Effect of individual SCFA on the epithelial barrier of sheep rumen under physiological and acidotic luminal pH conditions

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ABSTRACT: The objective of this study was to investigate whether individual short-chain fatty acids (SCFA) have a different potential to either regulate the formation of the ruminal epithelial barrier (REB) at physiological pH or to damage the REB at acidotic ruminal pH. Ruminal epithelia of sheep were incubated in Ussing chambers on their mucosal side in buffered solutions (pH 6.1 or 5.1) containing no SCFA (control), 30 mM of either acetate, propionate or butyrate, or 100 mM acetate. Epithelial conductance (G_t), short-circuit current (I_{sc}), and fluorescein flux rates were measured over 7 h. Thereafter, mRNA and protein abundance, as well as localization of the tight junction proteins claudin (Cldn)-1, -4, -7, and occludin were analyzed. At pH 6.1, butyrate increased G_t and decreased I_{sc} , with additional decreases in claudin-7 mRNA and protein abundance (each $P < 0.05$) and disappearance of Cldn-7 immunosignals from the *stratum corneum*. By contrast, the mRNA abundance of *Cldn-1* and/or *Cldn-4* were upregulated by 30 mM propionate, 30 mM butyrate, or 100 mM acetate ($P < 0.05$), however, without coordinated changes in protein abundance. At luminal pH 5.1, neither G_t , I_{sc} , nor TJ protein abundance was altered in the absence

of SCFA; only fluorescein flux rates were slightly increased ($P < 0.05$) and fluorescein signals were no longer restricted to the *stratum corneum*. The presence of acetate, propionate, or butyrate at pH 5.1 increased fluorescein flux rates and G_t , and decreased I_{sc} (each $P < 0.05$). Protein abundance of Cldn-1 was decreased in all SCFA treatments but 30 mM butyrate; abundance of Cldn-4 and -7 was decreased in all SCFA treatments but 30 mM acetate; and abundance of occludin was decreased in all SCFA treatments but 30 mM propionate (each $P < 0.05$). Immunofluorescence staining of SCFA-treated tissues at pH 5.1 showed disappearance of Cldn-7, discontinuous pattern for Cldn-4 and blurring of occludin and Cldn-1 signals in tight junction complexes. The fluorescein dye appeared to freely diffuse into deeper cell layers. The strongest increase in G_t and consistent decreases in the abundance and immunosignals of tight junction proteins were observed with 100 mM acetate at pH 5.1. We conclude that SCFA may contribute differently to the REB formation at luminal pH 6.1 with possible detrimental effects of butyrate at 30 mM concentration. At luminal pH 5.1, all SCFA elicited REB damage with concentration appearing more critical than SCFA species.

Key words: epithelial barrier, sheep rumen, short-chain fatty acids, subacute ruminal acidosis, tight junction, Ussing chamber

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INTRODUCTION

Microorganisms in the forestomach of ruminants convert carbohydrates to short-chain fatty acids (SCFA) (Bugaut, 1987; Allen, 1997). The concentration of total SCFA in the rumen varies between 60 and 160 mM with greater values being linked to easily fermentable, starch-rich diets. The molar proportions of the three SCFA acetate (45% to 70%), propionate (15% to 40%), and butyrate (5% to 20%) also vary (Bergman, 1990; Aschenbach et al., 2011) with fiber-limited, starch-rich diets favoring greater propionate and butyrate, but lower acetate proportions (Loncke et al., 2009).

While the onset of ruminal fermentation and SCFA production appears relevant for the establishment of the gastrointestinal barrier function after weaning (Malmuthuge et al., 2013), very high ruminal SCFA concentrations induced by starch-rich diets impair the ruminal epithelial barrier (REB) (Klevenhusen et al., 2013). Ruminal pH (Gäbel et al., 1989; Aschenbach et al., 2011) and total SCFA (Meissner et al., 2017) play an important role in this scenario. However, to what extent each individual SCFA contributes to REB failure at low luminal pH has never been systematically investigated.

Epithelial barrier function is primarily dependent on tight junction (TJ) proteins that limit paracellular permeation (Marchiando et al., 2010). In the multilayered ruminal epithelium, such junctions appear most structured in the *stratum granulosum* (Schnorr and Wille, 1972) where the TJ proteins claudin (Cldn)-1, Cldn-4, and occludin (Ocln) are concentrated, while Cldn-7 primarily cross-links cells in the *stratum corneum* (Stumpff et al., 2011).

Proceeding from the hypothesis that each SCFA may have different and pH-dependent effects on the REB integrity, the present study aimed to elucidate the influence of acetate, propionate, and butyrate on the expression and localization of claudins-1, -4, -7, and occludin in the ruminal epithelium, and their consequences for the REB integrity at physiological and acidotic luminal pH.

MATERIALS AND METHODS

Animals and Tissue Sampling

All experiments were conducted in compliance with the German legislation on the welfare of experimental animals and announced to the local authorities (Registration No. T 0360/12). Ruminal tissue harvest for subsequent Ussing

chamber experiments was performed as described by Meissner et al. (2017) using the same nonpregnant, nonlactating adult sheep prefed a hay-only diet plus mineral supplements.

Ussing Chamber Experiments

Ruminal epithelia were mounted in Ussing chambers and bathed with standard buffered solution of pH 7.4 at the serosal side containing (in mM): 10 NaCl, 24 NaHCO₃, 0.6 NaH₂PO₄, 2.4 Na₂HPO₄, 5.5 KCl, 10 2-(N-morpholino) ethanesulfonic acid, 1 L-glutamine, 10 D-glucose, 100 Na-gluconate, 1 CaCl₂, and 1.25 MgCl₂. A standard buffered solution titrated to pH 6.1 using gluconic acid was used at the mucosal side. After equilibration for 30 min, we applied 10 different solutions; 5 titrated to pH 6.1 and 5 titrated to pH 5.1 using gluconic acid. Titration of standard buffered solution to pH 6.1 or pH 5.1 without further modifications yielded mucosal incubation solutions for the two control groups **Con-pH6.1** and **Con-pH5.1**, respectively. In the treatment groups, the same solution recipe was used except that 30 mM Na-acetate (**Ace30**), 100 mM Na-acetate (**Ace100**), 30 mM Na-propionate (**Prop30**), or 30 mM Na-butyrate (**But30**) were included at the expense of equimolar omissions of Na-gluconate. Solutions containing any single SCFA were similarly titrated to pH 6.1 or pH 5.1 using gluconic acid, resulting in groups **Ace30-pH6.1**, **Ace100-pH6.1**, **Prop30-pH6.1**, and **But30-pH6.1**, as well as **Ace30-pH5.1**, **Ace100-pH5.1**, **Prop30-pH5.1**, and **But30-pH5.1**. Each treatment group was designed to include 12 observations. This was generally achieved by assigning two epithelia per sheep to each treatment. One exception was that treatment **Ace100-pH5.1** was not included in the first three experiments and therefore included at a higher frequency in later experiments.

All mucosal and serosal incubation solutions had a final osmolarity of 290 mOsmol/L. They were gassed with carbogen (95% O₂/5% CO₂), thermostated to 37 °C and supplemented with antibiotics (25 mg/L colistin methanesulfonate and 100 mg/L cefuroxime) to limit bacterial proliferations.

Transepithelial conductance (G_t) as a measure for passive ion permeability and short-circuit current (I_{sc}) as a measure for active electrogenic electrolyte transport were recorded every minute as described previously (Aschenbach and Gäbel, 2000; Meissner et al., 2017). After a 7-h incubation period, epithelia were removed from the Ussing chambers, cut into three pieces, and preserved for

later analyses. One piece was bathed at 4 °C in RNAlater (Sigma-Aldrich, St. Louis, MO) overnight and stored at -20 °C for mRNA analysis. A second piece of epithelium was frozen in liquid nitrogen and stored at -80 °C for western blotting. The third piece was fixed in 4% paraformaldehyde at room temperature for 1 h, washed in Dulbecco's phosphate-buffered saline (PBS) solution with Ca²⁺ and Mg²⁺ (pH 7.2; Pan-Biotech GmbH, Aidenbach, Germany), thereafter in 25 mM aqueous L-glycine solution and again in DPBS solution at room temperature. After incubation in a series of solutions with increasing sucrose concentration (10% for 1 h, 20% for 1 h, and 30% overnight) epithelia were frozen with methylbutane for 10 s, embedded in Tissue Tek OCT (Sakura Finetek Europe B.V., Alphen aan den Rijn, The Netherlands) and stored at -80 °C.

Because Ussing chamber experiments of this study were performed together with the experiments of Meissner et al. (2017), raw data for G_p , I_{sc} and fluorescein flux rates, as well as tissue sample material for molecular biology and immunohistochemistry, was shared for control epithelia among these two studies.

Fluorescein Flux Rates

Fluorescein flux rates from the mucosal to the serosal side ($J_{ms-fluor}$) were measured at a final mucosal concentration of 100 μ M fluorescein exactly as described in Meissner et al. (2017). They

represent the amount of fluorescein marker flux within a 1-h flux period and are normalized per cm² of epithelial area.

RNA Analysis

Whole-cell RNA was extracted from RNAlater-preserved samples with the Nucleo Spin RNA II kit (Macherey-Nagel GmbH & Co. KG, Düren, Germany). The RNA concentration was measured with a nanophotometer (Implen, München, Germany). Agilent 2100 Bioanalyzer electrophoresis (Agilent Technologies GmbH, Böblingen, Germany) was adopted to assess the RNA quality. An aliquot of 400 ng of total RNA (RIN $\geq$ 6) was converted in a total 40 μ L volume to cDNA with the commercial iScript cDNA Synthesis Kit (BioRad Laboratories, Hercules, CA). Primers and probes used to amplify the target genes *Cldn-1*, *Cldn-4*, *Cldn-7*, and *Ocln*, as well as the reference genes *Gapdh*, *Ywhaz*, and *Rps19* are listed in Table 1. They were designed based on GenBank database sequence information using the MWG Operon Tool (qPCR Probe Design; Eurofins MWG Operon, Ebersberg, Germany) and their optimum concentrations were determined in a primer-probe optimization experiment. An aliquot of 4.5 μ L with a total of 4.5 ng of the obtained cDNA was mixed with primers and probe for the respective target gene, 4.5 μ L mastermix iTaq Universal Probes Supermix (Bio Rad Laboratories) and water at

Table 1. Sequences of primers and probes used for quantitative real-time reverse-transcription PCR

Gene	Primer and probe sequences	nM
<i>Cldn-1</i>	Forward: 5'-TGCCAGGTATGAATTTGGTC-3'	500
	Probe: 5'-FAM-TCTTCATTTGG CTGGGCTGCTGCTTCT-TAMRA-3'	300
	Reverse: 5'-GGGATAGGGCCTGGGTGTTG-3'	1,500
<i>Cldn-4</i>	Forward: 5'-TTCATCGGCAGCAACATC-3'	500
	Probe: 5'-FAM-AGACCATCTGGGAG GGCCTATGGATGAACT-BHQ-3'	300
	Reverse: 5'-GTACACCTTGCACTGCATC-3'	1,500
<i>Cldn-7</i>	Forward: 5'-ATAGCTTGCTCCTGGTACGG-3'	1,500
	Probe: 5'-FAM-ATAACCC GTTGGTCCCCATGAATGTTA AGTATGAA-TAMRA-3'	300
	Reverse: 5'-CAGGATGATCAGAGCAGACC-3'	1,500
<i>Ocln</i>	Forward: 5'-TCAGGGAATATCCACCTATCAC-3'	500
	Probe: 5'-FAM-GAGCCTACA AGCAGAAGCTTGATGAG GTCAATAAA-TAMRA-3'	150
	Reverse: 5'-CATCCAGTTCTTTATCCAGACG-3'	3,000
<i>Gapdh</i>	Forward: 5'-AAGAAGGTGGTGAAGCAGGC-3'	1,500
	Probe: 5'-FAM-GGCATTCTAGGCTACACTGAGGAC CAGGTTG-TAMRA-3'	300
	Reverse: 5'-CTGTTGAAGTCGAGGAGAC-3'	3,000
<i>Rps19</i>	Forward: 5'-GGAAAAGGACCAAGATGGGG-3'	500
	Probe: 5'-FAM-ACAGAG AGATCTGGACAGAATCGC TGGACA-TAMRA-3'	300
	Reverse: 5'-CGAACGAGGCAATTTATTAACC-3'	3,000
<i>Ywhaz</i>	Forward: 5'-AGAGAGAAAATAGAGACCGAGC-3'	1,500
	Probe: 5'-FAM-CCAACG CTTACAAGCAGAGA GCAAA-TAMRA-3'	300
	Reverse: 5'-AGCCAAGTAGCGGTAGTAG-3'	1,500

BHQ = blackhole quencher; *Cldn* = claudin; FAM = 6-carboxy-fluorescein; *Gapdh* = glyceraldehyde 3-phosphate dehydrogenase; *Ocln* = occludin; *Rps19* = ribosomal protein S19; TAMRA = 6-carboxy-tetramethylrhodamine; *Ywhaz* = 14-3-3 protein zeta/delta.

a total volume of 12 μ L per well in triplicate for reverse transcription quantitative real-time PCR (RT-qPCR). A two-step temperature protocol with 40 cycles of 20 s at 60 °C and 1 s at 95 °C was run on an RT-qPCR performing thermocycler (ViiA7, Life Technologies, Carlsbad, CA). Data were normalized and elaborated with the software qbase PLUS (Biogazelle NV, Zwijnaarde, Belgium). Double gene normalization was achieved utilizing the two most stably expressed reference genes *Rps19* and *Ywhaz*.

Protein Extraction and Western Blot Analysis

Samples stored at -80 °C were homogenized in lysis buffer (10 mM Tris, 140 mM NaCl, 5 mM EDTA, 1% Triton X, 1 mM phenylmethanesulfonyl fluoride, 1 mM dithiothreitol) containing complete EDTA-free protease inhibitor cocktail (Roche Diagnostics, Mannheim, Germany). The whole cell protein concentrations were determined with a Biophotometer (Eppendorf, Wesseling-Berzdorf, Germany) and a prediluted protein standard series (Bovine Serum Albumin, Thermo Fischer Scientific, Waltham, MA), which was used to draft a reference curve for the protein concentration. Aliquots of 20 μ g protein lysate and 5 μ L of pre-stained molecular weight marker (Precision Plus Protein Dual Color Standards, Bio Rad Laboratories) were loaded on self-made polyacrylamide gels with 10% acrylamide concentration. After electrophoresis in running buffer (192 mM L-glycine, 25 mM Tris, 0.1% SDS, pH 8.3) at 150 V for 1 h, proteins were semidry transferred from the gel to a PVDF membrane (Bio Rad Laboratories) at 70 V for 60 min. Transfer buffer contained 48 mM L-glycine, 39 mM Tris, 0.037% SDS, 20% methanol, pH 9.2. Membranes were blocked with milk powder (5%; Carl Roth, Karlsruhe, Germany) dissolved in 0.1% Tween-containing Tris-buffered saline (50 mM Tris, 150 mM NaCl) (TBST) at room temperature for 2 h. After washing in TBST, membranes were incubated overnight at 4°C with the following primary antibodies: mouse anti-Cldn-4 and anti-Ocln (Invitrogen, Life Technologies, Carlsbad, CA), rabbit anti-Cldn-1 (Invitrogen, Life Technologies) and anti-Cldn-7 (Abcam, Cambridge, UK), and mouse anti- β -actin (Sigma Aldrich). Peroxidase-conjugated secondary anti-mouse and anti-rabbit IgG antibodies (Cell Signaling Technology, Danvers, MA) were incubated at room temperature for 45 min. Before use, all antibodies were diluted 1/1,000 in TBST containing 2.5% milk powder. Primary antibodies were preserved with 0.1% sodium azide (NaN_3). After washing in

TBST, 400 μ L of peroxidase substrate (SuperSignal West Dura Extended Duration Substrate, Thermo Fisher Scientific, Waltham, MA) were applied on the membranes and luminescence was imaged using ChemiDoc MP (Bio-Rad Laboratories). The Image Lab software (version 4.1, Bio-Rad Laboratories) was utilized for densitometry and normalization.

Immunofluorescence Staining and Confocal Laser Scanning Microscopy

Sections from paraformaldehyde-fixed and Tissue Tek OCT-embedded tissues were cut 5- μ m thin at -26 °C with the cryostat Leica CM 1900 (Leica Biosystems, Nussloch, Germany). The cross sections were boiled 15 min in 1 mM EDTA in PBS solution at pH 8.0 and then washed and incubated 5 min in 0.5% Triton-X in PBS solution. A blocking solution containing 6% goat serum in PBS was applied for 60 min. Tissue sections were incubated overnight at 4 °C in 1/250 dilution of primary antibodies: rabbit anti-Cldn-1 polyclonal (Invitrogen, Life Technologies, Carlsbad, CA), mouse anti-Cldn-4 monoclonal (Invitrogen, Life Technologies), rabbit anti-Cldn-7 polyclonal (Abcam), and mouse anti-Ocln monoclonal (Invitrogen, Life Technologies). Thereafter, secondary goat Alexa Fluor 594-conjugated anti-mouse IgG (Invitrogen, Life Technologies) and goat Cy5-conjugated anti-rabbit IgG antibody (Cell Signaling Technology, Danvers, MA) were incubated at 37 °C in 1/500 concentration for 60 min. DAPI dye (1 μ g/mL in PBS; Roche, Grenzach-Wyhlen, Germany) was used as nuclear counterstain. The sections were imaged with a confocal laser-scanning microscope (Zeiss LSM510, Carl Zeiss, Jena, Germany) at 488 nm for the fluorescein dye applied in the Ussing chamber, at 594 and 633 nm for antibody signals of Alexa Fluor 594 and Cy5, respectively, and at 405 nm for DAPI. Sample processing, imaging and comparative assessment of the obtained images was always performed by the same trained person without blinding.

Statistics

Statistical analysis was performed with the statistical software Sigma Plot 11.0 (Systat Software Inc, San José, CA). Because of expected diverging effects of SCFA at physiological vs. acidotic luminal pH, data obtained at pH 6.1 and 5.1 were tested separately. The Con-pH6.1 was used as time-dependent reference for baseline in both models. As such, the model at pH 6.1 included group Con-pH6.1 plus

the groups with SCFA additions at mucosal pH 6.1, whereas, the model at pH 5.1 included the groups Con-pH6.1 and Con-pH5.1 plus the groups with single SCFA additions at mucosal pH 5.1. Data from Ussing chamber experiments were analyzed with two-way analysis of variance for the fixed factors ‘treatment’ and ‘incubation time’ ($Y_{ij} = \mu + \alpha_i + \beta_j + \gamma_{ij} + \varepsilon_{ijk}$, where μ is the mean response, α_i is the effect due to the i^{th} level of factor treatment, β_j is the effect due to the j^{th} level of factor incubation time, γ_{ij} is the effect due to any two-way interaction between the factor levels of α and β , and ε is the random effect of a given measurement). The seventh hour of incubation was removed from the analysis of $J_{\text{ms-fluor}}$ because technical problems had made the data set for that period incomplete. Already by the sixth hour, data availability for $J_{\text{ms-fluor}}$ was reduced to only $n = 5$ in two groups (Prop30-pH5.1 and But30-pH5.1). Data from RT-qPCR and Western blot analysis were analyzed with one-way ANOVA or, in the case of non-normal distribution, one-way ANOVA on ranks. Differences between multiple means were separated by post-hoc Holm-Sidak’s test (one and two-way ANOVA) or Dunn’s test (one-way ANOVA on ranks). Differences were considered significant at $P < 0.05$. All data are reported as least square means (LSM; two-factorial data) or means (one-factorial data) and SEM.

RESULTS

Electrophysiology

All electrophysiological data are shown in Tables 2 and 3. At mucosal pH 6.1, G_t values remained generally stable over time with no treatment $\times$ time interaction. However, the effect of treatment was significant ($P < 0.01$). The G_t was greatest in But30-pH6.1 (i.e., greater than Con-pH6.1 and Ace30-pH6.1) and lowest in Ace30-pH6.1, with Prop30-pH6.1 and Ace100-pH6.1 showing intermediate values. A treatment effect was also observed for I_{sc} ; however, with lowest values in But30-pH6.1 (i.e., lower than Con-pH6.1, Ace30-pH6.1, and Prop30-pH6.1), greatest values in Prop30-pH6.1, and with Ace100-pH6.1 being intermediate. Time effect was significant for I_{sc} at pH 6.1 ($P < 0.01$), showing a decrease of I_{sc} over the 7 h of incubation with no treatment $\times$ time interaction.

At mucosal pH 5.1, G_t values increased over time ($P < 0.01$). In addition, G_t differed among treatments ($P < 0.01$) with G_t being greater in Ace100-pH5.1 compared to the groups incubated with only 30 mM of SCFA (Ace30-pH5.1,

Prop30-pH5.1, and But30-pH5.1). However, the latter three groups had even greater values than Con-pH5.1 and Con-pH6.1. Of note, G_t values of Con-pH5.1 and Con-pH6.1 did not differ from each other. A treatment $\times$ time interaction ($P < 0.01$) indicated that the time-dependent changes differed among treatments; with multiple comparisons showing that the divergence of G_t developed primarily in the last 4 h of incubation. Values of I_{sc} were also affected by treatment ($P < 0.01$) with Con-pH5.1 = Con-pH6.1 > Ace30-pH5.1 > But30-pH5.1 > Prop30-pH5.1 = Ace100-pH5.1. A treatment $\times$ time interaction ($P < 0.01$) with a trend for a time effect ($P = 0.067$) showed that the I_{sc} changes differed among treatments over the 7 h of incubation. During the first hour of incubation, all epithelia incubated in the presence of SCFA showed I_{sc} below the values of Con-pH5.1 and Con-pH6.1. Thereafter, I_{sc} stayed low in group But30-pH5.1 or even continued to decrease in groups Prop30-pH5.1 and Ace100-pH5.1. In contrast, I_{sc} of group Ace30-pH5.1 fully recovered to values not different from those of groups Con-pH5.1 and Con-pH6.1 (Table 3).

Fluorescein Flux Rates

Table 3 summarizes data for $J_{\text{ms-fluor}}$. At pH 6.1, $J_{\text{ms-fluor}}$ differed among treatments ($P < 0.05$); however, differences between individual groups were not great enough to be separated by the Holm-Sidak’s post-hoc test. Time was without effect on $J_{\text{ms-fluor}}$ at pH 6.1 with no treatment $\times$ time interaction.

At pH 5.1, $J_{\text{ms-fluor}}$ differed among treatments ($P < 0.01$) where Ace30-pH5.1, Prop30-pH5.1, and But30-pH5.1 were greater than Con-pH5.1, with Ace100-pH5.1 being intermediate. The $J_{\text{ms-fluor}}$ was greater for Con-pH5.1 than for Con-pH6.1. Time effect was significant ($P < 0.01$) with no treatment $\times$ time interaction. Corresponding multiple comparisons showed that the major part of acidification-induced increase in $J_{\text{ms-fluor}}$ occurred in the first 2 to 4 h of incubation (Table 4).

mRNA Abundance of TJ Proteins

At pH 6.1, treatment affected expression of *Cldn-1* mRNA ($P < 0.01$). Treatments Prop30-pH6.1, But30-pH6.1, and Ace100-pH6.1 increased the abundance of *Cldn-1* mRNA above Con-pH6.1, with Ace30-pH6.1 being intermediate (Fig. 1). Treatment effects of similar direction were obtained for *Cldn-4* mRNA ($P < 0.01$), which was increased by Prop30-pH6.1 and But30-pH6.1 above

Table 2. Effects of individual SCFA and mucosal pH on tissue conductance (G_t)

Gt, mS·cm ⁻² ; TRT at pH 6.1														Gt, mS·cm ⁻² ; TRT at pH 5.1					
Hour	Con ¹	Ace30	Ace100	Prop30	But30	LSM	SEM	Con ¹	Ace30	Ace100	Prop30	But30	LSM	SEM					
1	1.97	1.84	1.98	1.90	1.60	1.86	0.132	2.06	2.48	2.86	2.00	2.95	2.39 ^v	0.507					
2	1.92	1.79	1.94	1.89	1.77	1.86	0.132	2.03	3.70	5.39	3.06	4.79	3.48 ^{vw}	0.507					
3	1.89	1.80	2.09	2.00	2.19	1.99	0.132	2.13	5.14	6.79	4.61	6.41	4.49 ^{wx}	0.507					
4	1.88 ^B	1.77	2.22	2.07	2.53	2.09	0.132	2.26 ^B	6.54 ^{A,B}	8.85 ^A	6.23 ^{A,B}	9.17 ^A	5.82 ^x	0.507					
5	1.88 ^C	1.74	2.34	2.17	2.81	2.19	0.132	2.52 ^C	8.49 ^B	14.15 ^A	8.67 ^B	12.02 ^{A,B}	7.96 ^y	0.507					
6	1.77 ^C	1.71	2.45	2.23	3.04	2.24	0.133	2.70 ^C	10.78 ^B	18.40 ^A	11.61 ^B	11.31 ^B	9.43 ^{yz}	0.528					
7	1.77 ^C	1.67	2.53	2.24	3.25	2.29	0.133	2.82 ^C	12.58 ^B	23.24 ^A	12.40 ^B	13.64 ^B	11.07 ^z	0.528					
LSM	1.87 ^{b,c,C}	1.76 ^c	2.22 ^{ab}	2.07 ^{abc}	2.46 ^a			2.36 ^C	7.10 ^B	11.38 ^A	6.94 ^B	8.61 ^B		TRT × Time P = 0.58					
SEM	0.110	0.113	0.108	0.113	0.113			0.468	0.484	0.476	0.484	0.491		TRT × Time P < 0.01					
Factor TRT, P < 0.01 (^{a-c})														Factor TRT, P < 0.01 (^{A-C})					

Data are least square means (LSM) and SEM ($n = 10-12$). Differences between multiple means are indicated by letter coding; values differ if they do not share a common letter. Lower case letters are used for comparisons at pH 6.1 for factor Treatment (TRT) within the same row (encoded by a-e) and factor Time within the same column (encoded by x-z). Capital letters are used for comparisons at pH 5.1 (including Con-pH6.1 as additional reference) for the factors TRT (encoded by A-D within one row) and Time (encoded by V-Z within one column). Con, control; Ace30, acetate 30 mM; Ace100, acetate 100 mM; Prop30, propionate 30 mM; But30, butyrate 30 mM.

¹Raw data for Con-pH6.1 and Con-pH5.1 has been used previously in [Meissner et al. \(2017\)](#).

²Interactions for factor Time within TRT not shown for reasons of clarity.

Table 3. Influence of individual SCFA and mucosal pH on short circuit current (I_{sc})

I_{sc} , $\mu\text{Eq}\cdot\text{cm}^{-2}\cdot\text{h}^{-1}$; TRT at pH 6.1														
Hour	Con ¹	Ace30	Ace100	Prop30	But30	LSM	SEM	Con ¹	Ace30	Ace100	Prop30	But30	LSM	SEM
1	1.52 ^A	1.38	0.98	1.33	0.73	1.19 ^s	0.066	1.61 ^A	0.51 ^B	0.06 ^B	0.28 ^B	0.23 ^B	0.70	0.072
2	1.29 ^A	1.21	0.88	1.15	0.63	1.03 ^{xy}	0.066	1.27 ^A	0.42 ^B	-0.03 ^B	0.05 ^B	0.20 ^B	0.53	0.072
3	1.00 ^A	1.02	0.78	0.98	0.54	0.86 ^{xy}	0.066	1.09 ^A	0.48 ^{AB}	-0.09 ^B	0.01 ^B	0.25 ^B	0.46	0.072
4	0.80 ^A	0.87	0.72	0.91	0.50	0.76 ^{yz}	0.066	0.97 ^A	0.61 ^{AB}	-0.10 ^C	-0.02 ^{BC}	0.28 ^{ABC}	0.42	0.072
5	0.67 ^{AB}	0.77	0.70	0.89	0.54	0.71 ^{yz}	0.066	0.95 ^A	0.77 ^A	-0.09 ^C	-0.01 ^{BC}	0.30 ^{ABC}	0.43	0.072
6	0.57 ^{AB}	0.67	0.68	0.85	0.57	0.67 ^z	0.067	0.93 ^A	0.97 ^A	-0.01 ^B	-0.02 ^B	0.32 ^{AB}	0.46	0.073
7	0.50 ^{ABC}	0.58	0.63	0.79	0.61	0.62 ^z	0.067	0.87 ^{AB}	1.14 ^A	-0.21 ^{CD}	-0.20 ^D	0.38 ^{BCD}	0.42	0.073
LSM	0.91 ^{ab,AB}	0.93 ^{ab}	0.77 ^{bc}	0.98 ^a	0.59 ^c			TRT $\times$ Time $P = 0.71$	0.70 ^B	-0.07 ^D	0.01 ^D	0.28 ^C		TRT $\times$ Time $P < 0.01$
SEM	0.056	0.058	0.053	0.055	0.058			0.070	0.062	0.070	0.064	0.070		
Factor TRT, $P < 0.01$ (^{A-C})														
Factor TRT, $P < 0.01$ (^{A-D})														

Data are least square means (LSM) and SEM ($n = 10-12$). Differences between multiple means are indicated by letter coding; values differ if they do not share a common letter. Lower case letters are used for comparisons at pH 6.1 for factor Treatment (TRT) within the same row (encoded by a-c) and factor Time within the same column (encoded by x-z). Capital letters are used for comparisons at pH 5.1 (including Con-pH6.1 as additional reference) for the factors TRT (encoded by A-D within one row) and Time (encoded by V-Z within one column). Con, control; Ace30, acetate 30 mM; Ace100, acetate 100 mM; Prop30, propionate 30 mM; But30, butyrate 30 mM.

¹Raw data for Con-pH6.1 and Con-pH5.1 has been used previously in [Meissner et al. \(2017\)](#).

Table 4. Effects of individual SCFA and mucosal pH on fluorescein flux rates ($J_{ms-fluor}^1$)

Hour	$J_{ms-fluor}^2$, nmol·cm ⁻² ·h ⁻¹ ; TRT at pH 6.1										$J_{ms-fluor}^1$, nmol·cm ⁻² ·h ⁻¹ ; TRT at pH 5.1					Factor Time, $P < 0.01$ (χ^2)
	Con ²	Ace30	Ace100	Prop30	But30	LSM ¹	SEM	Factor Time, $P = 0.28$	Con ²	Ace30	Ace100	Prop30	But30	LSM	SEM	
1	0.20	0.18	0.25	0.20	0.13	0.19	0.035		0.71	0.84	0.30	0.45	0.53	0.51 ^z	0.232	
2	0.28	0.22	0.29	0.21	0.14	0.23	0.034		1.15	2.29	1.39	2.79	2.14	1.67 ^y	0.232	
3	0.31	0.19	0.35	0.22	0.19	0.25	0.034		1.51	2.11	1.60	3.09	3.07	1.95 ^{xy}	0.227	
4	0.34	0.24	0.35	0.18	0.17	0.26	0.034		2.11	3.81	1.97	4.88	3.88	2.83 ^x	0.227	
5	0.27	0.08	0.26	0.13	0.18	0.18	0.036		1.70	3.20	2.78	3.10	4.05	2.52 ^{xy}	0.241	
6	0.19	0.11	0.19	0.18	0.12	0.16	0.040		1.39	3.21	3.07	2.46	3.43	2.29 ^{xy}	0.272	
LSM	0.27 ^b	0.17	0.28	0.19	0.15			TRT × Time $P = 1.00$	1.43 ^c	2.58 ^{ab}	1.85 ^{bc}	2.80 ^a	2.85 ^a			TRT × Time $P = 0.24$
SEM	0.033	0.034	0.032	0.032	0.032				0.233	0.227	0.219	0.252	0.253			
Factor TRT, $P < 0.05$																
Factor TRT, $P < 0.01$ (χ^2)																

Data are least square means (LSM) and SEM ($n = 5-10$; the lowest representation of $n = 5$ applying to But30-pH5.1 and Prop30-pH 5.1 in the sixth hour due to technical reasons). Differences between multiple means are indicated by letter coding; values differ if they do not share a common letter. Capital letters are used for comparisons at pH 5.1 (including Con-pH6.1 as additional reference) for the factors treatment (TRT); encoded by A-D within one row) and Time (encoded by X-Z within one column). Con, control; Ace30, acetate 30 mM; Ace100, acetate 100 mM; Prop30, propionate 30 mM; But30, butyrate 30 mM.

¹ $J_{ms-fluor}$ represent the amount of fluorescein marker flux within a 1-h flux period and is normalized per cm² of epithelial area.

²Raw data for Con-pH6.1 and Con-pH5.1 has been used previously in Meissner et al. (2017).

Con-pH6.1 and Ace30-pH6.1, with Ace100-pH6.1 being intermediate (Fig. 2). The mRNA abundances of *Cldn-7* (Fig. 3) and *Ocln* (Fig. 4) were not significantly affected by treatment at pH 6.1.

At pH 5.1, treatment was without effect on the mRNA abundance of *Cldn-1* (Fig. 1), *Cldn-4* (Fig. 2), and *Ocln* (Fig. 4). The mRNA abundance of *Cldn-7* was affected by treatment ($P < 0.01$) with decreases of *Cldn-7* below the level of Con-pH6.1 being triggered by treatments Ace100-pH5.1, Prop30-pH5.1, and But30-pH5.1 (Fig. 3).

Abundance of TJ Proteins

At pH 6.1, abundances of *Cldn-1* (Fig. 1), *Cldn-4* (Fig. 2), and *Ocln* (Fig. 4) were not affected by treatment. However, abundance of *Cldn-7* responded to treatment ($P < 0.01$); being lower in group But30-pH6.1 compared with group Con-pH6.1 (Fig. 3).

At pH 5.1, expression of all tested TJ proteins was affected by treatment ($P < 0.01$). Compared to Con-pH6.1, the abundance of *Cldn-1* was decreased by Ace30-pH5.1, Ace100-pH5.1, and Prop30-pH5.1 (Fig. 1). The abundances of *Cldn-4* and *Cldn-7* were decreased by Ace100-pH5.1, Prop30-pH5.1, and But30-pH5.1 (Fig. 2 and Fig. 3) while the protein abundance of *Ocln* was decreased by Ace30-pH5.1, Ace100-pH5.1, and But30-pH5.1 below the level of Con-pH6.1 (Fig. 4).

Immunofluorescence Staining of TJ Proteins

Epithelia incubated at pH 6.1 either with or without various SCFA presented a sharply delineated paracellular network after staining for *Cldn-1* and *Cldn-4* with no noticeable differences between groups (Fig. 5). Staining for *Ocln* also resulted in a sharply delineated strand network in all groups incubated at pH 6.1, except for some blurring in group But30-pH6.1. The blurring indicated that *Ocln* had less condensation in the TJ and, as such, an incipient and subtle decrease in TJ net integrity in group But30-pH6.1. Staining for *Cldn-7* delineated the circumference of some cells in the *stratum corneum* in Con-pH6.1, Ace30-pH6.1 and Prop30-pH6.1. The specific signal for *Cldn-7* protein appeared less clear in group Ace100-pH6.1 and completely disappeared in group But30-pH6.1.

Epithelia incubated at pH 5.1 without SCFA (Con-pH5.1) showed a completely preserved paracellular network of *Cldn-1* and, with some blurring, also for *Cldn-4* and *Ocln*. The network of *Cldn-1* strands faded in epithelia incubated with

Figure 1. Expression of claudin-1 mRNA (A) and protein (B) in ruminal epithelia after 7 h of incubation with various SCFA at mucosal pH 6.1 or 5.1. Data are means $\pm$ SEM ($n = 10$ – 12). Letter code is used to indicate significant differences identified by multiple comparisons post-hoc test. Within each graph, columns differ at $P < 0.05$ if they do not share a common letter. Lower case letters are used for comparisons at pH 6.1 (encoded by a–c) while capital letters are used for comparisons at pH 5.1, the latter including Con-pH6.1 as additional reference (encoded by A–C). Ace30, acetate 30 mM; Ace100, acetate 100 mM; Prop30, propionate 30 mM; But30, butyrate 30 mM.

30 mM of either single SCFA at pH 5.1 (i.e., groups Ace30-pH5.1, Prop30-pH5.1, and But30-pH5.1) and appeared completely disrupted in group Ace100-pH5.1. Claudin-4 was no longer organized in strands in any of the groups incubated with SCFA at either 30 or 100 mM, whereas Occludin network strands were still detectable with some blurring in the same groups. Clearly delineated signals for Cldn-7 were not observed in any of the groups incubated at pH 5.1 (Fig. 5).

The fluorescein that had been applied as a permeability marker in the mucosal incubation solution to study fluorescein flux rates was also visible in tissue sections (Fig. 5). In all epithelia incubated at mucosal pH 6.1, fluorescence of fluorescein was limited to the *stratum corneum*, indicating an effective epithelial barrier to fluorescein permeation to the deeper epithelial cell layers. Epithelia incubated at mucosal pH 5.1 without SCFA (Con-pH5.1) were still able to partially limit the permeation of fluorescein dye into deeper epithelial layers. However, epithelia incubated with any single SCFA at pH 5.1 (Ace30-pH5.1, Ace100-pH5.1, Prop30-pH5.1, and But30-pH5.1) did not provide any visible barrier to fluorescein permeation. Of note, the fluorescein

signal was also detectable inside epithelial cells at pH 5.1.

DISCUSSION

The ruminal epithelium mediates the absorption of SCFA but it is also an effective barrier against microbes and toxins in the ruminal content (Galfi et al., 1981; Aschenbach et al., 2000; Emmanuel et al., 2007). It consists of four distinct cell layers (i.e., *stratum basale*, *stratum spinosum*, *stratum granulosum*, and *stratum corneum*) interconnected by a network of TJ proteins (Graham and Simmons, 2005; Stumpff et al., 2011). The TJ network is most complex in the *stratum granulosum* where a tight permeation barrier exists (Henrikson and Stacy, 1971; Meissner et al., 2017). A disruption of the TJ network leads to an increase of molecular permeability and a decrease of ions gradients; meaning that proinflammatory molecules, toxins, and microorganisms become capable of entering the body (Lerner and Matthias, 2015; Meissner et al., 2017). An impaired epithelial barrier may thus result in a threat to the animal's health and in economic losses for the farm (Enemark, 2008).

Figure 2. Expression of claudin-4 mRNA (A) and protein (B) in ruminal epithelia after 7 h of incubation with various SCFA at mucosal pH 6.1 or 5.1. Data are means $\pm$ SEM ($n = 10$ –12). Within each graph, columns differ at $P < 0.05$ if they do not share a common letter. Lower case letters are used for comparisons at pH 6.1 (encoded by a–b) while capital letters are used for comparisons at pH 5.1, the latter including Con-pH6.1 as additional reference (encoded by A–C). Ace30, acetate 30 mM; Ace100, acetate 100 mM; Prop30, propionate 30 mM; But30, butyrate 30 mM.

A variation in epithelial permeability can be related to a change in the general abundance of TJ proteins, their localization, and their interaction (Markov et al., 2015, 2017). Most of the TJ proteins support a high epithelial resistance by decreasing ion and macromolecular permeability, whereas only a few work as selective channels (Krug et al., 2012; Markov et al., 2015). The four TJ proteins that have been discovered in the ruminal epithelium include Cldn-1, -4, -7, and Ocln (Stumpff et al., 2011). Of these, Cldn-1 and Cldn-4 are major tightening proteins, and also Ocln and Cldn-7 have mostly, but not exclusively, been associated with barrier tightening (Markov et al., 2015).

The maturation of the ruminal epithelium after birth appears to be tightly coupled to the intake of solid feed and the commencement of ruminal fermentation (Meale et al., 2017). Epithelial maturation includes an establishment of a tight REB. It has been shown that tissue conductance and mannitol permeability as indicators of passive ion leak flux and paracellular leak flux, respectively, decrease to less than 20% from 6 wk of age (Wood et al., 2015) to 6 mo of age (Penner et al., 2014). Short chain fatty acids are considered the main

trigger for overall ruminal epithelial development (Meale et al., 2017) with butyrate being likely a key player (Niwinska et al., 2017). It has been suggested that the regulatory role includes a barrier-tightening effect, at least for butyrate, based on a comparative transcriptomics approach using ruminal biopsies from Holstein cows infused with 12.5 mole/d over 168 h and a bovine kidney epithelial cell line exposed to 10 mM butyrate for 24 h. In that study, the mRNA of 17 TJ or TJ-associated proteins were upregulated by butyrate in the kidney cell line; however, only one (cingulin) was also upregulated in the bovine rumen in vivo (Baldwin et al., 2012). The few existing functional studies are also not fully conclusive. While Lodemann and Martens (2006) showed a decreased paracellular leak flux of Na^+ during a hyperosmolar challenge in concentrate-adapted sheep, another study could not detect decreased passive permeability to ions (G_i) in the ruminal epithelium of lambs after targeted butyrate supplementation (1.25% or 2.50% butyrate as a proportion of DMI; Wilson et al., 2012). To bring light into this controversy, our first hypothesis was that SCFA aid to establish the REB.

Figure 3. Expression of claudin-7 mRNA (A) and protein (B) in ruminal epithelia after 7 h of incubation with various SCFA at mucosal pH 6.1 or 5.1. Data are means $\pm$ SEM ($n = 10$ – 12). Within each graph, columns differ at $P < 0.05$ if they do not share a common letter. Lower case letters are used for comparisons at pH 6.1 (encoded by a–b) while capital letters are used for comparisons at pH 5.1, the latter including Con-pH6.1 as additional reference (encoded by A–B). Ace30, acetate 30 mM; Ace100, acetate 100 mM; Prop30, propionate 30 mM; But30, butyrate 30 mM.

In contrast to the hypothesis that SCFA play a role in REB formation in the healthy rumen, it has also been shown that highly fermentable diets may increase REB permeability (Klevenhusen et al., 2013) by impairing adhesion and organization of cells of the *stratum granulosum* (Steele et al., 2011) where the REB resides (Henrikson and Stacy, 1971; Meissner et al., 2017). On a molecular level, the increased REB permeability induced by highly fermentable diets was caused by downregulation of the TJ proteins Cldn-4 and Ocln, as well as redistribution of Cldn-1, Cldn-4, and Ocln out of the TJ (Liu et al., 2013). The underlying mechanisms are still not completely understood. Ruminal pH, ruminal osmolarity, and ruminal LPS concentration have been suggested as triggering insults (Gäbel and Aschenbach, 2006; Penner et al., 2011; Liu et al., 2013) because they are known to be detrimental for the REB (Gaebel et al., 1989; Lodemann and Martens, 2006; Al-Sadi and Ma, 2007; Zebeli and Ametaj, 2009). Of these, ruminal pH has clearly the dominant role because the REB failure caused by low pH is not or not immediately reversible in contrast to the reversible increase of REB permeability

caused by osmolarity (Penner et al., 2010). Low pH is also accepted as the primary event preceding bacterial LPS release and LPS permeation across the REB during subacute ruminal acidosis (SARA) (Liu et al., 2013; Klevenhusen et al., 2013). However, we recently demonstrated that it is not low ruminal pH per se that damages the REB; instead, it is essentially the combination of low ruminal pH and permeable SCFA (Meissner et al., 2017), the latter acting as “Trojan horses” that facilitate bulk H^+ influx into the epithelial cells. According to the weak acid carrier model (Gutknecht, 1987), SCFA $^-$ anions associate with protons in the ruminal lumen, penetrate inside the cell and release their proton to acidify the cytosol. Alternatively, an exchange of SCFA $^-$ anions by HCO_3^- depletes the cell interior of HCO_3^- buffer and, thereby, equally acidifies the cytosol (Gäbel et al., 2002; Aschenbach et al., 2011).

In this study, we addressed the question whether effects on the REB may differ among the three main SCFA. This question was tested separately at mucosal pH 6.1 where we hypothesized a possibly enforcing effect on the REB and at pH 5.1 where we

Figure 4. Expression of ocludin mRNA (A) and protein (B) in ruminal epithelia after 7 h of incubation with various SCFA at mucosal pH 6.1 or 5.1. Data are means $\pm$ SEM ($n = 10-12$). Within each graph, columns differ at $P < 0.05$ if they do not share a common letter. Capital letters are used for comparisons at pH 5.1, the latter including Con-pH6.1 as additional reference (encoded by A–B). Ace30, acetate 30 mM; Ace100, acetate 100 mM; Prop30, propionate 30 mM; But30, butyrate 30 mM.

Figure 5. Influence of mucosal presence of various SCFA and mucosal pH on the localization of tight junction proteins in ruminal epithelia. Images are representative immunocytochemistry pictures of claudin (Cldn)-1, Cldn-4, Cldn-7, and occludin (Ocln) after 7 h of incubation at mucosal pH 6.1 or pH 5.1; either in the absence of SCFA (Control) or in the mucosal presence of either 30 mM acetate (Ace30), 100 mM acetate (Ace100), 30 mM propionate (Prop30), or 30 mM butyrate (But30). The lower panels depict fluorescence of fluorescein that was applied from the mucosal side to measure fluorescein flux rates. *Stratum corneum* is located top/top-right whereas *stratum basale* is located bottom/bottom-left.

hypothesized a damaging effect on the REB. The effects of the individual SCFA were tested at isomolar concentrations of 30 mM of either SCFA. The strategy behind this was to compare the effects of the three main SCFA acetate, propionate and butyrate 1) at the same concentration that 2) is also the highest concentration with physiological rationing. Regarding the latter, butyrate is the SCFA with the lowest molar proportion in the ruminal fluid of typically less than 20% (Aschenbach et al., 2011; Morvay et al., 2011); extrapolating this 20% to maximum ruminal SCFA concentrations of more than 150 mM (Aschenbach et al., 2011) yielded the 30 mM choice for experimentation. However, as acetate may reach much greater concentrations in the ruminal content, the effect of a dose increase for acetate from 30 mM to 100 mM was also studied. From the above, it is clear that, for the vast majority of feeding situations, concentrations of 30 mM butyrate and 100 mM acetate have to be regarded as very high (Aschenbach et al., 2011; Morvay et al., 2011).

The animals used in the present study were prefed a hay-only diet 1) to minimize pre-exposure to SCFA, i.e., to make specific barrier-enforcing effects of SCFA easier to monitor ex vivo and 2) to strictly avoid any impact of preceding SARA episodes on ruminal tissue integrity, i.e., to ensure a homogeneous and completely intact ruminal tissue for experimentation. Such feeding regimen results in a low-conductance epithelium (Klevenhusen et al., 2013) that is especially suitable for investigating insults on the REB integrity ex vivo. Using intact ruminal tissue with precise control of mucosal and serosal incubation conditions can nicely model the situation in vivo; however, the interpretation of the results will need to account for other factors like the missing epithelial blood flow and the different degree of epithelial adaptation in animals fed on different diets.

In agreement with our first hypothesis, the mRNA abundance of the two major barrier-forming claudins, *Cldn-1* and *Cldn-4*, were upregulated after 7 h incubation with 30 mM propionate or butyrate at pH 6.1; the mRNA expression of *Cldn-1* was additionally increased by 100 mM acetate at pH 6.1. Although these changes were not accompanied by coordinated increases in Cldn-1 and Cldn-4 protein abundance, they suggest, at least, a potential of these SCFA to contribute to a tightening of the permeation barrier in the *stratum granulosum*. The increased mRNA abundance of *Cldn-1* and *Cldn-4* after butyrate or propionate application, as such, could possibly be explained by inhibition of histone

deacetylase. Butyrate (Plöger et al., 2012) and to a lesser extent also propionate (Ohata et al., 2005) act as histone deacetylase inhibitors; the resulting DNA hyperacetylation facilitates nucleosomal relaxation and, thereby, gene transcription. Because histone deacetylase inhibitors are able to upregulate TJ protein transcription (Bordin et al., 2004), this may be one underlying mechanism by which especially butyrate and propionate increase the transcription of *Cldn-1* and *Cldn-4* mRNA. Considering that the mRNA abundance of *Cldn-7* was not increased but decreased in group But30-pH6.1, however, a possibility cannot be ruled out that other explanations apply also or alternatively.

It was surprising that the SCFA-induced upregulations of *Cldn-1* and *Cldn-4* mRNA at luminal pH 6.1 were not followed by similar changes of TJ protein abundance. One technical explanation could be that epithelial protein synthesis was restricted by a limited availability of amino acids during the long-term incubation in the Ussing chambers. However, previous studies have shown similar discrepancies among mRNA and protein expression also for several membrane transport proteins (MCT1, DRA, NHE2, ATP1A; Dieho et al., 2017) and membrane receptors (IGF type 1 receptor; Shen et al., 2004) of the ruminal epithelium. Moreover, in the present study, unchanged mRNA expressions for *Cldn-1*, *Cldn-4*, and *Ocln* were also discordant from the decreases in their protein abundances at luminal pH 5.1 where no protein synthesis was required. Collectively, these data underline the generally required caution when interpreting gene expression results at only the mRNA level (Connor et al., 2010). Such caution may have special relevance in the ruminal epithelium where cell maturation is more complex compared to the intestinal epithelium. In the ruminal epithelium, cells migrate upwards in a multilayered structure with rather complex three-dimensional cell-cell interaction where posttranscriptional influences may perceivably override the transcriptional regulation. This concept may also explain why transcriptional regulation may be (more) relevant for Cldn-7. The expression of Cldn-7 is characteristic for the terminal differentiation of ruminal epithelial cells; thus restricted to the upper layers of the epithelium and may, therefore, be less affected by posttranscriptional influences.

In agreement with the missing changes in the protein abundance of Cldn-1, Cldn-4, and Ocln, and in agreement with the unchanged immunohistochemical localization of these proteins, acetate (30 or 100 mM), and propionate (30 mM) influenced

neither G_i , nor I_{sc} , nor $J_{ms-fluor}$ at a simulated luminal pH of 6.1 compared with Con-pH6.1, indicating a largely unaltered REB. At variance, But30-pH6.1 increased G_i values with concurrent decreases in both I_{sc} and the abundance of the TJ protein Cldn-7. The increase in G_i with a concomitant decrease in Cldn-7 could possibly point to an interfering effect of butyrate on the REB. However, flux rates of fluorescein (as a proposed marker for paracellular permeability) were unchanged (or rather decreased) by mucosal butyrate application. The latter suggests that the permeability restriction by the REB was not measurably altered by butyrate. In a previous study on bovine ruminal epithelium, [Sehested et al. \(1996\)](#) had observed similar changes of G_i and I_{sc} when assessing the influence of the same SCFA (i.e., acetate, propionate or butyrate, at 20 mM each) on Na^+ transport across the ruminal epithelium of cattle. In their study, net absorption of Na^+ as the primary generator of ruminal I_{sc} did not differ between the three acids. This suggests that the butyrate-induced decrease of I_{sc} in both the present and the previous studies ([Sehested et al., 1996](#)) may not be attributable to an opening of the paracellular space but to an increased anion absorption that electrically neutralizes part of the Na^+ -carried I_{sc} . A concurrent increase in G_i suggests that this may be due to the opening of an anion conductance which is probably localized in the basolateral membrane of ruminal epithelial cells ([Stumpff et al., 2009](#); [Georgi et al., 2014](#)). Such conductance is known to be activated upon cell swelling ([Georgi et al., 2014](#)).

The incubation with either 30 mM butyrate or 100 mM acetate at mucosal pH 6.1 triggered the disappearance of Cldn-7 signals from the *stratum corneum* in immunohistological images and caused a compact appearance and diffuse fluorescein labeling of that *stratum* without clear delineation of individual cells. Incubation with 30 mM butyrate additionally decreased Cldn-7 protein abundance. This suggests that the expression and localization of Cldn-7 as a final differentiation step of the REB may be altered by butyrate and acetate when these acids are present in concentrations higher than those normally observed in the rumen. Interestingly, our findings plausibly extend findings from earlier investigations in non-lactating dairy cows where daily ruminal infusions of acetate (8.4 mole/10 h) or butyrate (2.6 mole/10 h) but not propionate (4.7 mole/10 h) induced what these authors called a “first-order *stratum corneum*” with plate-like cells (similar to the *stratum corneum* of nonruminating ruminants) that partly contained extremely

condensed keratins (i.e., hyperkeratosis) ([Kauffold et al., 1977](#)). This leads to the speculation that the mechanical stability of the cornified cell layer relies on tightly packed keratins in a “first-order *stratum corneum*” (as observed in Ace100-pH6.1 and But30 pH6.1); however, when cells balloon and form a “second-order *stratum corneum*,” Cldn-7 is induced as an additional stabilizing factor (as observed for Con-pH6.1, Ace30-pH6.1, and Prop30-pH6.1). The missing of this stabilizing factor could perceptibly explain the cell sloughing and parakeratosis induced by excess butyrate in vitro by [Gálfi et al. \(1983\)](#). In vivo, the same group ([Gálfi et al., 1986](#)) showed that continuous infusion of butyrate (2 g/kg BW per day) in sheep led to a similar hyperkeratosis as described by [Kauffold et al. \(1977\)](#). However, if proliferation was enforced by infusing the same amount of butyrate as a single dose once daily at 08:00, the cornified cell layer was shed prematurely, leading to parakeratosis ([Gálfi et al., 1986](#)). Interestingly, CLDN-7 protein is also downregulated in the upper layers of human epidermis during psoriasis, a disease that also involves parakeratosis ([Kirschner et al., 2009](#)). Furthermore, the deletion of *Cldn-7* profoundly disrupted architecture and induced inflammation in the mouse intestine ([Ding et al., 2012](#)). Commonly, these findings may suggest a structure-stabilizing function of Cldn-7 with possible relevance for keratinization disorders of cornifying epithelia.

For completeness, however, it should be mentioned that butyrate application (at markedly lower concentrations) mostly improved barrier integrity of intestinal epithelial models ([Mariadason et al., 1997](#); [Venkatraman et al., 1999](#); [Lewis et al., 2010](#)) and upregulated proteins located in or associated with the TJ like Cldn-1 and ZO-1 ([Ma et al., 2012](#)). In Caco-2 cells, 5 mM butyrate also increased the abundance of Cldn-7 protein ([Valenzano et al., 2015](#)). The reasons for this discordant response to butyrate among intestinal and ruminal epithelial cells could be either due to the higher dose of butyrate used in the present study or may be related to different posttranscriptional regulation of TJ protein expression in both cell types because the mRNA expression of, e.g., *Cldn-1* was upregulated by butyrate in the present study similar to the intestine ([Wang et al., 2012](#)).

With regard to our second hypothesis, the key findings were that acidification of the mucosal incubation solution to pH 5.1 without SCFA influenced neither G_i , nor I_{sc} , nor the protein expression of any of the tested TJ proteins. As already concluded by [Meissner et al. \(2017\)](#), these results confirm earlier

findings of Penner et al. (2010) who demonstrated no changes in G_t and mannitol flux rates during a 1.5-h period where isolated ruminal epithelia were exposed to a mucosal solution with pH 5.2 devoid of SCFA in Ussing chambers. Admittedly, the present study demonstrated some initial signs of barrier failure at pH 5.1 in the absence of SCFA (e.g., increased flux rates and epithelial staining for fluorescein, some blurring of Cldn-4 and Occludin immunosignals, absence of immunosignals for Cldn-7). However, these signs were rather moderate and not comparable to the profound impairment of the REB induced by mucosal acidification to pH values between 5.1 and 5.4 in several previous studies where a mixture of SCFA was present (Gaebel et al., 1989; Aschenbach et al., 2000; Meissner et al., 2017).

The unique question of the current study was whether the three major ruminal SCFA, i.e., acetate, propionate, and butyrate, may differ in their potential to induce REB failure at low pH. Our results demonstrate, for the first time, that the REB was rather equally affected by either of the three SCFA at pH 5.1. At equimolar concentrations of 30 mM, all three SCFA induced similar increases of G_t and $J_{\text{ms-fluor}}$, and largely coordinated decreases of TJ protein expression. There were gradual differences for the latter in that Cldn-1 expression was decreased by Ace30-pH5.1 and Prop30-pH5.1, but not But30-pH5.1; Cldn-4 and Cldn-7 were decreased by Prop30-pH5.1 and But30-pH5.1, but not Ace30-pH5.1; and Occludin was decreased by Ace30-pH5.1 and But30-pH5.1, but not Prop30-pH5.1. Whether these gradual differences may have any pathophysiological implication is not readily visible. At least for the functional readouts of REB integrity of the present study (G_t and $J_{\text{ms-fluor}}$), they were irrelevant under the experimental conditions applied. This does not exclude that these gradual differences may become relevant in the recovery phase from an acidotic insult as this regularly implies an aggravation of barrier failure (Penner et al., 2010; Meissner et al., 2017). However, studying the recovery phase from an acidotic insult was beyond the scope of the present investigations.

While the present study did not indicate any differences in the detrimental properties of the three individual SCFA at pH 5.1, it clearly demonstrated an effect of SCFA dose on REB failure. Increasing the concentration of acetate from 30 to 100 mM significantly increased G_t and decreased I_{sc} . Furthermore, the expression of TJ proteins was decreased without any exemption in group Ace100-pH5.1. It thus appears that SCFA dose

may be more important for REB failure during a specific SARA incident than the molar concentrations of individual SCFA, even when considering that representation of animals within epithelia used for Ace100-pH5.1 was not as evenly distributed as in the remaining treatments.

CONCLUSION

A divergent influence of luminal SCFA on the REB under normal vs. acidotic pH conditions is mostly accepted albeit direct support by experimental data is very scarce. In the present study, we demonstrated that all three SCFA upregulate the mRNA expression of *Cldn-1* and/or *Cldn-4* when applied at luminal pH 6.1. However, we also demonstrated that the corresponding protein expression of Cldn-1 and Cldn-4 remained unchanged. It can thus be concluded that SCFA have a potential to support the formation of a tight REB in the ruminating animal at physiological pH; however, the circumstances and physiological pH range under which such potential is utilized have yet to be discovered. We further confirmed that the combination of low pH and SCFA is critical for the impairment of barrier integrity during ruminal acidosis. The new finding of the present study is that the three major SCFA, acetate, propionate, and butyrate, do not measurably differ in their potential to impair barrier integrity at pH 5.1; however, SCFA dose matters because acetate elicited decreased TJ protein expression and increased permeability with increasing dose at luminal pH 5.1. Considering 1) the early onset of increases in $J_{\text{ms-fluor}}$ and G_t within 1 to 2 h of luminal acidification to pH 5.1 and 2) that mostly SCFA concentration far higher than 100 mM are found during SARA in vivo, and considering further 3) our previous finding that the decrease in REB integrity continues for hours after the acidotic insult, it becomes plausible that pH values well above pH 5.1 should be sufficient to set an initial insult on barrier integrity that aggravates during the recovery phase from acidosis to a functionally relevant leakiness of the REB. Finally, our study also showed that very high concentrations of butyrate and acetate may alter the final differentiation of ruminal epithelial cells already at a luminal pH of 6.1, as demonstrated by decreased Cldn-7 expression and/or altered Cldn-7 localization. We speculate that such alterations, which were observed at pH 5.1 even independent of SCFA species, may pathogenetically link to parakeratosis that has been observed with easily fermentable diets.

Conflict of interest statement. The authors declare no conflict of interest regarding the publication of this article.

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