pH, and (third) to disclose the role of CO_2 holdup in disease (SARA) and rumen function.

Materials and Methods

Ethical and experimental guidelines

The experimental protocols were approved and licensed by the Animal Care and Ethics Committee of Wageningen University and Livestock Research, WUR Dairy Campus, according to the Experiment in Animals Act, WOD, The Netherlands, with permit AVD401002015298. The care of all cattle involved in this experiment adhered to the guidelines of the ethical committee for the use of fistulated cattle in tied stall facilities.

The experimental setup

The diets and cattle performance were described previously[2]. In brief, three fistulated lactating dairy cattle (Bar Diamond Inc., Ida., USA; 10 cm diameter), ~100 days in milk (DIM), were housed in tied stalls. The cattle were milked twice daily with ad libitum access to drinking water. Three total mixed ration (TMR) diets were prepared daily using an automatic feeding system (Trioliet Feeding Technology, Oldenzaal, The Netherlands) and were served in equal parts, three times per day. The SARA-prone diets were a low physically effective neutral detergent fibre (Low-peNDF), a high ruminally degradable starch (High-RDS), and a combination of both (Combined); please see Laporte-Uribe[2] for details on the formulation. All cattle were fed the same diet simultaneously for two weeks (run): the second week was for ruminal sampling and sensor deployment. The cattle had a three-day rest period between runs on a standard production TMR diet (Dairy Campus, Wageningen University). Indwelling pH sensors and manual ruminal samples were used as references (Diagram 1).

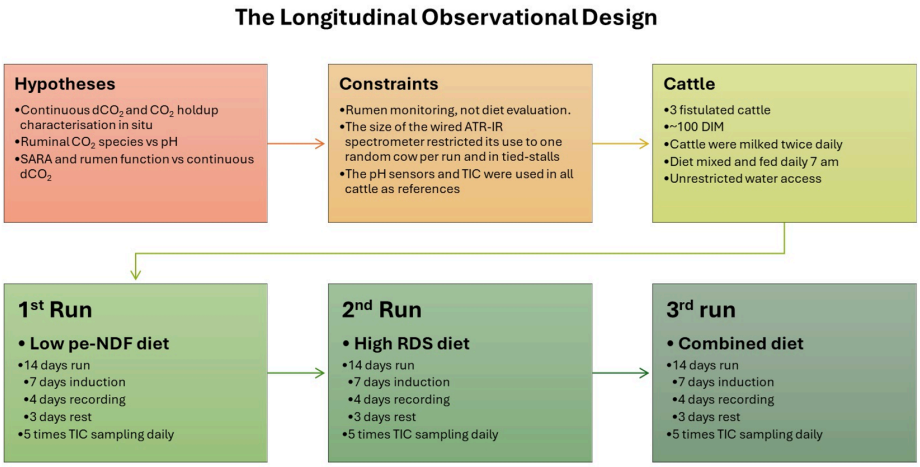

Diagram 1. The longitudinal observational design aimed to continuously monitor ruminal dCO_2 concentration. Total inorganic carbon (TIC) from rumen fluid samples and indwelling pH sensors were employed to corroborate the output of the attenuated total reflectance infrared (ATR-IR) sensor. The diets were the low physically effective neutral detergent fibre (Low-peNDF) in the 1st run, the high ruminally degradable starch (High-RDS) in the 2nd run, and a combination of both previous diets (Combined) in the 3rd run.

Sensor deployment

The pH from the ventral ruminal sac was recorded every 15 sec for three days with indwelling pH sensors in all cattle (DASCOR, Inc., CA, USA). For continuously monitoring the ruminal dCO_2 concentrations in one random sentinel cow per run, a wired ATR-IR sensor, VS-3000/3000E Sensor System, was employed (BevSense LLC, MA, USA, formerly VitalSensors Technologies LLC). The ATR-IR was placed into the ventral ruminal sac, and the dCO_2 was recorded every 10 seconds for three days. The wire was exteriorised through the cannula, sealed to reduce CO_2 losses, and connected to the sensor Management Station, VS-300 (BevSense LLC, MA, USA).

All pH sensors were calibrated before and after placement using a three-point calibration protocol (DASCOR, Inc., CA, USA). The ATR-IR sensor came calibrated for sensing dCO_2 specific IR signal at 4.27 µm in liquids ranging from 0 to 273 mM with a resolution of 0.02 mM, a repeatability of 0.36 mM, and an accuracy of 0.89 mM; see the product specification for details (BevSense LLC, MA, USA). Nevertheless, validation of the ruminal dCO_2 values and range was advised using a three-point alignment protocol developed for steady fermentative processes (BevSense LLC, MA, USA). The following modified protocol was adopted due to the dynamic nature of the ruminal environment.

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Ruminal fluid samples and calculations

The ventral ruminal sac fluid was manually sampled five consecutive times postprandially, with feeding starting at 07:00h (0.5 h, 1 h, 2 h, 4 h, 6 h), during the first three days of the experimental week in all cattle. The pH of the samples was recorded with a temperature-corrected handheld system (Seven2Go ProS8, Mettler-Toledo). Approximately 30 ml of rumen fluid was alkalised by the addition of 1 ml of 5 M sodium hydroxide (NaOH) solution and was frozen for subsequent total inorganic carbon (TIC) analysis (-20 °C). The goal was to retain TIC in HCO_3^- form by increasing the pH of the sample (pH ~10), according to the protocols given by the reference laboratory[10]. TIC was determined by gas chromatography at the Institute of Biochemical Engineering, University of Stuttgart.

Calculations of CO_2 species. The ruminal dCO_2 concentrations were computed from the TIC using the Bjerrum plot equation (Eq. 1) and described as the **observed dCO_2**. The **calculated dCO_2** was derived from the TIC as if only HCO_3^- was recovered. The **calculated HCO_3^-** was derived from the average pH and dCO_2 sensor reading for each minute in a day (1,440 records). Both the **calculated dCO_2** and **calculated HCO_3^-** were computed using the Henderson-Hasselbalch equation (Eq. 2).

$$dCO_2 = \frac{\left[H_3O^+\right]^2}{\left[H_3O^+\right]^2 + K_{a1} * \left[H_3O^+\right] + K_{a1} * K_{a2}} * \text{TIC}\,, \qquad \text{(Eq. 1)}$$

and,

$$-\log\left[H_3O^+\right] = -\log K_{a1} + \log\left(\frac{HCO_3^-}{dCO_2}\right) \qquad \text{(Eq. 2)}$$

where dCO_2 is the dissolved carbon dioxide (mM); HCO_3^- is bicarbonate (mM); TIC is the total inorganic carbon (mM); $[H_3O^+]$ is the hydrogen/hydronium activity derived from the pH of the sample (10^{-pH}); the 1st dissociation constant (K_{a1}) 4.45 × 10^{-7}; and the 2nd dissociation constant (K_{a2}) 4.69 × 10^{-11} at 25 °C.

Raw values from the ATR-IR sensor were expressed in parts per million per 100 g of H_2O (ppm/100 g H_2O), and the following formulas were used to convert these values to millimoles per litre (mM) of ruminal dCO_2.

$$x\left(\frac{ppm}{100gH_2O}\right) = y\left(\frac{mg}{100g\mathrm{H_2O}}\right) \qquad \text{(Eq. 3.1)}$$

and,

$$z(mM) = \frac{y}{44.01 * 10} \qquad \text{(Eq. 3.2)}$$

where *x* is the ruminal dCO_2 concentration in parts per million per 100 g of H_2O (ppm/100 g of H_2O), *y* is the dCO_2 in milligrams per one hundred grams of water (mg/100 g H_2O), and *z* is the dCO_2 in millimoles per litre (mM).

Analysis and statistics

All values from the dCO_2 and pH sensors were used in the development of the categorical analysis except for the records made one hour after deployment. A histogram method was used to detect outliers in the sensors' output[37]. The pH sensors yielded no outliers, and the ATR-IR yielded only a few values. Values for CO_2 and HCO_3^- from the ruminal manual samples were compiled together. All descriptive statistical analyses and graphics were conducted in Origin 2020 (Origin Lab Corporation, MA, USA).

Categorical analysis to observe ruminal CO_2 holdup

The area under the curve for ruminal pH (AUC, pH units per min) emphasises the duration of the acidotic bouts at specific thresholds[8]. AlZahal et al.[25] employed the cumulative time under the curve to define a cut-off point for half-day exposure. More recently, Villot et al.[26] normalised

ruminal pH recordings and described two optimal thresholds, the 30th and 50th percentiles, for SARA detection. Previously, a “categorical analysis” was proposed to observe ruminal pH in the New Zealand pastoral system[38]. Our assumption was that changes in sensor location, due to the mixing movements and by the influx and outflow of nutrients, led to the recording of distinct pH values. Nevertheless, with sufficient “iterations,” the pH category with the highest frequency was consistently identified, such as in several cattle, days, and short recording intervals (<15 seconds). The four categories for ruminal pH values were “Critical,” (pH <5.4), “Acidic” (pH between 5.4 and 5.8), “Optimal” (pH between 5.8 and 6.4), and “Suboptimal” (pH> 6.4), reflecting the state of the art on the effect of ruminal pH. For instance, cattle with pH values lower than 5.4 and 5.8 for 3 to 5 h/d have a high risk of ruminal acidosis and SARA[39][26]. Bacterial protein synthesis and fibre digestion diminish when the pH falls below 5.8, which is also recognised as a sign of ruminal dysfunction[19][9]. Values around 6.4 are in the upper range in cattle given a TMR and are optimal for fermentation in pasture-based diets[19][9].

To my knowledge, this is the first time that continuous recordings of ruminal dCO_2 have been performed, and thresholds for ruminal dCO_2 function remain undefined. However, CO_2 holdup can be identified by assigning a probability value derived from the normal cumulative distribution function (Eq. 4). Accordingly, four categories for ruminal dCO_2 were defined: "Low" for values below the 10th percentile, "Normal" for values between the 10th and 50th percentiles, "High" for values between the 50th and 90th percentiles, and "Critical" for values above the 90th percentile.

$$\mathrm{F}(x) = \frac{1}{2}\left[1 + \mathrm{erf}\left(\frac{x-\mu}{\sigma\sqrt{2}}\right)\right] \qquad \text{(Eq. 4)}$$

where “x” is the recorded dCO_2 value, “μ” is the overall dCO_2 mean, and “σ” is the overall standard deviation for the experiment.

To comprehend the daily variation in these parameters and monitor CO_2 holdup, the day was divided into discrete segments of 10 minutes, e.g., 0:00, 0:10..., 23:50, or 144 segments. The interval was visually chosen, i.e., details were lost with longer intervals, and intervals smaller than 10 minutes might require shorter sampling frequencies or more iterations. Therefore, the “frequency” for each category was calculated by adding all the recorded values throughout the experiment for the 10-minute interval. The AUC (%) for each category was the frequency divided by the total number of observations within that 10-minute segment, multiplied by one hundred. The graphical representation, a 100% stacked area, provided a succinct overview of the calculated AUC for pH, Fig. 2.1a-c, and dCO_2, Fig. 2.2a-c.

Results and Discussion

Repeated acidosis challenges can lead to SARA[39], and the diets were fed in subsequent two-week periods. Cattle during the first run on the Low-peNDF diet experienced increased milk yield, they developed SARA when fed the High-RDS diet in the second run, and returned to pre-trial performance when fed the Combine diet, third run[2]. Accordingly, this report focuses on ruminal dCO_2 monitoring with the ATR-IR spectrometer and does not reiterate these previously established facts, which are again summarised in Table 2.

This is the first time that ATR-IR was used to monitor continuously in situ ruminal dCO_2 concentrations. It was uncertain whether ruminal dCO_2 would exceed ~60 mM[30], whether CO_2 holdup would develop, or if the dietary treatments would produce signs of SARA. Early work revealed high and varied ruminal dCO_2[31][33][34], but confirming its presence by manually sampling the rumen was challenging, Table 1[7][2][35]. The TIC sampling protocols adhered to the laboratory's recommendations[10], recognizing that freezing and transporting ruminal samples could lead to dCO_2 losses[7]. However, this report relies instead on the more established and accurate ATR-IR technique targeting the specific IR signal of dCO_2 to confirm the ruminal dCO_2 range and presence[36]. Moreover, the widespread use of the ruminal pH scale[8][40] has obscured the well-established significance of dCO_2 in rumen function[31][41]. As you are about to observe, ruminal dCO_2 did exist in substantial quantities, and rather than solely attributing changes in rumen function to the diet or feeding sequence, we should also consider the role that these large variations in ruminal dCO_2 concentrations might elicit on the epithelium and bacterial activity.

Manual sampling versus continuous ruminal CO_2 monitoring. Table 1 summarises the values for pH, total inorganic carbon (TIC), and the **observed dCO_2** obtained through manual sampling of the ventral ruminal sac. Previously, it was stated that the manual TIC sampling protocol used in these experiments primarily recovered ruminal HCO_3^-.[2]. For instance, the marked difference between manual (0.5 points higher) and continuous pH monitoring[42][40] is attributed to dCO_2 losses during manual sampling[4][30]. To verify this assumption, **calculated HCO_3^-** was derived by averaging the continuous pH and dCO_2 measurements (Eq. 2). The **calculated HCO_3^-** closely resembled the TIC values for all diets, which confirmed that mostly HCO_3^- was recovered via manual sampling. Subsequently, **calculated dCO_2** was computed from TIC (now HCO_3^-) and compared to the **continuous dCO_2** values derived from the ATR-IR sensor, as presented in Table 1 (Eq. 1).

Discrete manual sampling and continuous measurement represent different techniques with distinct outcomes[42][40] and are not readily comparable due to variations in time scales and sampling locations. Acidification of ruminal fluid samples in the past has yielded substantial TIC recovery[34][33]; however, alkali addition cannot be recommended for manual TIC sampling[2]. Nevertheless, the good agreement between the **calculated HCO_3^-** and TIC values, as well as between the **calculated** and **continuous dCO_2** values (Table 1), highlights the suitability of the ATR-IR technique and sensor for continuously monitoring CO_2 holdup and dCO_2 concentrations.

The results also support, as a discrete sampling alternative, targeting ruminal HCO_3^- using the protocols described by Hille et al.[7], in conjunction with *in situ* pH measurements, to indirectly estimate ruminal dCO_2 using the equations described here (Eq. 1). However, the manual sampling technique will have limited predictive value in detecting CO_2 holdup formation or SARA onset compared with continuous ruminal dCO_2 monitoring.

Parameter	Dietsxrun	Nxn	Mean	SD	SEM	Percentile			Normality	Skewness	Kurtosis
						10	50	90			
pH	Low-peNDF 1st run	3x45	6.08	0.219	0.033	5.75	6.07	6.41	0.47	0.15	-0.36
	High-RDS 2nd run	3x45	6.31	0.349	0.052	5.76	6.41	6.72	0.01	-0.54	-0.83
	Combined 3rd run	3x45	6.22	0.193	0.029	6.01	6.24	6.46	0.25	-0.69	1.60
TIC, mM	Low-peNDF 1st run	3x43	28.9	8.19	1.25	19.1	27.3	40.6	0.04	0.79	0.41
	High-RDS 2nd run	3x45	33.6	12.44	1.85	20.8	31.4	54.1	0.03	0.47	-0.83
	Combined 3rd run	3x45	27.4	6.30	0.94	20.3	25.7	36.3	0.45	0.42	-0.18
observed dCO_2, mM	Low-peNDF 1st run	3x43	17.9	4.33	0.66	12.8	17.2	22.4	0.01	1.22	2.42
	High-RDS 2nd run	3x45	15.6	3.72	0.55	11.1	15.2	19.2	0.01	1.02	2.44
	Combined 3rd run	3x45	15.3	2.93	0.44	10.6	15.8	18.9	0.54	-0.38	-0.24
calculated dCO_2, mM	Low-peNDF 1st run	3x43	55.2	23.38	3.57	30.5	46.8	91.7	0.01	0.76	-0.44
	High-RDS 2nd run	3x45	40.9	31.43	4.68	18.1	31.3	62.1	0.00	3.04	11.76
	Combined 3rd run	3x45	38.7	16.50	2.46	22.0	37.4	57.2	0.00	1.97	6.74
continuous pH	Low-peNDF 1st run	3x51,368	5.77	0.293	0.001	5.37	5.78	6.16	-	- 0.17	- 0.56
	High-RDS 2nd run	3x51,212	5.91	0.433	0.002	5.36	5.86	6.53	-	0.33	- 0.64
	Combined 3rd run	3x51,092	5.65	0.305	0.001	5.25	5.63	6.07	-	0.16	- 0.81
continuous dCO_2, mM	Low-peNDF 1st run	1x25,511	74.7	12.38	0.08	58.1	75.7	88.7	-	- 0.26	1.38
	High-RDS 2nd run	1x25,929	73.0	12.64	0.08	58.2	71.8	90.5	-	0.08	0.21
	Combined 3rd run	1x25,474	59.1	14.86	0.09	39.9	59.4	77.7	-	0.37	1.18
calculated HCO_3^-, mM	Low-peNDF 1st run	1x1440	20.6	6.05	0.16	12.8	20.3	27.7	-	0.47	-0.29
	High-RDS 2nd run	1x1440	28.0	12.03	0.32	17.9	23.6	49.2	-	1.51	1.30

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Parameter	Dietsxrun	Nxn	Mean	SD	SEM	Percentile			Normality	Skewness	Kurtosis
						10	50	90			
	Combined 3rd run	1x1440	12.0	2.89	0.08	8.8	11.4	16.4	-	0.80	-0.06

Table 1. Descriptive statistics of ruminal parameters measured by manual and continuous sampling methods*.

**The diets were the low physically effective neutral detergent fibre (Low-peNDF) in the 1st run, the high ruminally degradable starch (High-RDS) in the 2nd run, and a combination of both previous diets (Combined) in the 3rd run. Cattle (N) and records/samples (n). Manual sampling of ruminal pH and total inorganic carbon (TIC). Continuous ruminal pH (continuous pH) and dCO_2 concentration (continuous dCO_2) measurements. The Observed dCO_2 was calculated with Eq 1. The dCO_2 derived from TIC (calculated dCO_2) and the HCO_3^- derived from the pH and dCO_2 sensor (calculated HCO_3^-) were computed using Eq 2. The median (50th percentile) and the 10th and 90th percentiles, respectively. Normality of discrete manual samples was assessed using the Shapiro-Wilk test (p = 0.05). For continuous measurements, descriptive statistics provide a reliable assessment of normality due to the Central Limit Theorem.*

Continuous ruminal dCO_2 monitoring. The law of large numbers justified the reliance on descriptive statistics for analysing the continuous sensor data rather than solely on statistical comparisons (Table 1). Multiple independent measurements of a physiological phenomenon typically follow a normal distribution, and the central value tends to be closer to the expected mean value, the Central Limit Theorem[43]. The agreement between discrete measurements of pH, **calculated dCO_2**, and **TIC** by manual sampling, and continuous measurements of pH, dCO_2, and **calculated HCO_3^-**, respectively (Table 1), suggests a high likelihood that all parameters originated from the same population. The small kurtosis, skewness, and similar central values, both mean and median, for all diets indicated that the continuous dCO_2 and pH recordings conformed to a Gaussian curve (Fig. 1 and Table 1). The normal distribution of these biological parameters justified normalisation for detecting disease, comparing diets, and eliminating drift or calibration errors[8][25][26]. Consequently, the goodness of fit of the output of the sensors employed in this study suggests that they accurately detected ruminal pH and dCO_2 within the physiological and pathological range (Fig. 1 and Table 1), supporting the first hypothesis that ATR-IR is well-suited for continuous ruminal dCO_2 monitoring.

The range of ruminal dCO_2 concentrations detected by ATR-IR was 0 to 130 mM (Table 1). These results were comparable to the values for ruminal dCO_2 described for sheep[33][34]. The average ruminal dCO_2 values for the Low-peNDF, High-RDS, and Combined diets were 74.7, 73.0, and 59.1 mM, respectively (Table 1). These values mirrored those described for intact cattle (69.7 mM) and fistulated cattle (43.6 mM) and were close to the theoretical average ruminal dCO_2 of ~60 mM[30][35]. The observed peak ruminal dCO_2 values for the Low-peNDF (171 mM), Combined (151 mM), and High-RDS (117 mM) diets cannot be dismissed as biologically implausible. These findings challenge the previously proposed static view of the ruminal buffering system and the saturation of the ruminal fluid at 60 mM of dCO_2[44][30].

To provide context, human blood dCO_2 rarely exceeds ~5% of the total CO_2 content, with venous dCO_2 levels at rest and during exercise being ~1.4 mM and ~2.4 mM, respectively[45]. Cattle venous dCO_2 levels, calculated from total CO_2 using Eq. 1, might range from 2.2 to 2.5 mM under SARA[46]. Further, dCO_2 concentrations at rest in the inner lining fluid of the alveolar region are ~1.3 mM, corresponding to a 5% end-tidal CO_2 gas content[47]. Ruminants exposed to over 5% CO_2 gas in metabolic chambers develop tachypnoea[48] and at >10% CO_2 gas exposure, alveolar dCO_2 might exceed blood levels, reaching over ~2.4 mM, which is considered toxic[49]. In contrast, ruminal

dCO_2 values above 80 mM were routinely observed in all diets (Table 1, Fig. 2.2abc). These values are 30 times higher than blood and are readily available for transepithelial absorption.

The rapid equilibrium between ruminal and blood dCO_2 pools is well established[28][29] primarily due to CO_2 diffusion[50][51] and the utilization of ruminal CO_2 for SCFA absorption[31][32]. These exceptionally high ruminal dCO_2 concentrations suggest that ruminants are constantly exposed to hypoxemic/hypercapnic conditions, which explains several known unique physiological adaptations, such as the high ruminal epithelial cholesterol content[52][53] which limits CO_2 diffusion[51]; the low oxygen affinity of adult ruminant haemoglobin[54] which improves peripheral tissue oxygenation, and the enhanced blood HCO_3^- carrying capacity due to the chloride shift[55]. Blood CO_2 is carried mainly as HCO_3^-[45]. Nevertheless, the development of CO_2 holdup might enhance CO_2 absorption and overwhelm the cellular buffering system, as the capacity to eliminate this dCO_2 excess is impaired by the low CO_2 gas fugacity from the fluid.

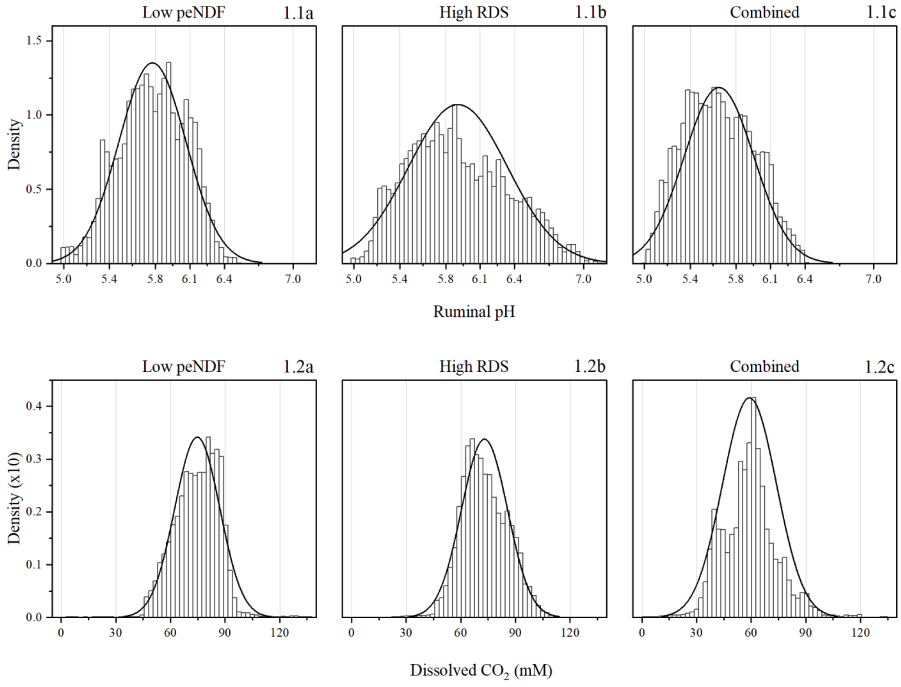

Figure 1. Histograms and normal curve fits for continuous measurements of ruminal pH and dissolved CO_2 concentrations (mM) are shown for lactating dairy cattle fed three diets in consecutive periods: Low-peNDF (low physically effective neutral detergent fiber, 1.1a and 1.2a), High-RDS (high ruminally degradable starch, 1.1b and 1.2b), and Combined (1.1c and 1.2c).

Ruminal pH cannot predict dCO_2 concentrations. In all the diets, high or critical dCO_2 levels were consistently observed postprandially, which were paralleled by a decline in ruminal pH (Fig. 2.2a-c). This phenomenon was attributed to the interconversion of HCO_3^- to dCO_2 during H_3O^+ buffering[1]. Nevertheless, pH is a quotient, limiting its ability to directly measure the specific concentrations of individual components; for instance, two HCO_3^-/dCO_2 solutions with the same pH (100/100 mM and 10/10 mM) exhibit distinct concentrations (Eq. 2). Feeding the combined diet resulted in the lowest pH (Fig. 1.1c) and minimum dCO_2 (Fig. 1.2c), whereas the high-RDS diet produced the highest pH (Fig. 1.1b) and maximum dCO_2 (Fig. 1.2b), corroborating the statement. The reduced pH in the combined diet can be attributable not only to lower dCO_2 but also to decreased HCO_3^- levels. Conversely, both HCO_3^- and dCO_2 concentrations were high in the high-RDS diet, bringing the pH closer to the equilibrium constant for CO_2 ($pK_{a1} \approx 6.1$). The distinctive

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feature of CO_2 holdup is that both ruminal dCO_2 and HCO_3^- concentrations were elevated. Therefore, CO_2 holdup explains why low ruminal pH does not always predict the clinical onset of SARA[26] or that SARA-affected cattle present larger variation in ruminal pH than healthy cattle[8][27][39]. The equilibrium between CO_2 species dictates the pH of the solution; if both molecules are in high concentrations, the ruminal pH might seem normal (Eq. 2), even when critical dCO_2 might be present. Therefore, while high dCO_2 can coexist with low pH, it is only during CO_2 holdup that critical dCO_2 concentrations persist for prolonged postprandial periods, a condition that cannot be accurately predicted by the ruminal pH scale but can be effectively monitored by the ATR-IR technique.

	Dietsxrun		
	Low-peNDF 1st run	**High-RDS 2nd run**	**Combined 3rd run**
Performance parameters, kg/d			
N	12	12	9
DMI	24.7 ± 0.85[a]	18.4 ± 0.85[b]	24.7 ± 0.98[a]
MY	36.1 ± 0.48[a]	32.8 ± 0.48[b]	33.2 ± 0.56[b]
ECM	37.2 ± 0.51[a]	34.6 ± 0.51[b]	35.6 ± 0.59[a]
Milk component yield, kg/d			
Fat	1.37 ± 0.02[a]	1.27 ± 0.02[b]	1.33 ± 0.02[ab]
Protein	1.22 ± 0.02[a]	1.15 ± 0.02[b]	1.18 ± 0.02[ab]
Lactose	1.62 ± 0.02[a]	1.49 ± 0.02[b]	1.48 ± 0.03[b]
Ventral ruminal sac parameters			
N	45	45	45
Acetate, mM	58.7 ± 0.29[a]	55.8 ± 0.29[b]	56.6 ± 0.29[b]
Propionate, mM	29.3 ± 0.29[a]	33.9± 0.29[b]	32.6 ± 0.29[c]
Butyrate, mM	11.7a ± 0.11[a]	8.9 ± 0.11[b]	9.9 ± 0.11[c]
A/P ratio	2.01 ± 0.028[a]	1.67 ±0.028[b]	1.79 ±0.028[c]
Total SCFAs, mM	114.6 ± 2.46[a]	125.5 ± 2.46[b]	114.9 ± 2.46[a]
Lactate, µM, n=27	9.2 ± 1.72	21.2 ± 2.21	41.1 ± 7.91
Viscosity, mPa.S	2.1 ± 0.18[a]	3.6 ± 0.18[b]	4.4 ± 0.18[c]
Surface Tension, mN/m	67.2 ± 0.50[a]	70.5±0.5[b]	71.5± 0.5[b]

Table 2. Summary of longitudinal trial results in fistulated cattle. Mean (±SEM) values for performance, milk components, and ruminal parameters across three runs with three fistulated cattle fed three diets*. This table consolidates findings described in the previous report of this experiment[2].

**The diets were low physically effective neutral detergent fibre (Low-peNDF) in the 1st run, high ruminally degradable starch (High-RDS) in the 2nd run, and the combination of both previous diets (Combined) in the 3rd run. Dry matter intake (DMI), milk yield (MY), short-chain fatty acids (SCFAs), and the ruminal acetate to propionate ratio (A/P ratio). The energy-corrected MY (ECM) = milk NEL output (Mcal/d)/0.7 Mcal of NEL/kg of milk, where milk NEL output (Mcal/d) = milk yield, kg/d × (0.0929 × milk fat % + 0.0563 × milk protein % + 0.0395 × milk lactose %). All comparisons were made at the 95%*

confidence level ($P < 0.05$), and means that do not share a letter are significantly different (Bonferroni).

The positive effect of high ruminal dCO_2. Ruminal CO_2 species play a pivotal role in epithelial metabolism. The majority of the intracellular HCO_3^- and H_3O^+ available for $SCFA^-$ and Na^+ exchange[56][57] are likely derived from ruminal dCO_2 [29][32], which is most likely absorbed into the epithelial cell with H_2O through aquaporins[50]. Aquaporins are abundantly expressed in the ruminal epithelia[58]. Therefore, high ruminal dCO_2 increases epithelial H_2O absorption[59] and carbonic anhydrase bound to the intracellular aquaporin domains[60][57] may expedite intracellular CO_2 hydration, leading to the formation of HCO_3^- and H_3O^+, which in turn enhances $SCFA^-$ uptake[31][41][32]. The rehydration by ruminal carbon anhydrase of the secreted intracellular HCO_3^- and H_3O^+ into dCO_2 provides the perfect (re)cycling system for nutrient uptake and explains the widespread expression of carbon anhydrase throughout the gastrointestinal tract[61][3].

The effect of high ruminal dCO_2 concentrations in this experiment confirms that CO_2 hydration plays a crucial role in nutrient uptake. For instance, cattle fed the Low-peNDF diet produced more milk (ECM, 37.2 vs. 35.6 kg/day) and lactose (1.62 vs. 1.48 kg/day) than cattle fed the Combined diet at a similar feed intake of 24.7 kg/day (Table 2). The diets were specifically formulated to provide similar amounts of energy and protein, and no significant differences in productivity were expected[2]. The rumen AUC maps for the Low-peNDF diet revealed a balanced pH (Fig. 2.1a) and consistently high dCO_2 levels (Fig. 2.2a) throughout the day. In contrast, cattle fed the Combined diet exhibited lower dCO_2 levels (Figure 2.2c) and a more acidic ruminal pH (Fig. 2.1c). The lower ruminal pH in the Combined diet might indicate a greater availability of SCFA, as they are passively absorbed as acids[5][56]. However, feeding the Combined diet did not result in a higher milk yield when compared with the Low-peNDF diet (Table 2). In fact, the reduced ruminal propionate levels with the Low-peNDF diet suggested enhanced SCFA absorption, as supported by a time series of propionate, see Laporte-Uribe[2]. Propionate absorption leads to glucose formation, which boosts lactose production and milk yield from the mammary gland[62][56]. Consequently, the higher milk and lactose yields with the Low-peNDF diet can be attributed to increased ruminal propionate absorption (Table 2), which was likely promoted by the high dCO_2 levels observed in the rumen AUC map (Figure 2.2a), confirming the positive effect of high dCO_2 on ruminal absorption[31][41].

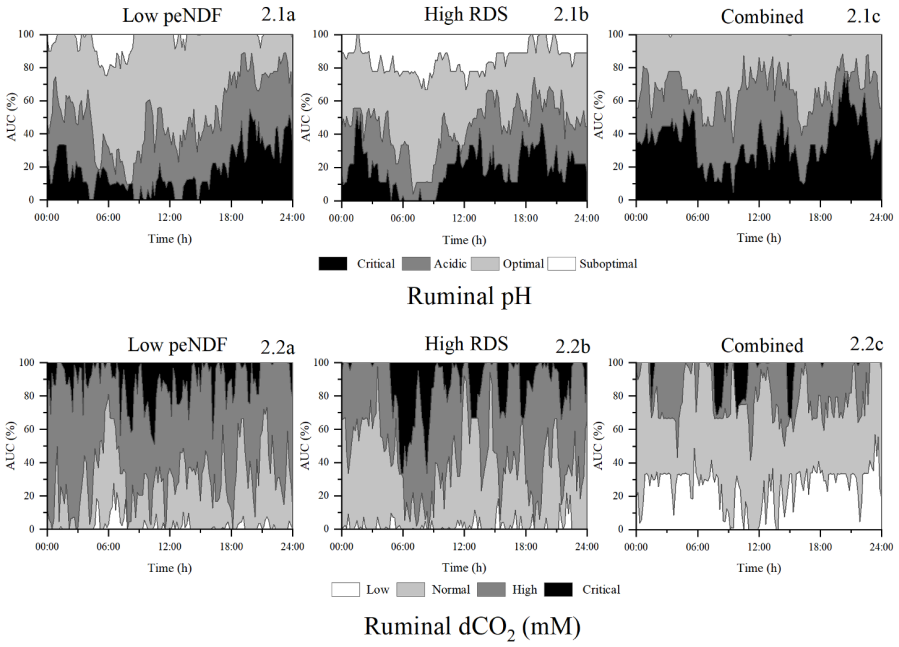

Figure 2. Rumen maps depicting the most frequent category (area under the curve, AUC, %) of ruminal pH (2.1ac) and dissolved CO_2 (dCO_2, 2.2ac) in lactating dairy cattle fed three diets: low physically effective neutral detergent fiber (Low-peNDF; 2.1a and 2.2a), high ruminally degradable starch (High-RDS; 2.1b and 2.2b), and a combination of both (Combined; 2.1c and 2.2c). The category descriptions are provided within the text.

CO_2 holdup might lead to clinical SARA signs. The ruminal AUC map for pH (Figure 2.1b) indicated that cattle had the lowest SARA risk when fed the High-RDS diet based on the conventional definition of SARA based on the pH scale[8][26]. However, cattle consuming the High-RDS diet exhibited typical SARA symptoms: reduced feed intake and milk yield, Table 2[8][39]. The rumen AUC map revealed that cattle fed the High-RDS diet experienced critical dCO_2 concentrations for extended postprandial periods, or CO_2 holdup (Spikes of critical values in Figure 2.2b). The high ruminal SCFA levels and the lower milk yield suggested impaired activity of the sodium-hydrogen exchanger (NHE) with the High-RDS diet[56][63]. Otherwise, high ruminal SCFA production and undisturbed absorption should increase milk yield, besides low feed intake with High-RDS should reduce SCFA production and not enhance it, Table 2[5].

Under normal conditions, NHE regulates intracellular H_3O^+ exchange with ruminal Na^+[56][63]. Since the ruminal epithelium is H_3O^+-impermeable, the intracellular H_3O^+ must originate from either ruminal CO_2 hydration or intracellular SCFA metabolism[56][32]. CO_2 holdup can lead to ruminal hyperosmolarity, which diminishes feed intake, H_2O absorption, and Na^+ absorption and is linked to the onset of SARA[59][64][52]. The impaired H_2O absorption resulting from hyperosmolarity could potentially reduce intracellular H_3O^+ formation and NHE activity, thereby impairing SCFA absorption. This could explain the elevated ruminal SCFA concentrations observed in cattle fed the High-RDS diet and during SARA[56].

Notably, the epithelial response to SARA involves increased intracellular SCFA metabolism and enhanced NHE expression[63], which might bolster intracellular H_3O^+ and HCO_3^- formation and SCFA absorption. Additionally, intracellular cholesterol synthesis and deposition are intensified[52][63], likely as a response to high dCO_2 exposure and as a mechanism to reduce dCO_2 diffusion[51]. Moreover, SARA courses with a strong inflammatory response[56] which mirrors the inflammation pathways triggered by CO_2 poisoning in the lungs[65][49]. Therefore, clinical SARA

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symptoms may come from CO_2 holdup development, as shown by the rumen AUC maps. Prolonged exposure to these critical dCO_2 conditions might elevate the risk of SARA or CO_2 poisoning.

Ruminal CO_2 holdup monitoring. The "rumen AUC maps" depict the daily ruminal fermentation pattern associated with ruminal dCO_2 and influenced by dietary components, daily feed intake, feed allowance, and management routines[38]. Therefore, these maps enable the monitoring of ruminal dCO_2 by classifying it into categories with biological significance. The dCO_2 detected by the ATR-IR sensor at these selected thresholds aligned with the established biological effect of CO_2. For instance, ruminal bacterial growth starts at 12 to 20 mM dCO_2[14], and the optimal succinate production, the primary ruminal propionate precursor, requires a greater than the ruminal average, > 60 mM[14][16]. A ruminal dCO_2 threshold over 80 mM might signal an increased risk of hyperosmolarity[64][52], impaired buffering capacity[4][7] and/or an increased risk of epithelial CO_2 poisoning[65]. Additionally, feeding consistent diets and adhering to stable feeding management routines enhance feed intake, milk yield, and lower the risk of nutritional disorders[66][67]. Consequently, rumen AUC maps provide valuable insight into the health and productivity of dairy cattle subjected to diverse diets and management practices. Furthermore, the (cross) tabulation of frequencies on daily "contingency tables" with the proposed categorical analysis streamlines the statistical comparison of ruminal patterns, i.e., the 144-time segment and 4-category matrix can be analysed utilising the Pearson chi-square (X^2), G-test, or Bayesian inference[43]. Consequently, rumen AUC maps establish the foundation for "precision ruminal fermentation": the selection of diets and management practices that optimise ruminal fermentation, reduce waste products, and prevent nutritional diseases associated with SARA by continuously measuring dCO_2 concentrations and CO_2 holdup formation.

Further work, and current limitations

To date, it is not possible to predict the impact of a specific feeding regimen or diet on dCO_2 concentrations or CO_2 holdup. For example, it was expected that the Combined diet would produce a stronger effect on dCO_2 retention, but the opposite happened (Table 1, Figure 2). Moreover, CO_2 holdup might be transient in pastoral systems, while it could be persistent in concentrate and corn-based diets[19][68]. It is this same unpredictability that might explain individual susceptibility to SARA[69]. Further work in this area should focus on those challenges based on the knowledge gathered from this trial. The diagram below (Diagram 2) proposes a schematic overview of the ruminal buffering system's function and dysfunction based on the results of this pilot experiment.

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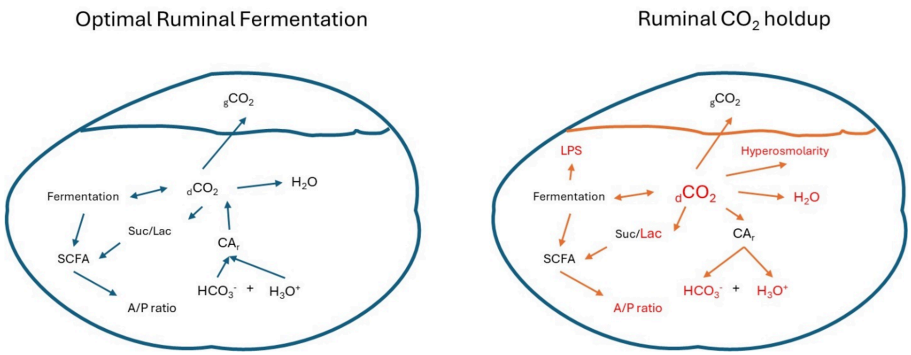

Diagram 2. During **optimal ruminal fermentation** (left), the buffering system relies on dCO_2 formation from bicarbonate (HCO_3^-) and protons (H_3O^+) to reduce water (H_2O) ionization and restore system equilibrium. Ruminal carbon anhydrase (CAr) catalyses this process. Impaired CO_2 gas (gCO_2) fugacity from the fluid results in dCO_2 accumulation and the development of **ruminal CO_2 holdup** (right). Accumulated dCO_2 might contribute to various clinical signs of ruminal acidosis and subacute ruminal acidosis, including hyperosmolarity of the ruminal fluid, lipopolysaccharide (LPS) formation, decreased acetate/propionate ratio (A/P ratio), lactate (Lac) accumulation at the expense of succinate (Suc), and an overall shift in short-chain fatty acid (SCFA) metabolism away from decarboxylation pathways.

Conclusions

Dissolved CO_2 is ubiquitous in the rumen environment, present in substantial and varied amounts. For the first time, ruminal dCO_2 presence and dynamics, including CO_2 holdup formation, were described *in situ*. Optimal rumen function relies heavily on dCO_2 concentrations as a key component of the ruminal buffering system. This crucial contribution has gone unrecognized. Conversely, disruption of the ruminal buffering system, leading to CO_2 holdup, could potentially heighten the risk of CO_2 poisoning and trigger clinical SARA signs. These results warrant further investigation. The novel methodology described here might help us to validate or refute this hypothesis.

Statements and Declarations

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Author contributions

JLU designed the study, performed the experiment, analysed the data, and wrote this manuscript.

Competing interests

The author is the inventor of the indwelling ruminal sensors. This research provides partial justification for the use of these systems for disease prevention and improved sustainability.

Data availability

Due to ethical and privacy considerations, the data that support this study cannot be publicly shared. However, upon reasonable request to the corresponding author, access to the data may be granted when appropriate.

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Declarations

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