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The intraruminal redox potential is stabilised by opposing influences during fermentation

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Abstract

An optimal fermentation process in the forestomach is pivotal for the wellbeing and performance of ruminants. Complex carbohydrates are broken down into short-chain fatty acids (SCFA) which form the major energy source for the animal. A strong interrelationship of this process with intraruminal pH and redox potential (Eh) exists. These parameters can be measured with intraruminal sensors, but the interpretation of the measurements, especially of Eh, and their meaning for intraruminal homeostasis is not completely clear. In this study, factors influencing intraruminal Eh were elucidated. We hypothesised that intraruminal Eh is influenced by the fermentation process as such, but not by its end products SCFA. We measured Eh and pH in ruminal fluid from fasting cannulated sheep after the addition of 0.06 M Na-acetate, -propionate, -butyrate or glucose in vitro. Furthermore, we assessed the interrelation of pH and Eh. Basal Eh and pH values were −120 ± 41 mV and 7.0 ± 0.3, respectively, in native ruminal fluid in vitro. While the addition of SCFA did not induce any changes, glucose addition caused a significant decrease in both pH and Eh compared to the values before the addition (paired Student's *t*-test, $p < 0.05$). We attribute the decrease in Eh to an increased production of H_2 in the process of generating SCFA, predominantly acetate. By titrating both native and particle-free ruminal fluid to more acidic and basic pH values (4.5–8.5), we found a non-linear inverse correlation of pH and Eh, counteracting the H_2-driven decrease of Eh during fermentation. Thus, the intraruminal Eh is influenced by pH and H_2 output during SCFA formation. The opposed character of these factors stabilises the intraruminal homeostasis which might help maintain symbiotic microbiota in the rumen. Understanding, monitoring, and supporting this system will be an essential part of modern cattle production.

KEYWORDS

carbohydrates, Eh, NAD^+/NADH, oxidoreduction potential, pH, SCFA

1 | INTRODUCTION

Ruminants evolved to use their forestomach as a huge fermentation chamber that allows the digestion of complex fibres beyond the scope of mammalian enzyme endowment. Nearly all carbohydrates reaching the rumen are broken down by micro-organisms into short-chain fatty acids (SCFA), mainly acetate, propionate and butyrate, in a ratio varying from 75:15:10 to 40:40:20 (Bergman, 1990). The SCFA are the major energy source for the ruminants' metabolism including performance such as growth or milk production (Baaske et al., 2020; Bergman, 1990). Lactating dairy cows absorb approximately 100 mol SCFA daily across the ruminal epithelium, covering up to 80% of their energy demand (Gäbel & Sehested, 1997; Storm & Kristensen, 2010; Storm et al., 2012). However, too rapid fermentation or a deviation of the stoichiometry will have detrimental effects on the ruminal integrity and subsequently also on the systemic health of the animal (Baaske et al., 2020; Plaizier et al., 2018). Thus, it is not surprising that the homeostasis of this complex system is one of the most important factors for wellbeing, health, and productivity in ruminants.

The optimal conditions for the fermentation processes in ruminal fluid have been under investigation for decades and this knowledge helped to improve diets and health monitoring especially for high yielding cattle. Sensors have been developed to monitor parameters such as intraruminal pH and redox potential (Eh) as basic parameters for microbial activity and fermentation dynamics (Dijkstra et al., 2020; Marden et al., 2005). While the pH is a well-accepted indicator for disorders such as (subacute) ruminal acidosis (Aschenbach et al., 2011), the implications of Eh measurement are less clear. There has been surprisingly little research regarding this parameter so far and a correct interpretation of Eh values in the ruminal fluid is difficult due to the paucity of data, so that the use of these sensors implies some uncertainty (Dijkstra et al., 2020). However, the oxidoreductive state is a pivotal parameter reflecting the metabolic pathways in an anaerobic system such as the rumen (Liu et al., 2013; Marounek et al., 1982). Oxidoreduction reactions involve the transfer of electrons from one partner to the other. While we live in a generally oxidising atmosphere, there are anaerobic niches such as the rumen where the milieu is predominantly reducing, that is, the Eh is negative, due to the anaerobic breakdown of carbohydrates with the corresponding release of reducing equivalents (Marden et al., 2008). Although small amounts of oxygen enter the forestomach with food and water, it is quickly consumed by facultatively aerobic micro-organisms (Müller & Kirchner, 1969a). Without the strong oxidant oxygen, other redox pairs become more important in this system. In the rumen, NAD^+/NADH is considered to be the major cofactor for oxidoreductive reactions and the key controller for the direction of fermentation, that is, the scale of SCFA produced (Dijkstra et al., 2020; Liu et al., 2013). NAD^+ is reduced to NADH by the uptake of two electrons and a proton. Thus, the NAD^+/NADH ratio reflects or rather follows the Eh (van Lingen et al., 2016). NAD^+/NADH and Eh in turn may reflect fermentation pathways. Depending on the hydrogenase, the degradation of one mol glucose to acetate or butyrate leads to a net release of up to 4 or 2 moles H_2, respectively, whereas the formation of propionate represents a H_2 sink (Ungerfeld, 2020; Figure 1). As the predominantly produced SCFA is acetate, this implies a net release of H_2, which is rapidly converted to CH_4, thus also modulating the intraruminal Eh.

As the intraruminal methanogenesis is not only a loss of energy for the animal but also increasingly considered to be a driving factor of climate change, the control of H_2 production and discharge via influencing the intraruminal Eh is tempting but difficult due to the complexity of the system (Ungerfeld, 2020). Understanding the pathways involved in and influence factors on the intraruminal Eh might benefit these attempts to manipulate the fermentation processes strategically and in accordance with animal health.

Factors influencing the Eh in ruminal fluid have been reported only in an anecdotic manner and contradictory in parts. Recurring findings are a connection between food uptake, diet composition or microbiota—factors that can hardly be differentiated, as one influences the others. There have been reports on an interrelation between microbiota composition and the Eh, at least regarding several subspecies (Friedman et al., 2017; Marounek et al., 1982; Ungerfeld, 2020; Wetzels et al., 2018). A connection between the composition of diet and intraruminal Eh can also be assumed (Müller & Kirchner, 1969b, 1969c). However, probably neither diet nor microbiota influence Eh directly, but rather the metabolic processes involving bacterial enzymes and metabolic substrates, that is, mainly the generation of SCFA from carbohydrates (Figure 1). In a recent review article, Huang et al. (2018) summarised data regarding Eh that had been published so far. Despite the difficulty that the original data were generated under very different conditions, they identified a negative correlation of Eh with total SCFA produced and especially acetate, and a positive correlation of Eh and propionate concentrations in ruminal fluid (Huang et al., 2018). However, it seems unlikely that the presence of SCFA themselves influences Eh under physiological conditions (anaerobic, reducing) in ruminal fluid. The current knowledge rather suggests that their production pathways influence the NAD^+/NADH ratio (see Figure 1) and thereby modify the Eh or that rather the Eh controls the glycolytic pathways via the NAD^+/NADH ratio.

Therefore, our study aimed to test the influence of metabolism or metabolic end products (SCFA) on the Eh in ruminal fluid in vitro. We observed no effects of the presence of SCFA on the Eh, but two opposing factors influencing the Eh during the fermentation of glucose. On the one hand, there is a strong (inverse) correlation between Eh and pH, increasing Eh when the pH drops. On the other hand, the liberation of H_2 during microbial fermentation of carbohydrates drives the Eh in the opposed direction, that is, to more reducing values.

2 | MATERIALS AND METHODS

2.1 | Animals and ruminal fluid sampling

Ruminal fluid was taken from three male rumen cannulated East Friesian sheep (*Ovis aries*) that were kept in one barn and fed with hay and water *ad libitum*, approximately 1 kg hay cobs (Deukavallo) and

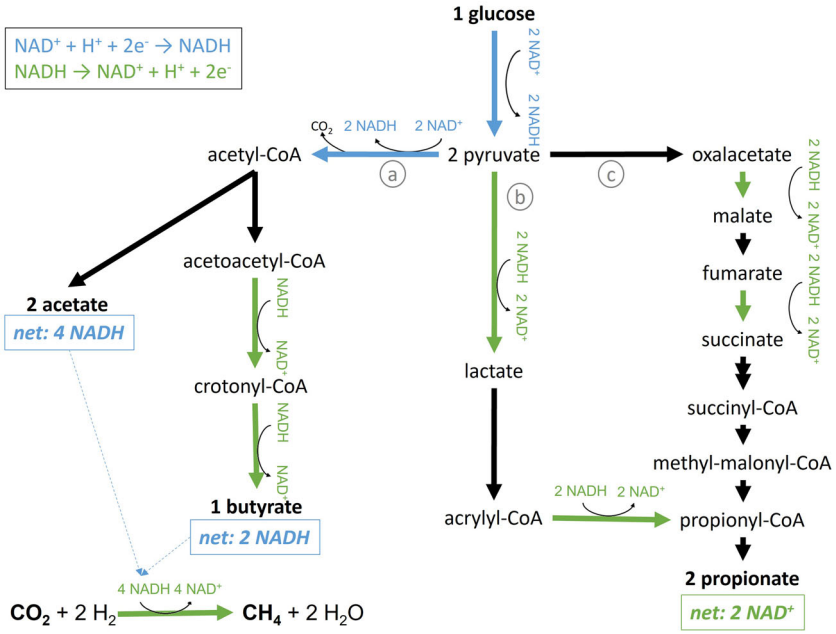

FIGURE 1 Reactions involved in intraruminal fermentation. Simplified overview over oxidoreduction pathways in the production of SCFA from glucose. Carbohydrates are broken down into monosaccharides which are quickly fermented via pyruvate to the SCFA acetate, propionate and butyrate (alternative pathways a, b or c), with acetate as the largest part. Thus, acetate and butyrate formation lead to a net release of H_2 in the form of NADH and CH_4, whereas propionate formation is a net sink of H_2. CO_2 is formed by decarboxylation reactions or via the bicarbonate buffer system. For a clearer arrangement, the stoichiometry is not balanced in the figure. In general, the amount of and the ratio between the SCFA that are produced during microbial fermentation varies but may be exemplified assuming a production of 100 mole SCFA with a hay diet as follows: 58 $C_6H_{12}O_6$ + 134 $NAD^+ \rightarrow$ 62 CH_3COOH + 22 CH_3CH_2COOH + 16 CH_3CH_2 CH_2COOH + 60.5 CO_2 + 33.5 CH_4 + 27 H_2O + 134 NADH. For a more comprehensive review of the reaction routes in intraruminal fermentation refer to (Ungerfeld, 2020; van Lingen et al., 2016). SCFA, short-chain fatty acid [Color figure can be viewed at wileyonlinelibrary.com]

100 g concentrate each per day (lamb and sheep muesli; Havens). They had free access to a salt lick stone.

We decided to conduct our measurements in vitro since in- and outflux of buffer substances, substrates and fluid impedes measurements under defined conditions in vivo (Müller & Kirchner, 1969a). Some of these problems might be overcome by inhibiting outflow and pumping out saliva, but this still leaves the issue of ruminal epithelial absorption and secretion and additionally the manipulation might cause distress in the animals. Besides, collecting a larger volume of ruminal fluid and dividing it for different setups allowed us to compare between different setups without having to consider day-to-day variations. All this added up to our decision to conduct the experiments in vitro.

The ruminal fluid was collected through the fistula directly from the ventral sac of the rumen with a tube connected to a metal syringe. The extraction of ruminal fluid always took place between 7 and 8 AM, approximately 16 h after the last and immediately before the morning feeding. After extraction, the fluid was transferred to a prewarmed thermos to keep its temperature.

For comparison, Eh and pH were measured in vivo at the time of collection once by inserting the electrodes into the ventral sac of the rumen through the fistula.

The experiments were conducted in accordance with the German legislation on the protection of animals and were reported to the Landesdirektion Leipzig as W12-19. The authors confirm that the ethical policies of the journal, as noted on the journal's author guidelines page, have been adhered to and the appropriate ethical review committee approval has been received. The authors confirm that they have followed EU standards for the protection of animals used for scientific purposes.

2.2 | Preparation of the ruminal fluid

In the laboratory, the ruminal fluid was divided in portions of 80 ml each into 100 ml vessels and closed tightly to maintain an anaerobic atmosphere. The handling of the ruminal fluid followed the recommendations of previous investigations (Müller & Kirchner, 1969a; Sauer & Teather, 1987). To avoid an accumulation of fermentation gasses with a rise in pressure and subsequent changes in the partial pressure of H_2 (pH_2), CO_2 or CH_4, a hollow needle (Gauge no. 20) was inserted in the lid. Before starting the measurements, the samples were equilibrated for at least 20 min to make sure that the anaerobic atmosphere was reconstituted after the sampling and dividing procedure. The vessels were kept in a water bath at 39°C with moderate shaking to keep the fluid warm and well mixed. For the measurements, the lid was replaced with a lid containing holes in which the measuring electrodes were fixed, thereby ensuring a constant position of the electrodes within the fluid.

2.3 | Measurement of pH and Eh

pH was measured using a SenTix 41-3 electrode, and the Eh was measured with a SenTix R ORP (3 M KCl) platinum electrode (WTW). The measured values (E_0) were corrected according to the equation Eh = E_0 + C, with C being the potential of the reference electrode relative to a hydrogen electrode = 199 mV at 39°C (Marden et al., 2008; Sauer & Teather, 1987). To elucidate a possible drift of the electrode, we measured Eh in a standard redox buffer (catalogue no. MT51350060; Merck) before and after measurements in ruminal fluid. After 40 min of incubation in ruminal fluid the Eh measured in the buffer solution was exactly the same as before (414 ± 0.8 mV before, 413 ± 1.5 mV after, at 39°C; values corrected to hydrogen electrode as described above). Thus, it was assured that the redox electrode had no drift.

All chemicals were obtained from Merck.

2.4 | Influence of SCFA on the Eh

The initial pH of all ruminal fluid samples was set to 6.5 using 1 M NaOH or 1 M HCl. After equilibration, a 1 M stock solution (pH 7.0) of Na-acetate, Na-propionate or Na-butyrate was added to one vessel each at a final concentration of 0.06 M. Subsequently, changes in Eh, pH and temperature were measured for 30 min. As control, 1 M NaCl, likewise leading to a concentration of 0.06 M, was used. The treatments were randomly assigned to the vessels and conducted in random order in each independent experiment.

2.5 | Influence of pH on the Eh

To evaluate changes that occur in the Eh due to changes in the pH of the ruminal fluid, the pH was titrated up or down stepwise. Therefore, the ruminal fluid was sampled and prepared as described before, this time using four portions of 60 ml. Two of the portions were centrifuged at 1500*g* for 5 min and the supernatant was used for further measurements in particle-free (and thus also largely free of microbiota) ruminal fluid and compared to native samples. The pH at the beginning of this experiment was 6.8 ± 0.13 in the native and 7.0 ± 0.07 in the particle-free ruminal fluid. To investigate the effect of pH, 1 M HCl was added to lower the pH to 6.0 and from this point further decreased to 4.5 in steps of 0.5. Similarly, the pH was increased from 7.0 to 8.5 using 1 M NaOH in steps of 0.5 in another portion. Between the steps the Eh was monitored for 10 min each.

2.6 | Influence of glucose on the Eh

To determine the influence of glucose (metabolism) on the Eh in the rumen, ruminal fluid was taken and prepared as described above. To each vessel 1 M glucose stock solution was added to provide a final concentration of 0.06 M and the changes in Eh and pH were measured for 30 min as well.

To isolate the effect of glucose on the Eh from the effect of changes in the pH, the pH was kept steady at 6.5 in one portion throughout the whole 30 min. This was achieved by adding 1 M NaOH when the pH started to change. Another portion in which pH was not adjusted served as a control.

2.7 | Statistics

The results are described as arithmetic means ± SD. Each of the three sheep was sampled twice in each experimental series, so that a total of $N = 6$ independent experiments was conducted for each setup. The significance is expressed as the probability of error (p) and the difference was considered significant at $p < 0.05$. The differences between the mean values of the treatment groups were tested using the paired Student's t-test (Sigma Plot 13.0; Systat Software). Regression analysis was also done with the Sigma Plot software.

3 | RESULTS

3.1 | SCFA do not affect the Eh in ruminal fluid in vitro

The first experimental series was conducted to evaluate the effect of the SCFA on Eh. Na-acetate, -propionate, -butyrate or NaCl were added to the ruminal fluid samples at a final concentration of 0.06 M and the changes in Eh and pH were measured for 30 min.

While ruminal fluid obtained from sheep 1 and 3 showed steady results, the samples from sheep 2 displayed higher Eh values compared to the others and in one experiment the measurements were very inconstant. Therefore, we excluded this experiment from the calculation of mean values over all three sheep, thus resulting in five independent experiments depicted in Figure 2 and used for statistical analysis.

The Eh was not affected by any SCFA addition compared to the controls incubated with NaCl or compared to the starting value before the addition (Figure 2). The pH remained stable in all setups.

3.1.1 | The Eh is correlated to pH in a non-linear manner

As the addition of SCFA themselves did not induce changes in Eh in our experiments, another mode of modulating Eh might be their influence on the intraruminal pH, either by acidifying the ruminal fluid during their production or alkalising it in the course of absorption. To assess the impact of pH on the Eh, we titrated the pH in ruminal fluid in steps of 0.5 either to a more acidic (4.5) or a more basic (8.5) value and measured the subsequent changes in the Eh. This experiment

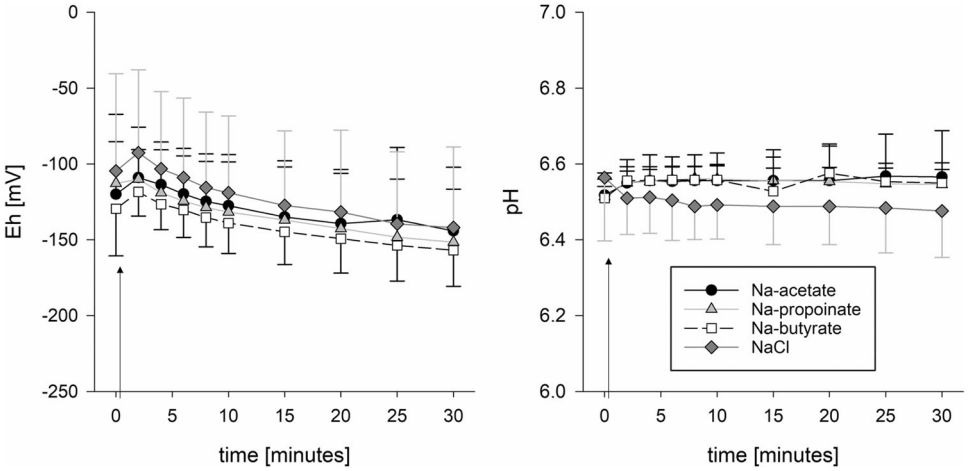

FIGURE 2 Eh and pH are not affected by the presence of SCFA. Graphs show means ± SD of the Eh and pH measured in five independent experiments ($N = 5$) in ruminal fluid from three different animals and incubated with 0.06 M Na-acetate, -propionate, -butyrate or NaCl (addition indicated by arrows). There was no effect of the addition in any of the treatment groups. SCFA, short-chain fatty acid

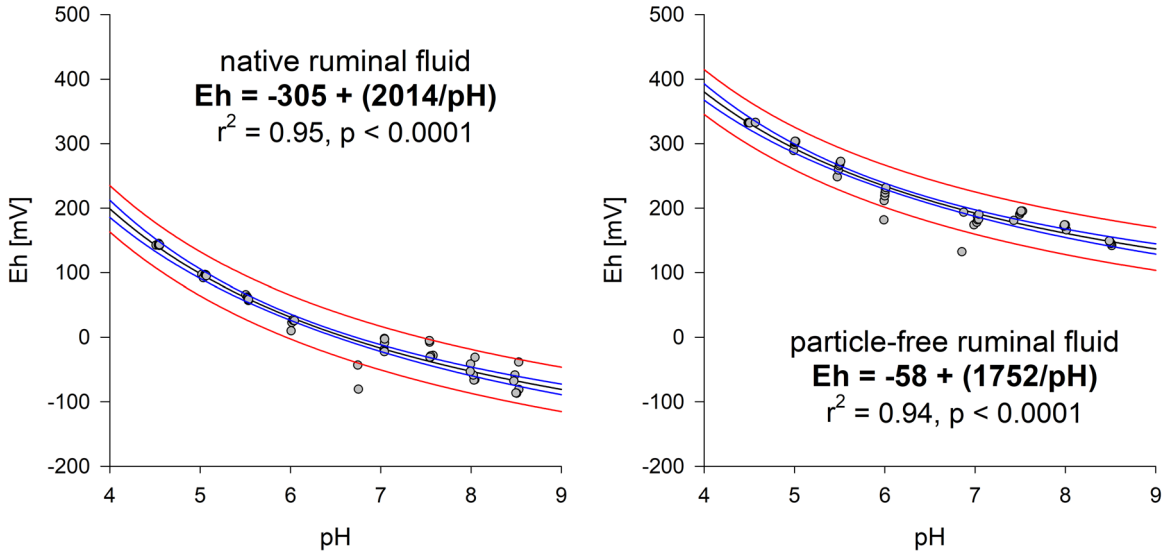

FIGURE 3 Non-linear regression (polynomial inverse first order) for the Eh and pH in native and particle-free ruminal fluid. The ruminal fluid samples were titrated in steps of 0.5 from pH 6.0 to 4.5 and from pH 7.0 to 8.5. From the resulting Eh we calculated the regression equations fitting best. Equations and correlation coefficients are given in the graphs, blue lines indicate 95% confidence band, red lines 95% prediction band; $N = 6$ [Color figure can be viewed at wileyonlinelibrary.com]

was done in parallel in native samples and in particle-free samples obtained as described in the materials and methods section. In the particle-free samples, we found generally higher Eh values. The interrelation between pH and Eh is shown in Figure 3. From these measurements, we calculated regression equations that describe an inverse non-linear relation between pH and Eh both in native samples ($r^2 = 0.95$, $p < 0.001$; Figure 3) and in particle-free samples ($r^2 = 0.94$, $p < 0.001$; Figure 3). Even though there was a slight variation in their slope (Student's t-test; $p < 0.001$), the resulting curves for native and particle-free ruminal fluid appear to be similar except for a different axis intercept (Figure 3).

3.1.2 | Glucose metabolism affects the Eh in ruminal fluid in vitro irrespective of pH

Because of the known effect of glucose metabolism on intraruminal pH and the strong relation of pH and Eh in turn we conducted another experiment to elucidate the effect of glucose addition on Eh independently of the pH, expecting a stronger effect of fermentation on the Eh without the influence of the pH. Therefore, after the addition of 0.06 M glucose the pH was either kept stable or not. Because of the correlation of pH and Eh, which we described before (Figure 3), we also corrected the Eh values measured after the addition of

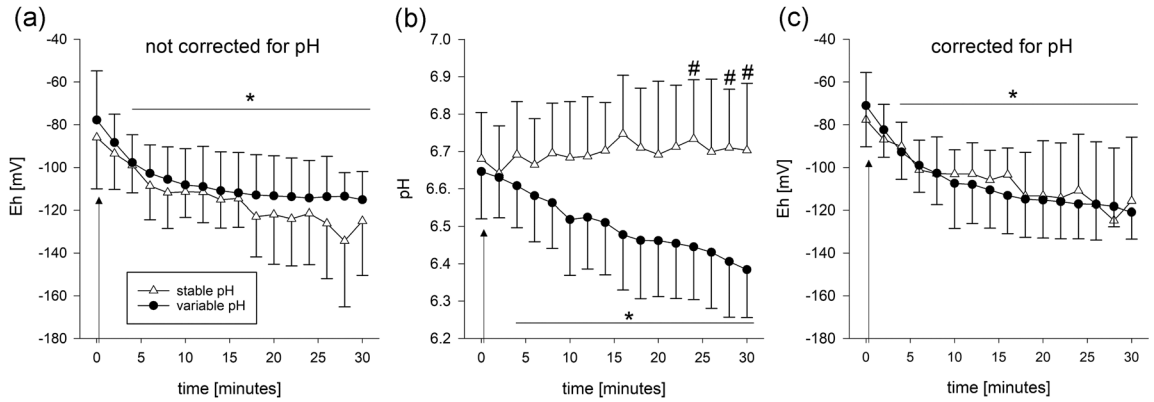

FIGURE 4 Fermentation of glucose decreases both pH and Eh. Course of the Eh after addition of glucose (indicated by arrows) at a final concentration of 0.06 M. pH (b) was either kept stable by titration (white triangles) or not (black dots). (a) Shows the uncorrected Eh as measured, (c) the Eh corrected for the influence of pH on Eh according to the equation from Figure 3. Mean ± SD, $N = 6$ for titrated and $N = 5$ for untitrated ruminal fluid. Asterisks indicate a significant difference compared to the starting value of the same group before the addition of glucose (0 min), rhombs indicate a significant difference between the groups (paired Student's t-test, $p < 0.05$)

glucose for pH according to the equation we found (Eh = −305 + 2014/pH) to a standardised pH of 6.5 (Figure 4c).

pH decreased quickly after glucose addition and was significantly different from the pH before the addition after 4 min of incubation (paired Student's t-test, $p < 0.05$; Figure 4b) in the group that was not titrated to keep the pH stable. Irrespective of the pH, the Eh dropped significantly compared to the values measured at the beginning of the experiment (paired Student's t-test, $p < 0.05$), but did not differ between the two groups (Figure 4a,c). The correction for pH led to a complete overlap of both curves for Eh (Figure 4c), confirming the accuracy of the equation from Figure 3.

4 | DISCUSSION

The rumen is a huge fermentation chamber offering unique conditions for the microbial digestion of carbohydrates. Most of the reactions involved in glycolysis and subsequent generation of SCFA are redox reactions requiring the transfer of electrons between carbon atoms either directly or via cofactors like NAD^+/NADH (van Lingen et al., 2016). Thus, the Eh of the intraruminal milieu is a pivotal factor deciding which reaction may or may not happen and thus which metabolic end products are generated (Marounek et al., 1991; van Lingen et al., 2016).

The Eh values measured in fermentation sites across different species and gastrointestinal compartments are in a similar negative range (Marounek et al., 1987). Literature reports on Eh in native ruminal fluid range from −440 to −50 mV (Friedman et al., 2017; Müller & Kirchner, 1969a). Regarding the values reported, it has to be kept in mind that by definition the Eh should be measured against a hydrogen electrode or corrected accordingly (Sauer & Teather, 1987) but it is not always stated clearly in the literature, how the Eh was measured or if the values were standardised. The lowest, that is, most negative, values reported result from uncorrected measurements with platinum electrodes, which are more common but give about 200 mV more negative values than measurements taken with a hydrogen electrode (Marden et al., 2008; Sauer & Teather, 1987). The Eh values we found after correcting for the differences between the platinum electrode and a hydrogen electrode correspond to the (corrected) Eh values reported before (Huang et al., 2018; Marden et al., 2008; Marounek et al., 1987). The initial Eh values in our study were in the upper range (compare Figures 2 and 4), which is presumably due to the collection time point 16 h after the last and immediately before the morning feeding. Several studies indicate that the intraruminal Eh is more positive before and decreases after feeding (Barry et al., 1977; Marden et al., 2008; Müller & Kirchner, 1969b, 1969c). The pH values measured (7.0 ± 0.3 directly after collecting the rumen fluid) also correspond to previously reported pH values after food deprivation (Gäbel et al., 1993). By collecting the ruminal fluid before the morning feeding, we standardised sampling as good as possible, although a ruminant can never be considered 'fasting'.

Yet, the initial Eh measured in vitro was less negative than the values we measured directly in the rumen fluid via the cannula. In vivo measurements were taken at the same time of the day as we collected the ruminal fluid for the in vitro experiments (Eh = −194 ± 16 mV, pH = 6.8 ± 0.11; $N = 3$ animals). This might be attributable to a loss of active microbiota after collecting the fluid. However, the stable Eh values measured after a relatively short equilibration period support the integrity of the redox system in vitro.

In particle-free-ruminal fluid (nominally free of bacteria, protozoa and fungi), we measured approximately 200 mV higher values compared to native ruminal fluid, that is, a positive Eh (Figure 3). This can be taken as a strong sign that mainly the fermentative activity of the micro-organisms (mostly attached to the particles) is responsible for generating a negative Eh.

Another determinant of the microbial fermentation and thus the Eh is the availability of metabolic substrates. However, not much is known

about the interrelationship of the Eh and metabolic end products, that is, SCFA, in the ruminal fluid. We measured the Eh in ruminal fluid after the addition of different metabolic substrates in vitro. To test if the sole presence of SCFA modulates Eh, we incubated ruminal fluid samples with different SCFA. However, the release of SCFA has also a strong impact on the pH of ruminal fluid. Thus, we also tried to elucidate the interplay between SCFA, pH and Eh. To simulate an increased glycolysis and subsequent transfer of pyruvate to SCFA we added high amounts of glucose to the ruminal fluid.

First, we tested the influence of acetate, propionate or butyrate on the Eh. Neither of the SCFA added led to changes in the Eh (Figure 2). This might be controversial to the findings of Huang et al. (2018) who postulated a connection between the Eh and acetate and propionate. However, it seems more plausible that not the presence of SCFA but the fermentation process with SCFA as end point and consisting of several reaction pathways affects the Eh. The process of SCFA production infers several oxidoreduction reactions that interact with the Eh. Due to the reciprocal effects on NAD^+/NADH during the glycolytic generation of acetate (H_2 net release) and propionate (H_2 sink), oppositional correlations of these processes with the Eh appear plausible to assume. With NAD^+/NADH as the central cofactor, its balance is naturally affected by intraruminal pH_2 and the availability of the cofactor for the respective reaction will then favour certain reaction pathways over others (van Lingen et al., 2016). Thus, at a high pH_2 the generation of propionate (and NAD^+) will be increased and that of acetate (and NADH) will be decreased (van Lingen et al., 2016). Consequently, not the SCFA as products, but rather the substrate and pathways underlying their generation are the factors interacting with the Eh. This is in accordance with our observation, that the addition of glucose, that is, a substrate for SCFA production, but not the SCFA themselves induced changes in the Eh in the ruminal fluid in vitro.

However, the fermentation of carbohydrates and the associated SCFA release does not only influence pH_2, but it also alters several parameters in the ruminal fluid. Most obviously, SCFA release is associated with a decrease in pH, which was also apparent in our experiments (Figure 4b). Thus, at least two parameters, pH_2 and pH, are altered in parallel during carbohydrate breakdown. This prompted us to investigate the relationship of Eh and pH independently from fermentation processes. We could clearly show a dependence of the Eh on pH in the ruminal fluid (Figure 3). A connection between the Eh and pH is obvious, as a majority of electron transfer reactions in the rumen infers a liberation of protons (Krishtalik, 2003). However, this would be a too simple truth for the complex ruminal system, as the activation energy for the single reaction, if considered uncoupled to preceding and subsequent processes, is not affected by pH (Krishtalik, 2003). In previous studies an inverse linear correlation of pH and Eh was described (Friedman et al., 2017; Huang et al., 2018; Marounek et al., 1987). We demonstrate for the first time, that the correlation is actually non-linear and does not depend on the activity of microbes. The curve fitting to our data acquired from measurements over a wide range of pH (4.5–8.5) shows a highly significant correlation not only in native but in particle-free ruminal fluid as well. In contrast to the assumption that the Eh is stable below a pH of 6 (Huang et al., 2018), the curve fitting our data is rather more dynamic in the acidic range compared to higher pH values.

This observation makes clear that it is necessary to observe Eh independently of pH or at least correct the measured values for pH. The use of Clark's exponent rH (rH = Eh/30 + 2 pH) in previous studies (Barry et al., 1977; Marden et al., 2008) might have been a first step towards this idea. Correcting the Eh values we measured after the addition of glucose to a pH of 6.5 led to an overlap with the values measured under stable pH, whereas the Eh values measured with a variable pH were slightly higher before (Figure 4). This strongly supports the fit of our equation and the inverse correlation of Eh and pH. However, the influence of pH on the Eh cannot explain why we observed a decrease of the Eh during the fermentation of glucose. A decreased pH would imply an increased Eh as shown in Figure 3. This discrepancy has been reported by others as well (Barry et al., 1977). By keeping the pH stable throughout the fermentation, we could show that the release of H_2 during the fermentation process induces a decrease of the Eh and thus antagonises the Eh elevating effect of a lowered pH. Combined, these effects might neutralise each other to a certain degree, unless one surmounts the other. This is supported by literature reports on changes in the Eh that cannot be attributed to the pH (only). For example, after feeding a high urea diet pH increased but the Eh was unaffected (Müller and Kirchner, 1969b), indicating an opposing effect of pH_2 counteracting that of pH. Contradictory findings of Eh modulation by different diets might be derived from varying effects of these diets on pH and pH_2. Eventually, the assumption that the Eh is stable in an acidic milieu (Huang et al., 2018) might also originate from counteracting effects of pH and pH_2 during active fermentation. As the formation of the SCFA, that is, approximately 70% acetate, 15% propionate and 10% butyrate (Bergman, 1990), shifts the balance towards NADH, this could explain a decreased Eh in the acute phase of fermentation. Thus, the opposing influences of pH and H_2 production keep a balance that prevents abrupt changes in the intraruminal milieu and thereby stabilise the intraruminal homeostasis. This may even be the mode of action of some of pro- and pre-biotics. Especially yeast, a 'rumen stimulant', has been shown to stabilise the Eh after concentrate feeding (Marden et al., 2008; Mathieu et al., 1996).

A stable milieu is crucial for the intraruminal micro-organisms' and thus the ruminant's welfare. Previous studies showed that the Eh is also interrelated with the intraruminal microbiota (Friedman et al., 2017; Wetzels et al., 2018). There is evidence suggesting that at least some microbial species are dependent on Eh and survive only in a specific redox milieu (Friedman et al., 2017; Marounek et al., 1991). Furthermore, if considered separately, single micro-organisms display specific Eh profiles during fermentation of their substrates (Liu et al., 2013). Thus, the Eh might not only be a parameter to evaluate homeostasis but also be a key to influence the microbiota and corresponding fermentation pathways in the rumen, and thereby impact methanogenesis or other (un)desirable pathways (Marounek et al., 1991). However, according to Liu et al. (2013) the Eh has less impact on the fermentation process than pH, temperature or substrate

composition and additionally the diverse and simultaneously redundant microbiota of the forestomach will be hard to decipher and orchestrate in the desired direction, so that this might rather be a theoretical experiment than a tool for practice.

In conclusion, the intraruminal Eh underlies a modulation by at least two different factors during the fermentation of carbohydrates. On the one hand, it is inversely non-linear correlated with pH and on the other hand it is determined by the H_2 output in the course of SCFA formation. The opposing nature of these factors stabilises the homeostasis and supports the microbiota in the rumen. Understanding, monitoring, and supporting this system is the key to modern cattle production and the translation of our findings to the in vivo level should be tested in future studies.

Our study underlines that not SCFA per se influence the Eh but the H_2 consumption or release during their production. Since the Eh is increasingly regarded as an important parameter controlling the efficiency of cattle feeding but difficult to record (Dijkstra et al., 2020), measuring SCFA concentration in the forestomach might offer a better alternative.

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