Aus dem Institut für Veterinär-Physiologie

des Fachbereichs Veterinärmedizin

der Freien Universität Berlin

Titel der Arbeit:

Regulation of the epithelial barrier in the rumen epithelium of sheep by incubation with single short-chain fatty acid at different pH, with glucagon-like peptide 1, glucagon-like peptide 2, and epidermal growth factor

Inaugural-Dissertation
zur Erlangung des Grades eines
Doktors der Veterinärmedizin
an der
Freien Universität Berlin

vorgelegt von

Gabriele Greco

Tierarzt aus

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List of abbreviations

AIDS	Acquired immunodeficiency syndrome
Akt	Protein kinase B
AMPK	AMP-activated protein kinase
ASICs	Acid sensing ion channels
ATP	Adenosine triphosphate
ATP1A3	Sodium/potassium-transporter ATPase subunit alpha-3
Caco cells	Colorectal adenocarcinoma cells
cAMP	Cyclic adenosine monophosphate
CAR	Coxsackie virus and adenovirus receptor
CDK2	Cyclin-dependent kinase 2
CDX	Caudal type homeobox
Cldn	Claudin
CLMP	CAR-like membrane protein
COX2	Cyclooxygenase-2
CTX	Cortical thymocyte marker in Xenopus
DMI	Dry matter intake
DPP-IV	Dipeptidyl peptidase-IV
DRA	Down-regulated in adenoma chloride anion exchanger
EB	Epithelial barrier
E-box	Enhancer box
EC_{50}	Half maximal effective concentration
EGF	Epidermal growth factor
EGF-m	Epidermal growth factor incubated from the mucosal side
EGF-s	Epidermal growth factor incubated from the serosal side
EGFR	Epidermal growth factor receptor
ELF3	Epithelial-specific E74 like ETS transcription factor 3
EMT	Epithelium-mesenchyme transition
ERK	Extracellular signal-regulated kinase
ETS	E26 transformation-specific sequence
FAM	6-Carboxy-fluorescein
GH	Growth hormone
GPCRs	G protein-coupled receptors
GPR4	G protein-coupled receptor 4
GLP-1	Glucagon-like peptide 1
GLP-2	Glucagon-like peptide 2
IEC monolayers	Intestinal epithelial monolayers
IE-IGF-1	Intestinal epithelial IGF-1
IGF-1	Insulin-like growth factor
IGF-1R	Insulin-like growth factor-1 receptor
HDAC	Histone deacetylase
HK2	Human kidney 2 cells
HNF-4α	Hepatocyte nuclear factor 4 alpha
hTERT-HPDE cells	Human telomerase reverse transcriptase-transfected human pancreatic epithelial cells
HUVECs	Human umbilical vein endothelial cells
iNOS	Inducible nitric oxide synthase
IPEC-J2	Intestinal porcine enterocytes J2

JAM-A	Junctional adhesion molecule
LNX1p80	Ligand of Numb-protein X1
MAGI-1	Membrane-associated guanylate kinase with inverted orientation
MAGUK	Membrane-associated guanylate kinase
MAPK	Mitogen-activated protein kinase
MARVEL	Myelin and lymphocyte (MAL) and related proteins for vesicle trafficking and membrane link
MCP-1	Monocyte chemoattractant protein 1
MCT1	Monocarboxylate transporter 1
MDCK monolayer	Madin-Darby Canine Kidney cells
MEK	Mitogen-activated ERK kinase
mRNA	Messenger RNA
mTOR	Mammalian target of rapamycin
NALP3	NACHT, LRR and PYD domains-containing protein 3
MWG Operon Tool	Molecular weight Operon Tool
NHE:	Na^+/H^+ transporter/exchanger
NK cells	Natural killer cells
PE:	Pansenepithel
PDZ-Domain	Post synaptic density protein (PSD95), drosophila disc large tumor suppressor (Dlg1), and zonula occludens-1 protein (zo-1)
PI3K	Phosphoinositide 3-kinase
pH	Potential of hydrogen
PKA	cAMP-dependent protein kinase
PKC	Protein kinase C
pKa	Negative base 10 logarithm of the acid dissociation constant
PLC-γ	Phosphoinositide-phospholipase C γ type
PPAR-γ	Peroxisome proliferator-activated receptor gamma
PVDF	Polyvinylidene difluoride
Raf	Rapidly accelerated fibrosarcoma kinase
Ras	Rat sarcoma/small GTPase
RE	Rumen epithelium
REB	Rumen epithelial barrier
RNA	Ribonucleic acid
ROCK	Roh-associated protein kinase family
RPS19	Ribosomal protein S19
SARA	Subacute ruminal acidosis
SCFA	Short-chain fatty acid
SNAI1P	Human SNAI Gene and related processed pseudogene
Snail	Zinc finger protein SNAI1
Sp-1	Specificity protein-1
STAT3	Signal transducer and activator of transcription 3
SUMO	Small ubiquitin-related modifier
TAMPs	TJ-associated MARVEL proteins
TNF-α	Tumor necrosis factor α
TJ	Tight junction
TRPV	Transient receptor potential vanilloid
WNK	Serine/threonine-protein kinase
ZO	Zona occludens

1. Introduction

1.1. Ruminant animals and their rumen

This work was aimed at understanding the epithelial barrier of the ruminal epithelium. It tried to amalgamate the knowledge on the complex mechanisms regulating the epithelial barrier of this epithelium in order to lay down the base for new successful management strategies in livestock. To better contextualize the relevance of the findings, the Introduction chapter will give a short overview of the context framing the topics and the related problems. The special digestive system of the suborder *ruminantia* encompasses, inter alia, a symbiotic bacterial population that converts large quantities of low quality carbohydrates into short-chain fatty acids (SCFA) in the forestomach (Bugaut, 1987). Ruminants are adapted to use these SCFA as a primary source of metabolic energy, therefore, large amounts of these acids are released on a daily basis and adsorbed primarily in the rumen (Allen, 1997). Such complex symbiosis is possible thanks to the coordination of highly specialized cells, tissues, and organs of the gastrointestinal system, which maintain a balance between production, absorption and removal of acids on the one side and the secretion of pH-buffering molecules on the other side (Allen, 1997). Such a task becomes particularly challenging in the case of high-energy demand in intensive farming, where animal productivity is intentionally increased (Plaizier et al., 2008; Wanapat et al., 2015). Intensive farming is nowadays largely established around the globe (Ehrlich & Pringle, 2008) as a strategy to maximize economic advantage (Anderson, 1982) or to improve land production efficiency (Wilkinson, 2011). In contrast, this strategy also increases animal stress and stress-associated sickness, which leads to economical loss and ethical concerns (Krause & Oetzel, 2006). Data from the USA, for example, shows that 25% of dairy cows are replaced after just 60 days of lactation (USDA, 2007). In Germany it is known that 10,7% and 20,9% of dairy cows are culled 300 and 450 days after first calving, respectively (Bergk & Swalve, 2011). Disturbances of the digestive system are the second reason for decreased productivity of cattle (Nagaraja et Al., 1998). In response to that, appropriate management strategies may be introduced to reduce the stress of these farming animals (Penner et al., 2011). In order to achieve effective management strategies, which can improve animal production and at the same time respect animal welfare and ethics, deep knowledge of the mechanisms regulating the physiological and pathophysiological adaptation of the animal and its tissues at cellular and even molecular level is necessary and should be considered. The specialized gastrointestinal system of ruminant animals has three non-glandular forestomachs in line before the

glandular stomach. Among all these, the rumen is the most voluminous organ (Reynolds et al., 2004). It is responsible for ~65% of total digestion (Hogan & Weston, 1967) and its size and capacity can influence the productivity of the animal (Beecher et al., 2014). The rumen volume of an adult cow can reach and exceed 100 liters (Jackson & Cockcroft, 2002). Such volumes encompass a gas component, made mainly of CO_2 and methane, a fluid component, made mainly from saliva, mixed with the *ingesta* (Kay, 1966; Frandson et al., 2009). This chamber is directly connected with the two other forestomachs, *reticulum* and *omasum,* and cooperates with these in the regulation of fermentation and digestion of feed in the first part of the alimentary canal (Cecava, 1995). Coordinated contractions of the reticulorumen promote the motility of the *ingesta* from the rumen to the omasum. That allows ruminal fluid turnover and the further processing of the *ingesta* in the following compartments of the gastrointestinal system. The contractions are also useful to mix the content of the rumen milieu and to expel the exceeding gas arising from bacterial fermentation through eructation (Hungate, 1966; Stone, 2004). During the rumination, ruminal content is regurgitated in the mouth where the coarse particles are mechanically minced by chewing (Dehorithy, 2002). Rumination and chewing increase the production of saliva, which is swallowed and reaches the ruminal milieu (Allen, 1997). Saliva production in adult cows may reach up to 250 liters per day (Maekawa et al., 2002). This large amount of saliva is not only the main fluid component of ruminal milieu but it also conveys a large quantity of buffer, mainly HCO_3^- and HPO_4^{-2}, which regulates the ruminal pH (Meyer et al., 1964; Kay, 1966; Allen, 1997). Reticulorumen contractions are controlled from the neuro-vegetative system under feeding stimulation (Frandson et al., 2009). But they are also influenced by the environment, diet composition and formulation (Stone, 2004). The diet regime impacts on the development of the rumen and its epithelium (Gäbel et al., 2002). Changes in one of any of these factors may influence ruminal health and trigger the development of ruminal disorders, which may also later affect different organs (Stone et al., 2004; Penner et al., 2009).

Dietary requirements of ruminants can be grouped into five points: crude protein, energy, fat, water soluble vitamins and minerals (McGrath et al., 2018). The microbe population in the forestomach of ruminants permits the conversion of plant cell wall components and non-amino nitrogen into nutrients. That should allow to feed these animals with only roughage and avoid the cereal competition between ruminant and monogastric animals and humans. However, the trend of the last decades shows that farmers have increased the use of energy-rich diets in their livestock (Jouany, 1991). Feed costs can account for up to 70% of total farm expenditure. Therefore, farmers pay particular attention to the economic benefit behind the dietary regimes used (Bach, 2012). High production in ruminant livestock is achieved by increasing the concentrated feed ratio. This accelerates microbial conversion of carbohydrates to SCFA in the rumen. The rapid increase of SCFA induces a drop in ruminal

pH, which can generate distress for the rumen (Penner et al., 2011). The ruminal pH of grass-eating cattle normally ranges between 6-7. But, it can drop to 5.5-6.0 in concentrate feeding animals (Grünberg & Constable, 2009). Abnormal production of SCFA may decrease the ruminal pH to 5.5-5.0, which is generally referred to as a subacute ruminal acidosis (SARA), or to a pH lower than 5.0, which is considered to be ruminal acute acidosis (Nagaraja & Titgemeyer, 2007). Ruminal acute acidosis is a severe condition which can easily evolve into rumen stasis, ruminitis, systemic acidosis and finally death. The extreme drop in pH alters the microbial population in the rumen and supports the proliferation of lactate-producing bacteria, which exacerbates the pH reduction and promotes systemic lactate absorption (Huber, 1976). It is generally triggered by a rapid change from a low to a high fermentable diet (Aschenbach et al., 2011). Ruminal acidosis was reported mostly in feedlot (Owens et al., 1998; Krause & Oetzel, 2006). However, thanks to improved management standards, acute ruminal acidosis is no longer so prevalent in the livestock husbandry (Krause & Oetzel, 2006). On the other hand, SARA is a more common condition (Kleen et al., 2009; McCann et al., 2016). It affects mostly dairy cows during early and mid-lactation (Humer et al., 2018). At risk of acidosis are also primiparous cows (Krause & Oetzel, 2006), weaning calves (Laarman & Oba, 2011), animals eating really high quality pasture (O'Grady et al., 2008), and animals with deviated eating behaviors like picking or selecting of feed (Leonardi et al., 2003). Diagnosis of SARA may be difficult due to the daily variation of the ruminal pH, as shown in Figure 1 (page 3); and the difficulties in detecting precise pH values in field (Gozho et al., 2006; Nagaraja, 2007; Abdela, 2016).

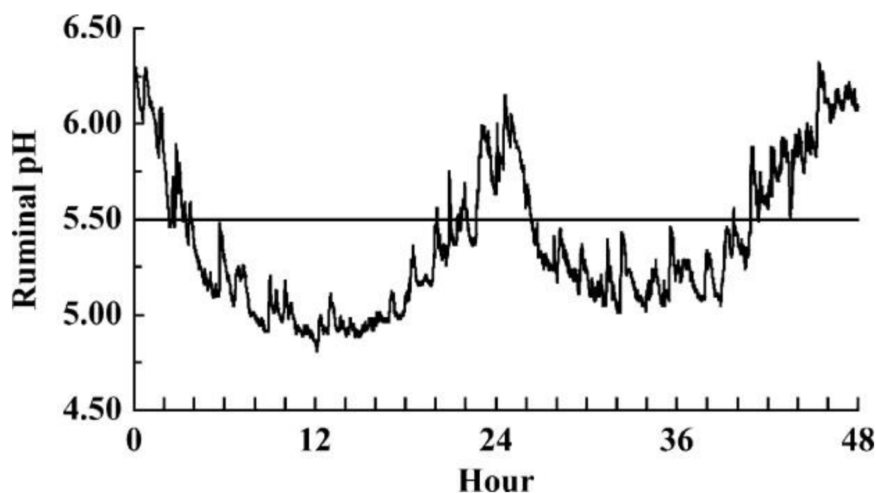

***Figure 1**: A graph showing the variation of ruminal pH of steers during a 48 hours monitoring. The ruminal pH ranged from a higher pH of almost 6.5 to a lower pH of less than 5.0 in 24 hours. The large daily variation of ruminal pH complicates the diagnosis of SARA in field. Figure from Cooper et al., 1998 in Nagaraja & Titgemeyer, 2007.*

A ruminal pH lower than 5.6 for 3 hours per day is already considered a SARA condition (Gozho et al, 2005). However, recurrent episodes of low ruminal pH during the day may also trigger it (Krause & Oetzel, 2006). Such a pattern is often complicated by the absence of clinical signs (Krause & Oetzel, 2005) which may appear later as a result of secondary pathologies (Krause & Oetzel, 2006), as happens in the case of laminitis (Nocek, 1997). Low ruminal pH can trigger rumenitis, parakeratosis, erosion, ulceration and inflammation of the ruminal epithelium (Garry, 2002). In some cases, the increased bypass of SCFA to the lower parts of the gastrointestinal tract may also trigger a hindgut acidosis (Gressley et al., 2011). Inflammation of the rumen epithelium facilitates the passage of bacteria and toxins in the portal circulation (Vermunt, 1992; Aschenbach & Gäbel, 2000; Guo et al., 2017). Through this way, bacteria may form abscesses in the liver and spread further colonizing lungs, heart valves (Figure 2a and 2b, page 4), kidneys (Figure 2c, page 4), and joints (Nocek, 1997; Oetzel, 2007); whereas, active amine toxins, like histamine, may induce laminitis (Nocek, 1997; Nagaraja, 2007).

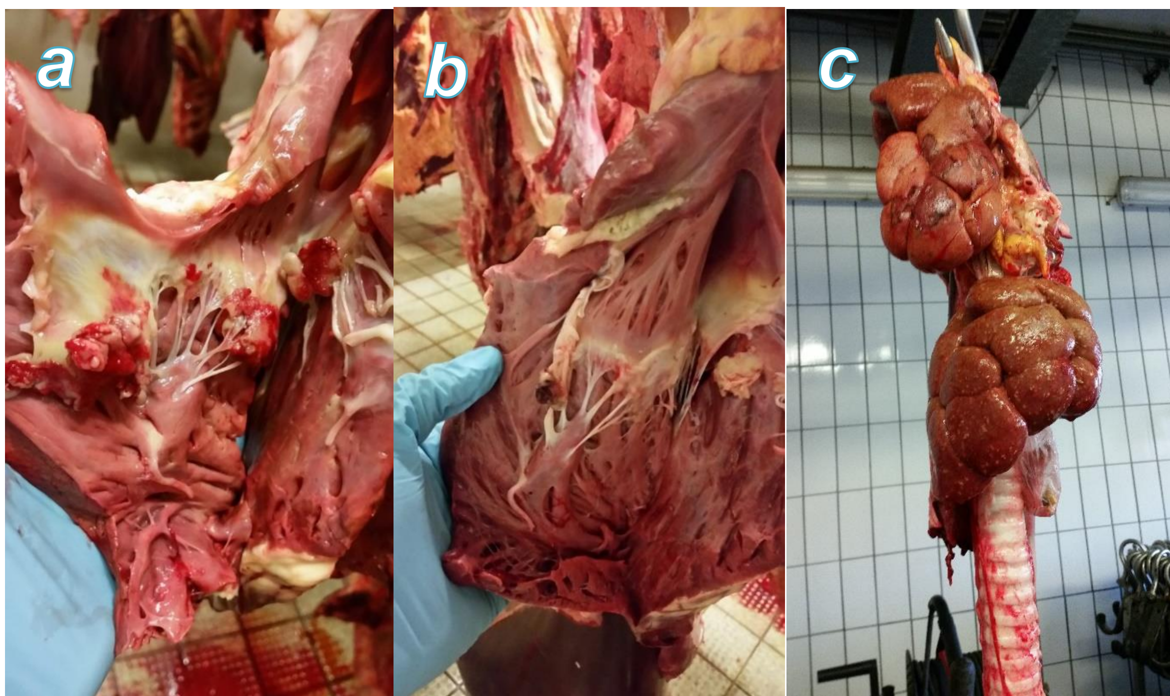

***Figure 2**: Endocarditis (a)(b) and pyelonephritis (c) in dairy cattle. These two conditions are often the long term consequences of dietary disorders like SARA. That means that the primary infection point for these bacteria was probably the ruminal epithelium. The animals showed only low productivity and a slight reduction in appetite as clinical signs. Source: Greco G.*

It is noteworthy that in contrast to acute ruminal acidosis, rumen lactate concentration in SARA is low, whereas SCFA concentration becomes elevated (Burrin & Britton, 1986). The concentration of SCFA in the rumen normally varies from 60 to 160 mM. The total amount consists of ~65% of acetate, ~20% of propionate and ~15% of butyrate. Different diet strategies may affect the quantities of SCFA but also the ratio of the SCFA (Sutton et al., 2003). The increase in total SCFA concentration is often associated with a higher percentage of propionate and a lower percentage of acetate (Krause et al., 2002; Penner et al., 2011). These acids are mostly absorbed in the rumen and only a small amount reaches the omasum (Bergman, 1990). Therefore, the ruminal wall and its epithelium work as a primary absorption point for SCFA and as a result they play a central role in the regulation of the ruminal pH (Allen, 1997). The ruminal epithelium folds itself in digital extensions increasing the absorptive surface. Such digitations are called papillae. These protrude in the lumen and their development is affected by diet formulation (Graham & Simmons, 2005; Penner et al., 2011). Well-developed rumen epithelia show high, dense and large papillae with an increased absorbing surface (Dirksen et al., 1984).

A well-developed rumen epithelium is adapted to absorb high SCFA quantities, however, such diet-induced morphological adaptation of the mucosa is reversible (Gäbel et al., 2002). The increasing concentration of SCFA in the rumen seems to be a factor triggering directly, or indirectly, papillae growth (Tamate et al., 1962; Dirksen et al., 1984; Steel et al., 2015). Such a beneficial consequence of high SCFA on the ruminal epithelium is in contradiction to the previous discussion on the mechanisms that lead to ruminal acidosis and the negative consequences of that. The undesirable effects can be decreased by adapting the epithelium to high concentrations of SCFA through gradual feed changes (Kleen et al., 2003). This strategy is particularly useful as a prevention against SARA, if it is applied before critical periods (Humer et al., 2018). However, also in adapted ruminal epithelia, the prolonged use of a highly fermentable diet can impair the mucosal permeability and increase the risk of transferring noxious agents in the blood circulation (Klevenhusen et al., 2013). Therefore, the ruminal epithelium not only mediates the absorption of SCFA, but it is also an effective permeability barrier between the ruminal content and its host (Graham & Simmons, 2005). More detailed information on the functions and structures of the rumen epithelium are offered in the following chapter.

1.2. The rumen epithelium

The rumen epithelium is classified as a stratified squamous epithelium and it is quite similar to the skin. Its cells are organized in four distinct overlapping cell strata. The deeper layer is called *stratum basale*. The cells of this *stratum* are columnar and rich in mitochondria. The layer above this is the *stratum spinosum*, which presents several intercellular bridges between contiguous cells. Next to this is the *stratum granulosum*, which also presents intercellular bridges, but also shows intracellular vesiculations and some flattening of cells. The uppermost layer is the *stratum corneum*. In this *stratum* the cells are flattened, keratinized, and tend to defoliate in the lumen (Dobson et al., 1956; Graham & Simmons, 2005). The number of cells in each *stratum* and the total amount of cells forming the epithelium may strongly differ according to diets, ruminal development, and feeding strategy (Baldwin & Connor, 2007). Even though cells of different strata have different shape, enzyme asset, and function, they work in synergy forming a functional syncytium (Graham & Simmons, 2005). Thus, cells in the lower part of the epithelium are metabolically more active. They show numerous Na^+-K^+ pumps and numerous gap-junctions, which explain the high energy requirement. Through its numerous ion enzymatic pumps, this part of the epithelium creates an ionic gradient that facilitates the absorption of nutrients from the rumen content (Graham & Simmons, 2005). The more superficial cells, poor in metabolic activity, mostly account for barrier, protective, and absorptive functions. Thanks to the barrier function, the ion gradient, which is formed in the lower strata, may work as a driving force for absorption of semi-selected molecules, also avoiding the retrograde flux of absorbed molecules in the ruminal milieu (Farquhar & Palade, 1963; Graham & Simmons, 2005). More specifically, the *stratum corneum* works mostly as a protection against mechanical and chemical insults, and through exfoliation as a first barrier against bacterial colonization (Wilhelm et al., 1990; Harding, 2004). It partially supports the maintenance of the permeability barrier (Andrews et al., 2011) and it hosts an epithelia-associated microflora (Graham & Simmons, 2005), which is considered a defensive mechanism against the colonization of pathogenic bacteria (Shen et al., 2019). The immune-cells of the epithelium are also considered part of the complexes supporting the defense and therefore, the epithelial barrier function (Shen et al., 2019). The barrier is mostly granted from a dense net of kissing junction proteins, called tight junctions (TJ), surrounding all the cells of the *stratum granulosum*, the *stratum spinosum* and to a lesser amount in the *stratum basale*. However, an effective permeability barrier seems to be present only in the *stratum granulosum* (Graham & Simmons, 2005). In this *stratum*, at least the following three different TJ proteins are expressed: claudin-1, claudin-4 and occludin. In addition to these, only in the *stratum corneum,* the expression of claudin-7 was demonstrated but no other TJ proteins (Stumpff et al., 2011). A simplified draft of their distribution in the rumen epithelium is reported in Figure 3 (Page 8).

1.3. The TJ proteins and the epithelial barrier

TJ proteins close the gaps between cells and seal the so-called paracellular space. The movement of solutes and solutions through these gaps is referred to as paracellular permeation (Farquhar & Palade, 1963; Tsukita et al., 2001). On the other hand, molecules moving across the phospholipid cell membranes take the so-called transcellular route (Spring 1998; Tsukita et al., 2001). The first authors studying cell-to-cell adhesions thought that the junctional point of two contiguous cells forming the epithelial barrier was a fusion of two cellular membranes. Today we know that the epithelial barrier is not completely impermeable (Gumbiner, 1993). The paracellular space is a 1 to 5 nm large lacuna between two cells (Figure 4, page 11) (Rubas et al., 1996). The size of this lacuna can be physiologically regulated and it varies among different epithelia (Gumbiner, 1993). Such a space is confined by selective-charged TJ proteins which can modulate the permeation of small molecules (Anderson & Van Itallie, 2009). Changes in epithelial permeability can be related to variation in the amount, interaction or localization of TJ proteins. Some of these TJ proteins, for instance, are free to move in different paracellular locations (González-Mariscal et al., 2008) or to be included in cytoplasmic vesicles under specific stimuli such as oxidative stress or inflammation and consequently affect the epithelia permeability (Shen et al., 2008). In the rumen epithelium, these proteins form an extra-membrane fence which blocks the arbitrary transition of solutes and solutions across the mucosa (Graham & Simmons, 2005). The disruption of the TJ net leads to a decrease in ion gradient and an increase in molecular permeability, ergo pro-inflammatory molecules, toxins, and microorganisms are facilitated to enter the blood circulation (Lerner & Matthias, 2015). That explains the pathophysiological changes of the ruminal epithelium with an impaired epithelial barrier and it explains why it may result in a threat to the animal´s health and in an economic loss for the farm (Enemark, 2008).

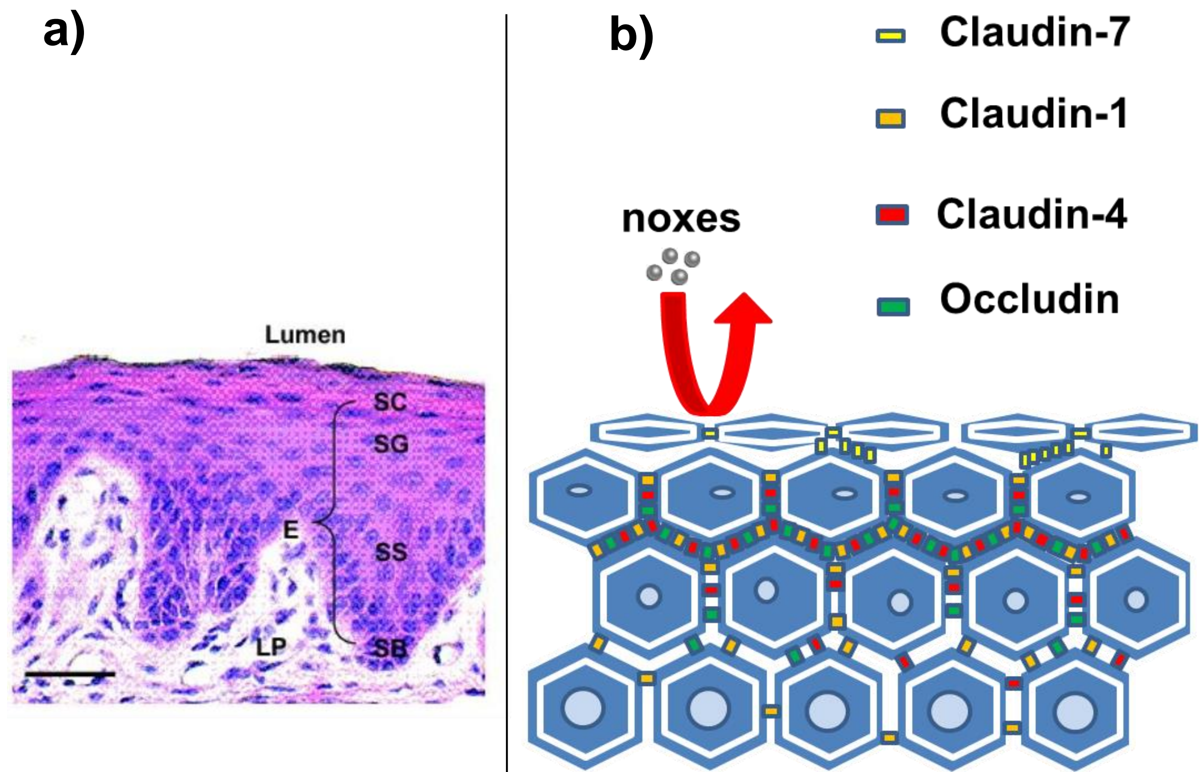

Figure 3: *An histological image of the rumen epithelium on microscope and a model of the epithelial barrier and the distribution of the tight junction proteins in the rumen epithelium. The picture a) shows a schematic differentiation of the cell strata of the epithelium: E: Epithelium; SC: Stratum corneum; SG: Stratum granulosum; SS: Stratum spinosum; SB. Stratum basale; LP: Lamina propria. The drawing on the right b) is a simplified model of the epithelium and its epithelial barrier showing the localization of the different TJ proteins. Each cell stratum is schematized by a single layer of cells. Each different TJ protein is represented with a different color. Source: a) Schweigel et al., 2009; b) Greco G.*

The epithelial barrier structure was for the first time elucidated by Farquhar and Palade (1963) in the tissues of rats and guinea pigs. They discovered the TJ belt in a tripartite junctional complex, which they described at the apical pole of epithelial cells. This complex was differentiated in an occluding zonule, an adherens zonule, and the desmosome. The last two components of this complex are positioned below the TJ belt. They carry an adhesive function as an anchor for contiguous cells, but they do not bear effective barrier function (Farquhar & Palade, 1963; Buckley & Turner, 2018). Subsequent studies using monoclonal antibodies discovered the first TJ-associated protein which was called zonula occludens (ZO-1) (Stevenson et al., 1986). The zonula occludens, which was also called tight junction, was previously recognized as a protein forming an effective barrier (Stevenson et al., 1988). More TJ-associated or peripherally associated membrane proteins were thereafter discovered: ZO-2, and ZO-3, 7H6, AF6, vinculin, symplekin, and cingulin (Aijaz et al., 2006; González-

Mariscal et al., 2008). These proteins are members of the membrane-associated guanylate kinase (MAGUK) also called MAGI-1 membrane-associated guanylate kinase with inverted orientation and are characterized by carrying a PDZ domain. They work as a scaffold between the TJ proteins, the underlying apical perijunctional F-actin belt, and also different intracellular complexes (Tsukita et al., 2002; González-Mariscal et al., 2008) and are indispensable for a correct epithelial barrier formation (Umeda et al., 2006). Seven years after the discovery of the first TJ-associated protein, Furuse et al. (1993) discovered the first tight junction protein and this was named occludin. Occludin protein has a tetraspan structure which crosses the membrane four times forming a helix-form MARVEL domain. Such a domain has been identified in myelin and lymphocyte (MAL), but also in physins, gyrins and occludin families, hence, the name MARVEL (MAL and related proteins for vesicle trafficking and membrane link) (Sánchez-Pulido, 2002). Other proteins in the occludin family are the MARVEL-D3 and the MARVEL-D2 which is also called tricellulin (Cummins, 2012). The TJ proteins of this family may also be referred to as TAMPs: TJ-associated MARVEL proteins (Garcia-Hernandez et al., 2017). Occludin shows a 90% homology among mammals (Ando-Akatsuka et al., 1996). It contributes to the formation of an effective epithelial barrier, but its role in the barrier may differ in epithelia of different tissues and animals. In some cases, its depletion can be compensated by other TJ proteins (Saitou et al., 1998). In other cases, occludin was shown to be essential for the formation of the epithelial barrier (Cummins, 2012) and its overexpression increases the epithelial barrier (Balda et al., 1996). It is able to copolymerize with other tight junction proteins, like claudins (Furuse et al., 1998) and tricellulin (Steed et al., 2009). But it is also involved in cellular adhesion, apoptosis, cell differentiation and Ca^{2+} homeostasis (Rachow et al., 2013). A short time after it was discovered that occludin-knock out embryonic stem cells still have an effective epithelial barrier (Saitou et al., 1998). Furuse et al. (1998) discovered the existence of two other TJ proteins with four transmembrane domains: claudin-1 and claudin-2. Claudins are expressed in all discovered epithelia (Günzel & Yu, 2013). The claudin family includes at least 27 different proteins (Mineta et al., 2011) which are differently distributed and expressed in epithelia of different tissues (Günzel & Yu, 2013). Most of these proteins improve the epithelial barrier generally by decreasing cations permeability (e.g., claudins-1, -4, -5, -7, -8, and -14). But some of them form selective channels (e.g., claudin-2, -10, -15 -17) (Krug et al., 2012). Claudin-1 was demonstrated to be essential for the maintaining of a functional epithelial barrier in squamous multilayer epithelia as well as in monolayers (Furuse et al., 2002; Wang et al., 2012). In a study by Yumine et al. (2019), it was demonstrated that the presence of claudin-1 on the epithelial barrier may be crucial for the prevention of virus diffusion. This extends the potential functions of the TJ. On the contrary, some viruses may

use the same claudin-1 as a target for getting an entrance point (Evans et al., 2007). Claudin-1 is also studied as a marker for graduating tumors malignity (Kim et al., 2019).

Claudin-4 is also recognized as a TJ protein responsible for maintaining the barrier function (Wray et al., 2009; Radloff et al., 2017). In the kidney, it was found that this claudin forms a selective pathway together with claudin-8 supporting the flux of chloride (Gong & Hou, 2017), but at the same time it decreases the sodium permeability (Van Itallie et al. 2001). Claudin-4 was discovered to be a target receptor for the toxins of *Clostridium perfringens*. The target of the toxins is a binder domain that could be also used as a key point to trigger the permeability of the epithelial barrier for promoting absorption of drugs (Watari et al., 2017). Claudin-4 expression and activity may be dysregulated in different types of neoplasia (Ma et al., 2015; Liu et al., 2018) and allergies (Lee et al., 2018).

Claudin-7 has displayed an unclear role in the maintenance of the epithelial barrier. Some studies suggest that this protein supports the sealing activity of the epithelial barrier (Tanaka et al., 2015; Nguyen et al., 2018). According to other authors, it may form anion pores (Alexandre et al., 2007; Garcia-Hernandez et al., 2017). In the most recent literature, it was studied for qualities not properly related with the barrier function. Using claudin-7 knockdown mice, it was demonstrated that this claudin can control abnormal proliferation in the intestinal cells (Xu et al., 2019). Comparable results were found from Lu et al. 2015, which reported similar anti-proliferative and pro-adhesive functions. Claudin-7 is also studied as a marker for tumor aggressiveness (Kominsky et al., 2003; Xu et al., 2018).

Apart from MARVEL and claudin, another TJ family is called cortical thymocyte marker in Xenopus (CTX). This family includes junctional adhesion molecule (JAM-A), coxsackie virus and adenovirus receptor (CAR) and CXADR-like membrane protein also called CAR-like membrane protein (CLMP) (Raschperger et al., 2004; Quiros & Nusrat, 2014; Garcia-Hernandez et al., 2017). These proteins are part of the immunoglobulin superfamily which show a single spanning structure. They are expressed in a variety of cells, e.g., platelets, T cells, and NK cells, displaying different functions. In epithelial and endothelial cells, they are involved in the barrier function (Raschperger et al., 2004). Also worth mentioning is the protein family of angulin, which includes three proteins that co-localize with tricellulin. These proteins have a single transmembrane region and are involved in the barrier forming mechanisms in the blood-brain barrier (Sohet et al., 2015).

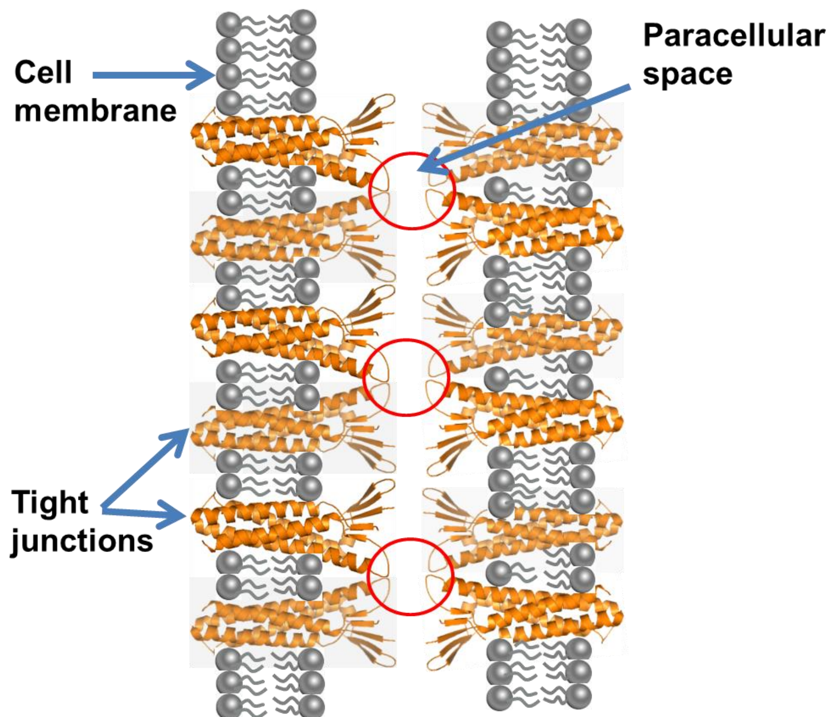

Figure 4: *Model of an epithelial barrier made on the base of claudin-15 protein. The image shows the phospholipid chains of the cell membrane in gray interrupted by the tetraspanning chains of the TJ proteins in orange; the red circles delineate the extracellular space forming the paracellular pathway. The model was drafted using the 3D graphing molecular visualization system PyMOL, which had only the amino acid sequences of the TJ protein claudin-15 available in the free software version. Source: Greco G.*

Tight junction proteins have additional functions apart from barrier properties. They can be summarized as: the regulation of cell proliferation (González-Mariscal et al., 2008), differentiation, migration, signal transduction, gene expression (Cummins, 2012), and adhesive connection (Günzel & Yu, 2013). As a consequence of so many properties and their large distribution, TJ anomalies may trigger, or be involved, in a variety of diseases like stroke, pulmonary disorder, diabetic retinopathy, inflammatory bowel disease, skin disorders, cancer, allergies, acquired immunodeficiency syndrome (AIDS), celiac disease, and multiple sclerosis (van Elburg et al., 1993; Yacyshyn et al., 1996; Sharpstone et al., 1999; Förster, 2008; Gruber et al., 2015; Lee et al., 2018). The present study primarily investigated the role of the TJ proteins in the formation and maintenance of the EB of the RE. However, it cannot be ruled out that these proteins also have other functions or properties in the RE. Further studies are necessary to investigate whether the TJ proteins of the RE are involved in mechanisms not related to barrier formation.

The tight junction regulation is a complex topic that is nowadays developing. The regulation includes transcriptional and post-translational events. Intracellular trafficking and stability in the plasma membrane may also influence TJ remodeling (Garcia-Hernandez et al., 2017). Transcriptional mechanisms include Snail factors, Grainyhead-like 2, nuclear factor (NF)-κB (Shigetomi & Ikenouchi, 2018), PPAR-γ, SP1, HNF-4α, CDX1, CDX2, and GATA-4 (Günzel & Yu, 2013). Post-translational mechanisms include phosphorylation, which can be mediated by the mitogen-activated protein kinase (MAPK), the AMP-activated protein kinase (AMPK), the serine/threonine-protein kinase (WNK4), cAMP-dependent protein kinase (PKA), atypical protein kinase C, ephrin receptor A2, the Rho-associated protein kinase family (ROCK), and other unknown kinases (Shigetomi & Ikenouchi, 2018). Other post-translational mechanisms are ubiquitination through the E3 ubiquitin ligase ligand of Numb-protein X1 (LNX1p80) (Takahashi et al., 2009) but also small ubiquitin-related (like) modifier (SUMO)ylation (Van Itallie et al., 2012), palmitoylation (Van Itallie et al., 2005; Shigetomi & Ikenouchi, 2018), and O- and N-linked glycosylation (Günzel & Yu, 2013). Intracellular trafficking and stability include the confinement of TJ proteins in endocytoplasmatic vesicles (Subramanian et al., 2007), changes in interaction between the TJ and scaffolding proteins, different assembling of TJ proteins of the same membrane site (cis-interaction) or of opposite membranes (trans-interaction) (Günzel & Yu, 2013). Intracellular trafficking is the translocation of TJ proteins from their supposed location to a different position in the same epithelium (Garcia-Hernandez et al., 2017). It has been demonstrated for claudins in the intestine of rats and in human fetal hindgut (Garcia-Hernandez et al., 2017), but also for occludin (Subramanian et al., 2007).

In physiological conditions, different TJ proteins cooperate together to maintain a functional epithelial barrier. The interaction between occludin and claudins, for example, normally results in a stronger barrier (Raleigh et al., 2011). Numerous TJ proteins, numerous regulating mechanisms, and the interactions among these, complicate the comprehension of the physiological and pathological mechanisms ruling the epithelial barrier.

Tight junction regulation in the rumen epithelium is still poorly studied. As mentioned earlier, a highly fermentable diet may be associated with an increased rumen epithelial permeability. Epithelia of cows under such a diet showed a reduction in cell adhesion, cell organization, and increased thickness of the cells of the *stratum granulosum* (Steele et al., 2011). These morphological changes of the rumen epithelium are often associated with compromised tight junction proteins (Liu et al., 2013). The pathophysiological mechanisms explaining how the high concentrated diets lead to epithelial barrier failure are still not completely understood. It has been suggested that some of the molecules released during the accelerated carbohydrate fermentation could be responsible for the increase in rumen epithelial permeability (Penner et al., 2011). Under such a dietary regime several molecules change

their proportion. Among these, some are considered detrimental or potentially detrimental to the epithelial barrier. This was proposed for the increased amount of SCFA (Meissner et al., 2017), which is associated with an increase in the relative proportion of propionic and butyric acids at the expense of acetic acid (Bergman, 1990; Pourazad et al., 2016); for the decrease of ruminal pH (Gäbel et al., 1989), for the increase of osmolarity (Lodemann & Martens, 2006) and for the increase of LPS concentration (Gozho et al., 2006; Zebeli & Ametaj, 2009). On the other hand, a multitude of molecules support the rumen epithelial adaptation and help to compensate the distressing condition. Among these are SCFA, hormones, e.g., insulin, glucagon, and GH; and growth factors, e.g., IGF-1, epidermal growth factor (EGF) (Penner et al., 2011).

In the results of a previous study in rumen epithelia *ex vivo*, it was demonstrated that the SCFA are the main trigger for the epithelial barrier failure during acidic stress and that the rumen would be relatively resistant to the drop in pH in the absence of SCFA (Meissner et al., 2017). As previously reported, ruminal acetate, propionate, and butyrate typically change their relative concentration during acidosis (Bergman, 1990). Therefore, the present study was focused on the effect of single SCFA on the epithelial barrier in two different pH environments. Furthermore, the effect of the hormones glucagon-like peptide 1 (GLP-1) and glucagon-like peptide 2 (GLP-2), and the effect of the growth factor EGF were tested on the epithelial barrier of rumen epithelia *ex vivo*.

1.4. The SCFA

The SCFA are organic acids containing one to seven atoms of carbon. These acids are predominantly represented by acetate, propionate and butyrate which have two, three, and four carbon atoms, respectively. They mostly originate from the bacterial fermentation of various carbohydrates in the rumen. Small amounts of these originate from the microbial degradation of proteins (Bergman, 1990). Their ratio in the rumen may vary between 70-45, 15-40, 5-20%, respectively, for acetate, propionate and butyrate (Bergman, 1990; Aschenbach et al., 2011). They are rapidly absorbed from the rumen and the absorption rate is faster for butyrate than for propionate and finally for acetate. However, their presence in the portal veins is inversely represented in proportion (Bergman, 1990). Indeed, 90% of the butyrate absorbed in the rumen epithelium is metabolized in loco to beta-hydroxybutyrate (Weigand et al., 1972) and acetoacetate or it is oxidized to CO_2 (Bergman, 1990). Propionate may be used as a direct source of energy by the cells and converted to lactate and CO_2 (Bergman, 1990). Of the total absorbed propionate, 15% is metabolized in the rumen epithelia of cows (Kristensen & Harmon, 2004) and 50% in rumen epithelia of sheep,

whereas, only 30% of the acetate absorbed in the rumen epithelium did not reach the portal blood (Bergman, 1990). SCFA are weak acids and have a pK_a value of circa 4.8 (Bergman, 1990). That means that they are mostly in their dissociated form in the rumen milieu at physiological pH ranges and that the undissociated SCFA ($SCFA^-$) can work as a buffer accepting new free protons in case of reduction of pH (Aschenbach et al., 2011). The absorption of dissociated SCFA from the luminal side occurs through an exchange protein. One molecule of dissociated SCFA from the lumen is exchanged with a molecule of intracellular HCO_3^-. Through this mechanism, the epithelium removes an acid and adds a molecule of buffer in the ruminal milieu. In this way, the absorption of SCFA may contribute to the regulation of ruminal pH more than the large amount of buffers deriving from the saliva (Aschenbach et al., 2011). The SCFA in the undissociated form, which normally constitute circa 15% of total SCFA (Allen, 1997), can easily cross the cellular membranes of ruminal epithelial cells. Finally, a further $HCO3^-$-independent mechanism, seems to exist for the transport of dissociated SCFA. However, this has still not been finally characterized (Penner et al., 2009; Aschenbach et al., 2011). In the case of decreasing ruminal pH, the balance between dissociated and undissociated SCFA shifts towards an increase in the protonated SCFA and this facilitates the passive absorption of SCFA (Aschenbach et al., 2011). Once absorbed in the cells, SCFA have to cross a second time the cell membrane at the basolateral side in order to get to the blood circulation. Due to the higher intracellular pH inside the cells, most of the passively absorbed undissociated SCFA dissociate to $SCFA^-$ and free H^+, which have to be removed from the cells (Aschenbach et al., 2011). Whereas the transport of ions across the rumen epithelium has been rather well elucidated (Lodemann & Martens, 2006; Leonhard-Marek et al., 2007; Aschenbach et al., 2011), e.g., the exceeding H^+ are removed from the intracellular space through Na^+/H^+ exchangers, the basolateral transport of SCFA is not completely understood. It has been suggested that the basolateral transport of SCFA may occur through the monocarboxylate transporter 1 (MCT1), which is already known as a transporter for SCFA metabolites (Graham et al., 2007; Aschenbach et al., 2011). Other transport mechanisms proposed are lipophilic diffusion, a $SCFA^-/HCO3^-$ protein exchanger, and an anion channel (Gäbel et al., 2002; Stumpff et al., 2009; Aschenbach et al., 2011). SCFA are not only a source of energy for the organism. They may directly influence the absorbing cells, stimulating genes responsible for their metabolism, but also altering cell proliferation rate, inhibiting cell apoptosis, and changing the epithelial morphology (Penner et al., 2011). In non-ruminant animals, SCFA were also shown to improve the epithelial barrier and the TJ protein expression (Wang et al., 2012; Feng et al., 2018). Contrasting results between *in vivo* and *in vitro* studies support the hypothesis that some of the effects of the SCFA are indirectly induced, namely, through the release of growth factors or hormones (Penner et al., 2011; Liu et al., 2017). The first part of this study

started with the investigation on the effect of single SCFA on the epithelial barrier by different pH. The corresponding publication contains detailed information and justifications for the procedures and chemical concentrations used in the investigation (see First Publication, Greco et al., 2018).

1.5. The EGF

One of the most interesting growth factors interacting with the rumen epithelium is the epidermal growth factor (EGF). Indeed, it is released from the salivary glands and through the ingestion of the saliva it reaches the rumen milieu and the rumen epithelium (Onaga et al., 2006). EGF may also reach the rumen epithelium serosally. In humans, EGF is present in most of the biological fluids and it is released mostly by paracrine and less by endocrine mechanism (Carpenter et al., 1986). Although epithelial cells in the gastrointestinal tract do not normally secrete this growth factor, they express EGF-receptors (EGFR), which can interact with the EGF secreted from submucosal cells or glands (Chen et al., 2016). EGF was first discovered by Cohen in 1961. He observed that daily injections of salivary gland extract in newborn mice accelerated teeth eruption and eyelids opening (Cohen 1961). Today we know that EGF is a 53-amino acid peptide involved in cell migration, apoptosis, differentiation, growth and proliferation (Tang et al., 2016). As a mitogenic factor it may trigger the proliferation of epithelial cells, but also a large variety of other cells (Wong & Guillaud, 2004). Once the molecules of EGF bind their membrane receptors, the complexes are internalized and the receptors are down-regulated for several hours (Aharonov et al., 1978). The EGFR were the first discovered tyrosine kinase receptors. They are made up of an extracellular binding domain, a single membrane-spanning region, a cytoplasmic protein tyrosine kinase domain, and a COOH-terminal. After activation of intracellular cascades, part of the endocytosed EGFR are recycled back to the cell membrane, part of these are degraded in lysosomes, and part of these are transported to the nucleus. Here the EGFR regulates cell metabolism through gene stimulation and through the activation of different kinases (Chen et al., 2016). Several studies demonstrated that EGF is also involved in the regulation of the TJ in different tissues and it showed protective effects on the epithelial barrier of the gastrointestinal tract of monogastric species (Tang et al., 2016). In ruminant animals, very few studies have focused on the role of EGF. It is known that it may support cell metabolism and proliferation of ruminal cells *in vitro* (Baldwin, 1999; Penner et al., 2011). Such comparable effects between monogastric and ruminant support the assumption of a similar role of EGF in the regulation of the epithelial barrier in the rumen epithelium. Therefore, part of this study tried to understand if EGF may have some protective effects on the epithelial barrier of rumen epithelia. Because of the previously reported mechanisms of

secretion of this growth factor, we decided to separately test the effect of EGF applied on the serosal side and on the mucosal side. Due to the variable availability of EGF in the rumen milieu, we tested different concentrations of this growth factor on the mucosal side. The exact concentrations of EGF used in the experiments and the explanation for their adoption are reported in the corresponding publication (see Second Publication; Greco et al., 2019).

1.6. The GLP-1 and GLP-2

Other molecules with a known protective effect on the epithelial barrier of epithelia in monogastric animals are glucagon-like peptide 1 and 2. GLP-1 was demonstrated to restore the TJ in the endothelial barrier of cultured lung human microvascular endothelial cells (Xu et al., 2019) and to improve the epithelial barrier of other endothelia (Gonçalves et al., 2016) and epithelia (O´Brien & O´Malley, 2019). GLP-2 was showed to improve the epithelial barrier of epithelial cells in the intestine of mice (Benjamin et al., 2000) and to increase the mRNA and protein expression of different TJ proteins in intestinal epithelial cells of piglets (Yu et al., 2014). The results of a study of jejunum of dairy calves showed that the effects of GLP-2 treatment in ruminants are consistent with the results in monogastric animals (Walker et al., 2015). It increases the intestinal mass, villous length, crypt depth and mucosal size (Taylor-Edwards et al., 2011). In monogastric animals, GLP-1 and -2 are released from enteroendocrine L-cells in the intestine after the ingestion of nutrients (Janssen et al., 2012). In ruminant animals, their concentration is also influenced by nutrients and particularly by SCFA (Elsabagh et al., 2017; Inabu et al., 2017). GLP-1 and GLP-2 hormones originated from the same precursor proglucagon, therefore it is commonly assumed that the mechanisms triggering their secretion are similar (Janssen et al., 2012). Both hormones are quickly inactivated by the ubiquitous enzyme dipeptidyl peptidase-IV (DPP-IV), but GLP-1 is more sensitive than GLP-2. Indeed, only 25% of the intestinally produced GLP-1 can be found active in the blood circulation whereas all intestinally released GLP-2 can reach the blood circulation in its active form. As a consequence, the half-life in the circulation is 2 and 7 minutes, respectively (Janssen et al., 2012). Although these hormones are quickly metabolized, their plasma concentration increases after a meal, however, a basal plasma concentration is detectable also in fasting animals (Xiao et al., 1999; Janssen et al., 2012). The functions of GLP-1 and GLP-2 are diverse and involve different organs. GLP-1 acts as incretin hormone. It modulates plasma glucose concentration by regulating the secretion of insulin, glucagon, and somatostatin. It influences fluid intake, gastric motility and secretion (Janssen et al., 2012; ThanThan et al., 2012). GLP-2 regulates gastric and intestinal secretions, but it doesn’t act as an incretin hormone. It shows intestinotrophic function and anti-inflammatory activity. It increases nutritional absorption and supports the mucosal

integrity by strengthening the epithelial barrier (Janssen et al., 2012). GLP-1 and GLP-2 have different specific receptors, which are G protein-coupled receptors (Janssen et al., 2012). These kinds of receptors are known to regulate the epithelial barrier of different tissues (González-Mariscal et al., 2018). GLP-2R mRNA was detected in low expression in the rumen epithelium (Taylor-Edwards et al., 2010). That supports the idea that GLP-2 may have some function in the physiological mechanism of this epithelium. The expression of GLP-1R was never detected in this epithelium. However, it is not possible to exclude a priori any biological effect of these hormones on the rumen epithelium. Therefore, the effect of these hormones on rumen epithelia of sheep *ex vivo* was tested in the second part of this study. Two different concentrations for each hormone were introduced on the serosal side of epithelia incubated in Ussing chamber devices. The used concentrations are reported in the Second Publication (Greco et al., 2019) and justified in the Discussion chapter. Supraphysiological concentrations were also tested for both hormones. In these experiments, inhibitors for DDP-IV were not used because such protease seems to be absent in the rumen (Connor et al., 2010) and in related organs such as esophagus and stomach of humans (Uhlen et al., 2010). However, part of the original incubating buffer was regularly replaced with a new buffer containing new hormones and growth factors. More details will be given in the Methods section of the Second Publication (Greco et al., 2019).

1.7. Hypothesis and research questions

At this point the key information for understanding the focus of this manuscript has been provided. Therefore, given this background, the present work proceeded from the hypothesis that SCFA but also GLP-1, GLP-2 and EGF may be involved in the regulation of the epithelial barrier of rumen epithelia.

The deducted research questions were the following:

First Publication: Do single SCFA differently affect the epithelial barrier of rumen epithelia in sheep under physiological and acidotic ruminal pH?

Second Publication: Do GLP-1, GLP-2 and EGF play a role in the regulation of the epithelial barrier of rumen epithelia in sheep?

2. First Publication

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

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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 $NaHCO_3$, 0.6 NaH_2PO_4, 2.4 Na_2HPO_4, 5.5 KCl, 10 2-(N-morpholino) ethanesulfonic acid, 1 L-glutamine, 10 D-glucose, 100 Na-gluconate, 1 $CaCl_2$, and 1.25 $MgCl_2$. 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-pH6.1**. 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% O_2/5% CO_2), 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 Ca^{2+} and Mg^{2+} (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_t, 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 ($\boldsymbol{J}_{\text{ms-fluor}}$) were measured at a final mucosal concentration of 100 µM 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 cm^2 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 ≥ 6) was converted in a total 40 µL 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 µL with a total of 4.5 ng of the obtained cDNA was mixed with primers and probe for the respective target gene, 4.5 µL 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
Cldn-1	Forward: 5′-TGCCAGGTATGAATTTGGTC-3′	500
	Probe: 5′-FAM-TCTTCATTGG CTGGGCTGCTGCTTCT-TAMRA-3′	300
	Reverse: 5′-GGGATAGGGCCTGGGTGTTG-3′	1,500
Cldn-4	Forward: 5′-TTCATCGGCAGCAACATC-3′	500
	Probe: 5′-FAM-AGACCATCTGGGAG GGCCTATGGATGAACT-BHQ-3′	300
	Reverse: 5′-GTACACCTTGCACTGCATC-3′	1,500
Cldn-7	Forward: 5′-ATAGCTTGCTCCTGGTACGG-3′	1,500
	Probe: 5′-FAM-ATAACCC GTTGGTCCCCATGAATGTTA AGTATGAA-TAMRA-3′	300
	Reverse: 5′-CAGGATGATCAGAGCAGACC-3′	1,500
Ocln	Forward: 5′-TCAGGGAATATCCACCTATCAC-3′	500
	Probe: 5′-FAM-GAGCCTACA AGCAGAACTTGATGAG GTCAATAAA-TAMRA-3′	150
	Reverse: 5′-CATCCAGTTCTTTATCCAGACG-3′	3,000
Gapdh	Forward: 5′-AAGAAGGTGGTGAAGCAGGC-3′	1,500
	Probe: 5′-FAM-GGCATTC TAGGCTACACTGAGGAC CAGGTTG-TAMRA-3′	300
	Reverse: 5′-CTGTTGAAGTCGCAGGAGAC-3′	3,000
Rps19	Forward: 5′-GGAAAAGGACCAAGATGGGG-3′	500
	Probe: 5′-FAM-ACAGAG AGATCTGGACAGAATCGC TGGACA-TAMRA-3′	300
	Reverse: 5′-CGAACGAGGCAATTTATTAACC-3′	3,000
Ywhaz	Forward: 5′-AGAGAGAAAATAGAGACCGAGC-3′	1,500
	Probe: 5′-FAM-CCAACG CTTCACAAGCAGAGA 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 µL 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 µg protein lysate and 5 µL 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 µL 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-µm 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 µg/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 ith level of factor treatment, β_j is the effect due to the jth 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 $J_{ms\text{-}fluor}$ because technical problems had made the data set for that period incomplete. Already by the sixth hour, data availability for $J_{ms\text{-}fluor}$ 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 × 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 × 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 × 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 × 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 $J_{ms\text{-}fluor}$. At pH 6.1, $J_{ms\text{-}fluor}$ 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 $J_{ms\text{-}fluor}$ at pH 6.1 with no treatment × time interaction.

At pH 5.1, $J_{ms\text{-}fluor}$ 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 $J_{ms\text{-}fluor}$ was greater for Con-pH5.1 than for Con-pH6.1. Time effect was significant ($P < 0.01$) with no treatment × time interaction. Corresponding multiple comparisons showed that the major part of acidification-induced increase in $J_{ms\text{-}fluor}$ 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	Factor Time, $P = 0.10$	2.06	2.48	2.86	2.00	2.95	2.39^{V}	0.507	Factor Time, $P < 0.01$ $^{(V\text{-}Z)}$[2]
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	$4.49^{W,X}$	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}	$9.43^{Y,Z}$	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}	$2.22^{a,b}$	$2.07^{a,b,c}$	2.46^{a}			TRT × Time $P = 0.58$	2.36^{C}	7.10^{B}	11.38^{A}	6.94^{B}	8.61^{B}			TRT × Time $P < 0.01$
SEM	0.110	0.113	0.108	0.113	0.113				0.468	0.484	0.476	0.484	0.491			
	Factor TRT, $P < 0.01$ $^{(a\text{–}c)}$								Factor TRT, $P < 0.01$ $^{(A\text{–}C)}$							

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-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.

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

[2]Interactions 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}, µEq·cm⁻²·h⁻¹; TRT at pH 6.1								I_{sc}, µEq·cm⁻²·h⁻¹; TRT at pH 5.1							
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^{x}	0.066	Factor Time, $P < 0.01$ $^{(x\text{-}z)}$	1.61^{A}	0.51^{B}	0.06^{B}	0.28^{B}	0.23^{B}	0.70	0.072	Factor Time, $P = 0.06$
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 × Time $P = 0.71$	1.10^{A}	0.70^{B}	-0.07^{D}	0.01^{D}	0.28^{C}			TRT × 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, $P < 0.01$ $^{(a\text{-}c)}$								Factor TRT, $P < 0.01$ $^{(A\text{-}D)}$							

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). Con, control; Ace30, acetate 30 mM; Ace100, acetate 100 mM; Prop30, propionate 30 mM; But30, butyrate 30 mM.

[1]Raw 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 ($J_{ms\text{-}fluor}$)[1]

	$J_{ms\text{-}fluor}$, nmol·cm^{-2}·h^{-1}; TRT at pH 6.1								$J_{ms\text{-}fluor}$, nmol·cm^{-2}·h^{-1}; TRT at pH 5.1							
Hour	Con[2]	Ace30	Ace100	Prop30	But30	LSM[1]	SEM		Con[2]	Ace30	Ace100	Prop30	But30	LSM	SEM	
1	0.20	0.18	0.25	0.20	0.13	0.19	0.035	Factor Time, $P = 0.28$	0.71	0.84	0.30	0.45	0.53	0.51^{Z}	0.232	Factor Time, $P < 0.01$ ($^{X\text{-}Z}$)
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^{D}	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, $P < 0.01$ ($^{A\text{-}D}$)							

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] $J_{ms\text{-}fluor}$ represent the amount of fluorescein marker flux within a 1-h flux period and is normalized per cm^2 of epithelial area.

[2] Raw 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

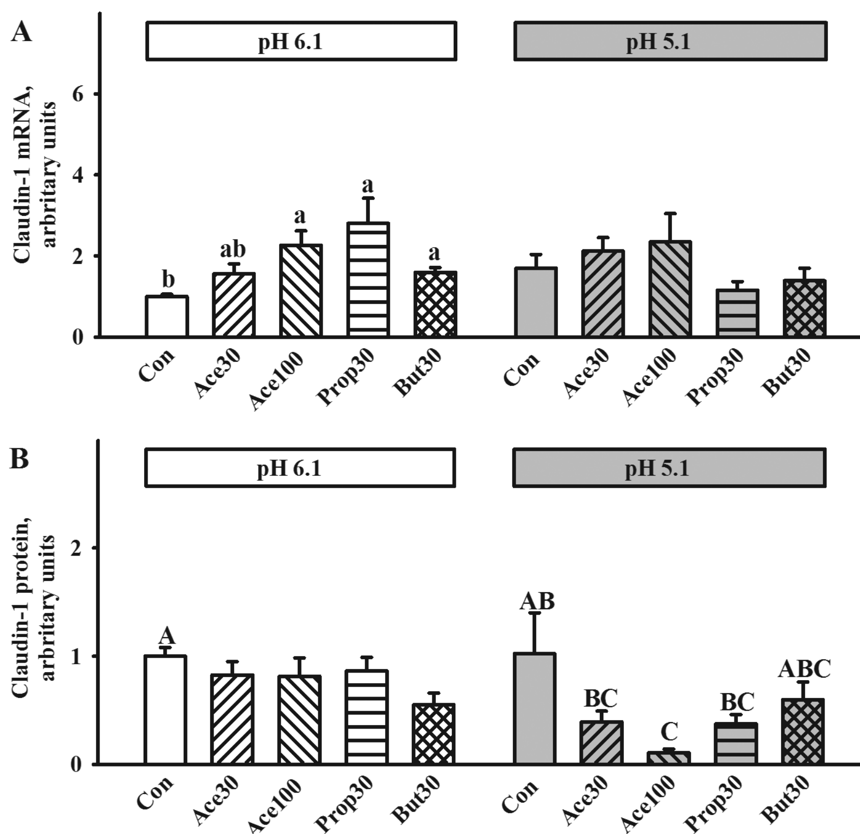

Figure 1. Expression of claudin-1 mRNA (A) and protein (B) in ruminal epithelia after 7 h of incubation with various SCFA at mucosal pH 6.1 or 5.1. Data are means ± SEM (n = 10–12). Letter code is used to indicate significant differences identified by multiple comparisons post-hoc test. Within each graph, 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 Ocln 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).

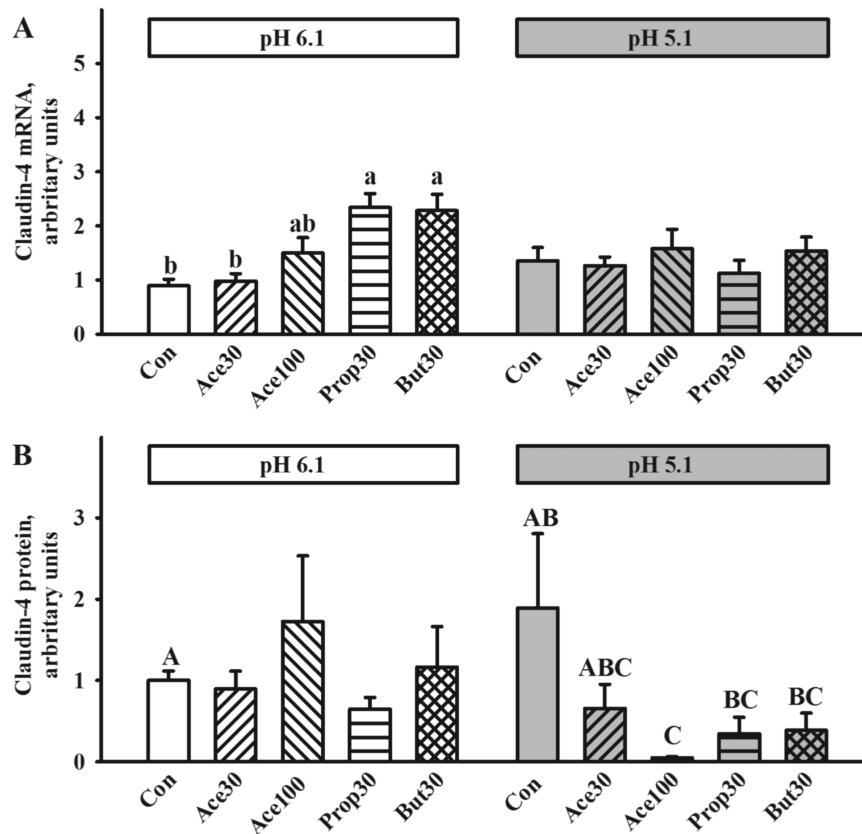

Figure 2. Expression of claudin-4 mRNA (A) and protein (B) in ruminal epithelia after 7 h of incubation with various SCFA at mucosal pH 6.1 or 5.1. Data are means ± SEM (n = 10–12). Within each graph, columns differ at $P < 0.05$ if they do not share a common letter. Lower case letters are used for comparisons 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 (Niwińska 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_t) 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.

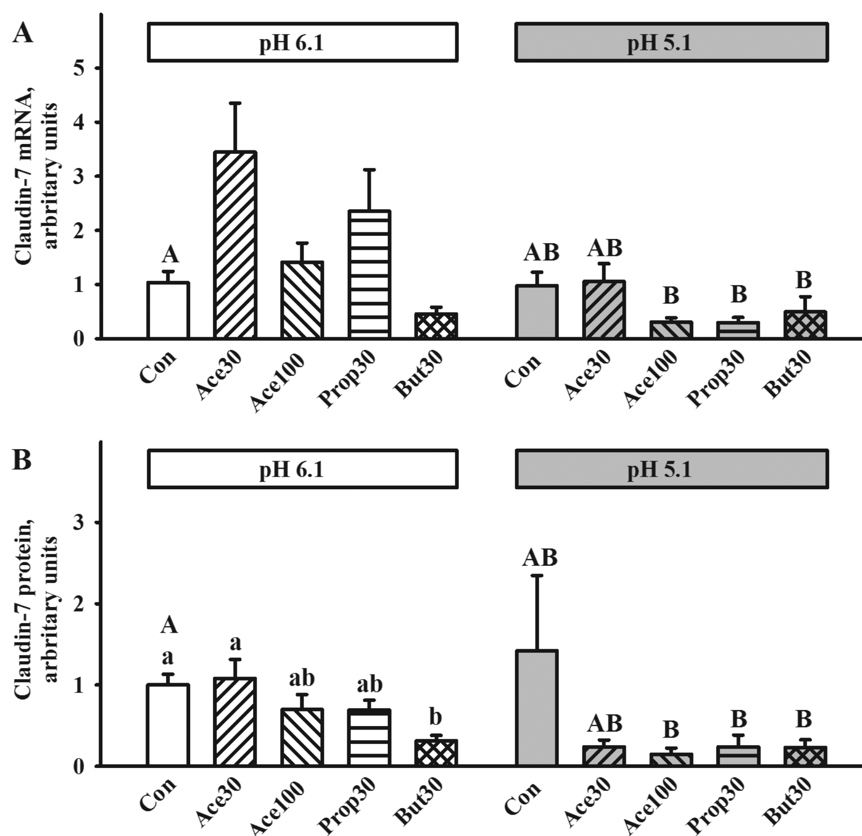

Figure 3. Expression of claudin-7 mRNA (A) and protein (B) in ruminal epithelia after 7 h of incubation with various SCFA at mucosal pH 6.1 or 5.1. Data are means ± SEM (n = 10–12). Within each graph, columns differ at $P < 0.05$ if they do not share a common letter. Lower case letters are used for comparisons 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

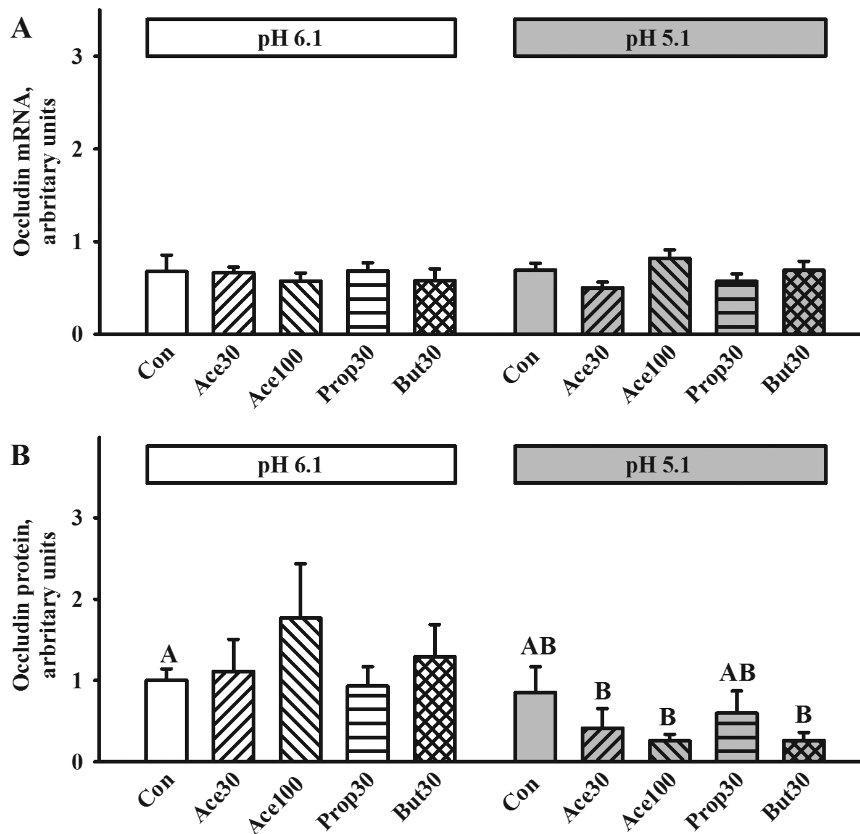

Figure 4. Expression of occludin mRNA (A) and protein (B) in ruminal epithelia after 7 h of incubation with various SCFA at mucosal pH 6.1 or 5.1. Data are means ± SEM (n = 10–12). Within each graph, columns differ at $P < 0.05$ if they do not share a common letter. Capital letters are used for comparisons 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.

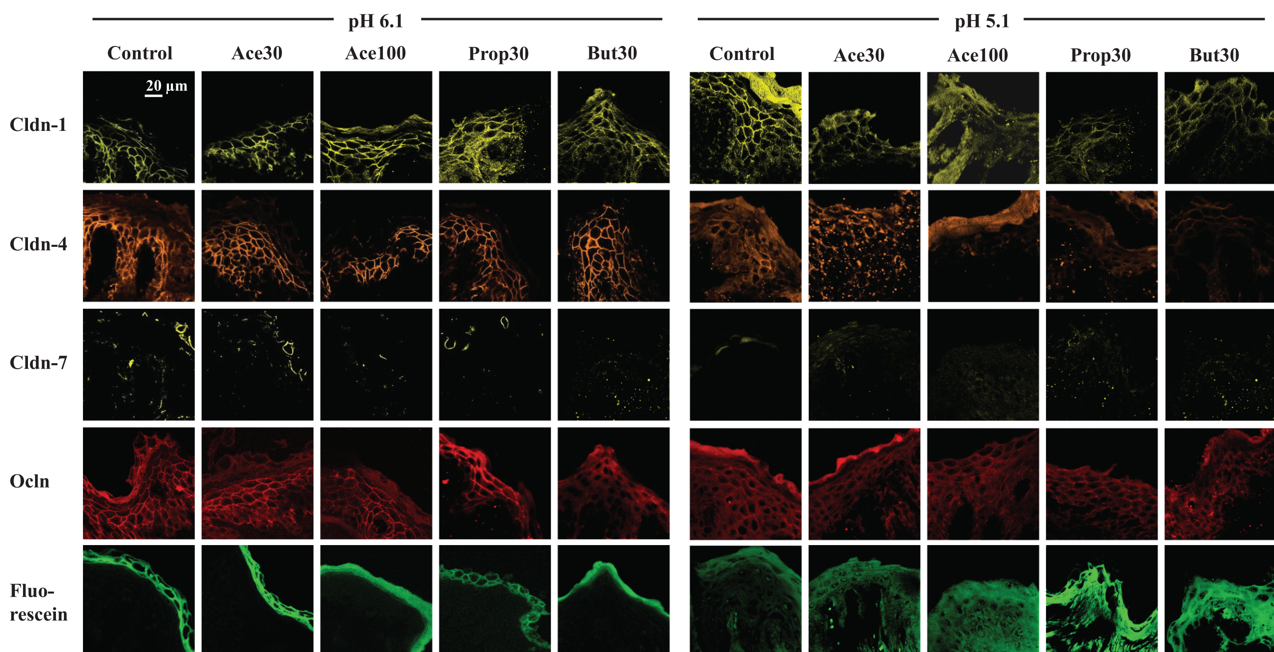

Figure 5. Influence of mucosal presence of various SCFA and mucosal pH on the localization of tight junction proteins in ruminal epithelia. Images are representative immunocytochemistry pictures of claudin (Cldn)-1, Cldn-4, Cldn-7, and occludin (Ocln) after 7 h of incubation at mucosal pH 6.1 or pH 5.1; 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_t, nor I_{sc}, nor $J_{ms\text{-}fluor}$ 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_t values with concurrent decreases in both I_{sc} and the abundance of the TJ protein Cldn-7. The increase in G_t 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_t 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_t 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 perceivably 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_t, 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 Ocln 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 $J_{ms\text{-}fluor}$, 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 Ocln 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 $J_{ms\text{-}fluor}$), 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 $J_{ms\text{-}fluor}$ 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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3. Second Publication

Effects of glucagon-like peptides 1 and 2 and epidermal growth factor on the epithelial barrier of the rumen of adult sheep

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ORIGINAL ARTICLE

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Effects of glucagon-like peptides 1 and 2 and epidermal growth factor on the epithelial barrier of the rumen of adult sheep

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Abstract

Epidermal growth factor (EGF) and glucagon-like peptides (GLP) modulate the tight junctions (TJ) of the intestinal epithelial barrier (EB) of monogastric animals. This work tried to elucidate whether GLP-1, GLP-2 and EGF can affect the EB of the rumen. Ovine ruminal epithelia were incubated in Ussing chambers for 7 hr with 25 or 250 nM of either GLP-1 or GLP-2 on the serosal side, with 2.5 nM of EGF on the serosal side or with 0.25 or 2.5 nM EGF on the mucosal side. No treatment affected tissue conductance. Short-circuit current (I_{sc}) was affected by time and treatment and their interactions. Only 250 nM of either GLP-1 or GLP-2 decreased I_{sc} in certain periods compared with 25 nM GLP-1 or 0.25 nM mucosally applied EGF; however, not when compared to control epithelia. Fluorescein flux rates (J_{fluor}) of ruminal epithelia were affected by treatment, time and time × treatment interaction. The time × treatment interaction was based on an increase in J_{fluor} between the first and last hour in epithelia incubated with 25 nM GLP-1 or GLP-2 and in epithelia incubated with EGF. After 7 hr incubation, claudin-7 mRNA expression was downregulated in all treatments. Claudin-1 mRNA was upregulated after incubation with 2.5 nM EGF on the serosal side, claudin-4 mRNA was downregulated by 2.5 nM EGF on the mucosal side, and occludin mRNA was increased after incubation with 250 nM GLP-2. The protein abundance of all tested TJ proteins was not influenced by treatment. We conclude that GLP-1, GLP-2, and EGF have no obvious acute effects on the EB of ruminal epithelia under simulated physiological conditions ex vivo. However, by decreasing the mRNA expression of claudin-7 and partly affecting other TJ proteins, they may modulate EB in the longer term or under certain conditions.

KEYWORDS
epidermal growth factor, epithelial barrier, glucagon-like peptide, rumen, tight junction

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1 | INTRODUCTION

The epithelial barrier (EB) of the rumen provides effective protection against free noxious agents in the ruminal milieu (Aschenbach et al., 2019; Penner, Steele, Aschenbach, & McBride, 2011). However, it can become ineffective under pathological conditions, e.g., when luminal accumulation of short-chain fatty acids and protons challenge key epithelial functions (Aschenbach & Gabel, 2000; Greco et al., 2018; Meissner et al., 2017). Different studies proposed a loss of barrier integrity and selectivity under such circumstances (Liu, Xu, Liu, Zhu, & Mao, 2013; Steele, AlZahal, Hook, Croom, & McBride, 2009). The health consequences of such loss of barrier function appear directly related to the nature and amount of toxins and micro-organisms absorbed into the blood circulation across the leaky epithelium (Aschenbach et al., 2019; Plaizier, Krause, Gozho, & McBride, 2008).

To develop strategies of avoiding or ameliorating a loss of barrier integrity and selectivity, the present study aimed at identifying molecules that may potentially improve the tightness of the EB in ruminal epithelia (RE). Hormones like glucagon-like peptide-1 (GLP-1) and glucagon-like peptide-2 (GLP-2) have been shown to modulate EB of different epithelia (Fukuda et al., 2016; Yu et al., 2014). Such hormones are normally secreted from enteroendocrine cells in the intestine and released into the blood circulation (Gorka et al., 2011; Janssen, Rotondo, Mule, & Tack, 2013). Once reaching the RE, they can directly stimulate their receptors (Taylor-Edwards et al., 2010) or may indirectly affect the EB (Steele, Penner, Chaucheyras-Durand, & Guan, 2016). Likewise, epidermal growth factor (EGF) has also potential to improve the EB of different epithelia (Lamb-Rosteski, Kalischuk, Inglis, & Buret, 2008; Ogawa et al., 2012). It is normally released from a variety of tissues; the release into saliva being of proposed relevance for the RE (Onaga et al., 2006). Hence, EGF may reach the RE from both the luminal and blood side.

To assess the effects of the mentioned GLP hormones and EGF on the ruminal EB, we chose an Ussing chamber approach for the present study. The great advantage of this approach is that functional readouts of barrier integrity like tissue conductance (G_t) and permeability to macromolecules like fluorescein can be monitored continuously over several hours. Secondly, supraphysiological hormone concentrations can be applied without animal welfare concern and compared on epithelia from the same animal. Thirdly, the tissue can be easily harvested at the end of the procedure to study the influence of treatment on the expression of tight junction (TJ) proteins, which physically form the EB (Gonzalez-Mariscal, Betanzos, Nava, & Jaramillo, 2003). Tight junction proteins with a proven role for the ruminal EB are claudin-1, -4, -7, and occludin. Of these, claudin-1 and claudin-4, together with occludin, directly contribute to the formation of the permeation barrier at the level of the *stratum granulosum*; whereas, claudin-7 appears to support the mechanical barrier against abrasion by connecting cells of the *stratum corneum* (Aschenbach et al., 2019; Stumpff et al., 2011).

2 | MATERIALS AND METHODS

2.1 | Animals and tissue sampling

Six healthy adult sheep (*Ovis aries*) with a weight ranging from 65 to 92 kg were purchased from a commercial farm. Animals were fed with hay ad libitum and 200 g concentrate per day for at least 15 days prior to the experiment. The concentrate supply was divided into two equal portions, one in the morning and one in the afternoon. Water was supplied with no restriction. After ≥15 days on the controlled diet, one sheep per day was slaughtered and RE were collected for the experiments. Sheep were stunned with a captive bolt and killed by exsanguination. The abdomen was open by midline incision; the viscera were externalized. A portion of ruminal wall of approximately 30 × 30 cm was cut from the ventral ruminal sac. It was washed first with pre-warmed (38°C) 0.9% NaCl isotonic solution and thereafter in a standard buffered solution containing 10 mM NaCl, 24 mM $NaHCO_3$, 0.6 mM NaH_2PO_4, 2.4 mM $NaHPO_4$, 5.5 mM KCl, 10 mM 2-(N-morpholino) ethanesulfonic acid (MES), 1 mM L-glutamine, 10 mM D-glucose, 100 mM Na-gluconate, 1 mM $CaCl_2$ and 1.25 mM $MgCl_2$ (290 mOsm/L) warmed to 38°C and gassed with carbogen (95% O_2 and 5% CO_2). The washing solution was discarded and the tissue was bathed in fresh standard buffered solution (38°C, carbogen-gassed) in which the *Tunica serosa* and *muscularis* were manually stripped from the *Tunica mucosa* and discarded. The remaining *tunica mucosa* (called epithelium hereafter) was immediately transported to the laboratory in a Dewar container filled with fresh standard buffered solution (38°C, carbogen-gassed).

2.2 | Ussing chamber experiments

Epithelial samples were cut into squares with circa 3.5 cm sides. These epithelial pieces were mounted in Ussing chambers with an inner aperture of 3.14 cm^2 as described by Aschenbach and Gäbel (2000). Ussing chambers were connected to a multichannel voltage-current clamp device (Ing.-Büro Mussler, Aachen, Germany) via platinum wire electrodes for direct current application and Ag/AgCl electrodes for voltage measurement, the latter being connected with the incubation solution via 3 M KCl-agar bridges. The device allowed the continuous recording of short-circuit current (I_{sc}) and G_t (Aschenbach & Gabel, 2000).

The incubation solution on the serosal (i.e. blood-directed) side was 16 ml standard buffered solution (see previous section). The 16 ml of incubation solution on the mucosal (i.e. luminally directed) side were of similar composition, except that 40 mM Na-gluconate was replaced by 24 mM Na-acetate, 12 mM Na-propionate, 4 mM Na-butyrate and that pH was titrated to pH 6.1. Before use, all incubation solutions were added 100 mg/L bovine serum albumin to prevent unspecific binding of hormones and EGF to the glassware and with the antibiotics colistin methanesulfonate (25 mg/L) and cefuroxime (100 mg/L). During the whole experiment, mucosal and serosal solutions were thermostated to 38°C and gassed with carbogen.

After an equilibration period of ~45 min, the hormones GLP-1 and GLP-2 (Sigma-Aldrich, St. Louis, MO), and the growth factor EGF (Genscript Biotechnology; Piscataway, NJ) were added in a total of seven treatments plus a control group that received neither hormones nor EGF. Two epithelia per animal were allocated to each group. Treatments with serosal additions of hormones and EGF were 25 and 250 nM GLP-1, 25 and 250 nM GLP-2, and 2.5 nM EGF. Because EGF reaches the RE predominantly from the luminal side in vivo, EGF was also applied at 0.25 and 2.5 nM on the mucosal side. All given concentrations are final concentrations in the incubation solutions. Stem solutions for hormones and EGF were prepared in deionized water containing 100 mg/L bovine serum albumin. The pH values of all incubation solutions were checked after 1 and 6 hr; they did not change. After 7 hr incubation, epithelia were removed from the Ussing chambers. The solution-exposed area was cut out and split into two pieces. One-piece was preserved in 1 ml mRNAlater, stored at 4°C overnight and at −80°C thereafter. The second piece was harvested in a cryovial, shock frozen in liquid nitrogen and stored at −80°C.

2.3 | Fluorescein flux rate monitoring

Measurement of fluorescein flux rates (J_{fluor}) was performed according to Meissner et al. (2017). Immediately after mounting, that is ~45 min before application of hormones and EGF, Na-fluorescein (Carl Roth, Karlsruhe, Germany) was added to a final concentration of 100 µM to the mucosal side of those chambers that received hormones or EGF serosally. Chambers that were treated with EGF mucosally received 100 µM Na-fluorescein on the serosal side.

Immediately after addition of Na-fluorescein and shortly before the end of the experiment, 50 µl of the incubation solution with added Na-fluorescein (hot samples) were collected and stored in a dark vial at 4°C. Starting at the time of hormone addition, samples of the contralateral incubation solution (cold samples) were collected at hourly intervals and two cold sample aliquots for each hour were stored at 4°C in the dark. To continuously refresh the hormone and EGF concentrations in the solutions, a comparatively large sample volume of 2 ml was collected from the cold side at each hourly sampling and replaced with 2 ml fresh solution containing the respective hormone or EGF.

Fluorescence intensity of each sample was measured in 96-well plates for fluorescence-based assays (Thermo-Fischer Scientific, Waltham, MA) in duplicate in three dilution steps (undiluted, 1 in 10, and 1 in 100). Blanks contained incubation solution without Na-fluorescein. Fluorescence measurements were performed at 490 nm (excitation) and 525 nm (emission) using an EnSpire Multimode Plate Reader (Perkin Elmer, Waltham, MA). The total amount of fluorescein in the cold solution was calculated by calibrating the fluorescence intensity of the cold samples to the specific fluorescence intensity of the hot samples from the same chamber. The increase in the total amount of fluorescein between two samplings, corrected for the fluorescein withdrawn by the sampling procedure itself, represented J_{fluor}, either in serosal-to-mucosal (sm; mucosal EGF treatments) or in mucosal-to-serosal (ms; all other treatments) direction, and was normalized per cm^2 of epithelial area.

2.4 | Total RNA extraction, cDNA production and RT-qPCR

Total RNA was isolated with the Nucleo Spin RNA II kit (Macherey & Nagel, Dueren, Germany). and its concentration measured with a nanophotometer (Implen GmbH, Munich, Germany). Quality of RNA was assessed by microchip electrophoresis using RNA 6000 Nano Kit in an Agilent 2100 Bioanalyzer (Agilent, Santa Clara, CA). An amount of 100 ng of total RNA, with RIN values ranging from 8 to 10 for most samples, were converted to cDNA with the iScript cDNA Synthesis Kit (Bio-Rad Laboratories, Hercules, CA) and adjusted to a concentration of 0.5 ng/µl. A volume of 3.5 µl of such cDNA was pipetted in 384-well plate (Thermo Fisher Scientific) together with the 5.5 µl mastermix which contained iTaq Universal Probes Supermix (Bio-rad Laboratories) and target-specific primers and probes in a final volume of 9 µl. Gene targets were claudin-1, -4, -7, occludin, YWHAZ, RPS 19 and GAPDH, the last three genes were chosen as non-regulated reference genes. Primer sequences and concentrations are listed in Greco et al. (2018). Real-time quantitative PCR was run in triplicates on a thermal cycler ViiA7 (Life Technologies, Carlsbad, CA). After initial denaturation at 95°C for 20 s, a two-step protocol of 40 cycles at 95°C for 1 s and 60°C for 20 s was used for cDNA amplification. Each 384-well plate contained three inter-run calibrators, three blank controls with only deionized water, three negative no-RT controls, and a standard curve to assess amplification efficiencies. Expression values of target genes were normalized with the software qBase (Biogazelle NV, Zwijnaarde, Belgium), which automatically selected RPS 19 as the most stably expressed reference gene. The same software automatically adjusted the C_q values of the amplification curves to the inter-run calibrator.

2.5 | Protein extraction and Western blot analysis

The procedures for protein extraction and Western blot analysis of claudin-1, -4, -7 and occludin were performed as described previously (Greco et al., 2018), except that blotting was performed at 80 V for 60 min and that antibodies for the reference gene (anti-β-actin) were diluted 1 in 2000.

2.6 | Statistical analysis

Time series data (G_t, I_{sc}, J_{fluor}) was analyzed for effects of time and treatment and their two-way interactions using two-way repeated measurement analysis of variance. The results of Western blot and RT-qPCR were analyzed using a one-way analysis of variance. If analysis of variance indicated differences among time points or treatments, post-hoc Holm-Sidak method was used for all-pairwise comparison. Data are reported as standard error of mean (*SEM*).

TABLE 1 Influence of GLP hormones and EGF on short-circuit current (I_{sc}, in µEq·cm^{-2}·h^{-1}; pooled *SEM* = 0.040)

Hour	Control	GLP–1 25 nM	GLP–1 250 nM	GLP–2 25 nM	GLP–2 250 nM	EGF-m 0.25 nM	EGF-m 2.5 nM	EGF-s 2.5 nM
1	1.31	1.43	1.08	1.28	1.07	1.27	1.31	1.23
2	1.20^{ab}	1.31^{a}	0.88^{b}	1.14^{ab}	0.90^{b}	1.16^{ab}	1.18^{ab}	1.14^{ab}
3	1.08^{ab}	1.17^{a}	0.75^{b}	0.94^{ab}	0.76^{ab}	1.06^{ab}	1.09^{ab}	1.03^{ab}
4	0.99^{abc}	1.08^{a}	0.66^{bc}	0.79^{abc}	0.63^{c}	0.94^{abc}	1.02^{b}	0.93^{abc}
5	0.93	1.00	0.66	0.80	0.63	0.96	1.00	0.97
6	0.87	0.92	0.60	0.72	0.59	0.95	0.96	0.89
7	0.74^{ab}	0.77^{ab}	0.52^{b}	0.68^{ab}	0.50^{ab}	0.90^{a}	0.90^{ab}	0.79^{ab}

Note: Data are least square means (LSM; *n* = 9 to 12). The following *P*-vales were calculated: factor treatment, *p* = .004; factor time, *p* < .001; interaction treatment × time, *p* = .018.

[a-c]Differences between multiple treatment means (at *p* < .05) are indicated by letter coding; values within a row differ if they do not share a common letter.

GLP hormones were always applied from the serosal side. EGF was applied either from the mucosal (EGF-m) or the serosal side (EGF-s).

TABLE 2 Influence of GLP hormones and EGF on tissue conductance (G_t, in mS/cm^2; pooled *SEM* = 0.093)

Hour	Control	GLP–1 25 nM	GLP–1 250 nM	GLP–2 25 nM	GLP–2 250 nM	EGF-m 0.25 nM	EGF-m 2.5 nM	EGF-s 2.5 nM
1	2.29	2.30	1.98	2.29	1.95	2.26	2.46	2.35
2	2.56	2.61	2.31	2.67	2.28	2.57	2.83	2.66
3	2.77	2.85	2.54	2.94	2.50	2.78	3.11	2.87
4	2.90	3.00	2.73	3.11	2.68	2.89	3.34	2.85
5	3.02	3.15	2.90	3.27	2.80	3.02	3.54	3.01
6	3.13	3.31	3.07	3.42	2.90	3.12	3.75	3.23
7	3.30	3.58	3.27	3.65	3.02	3.24	4.03	3.35

Note: Data are least square means (LSM; *n* = 12). The following *p*-vales were calculated: factor treatment, *p* = .74; factor time, *p* < .001; interaction treatment × time, *p* = .88.

GLP hormones were always applied from the serosal side. EGF was applied either from the mucosal (EGF-m) or the serosal side (EGF-s).

The zero hypothesis was considered disproved with *p*-values < .05. The software package used for data assessment and plotting of graphs was SigmaPlot 11 (Systat Software, San José, CA).

3 | RESULTS

3.1 | Electrophysiological data

Values of I_{sc} were affected by the factors time of incubation (*p* < .01) and treatment (*p* < .01) with significant time × treatment interaction (*p* < .05; Table 1). The I_{sc} decreased over time in all treatments. Thus, the interaction was primarily based on lower I_{sc} values of epithelia incubated with 250 nM GLP-1 or GLP-2 compared to those incubated with 25 nM GLP-1 during the second, fourth and partly the third hour of incubation (*p* < .05), and due to lower I_{sc} values of epithelia treated with 250 nM GLP-2 compared to epithelia incubated with 0.25 nM of EGF on the mucosal side in the seventh hour (*p* < .05). However, I_{sc} values of all hormone or EGF-treated groups were not different from Control at any time (Table 1). Values of G_t gradually increased over time (*p* < .01) with no difference among and no interaction with treatments (Table 2).

3.2 | Fluorescein flux rates

Fluorescein flux rates of epithelia were affected by treatment and time of incubation (*p* < .01 each) with significant time × treatment interaction (*p* < .05; Table 3). The main effect of treatment was exclusively attributable to the much lower flux rates in sm direction measured for the mucosal EGF treatments as opposed to the flux rates in ms direction measured for all other treatments (Table 3). The statistical significance of the treatment effect disappeared when ms and sm flux rates were analysed separately (data not shown). The time effect with a time × treatment interaction was mainly caused by an increase in J_{fluor} between the first and last hour of incubation occurring in epithelia incubated with 25 nM of GLP-1 or GLP-2 and in all epithelia incubated with EGF either on the mucosal or on the serosal side (*p* < .05; significances for time within treatment not shown in Table 3 for reasons of clarity). Due to these time-dependent increases, J_{fluor} was greater in epithelia treated serosally with EGF compared to Control in the seventh hour of incubation (*p* < .05). Flux rates of GLP-treated tissues were at no time point different to Control (Table 3).

TABLE 3 Influence of GLP hormones and EGF on fluorescein flux rates (J_{fluor}, in nmol·cm^{-2}·h^{-1}; pooled *SEM* = 0.083)

Hour	Control	GLP–1 25 nM	GLP–1 250 nM	GLP–2 25 nM	GLP–2 250 nM	EGF-m 0.25 nM	EGF-m 2.5 nM	EGF-s 2.5 nM
1	0.52[a]	0.56[a]	0.56[a]	0.53[a]	0.63[a]	0.06[b]	0.07[b]	0.58[a]
2	0.78[a]	0.73[a]	0.84[a]	0.70[a]	0.78[a]	0.12[b]	0.10[b]	0.80[a]
3	0.74[a]	0.82[a]	0.58[a]	0.70[a]	0.61[a]	0.10[b]	0.12[b]	0.83[a]
4	0.51[ab]	0.75[a]	0.51[ab]	0.66[a]	0.70[a]	0.11[b]	0.15[b]	0.81[a]
5	0.52[ab]	0.82[a]	0.61[ab]	0.76[a]	0.60[ab]	0.17[b]	0.19[b]	0.86[a]
6	0.83[a]	0.62[abc]	0.61[abc]	0.59[abc]	0.66[ab]	0.20[c]	0.22[bc]	0.94[a]
7	0.75[bc]	1.08[ab]	0.64[cd]	1.07[ab]	0.63[cd]	0.15[e]	0.29[de]	1.25[a]

Note: Data are least square means (LSM; *n* = 10 to 12). The following *p*-vales were calculated: factor treatment, $p < .001$; factor time, $p = .009$; interaction treatment × time, $p = .048$.
Differences between multiple treatment means (at $p < .05$) are indicated by letter coding; values within one row differ if they do not share a common letter.
GLP hormones were applied from the serosal side, and flux rates were measured in mucosal-to-serosal direction. EGF was applied either from the mucosal (EGF-m) or from the serosal side (EGF-s). Flux rates after mucosal application of EGF were measured in serosal-to-mucosal direction, whereas flux rates after serosal EGF application were measured in mucosal-to-serosal direction.

3.3 | RT-qPCR and mRNA quantification

Results of RT-qPCR are shown in Figure 1 for claudin-1 and claudin-4, and in Figure 2 for claudin-7 and occludin. The mRNA expression of all tested TJ proteins was affected by treatment ($p < .05$). Incubation with 25 nM or 250 nM GLP-1, or 25 nM GLP-2, or 0.25 nM mucosally applied EGF resulted in levels of claudin-1, claudin-4, and occludin mRNA expression not different from control epithelia. Epithelia incubated with 250 nM GLP-2 also showed claudin-1 and claudin-4 mRNA expression similar to control epithelia (Figure 1); however, occludin mRNA expression was upregulated above the level of Control and all other treatments ($p < .05$). Epithelia incubated with 2.5 nM EGF on the mucosal side showed claudin-1 and occludin mRNA expression similar to control epithelia, but claudin-4 mRNA expression was lower than control epithelia and epithelia incubated with GLP-1 or serosally applied EGF ($p < .05$). Finally, epithelia incubated with 2.5 nM EGF on the serosal side showed mRNA expression of claudin-4 and occludin not different from control epithelia; however, claudin-1 mRNA expression was higher than for control and all other treatments, except 0.25 nM mucosally applied EGF ($p < .05$). The mRNA expression of claudin-7 was downregulated in all treatments compared with control epithelia, most prominently by GLP-1, 0.25 nM mucosally applied EGF and 2.5 nM serosally applied EGF ($p < .05$).

3.4 | Western blot analysis

Protein abundance of claudin-1, -4, -7 and occludin were not different among treatments and control epithelia (Figure 1 and Figure 2).

4 | DISCUSSION

In order to understand the factors that modulate the tightness of the ruminal EB, the present study aimed at elucidating the influence of GLP-1, GLP-2 and EGF on ruminal permeability characteristics and TJ protein expression. Our research group had previously studied the effects of pH and SCFA on the EB in the ovine rumen using a comparable experimental setup (Greco et al., 2018; Meissner et al., 2017). Key advantages of the Ussing chamber approach have been mentioned in the Introduction. The present results verified the appropriateness of such approach; data on I_{sc} and G_t confirmed that tissues remained vital throughout the experimental period of 7 hr, which proofed long enough to induce mRNA expression changes of selected TJ proteins.

During the experiments, an unexpected technical peculiarity of fluorescein emerged: The J_{fluor} differed greatly in ms versus sm direction. When planning the study, we had assumed that fluorescein as an established paracellular marker should yield comparable flux rates in either flux direction. Consequently, we had initially interpreted the greatly reduced J_{fluor} after mucosal EGF treatments as barrier-tightening effects of EGF. However, the fact that the discrepancy between ms and sm flux rates was greatest already in the first flux hour and the fact that G_t and TJ protein expression did not mirror this 'effect', strongly argued against a true EGF effect of such magnitude on J_{fluor}. The unequal flux rates of fluorescein in ms and sm direction can have two causes. Firstly, there could be active (transcellular) transport of fluorescein across the ruminal epithelium in ms direction. Secondly, lower sm flux rates could be a measuring artefact because fluorescein is a weak acid and changes from the divalent anion (of greater fluorescence) to a monovalent anion (of lower fluorescence) with a pK_a of ~6.4 (Lakowicz, 2006). As a consequence, fluorescein yielded lower fluorescence signals in buffer samples from the mucosal side, which had a pH of 6.1. For the results of the present study, these limitations have the consequence that sm flux rates of fluorescein (mucosal EGF treatments) are not further discussed and results of the ms flux rates of fluorescein will be interpreted cautiously.

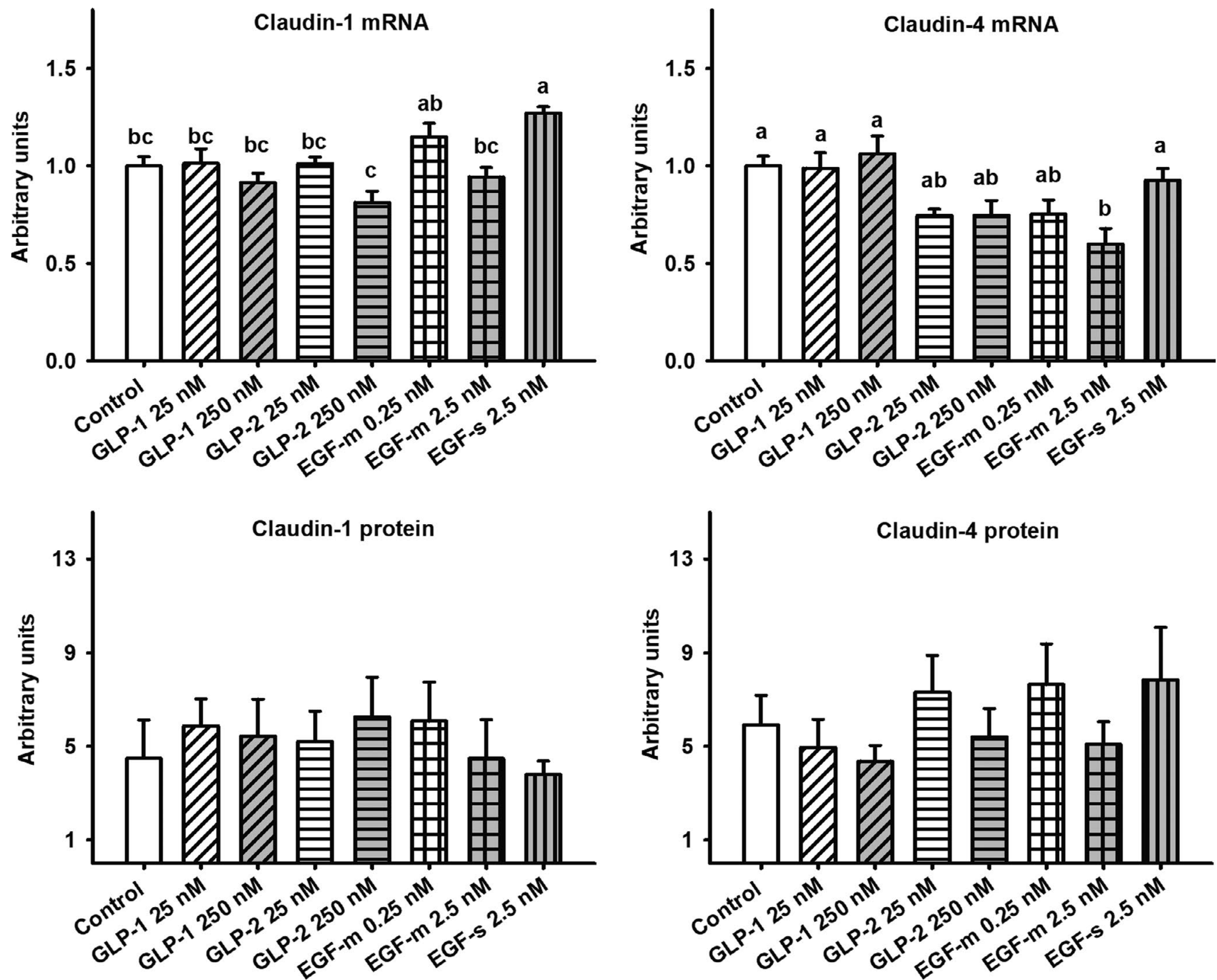

FIGURE 1 Expression of claudin-1 (left panels) and claudin-4 (right panels) at the level of mRNA (upper panels) and protein (lower panels) in ruminal epithelia. Expression was measured after 7-hr incubation with 25 nM and 250 nM GLP-1 on the serosal side, 25 and 250 nM GLP-2 on the serosal side, 0.25 and 2.5 nM EGF on the mucosal side (EGF-m), and 2.5 nM EGF on the serosal side (EGF-s). Data are means ± *SEM* ($n = 12$). Letter code is used to indicate significant differences identified by multiple comparisons post-hoc test. Within each graph, columns differ at $p < .05$ if they do not share a common letter

A great challenge of the experimental design was to choose the right GLP and EGF concentrations. The concentrations of GLP-1 and GLP-2 in blood serum or plasma of ruminants are typically below 100 pM, where they are increased by feed intake (Castro et al., 2016; McCarthy, Faulkner, Martin, & Flint, 1992; Suominen et al., 1998) and further influenced by metabolic and reproductive status (Larsen, Relling, Reynolds, & Kristensen, 2010; Marti, Perez, Aris, Bach, & Devant, 2014; Relling & Reynolds, 2007; Zapata, Salehi, Ambrose, & Chelikani, 2015). Dose-response data for GLP are not available for gastrointestinal epithelia of ruminants; however, GLP-1 and GLP-2 have been tested for dose effects on chloride secretion in the guinea-pig ileum mounted in Ussing chambers with maximum responses at ~100 nM for GLP-1 (Baldassano, Wang, Mulè, & Wood, 2012) and ~30 nM for GLP-2 (Baldassano, Liu, Qu, Mulè, & Wood, 2009). Importantly, those previous experiments observed GLP effects without applying any protease inhibitor to protect against GLP degradation by DDP-4. The latter is a widely distributed GLP-degrading enzyme in the intestine but apparently absent in stomach and oesophagus (Uhlen et al., 2010). Considering that rumen is rather related to stomach and oesophagus than intestine, we assumed that DPP-4 degradation should not be a major issue in our experimental setup and chose 25 and 250 nM as our test concentrations. To yet account for any possibility of GLP degradation, we replenished 12.5% of the incubation solution hourly with fresh solution containing the correct GLP concentration.

Plasma concentrations of EGF are apparently not documented for ruminants in literature. This is due likely to the fact that EGF plasma concentrations are very low and barely measurable in ruminants. In one study on goats, all plasma concentrations were below the detection limit of the assay (<50 pM; Dehnhard, Claus, Munz, & Weiler,

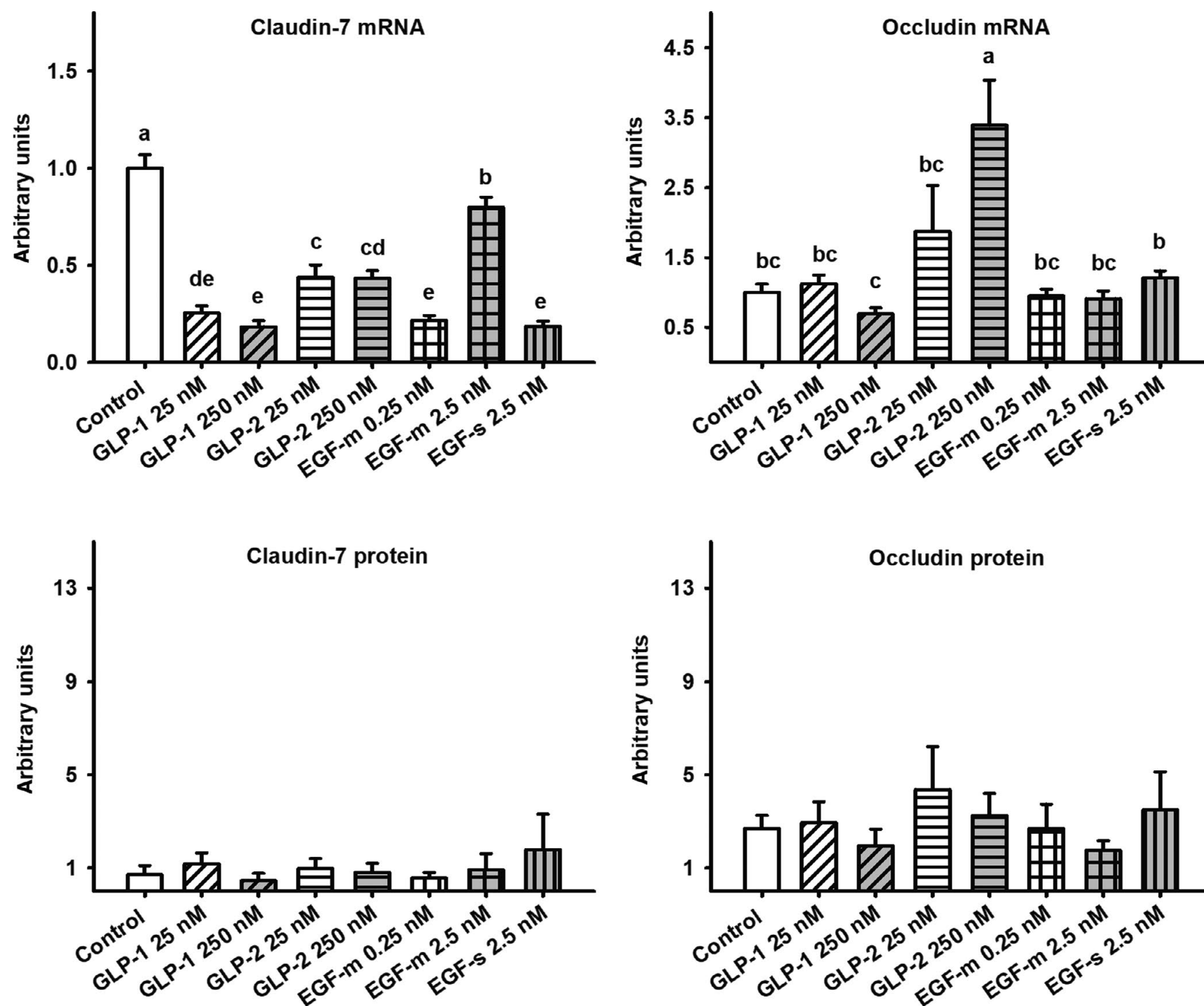

FIGURE 2 Expression of claudin-7 (left panels) and occludin (right panels) at the level of mRNA (upper panel) and protein (lower panel) in ruminal epithelia. Expression was measured after 7-hr incubation with 25 nM and 250 nM GLP-1 on the serosal side, 25 and 250 nM GLP-2 on the serosal side, 0.25 and 2.5 nM EGF on the mucosal side (EGF-m), and 2.5 nM EGF on the serosal side (EGF-s). Data are means ± *SEM* (*n* = 12). Letter code is used to indicate significant differences identified by multiple comparisons post-hoc test. Within each graph, columns differ at $p < .05$ if they do not share a common letter

2000). By contrast, EGF concentrations of ruminal fluid were readily measurable in sheep (73 pM; Onaga et al., 2006), implying that these luminal concentrations are much higher than plasma concentrations. Using the Ussing chamber approach, ion currents in a human colonic adenocarcinoma cell line were influenced by EGF with an EC_{50} of 0.25 nM. Accordingly, we chose concentrations of 0.25 and 2.5 nM for our ruminal permeability measurements. Stability appears less a concern for EGF compared with GLP because EGF is catabolized primarily through internalization together with its receptor. In 3T3 cells, maximal binding between EGF and its receptor occurred after 30–40 min incubation and decreased to 40% after 6 hr (Ahrnonv, Pruss, & Herschman, 1978).

In the present study, GLP-1 and GLP-2 had subtle dose-dependent effects on the active electrogenic current flow (I_{sc}) and the passive permeability to the large anion fluorescein (J_{fluor}) across the RE. The high-dose of either GLP-1 or GLP2 was associated with lower values of both I_{sc} and J_{fluor} compared with the low dose, at least, at certain time points. Decreasing effects of GLP-1 and GLP-2 on I_{sc} had previously been observed in electrical field-stimulated guinea-pig small intestine and were explained by a GLP-mediated depression of acetylcholine release from enteral nerves (Baldassano et al., 2009, 2012). Although the stripped RE preparations used in the present study were devoid of neuronal ganglia, the observed effects on J_{fluor} and I_{sc} yet suggest that both peptides act on ion flow across the epithelium by either direct effects on RE cells or influencing paracrine signals. Given the subtle nature of these responses, interpretations should be seen with care. Nonetheless, the present results appear to extend previous findings, suggesting that receptors

for GLP-1 and GLP-2 are functional in the RE despite comparatively low levels of mRNA expression (Gorka et al., 2011; Pezeshki, Muench, & Chelikani, 2012; Taylor-Edwards et al., 2010).

Alterations in J_{fluor} and G_t are commonly used to assess the permeability of the paracellular space to large (J_{fluor}) and small ions (G_t), assuming that the transcellular route has negligible (J_{fluor}) or rather constant (G_t) contribution to these functional barrier readouts. Nevertheless, we have demonstrated in previous studies that fluorescein is also able to use the transcellular route for permeation across RE, for example, after an acid challenge (Greco et al., 2018; Meissner et al., 2017). Based on the fact that G_t and the protein expression of all tested TJ proteins were unchanged by GLP-1 and GLP-2 in the present study, we thus propose that paracellular barrier function of RE was not affected by these hormones during the 7-hr incubation, despite the fact that J_{fluor} was changed in the last flux period by either of them.

Nonetheless, the mRNA expression of TJ proteins was affected by both GLP-1 and GLP-2. The incubation of RE with GLP-2 and, especially, GLP-1 decreased the mRNA expression of claudin-7 at both tested concentrations. Incubation with GLP-2 simultaneously increased the mRNA expression of occludin mRNA, which was statistically significant at a concentration of 250 nM. Our results align with a previous study where twice-daily injection of GLP-2 (50 µg/kg of BW) selectively increased the mRNA expression of occludin in the jejunum and caecum of calves but had no significant effects on claudin-1 and claudin-4; with claudin-7 being not measured in that previous study (Walker, Evock-Clover, Elsasser, & Connor, 2015).

Earlier studies from our institute have identified claudin-7 as a marker for the terminal differentiation of the RE that is induced in cornifying cells (Greco et al., 2018; Stumpff et al., 2011). Claudin-7 is localized in the *stratum corneum* of the RE and acts as anchor between the cells of the same *stratum corneum* and between the cells of this *stratum* with the cells of the lower *stratum granulosum*. Connection by claudin-7 appears essential to guarantee continuous epithelial integrity under intensive feeding conditions when cornified cells start to balloon. Accordingly, we have suggested previously that decreased expression of claudin-7 may predispose the RE to hyperkeratosis and parakeratosis, especially, when proliferative stimuli are present simultaneously (Greco et al., 2018). Such thesis is reinforced from a study on neoplastic cell models, in which claudin-7 expression was linked to a reduction of cell proliferation and increased cell adhesion (Lu et al., 2015). In contrast to claudin-7, occludin is expressed in all 'living' cell layers of the RE from the *stratum basale* to the *stratum granulosum* (Greco et al., 2018; Meissner et al., 2017; Stumpff et al., 2011). The additional upregulation of occludin mRNA expression in epithelia incubated with higher concentrations of GLP-2 may possibly indicate an increased requirement for junction molecules in the lower strata of the RE due to the proliferative action of GLP-2, as deductible from the proliferative action of GLP-2 in porcine intestine (Sigalet et al., 2014) and the intestine of feed-restricted cows (Kvidera et al., 2017).

Considering that mRNA changes were not followed by changes in TJ protein expression, one may assume that either the time frame of 7 hr GLP treatment was insufficient to initiate changes in TJ protein expression or that additional signals (Yang, Rao, & Wang, 2014) are required to translate the mRNA signals into changes of TJ protein expression. Due to its positive effect on the mRNA expression of occludin, GLP-2 appears to carry a potential to elicit beneficial effects on the EB in RE, albeit the circumstances that translate this into functional protein are currently unknown and despite the risk of parallel induction of parakeratosis via downregulation of claudin-7. Such beneficial effects of GLP-2 have been proposed previously for the development of the EB in the rumen in growing ruminants (Gorka et al., 2011) and could possibly apply to feed adaptation in later life, although studies in vivo clearly indicate that the level of feeding has more prominent effects on the intestinal GLP system (Taylor-Edwards et al., 2010). To further evaluate this, future experiments should test the effect of GLP hormones in premature RE and under challenge conditions. The latter acknowledges that both GLP-1 and GLP-2 display their beneficial effects on intestinal epithelia most prominently under stress conditions like inflammation (Insuela & Carvalho, 2017; Nakame, Kaji, Mukai, Shinyama, & Matsufuji, 2016) and feed restriction (Kvidera et al., 2017; Sigalet et al., 2014).

The effects of GLP-1 and, especially, GLP-2 observed in the present study apparently bear some overlap with the effects of SCFA observed in one of our earlier studies. For example, luminal application of 30 mM butyrate also induced a decrease in I_{sc}, an increase in claudin-1 mRNA expression and a decrease in claudin-7, albeit the latter at the protein level (Greco et al., 2018). It is also accepted that GLP-1 and GLP-2 are released in response to short-chain fatty acids in ruminants (Elsabagh, Inabu, Obitsu, & Sugino, 2017; Fukumori, Mita, Sugino, Obitsu, & Taniguchi, 2012). It can be excluded, however, that the effects of SCFA observed in our previous study were, in part, mediated by GLP hormones. It has been clearly shown that RE has no to negligible mRNA expression of proglucagon (Pezeshki et al., 2012; Taylor-Edwards et al., 2010) and glucagon immune-reactive cells are absent (Bunnett & Harrison, 1986). As such, any GLP-1 or GLP-2 acting on the RE has to be of distant, most likely, intestinal origin (Taylor-Edwards et al., 2010).

With regard to EGF, no changes in electrophysiology were observed during the 7-hr incubation period and, except for the last hour of incubation, no changes were observed for J_{fluor}. Based on the fact that TJ protein expression and G_t were unchanged, we take this as indication that paracellular barrier function was not altered by EGF as extrapolated earlier for GLP-1 and GLP-2. Nevertheless, EGF influenced the mRNA expression of TJ proteins in this study. It appeared that incubation with 0.25 nM of EGF on the mucosal side and 2.5 nM of EGF on the serosal side had comparable effects that were dissimilar to the effects after mucosal application of 2.5 nM EGF. Mucosal application of 0.25 nM EGF and serosal application of 2.5 nM EGF increased or tended to increase claudin-1 mRNA expression and very prominently decreased claudin-7 mRNA expression. At variance, mucosal application of 2.5 nM EGF had no effect on claudin-1 mRNA expression, only moderately decreased claudin-7 mRNA expression and decreased claudin-4 mRNA expression. Previous studies had already demonstrated that EGF may differently affect

the TJs of the same epithelium when changing the epithelial side of growth factor application (Chen, Solomon, Kui, & Soll, 2002; Xiao et al., 2011). One possible explanation for the similar effects of mucosal application of 0.25 nM EGF and serosal application of 2.5 nM EGF in the present study could be that EGF receptors are located primarily on the mucosal (i.e. luminal) side of the RE to bind EGF secreted with saliva (Onaga et al., 2006). In such concept, the similarity of the effect of high-dose serosal EGF with low-dose mucosal EGF could be explained by spill-over of serosally applied, high-dose EGF to the mucosally located receptors. The effect of high-dose (2.5 nM) mucosal EGF is less clear because it ameliorated the decrease in claudin-7 mRNA expression, abolished the stimulation of claudin-1 mRNA expression and, alternatively, caused a decrease in claudin-4 mRNA expression. In support of our findings, however, an increase from 1.6 to 3.3 nM EGF had also reversed the stimulating effect of EGF on transepithelial resistance in previous studies on bronchial epithelium (Xiao et al., 2011). Steele et al. (2015) have concluded from their results that ruminal EGF receptors are downregulated upon sustained exposure to increased EGF concentrations, which could provide one possible explanation for the reversal of EGF effects at very high EGF concentrations.

In the published literature, the role of EGF is mainly seen in a protection of the EB against stressing conditions rather than directly improving the EB (Banan et al., 2003; Basuroy, Seth, Elias, Naren, & Rao, 2006; Guntaka, Samak, Seth, LaRusso, & Rao, 2011; Koepke et al., 2015; Okuyama et al., 2007; Sheth, Seth, Thangavel, Basuroy, & Rao, 2004). It often prevented or decreased the detrimental effect of micro-organisms on the epithelial barrier (Buret, Mitchell, Muench, & Scott, 2002; Kaur, Vaishnavi, Ray, Singh, & Kochhar, 2014; Lamb-Rosteski et al., 2008). EGF does so by stimulation of several intracellular pathways involved in TJ modulation. The MEK/ERK is a frequently cited pathway involved in the TJ modulation after EGF stimulation (Grande et al., 2002; Ikari, Takiguchi, Atomi, & Sugatani, 2011). In other cases, the TJ adaptation was related to PKC, PIK-3, src and STAT signalling (Garcia-Hernandez et al., 2015; Wang et al., 2006; Yoshida et al., 2005). Few studies also reported negative effects of EGF on the EB (Soler, Laughlin, & Mullin, 1993), including the EB of epidermal keratinocyte cultures (Tran et al., 2012). As such, the negative effect of EGF on claudin-7 mRNA and, at very high luminal concentrations, also on claudin-4 mRNA may suggest that EGF may possibly contribute to a weakening the RE barrier under certain conditions in vivo. In this regard, it is interesting to note that the RE down-regulates EGF receptors during the transition to high-energy diets after parturition of dairy cows (Steele et al., 2015).

5 | CONCLUSION

The present study evidenced that a 7-hr exposure to GLP-1, GLP-2 and EGF had no effect on G_t and on the expression of TJ proteins in RE isolated from healthy sheep on a hay-based, low-concentrate diet. This indicates that the paracellular EB of RE was functionally not affected under the experimental conditions, although very high serosal concentration of GLP-1, GLP-2 and EGF increased J_{fluor} in the 7th hour of incubation; the latter may likely be attributable to increased transcellular passage of this permeability marker. Despite no measurable paracellular barrier changes, GLP-2 and EGF increased occludin and claudin-1 mRNA expression respectively, which may be seen as a potential to contribute to barrier stabilization under certain, currently unknown circumstances. However, all tested application modes and concentrations of GLP hormones and EGF decreased the expression of claudin-7 mRNA, which may potentially predispose to disturbed function of the *stratum corneum*, that is, hyperkeratosis and parakeratosis, if translated into similar changes in claudin-7 protein expression. Together with the literature finding that RE downregulate the expression of EGF receptors upon transition to highly fermentable diets, our results suggest that one cannot simply postulate beneficial versus detrimental effects of the tested hormones and EGF on the RE barrier. Further studies are necessary to test the effects under several different conditions and possibly in combination with other endocrine or metabolic signals.

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ANIMAL WELFARE STATEMENT

All procedures were conducted in compliance with the German legislation on the welfare of experimental animals and communicated with the local authorities, the State Office for Health and Social Affairs Berlin (LAGeSo, Registration No. T 0360/12).

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4. Discussion

The focus of these studies was to test molecules which may potentially influence the epithelial barrier (EB) of the rumen epithelium (RE) by either improving the barrier, like EGF, GLP-1, GLP-2, or impairing it, like SCFA associated with low pH environment. The obtained results offer new information about the complex and rarely studied mechanisms regulating the EB of the cornified multilayer rumen epithelium. The first part of this study investigated the effect of individual SCFA in combination with two different mucosal pH values on rumen epithelia *ex vivo,* whereas the second part examined the effect of different concentrations of GLP-1, GLP-2, and EGF, using the same methodology under simulated physiological pH conditions. Part of the results of the first study demonstrated some potentially beneficial effects of the SCFA on the EB of RE incubated at physiological pH. These effects were limited to the mRNA expression, but they were not translated to corresponding protein expression. In the First Publication, the possibility was discussed that the experimental conditions, which were *ex vivo*, may have limited the translation of SCFA-induced changes from mRNA to protein whereas the *in vivo* circumstances might have probably shown a better correspondence between mRNA and protein changes. That may also be related to the limited incubation time. Also the absence of proteins, amino acids or a more effective or simply different clearance of cellular metabolites may also be considered as limiting factors in *ex vivo* experiments. The Ussing chamber technique cannot completely simulate the close contact between epithelium and blood circulation under *in vivo* conditions. A longer incubation time and the introduction of amino acids in the buffer solutions could be considered as points of improvement for possible new experiments trying to demonstrate the effects of hormones and growth factors on the EB of the RE. Similar considerations could be addressed to the results obtained in epithelia incubated with GLP-1, -2, and EGF in the Second Publication. GLP-2 and EGF increased the mRNA expression of the TJ proteins occludin and claudin-1 without affecting their respective protein abundance and therefore the epithelial barrier permeability. In the literature on this subject, it is reported that different correlations between mRNA and protein expression are not unusual and can be often ascribed to mechanisms involved in the translational regulation, to different protein half-lives *in vivo*, and to experimental errors (Nie et al., 2006). According to some authors, the discrepancy between the mRNA and protein expression may occur so often that a linear relation between the two expressions cannot be reliably assumed (Mackay et al., 2004). On the other hand, other authors suggest to use the mRNA expression as a reliable tool for the prediction of protein expression (Guo et al., 2008). Accordingly, it is possible to use the results on the regulation of mRNA expression of TJ proteins in the present work for further discussion on the mechanism involved in the EB modulation in the RE. As already mentioned in the Introduction chapter, TJ proteins forming the EB may be regulated at transcriptional

and at post-translational level. The post-translational modifications consist of covalent alterations of a single or more amino acids of the TJ proteins (Kumar & Prabhakar, 2008). Therefore, only the mechanisms involved in the transcriptional regulation can be considered to explain the mRNA upregulation in the experiments on the effect of EGF and GLP-2 on the EB of RE. In the First Publication on the effect of SCFA on the EB, the expressions of the TJ proteins were modified either at mRNA or at protein level. In this case, post-translational regulations may also be taken into account.

The studies under *ex vivo* conditions have the advantage of testing the effect of single molecules on the epithelial barrier of rumen epithelia in a strongly controlled environment. This minimizes the influence of other disturbing factors, which are inevitable in *in vivo* studies. The Discussion chapters of the two publications elucidated the relevance of the results and related these to the current literature. These discussions put forward new useful perspectives on the complex mechanisms regulating the EB of the RE. At this stage, it is useful to review the results of the two studies as a part of comprehensive discussion on the mechanisms involved in the regulation of the EB from another perspective. The results of the First Publication on the effect of SCFA on the EB, showed an increase of claudin-1 mRNA expression after incubation with 100 mM acetate, 30 mM propionate, and 30 mM of butyrate and an increase of claudin-4 mRNA expression after incubation with 30 mM propionate and 30 mM butyrate at the same pH of 6.1. The upregulation of claudin-1 and claudin-4 mRNA was ascribed to the activation of the transcriptional mechanisms by an SCFA-mediated inhibition of the histone deacetylase. In the Second Publication on the effect of GLP-1, -2, and EGF on the EB, the mRNA expression of claudin-1 was also upregulated after incubation with 2.5 nM EGF on the serosal side, while claudin-4 mRNA expression was downregulated after incubation with 2.5 nM EGF on the mucosal side. The Discussion chapter in the Second Publication referred to some of the intracellular pathways that could be potentially activated in different cell lines after EGF incubation. Given this data, it is still possible to discuss the mechanisms that may potentially mediate claudin-1 and -4 mRNA transcription after stimulation of an SCFA or SCFA-induced histone deacetylase inhibition; about the mechanisms leading to GLP-2-induced occludin mRNA upregulation, and about the EGF-triggered intracellular pathway that may be taken into account for the regulation of claudin-4 and -7 mRNA expression.

4.1. SCFA pathways

In monolayer models, SCFA have mainly displayed beneficial effects on the EB and these effects were attributed to the histone deacetylase (HDAC) inhibitor activity of SCFA, which

are known to suppress inflammatory reactions. How HDAC inhibition improves the EB has not been discussed in the First Publication. In a study in Caco-2 cells, Feng et al. (2018) suggested that SCFA, thanks to their HDAC inhibitor activity, repressed the LPS and ATP-induced NLRP3 inflammasome. As result of that repression, SCFA protected the epithelial barrier and avoided the downregulation of the TJ proteins occludin and claudin-1 (Feng, et al., 2018). In contrast, in the gingival epithelium it was found that the ATP-induced NLRP3 inflammasome enhanced the gene expression of claudin-1 and decreased the gene expression of occludin in the early stage of activation. This was interpreted as a defense reaction of the cells. However, the ATP-induced NLRP3 inflammasome decreased the protective capacity of the EB and increased the bacterial invasion subsequently (Guo et al., 2018). Unfortunately, very little is known about the expression and role of the NLRP3 in ruminant animals and in the rumen epithelium. The few studies focusing on NLRP3 in ruminants were concerned with the relation between these inflammasome components and the efficacy of the immune system to react against bacteria and parasite infections (Vrentas et al., 2018). Other molecules were also proposed as mediators of SCFA effects on the EB. In a study on Caco-2 cells, SCFA, with butyrate in first line, improved the EB by activating three isoforms of lipoxygenases. Their activation was triggered by SCFA-mediated HDAC inhibition (Ohata et al., 2005). In another study on Caco-2 cells, butyrate improved the epithelial barrier function but without stimulating the transcription of TJ proteins. In this case, it triggered TJ assembly and induced TJ reorganization (Peng et al., 2009). According to Wang et al. (2012), sodium butyrate improves claudin-1 transcription by facilitating the interaction between the SP1 transcription factor and claudin-1 promoter in cdx2-IEC monolayers (Wang et al., 2012). In line with this finding, some authors showed that an Sp1-induced upregulation of claudin-4 was in parallel with high histone acetylation in ovarian cancer cells (Honda et al., 2006; Günzel & Yu, 2013). In IPEC-J2 cells, butyrate upregulated claudin-4 expression and decreased the epithelial permeability. The authors suggested that the akt/mTor signaling pathway was the mechanism mediating the transcriptional activation. In the same study, they also detected increased intracellular concentration of ATP after butyrate treatment (Yan & Ajuwon, 2017). In an *in vivo* study, gastric SCFA administration increased claudin-1 mRNA expression in jejunum and claudin-1 and occludin mRNA expression in duodenum and ileum of piglets. Along with that, gastric SCFA administration upregulated the mRNA expression of GLP-2 in jejunum, GLP-2R in jejunum and colon, and EGF in colon (Diao et al., 2019). However, it is known that GLP-2 is not synthesized in the rumen epithelium, thus any involvement of GLP-2 was rejected in the discussion on the effects of SCFA in the First Publication. A similar assumption can be made for EGF. Even though it was detected in many body fluids (Zeng & Harris, 2014), there is no evidence supporting its expression in the rumen epithelium.

4.2. GLP-2 and GLP-1 pathways

GLP-2 was shown to increase occludin and claudin-1 expression in cultured cells derived from the jejunum of piglets. The authors suggested that the intracellular signaling pathways triggering this transcription was the MAPK kinase pathway (Yu et al., 2014). In stressed IPEC-J2 cells, the same authors discovered that GLP-2 increases the mRNA and protein expression of occludin and claudin-1 by activating the PI3K/Akt/mTOR pathway (Yu et al., 2016). These results suggest that claudin-1 upregulation induced by GLP-2 may be mediated by different intracellular pathways in specific intestinal cell lines. It is known that GLP-2 receptors (GLP-2R) are mainly expressed in the intestine, but their distribution in this organ is mostly limited to fibroblasts, endocrine cells, and enteric neurons. In contrast, enterocytes and crypt epithelium, which are the cells forming the epithelial barrier in the small intestine (Schellekens et al., 2017), show no expression of GLP-2R (Yusta et al., 2000; Dong et al., 2014). This suggests that the effect of GLP-2 in the intestine may be mediated by other substances (Dubé et al., 2006; Dong et al., 2014). In jejunal epithelia of mice treated with GLP-2, some authors reported an increase of claudin-7 expression. Of note is that not only the upregulation of claudin-7, but also the GLP-2-induced restoration of the barrier function were partially mediated by IGF-1 receptors (IGF-1R) (Dong et al., 2014). This suggests a role of IGF-1R in the GLP-2-mediated effects on the EB. IGF-1R are expressed and functional in the rumen epithelium (Shen et al., 2004), therefore, a comparison with the intestinal barrier may be appropriate. Similar speculations may be proposed in the rumen epithelium. These may offer new explanations for the results of the present study, particularly, on the increase of occludin mRNA expression after GLP-2 incubation. Further studies would be useful to demonstrate the effects of IGF-1 on the epithelial barrier in the first place and later to investigate possible relations between GLP-2 and IGF-1R in the RE.

The results obtained after incubation with GLP-1 differed from the results obtained after incubation with GLP-2. As described in the introduction, GLP-1 shows many similarities with GLP-2, but these were not confirmed by the results of the present study. In the literature, it was reported that GLP-1 has been shown to increase TER and occludin expression in endothelial cell cultures of rat brain by activation of the cAMP/PKA signaling pathway (Fukuda et al., 2016; González-Mariscal et al., 2018). However, this was not confirmed in the present study, where GLP-1 did not affect the EB of the RE and it also did not increase the mRNA expression of any TJ proteins. The only change induced by GLP-1 in the present study was the downregulation of claudin-7 mRNA expression.

4.3. EGF pathways

In comparison to GLP-1, the literature on the effects of EGF on the EB is more abundant, but the role of EGF in TJ modulation appears more complex rather than clear. A study on ovarian cancer cell lines was in line with results on the RE in the Second Publication. In that study, EGF downregulated claudin-4 expression by activating the MEK/ERK or PI3K/Akt signaling pathway (Ogawa et al., 2012). In contrast to this, EGF more frequently induced upregulation of claudin-4 in different cell lines. In MDCK monolayers, it upregulated claudin-4 at transcriptional level by activating the Src and STAT3 signaling pathways (García-Hernández et al., 2015). In another study also in MDCK cell lines, EGF improved the expression of claudin-4, but not claudin-1 by activating Sp1, which was, in turn, triggered by the MEK/ERK signaling pathways (Ikari et al., 2009). Most of the experiments studying the effect of EGF on the epithelial barrier were performed on challenged epithelia and they were focusing on EGF's ability to prevent epithelial barrier failure and to decrease the loss of TJs. In such circumstances, different signaling pathways were involved in the TJ regulation: Ras/MAPK, PI3K/AKT, PLC-γ/PKC, and STAT (Tang et al., 2016). On the other hand, EGF may also exacerbate the harmful effects of some noxious agents on the epithelial barrier (Heijink et al., 2010). Signaling pathways, like ERK, activated by EGF may produce opposite effects on the barrier activity (Lechner et al., 2007). In some cases, such contrasting effects may be related to a different response between differentiated and undifferentiated cells on the ERK-mediated EGF effects (Aggarwal et al., 2011). Such a hypothesis may fit well in the cornified multilayer epithelium of the rumen and explain the differential TJ adaptation after applying the same dose of EGF on the two opposite sides of the epithelium. From the current literature on the effects of EGF on the EB, it is possible to hypothesize that the downregulation of claudin-4 mRNA in the RE may result from a modulation of the MEK/ERK signaling pathway, also known as Ras-Raf-MEK-ERK pathway, or alternatively from the PI3K/Akt signaling pathways (Ogawa et al., 2012). These two signaling pathways together with protein kinase C (PKC) are the most commonly studied intracellular pathways involved in the EB regulation. Hence, these could be potential candidates for further investigations into the intracellular regulatory mechanisms in the RE. In hTERT-HPDE cells, PKC modulated the TJ proteins claudin-1, -4, -7, and occludin differently. In these cells, claudin-4 was specifically regulated by PKC-α, claudin-7 and occludin by PKC-δ, while the specific regulation of claudin-1 was not clearly elucidated. PKC regulation of claudin-7 in these cells was mediated by ELF3 (Yamaguchi et al., 2010). Analyzing the results of the latter, but also of different studies in the literature cited in this Discussion chapter, it is possible to extrapolate that different intracellular signaling pathways may run in parallel to each other, activating cascades targeting the same gene.

4.4. Claudin-7 regulation

The TJ protein claudin-7 was downregulated at the protein level in epithelia treated with 30 mM of butyrate at pH 6.1. Immunohistological investigations confirmed the decrease of claudin-7 in these epithelia but also in epithelia incubated with 100 mM acetate. However, claudin-7 protein expression was not changed in epithelia incubated with 100 mM acetate and claudin-7 mRNA expression was not decreased in both treatment groups. The *stratum corneum* of the epithelia of both treatment groups appeared more compact in comparison with those of the remaining treatment groups. Together with decreased abundance of claudin-7 this may promote increased exfoliation rate of the cells in the *stratum corneum* in these epithelia. A possible explanation for this outcome may be the result of altered differentiation of the REB under high concentration of butyrate and acetate which leads to negative post-translational claudin-7 modification. This TJ protein could work as a stabilizing factor for the cornified cell layers to support cell adhesion. The absence of this TJ protein could potentially predispose the epithelium to the development of parakeratosis and hyperkeratosis (Greco et al., 2018). In the Second Publication, claudin-7 mRNA expression, but not protein expression, was decreased in all epithelia treated with GLP-1, GLP-2, and EGF at all tested concentrations. MAPK and PKC pathways appear mostly involved in the transcriptional regulation of claudin-7 in the literature. It was reported that claudin-7 transcription may be regulated by the E74-like factor 3 (ELF3) which is a member of the E26 transformation-specific sequence (ETS) family. The proteins of this family typically regulate cell proliferation, differentiation, and transformation, and they are regulated by the MAPK (Khan & Asif, 2015). In hTERT-HPDE cells, PKC was involved in the regulation of claudin-7 at the transcriptional level (Yamaguchi et al., 2010). A possible explanation for the decrease in claudin-7 mRNA expression obtained in the epithelia treated with GLP-1, -2, and EGF may be extrapolated from a study on the epithelium-mesenchyme transition. In this study on different cell lines of mice, it was found that Snail, a transcription factor activating the epithelium-mesenchyme transition, directly linked the E-boxes of the claudin-7 gene promoter and repressed the transcription. That resulted in downregulation of claudin-7 (Ikenouchi et al., 2003). Epithelium-mesenchyme transition consists of a phenotype modification of polarized cells that increases their migratory capacity, invasiveness, and resistance to apoptosis (Kalluri & Neilson, 2003). Such phenotype change occurs under different physiological and pathological conditions, which are also involved in wound healing, tissue regeneration, and organ fibrosis (Kalluri & Weinberg, 2009). Epithelium-mesenchyme transition (EMT) may be activated by EGF (Kalluri & Neilson, 2003), GLP-1 (Chen et al., 2019), by GLP-2 mediated kinases (Yusta et al., 2002; Vijay et al., 2019), and other growth factors (Kalluri & Neilson, 2003). There is no available information about the EMT in the RE, but it could be speculated that a similar cell behavior may explain the transcriptional

repression of claudin-7 in epithelia incubated with EGF, GLP-1, and GLP-2. This is in line with the assumption that claudin-7 functions as a stabilizing factor in the *stratum corneum*. In a study on human breast cancer cells, it was found that HDAC1 mediated the SNAI1P-induced repression of claudin-7 gene promoter and the SNAI1P-induced repression of claudin-7 gene promoter disappeared in the presence of HDAC1 inhibitor trichostatin A. SNAI1P is a human homologous of SNAIL classified as a retro-transcribed gene (Mittal et al., 2010). As previously reported, SCFA act as HDAC inhibitors. In the Second Publication, SCFA were used at a concentration of 30 mM in all mucosal incubation solutions. Nevertheless, the SCFA did not prevent the downregulation of claudin-7 mRNA in epithelia treated with EGF, GLP-1, and GLP-2. More information about claudin-7 regulation may lead to a better understanding of the mechanisms behind the development of parakeratosis in the rumen epithelium.

4.5. Interaction of SCFA and low pH level

The results of the First Publication on the effect of SCFA at low pH showed a marked increase of epithelial permeability associated with decreased expression of TJ proteins in epithelia incubated with single SCFA at low pH, but not in epithelia incubated at low pH without SCFA. The pathophysiological mechanisms triggering the outcomes of these experiments were elucidated in the Discussion chapter of the First Publication. The Discussion chapter reported that the detrimental effects of SCFA at low pH on the EB of RE are independent of the quality, but are dependent on the quantity of SCFA dissolved in the incubation solution. Confirming the outcomes of a previous publication by Meissner et al. (2017), the results of the First Publication showed that the deleterious effects on the RE were triggered by the combination of low pH and SCFA, whereas low pH alone is rather well tolerated by the RE. This assumption was based on the G_t and I_{sc} values together with the fluorescein flux rates and protein expressions of the TJ proteins observed in the experiments. The results suggested that the combination of these two factors, that is, low pH and SCFA, enhances the harmful potential of the two factors to induce EB failure in the RE. As an explanation for these results, the Discussion chapter of the First Publication reported the weak acid carrier model of Gutknecht (Gutknecht, 1987) in which according to the Henderson–Hasselbalch equation, the low pH increases the ratio of protonated SCFA. These are free to cross the cell membranes and passively penetrate inside the cells, where they dissociate again because of the higher intracellular pH. In this way, SCFA act like a carrier of H^+ in a “horse troy” mechanism, which leads to an increase of free intracellular H^+ that, in turn, decreases the cytoplasmic pH. As previously mentioned, this passive mechanism of SCFA absorption works in parallel with the $SCFA^-/HCO3^-$ exchanger and both mechanisms

lower the intracellular pH (Aschenbach et al., 2011). At this point, there are not many explanations about the intracellular adaptations following this condition. The ruminal cells have to cope with extraordinary intracellular concentration of H^+ and $SCFA^-$, but at the same time, some mechanisms start to dismantle the TJ proteins and lead to EB failure. No attempts have been made to elucidate whether the TJ downregulation is a direct consequence of excessive intracellular H^+ accumulation or if it is a result of specifically induced intracellular signaling mechanisms. In a review of the effects of gastric reflux on the esophageal epithelium, it was reported that the esophageal epithelium appeared particularly resistant to low pH environment in the absence of pepsin and organic acids. In such a scenario only a pH of 1.0-1.3 was able to disrupt the esophageal epithelium (Vaezi et al., 1995). On the other hand, the esophageal mucosa was easily damaged by the combination of low pH and pepsin or bile acids. It is worth noting that the mechanisms described for the bile acids are partially comparable with the SCFA behavior that was discussed in the First Publication. The conjugated acids were more detrimental at lower pH while the unconjugated acids were more harmful at higher pH (Vaezi et al., 1995). At low pH, conjugated acids were able to cross the cellular membrane more efficiently than non-conjugated acids (Nehra et al., 1999). The harmful effects of low pH alone on the mucosa were tested in the esophagus of the mouse. The injuries seemed to be mediated directly from the H^+ ions, which inhibit the Na^+/K^+-ATPase in the basolateral membranes of the cells in the *stratum spinosum* of the epithelium. This finally causes an increase of cell volume which, in that case, ended with the death of the cells (Orlando et al., 1984; Vaezi et al., 1995). Other authors studying patients suffering with gastrointestinal reflux disease also suggested that after low pH distress, cell dysfunctions originate in the more basal layers of the esophageal epithelium (Blevins et al., 2018). In the ruminal epithelium, amiloride-sensitive Na^+/H^+ transporters (NHE) are also present (Martens & Gäbel, 1988). These are involved in the regulation of the intracellular pH, in the regulation of fluid balance, cell volume, and transcellular absorption of NaCl (Müller et al., 2000; Lu et al., 2016). At least three isoforms of these transporters have been discovered in the rumen epithelium (Schweigel et al., 2005). NHE distribution is generally tissue and isoform-specific (Lu et al., 2016). In the rumen epithelium NHE was detected from the stratum basale to the stratum granulosum (Graham et al., 2007; Rabbani et al., 2011). This epithelium normally upregulates its NHE expression in the case of increased H^+ and SCFA concentration (Yang et al., 2012) in order to increase the exchange of intracellular H^+ with extracellular Na^+ and in order to maintain an optimal intracellular pH (Uppal et al., 2003). According to that, the intracellular H^+ concentration is crucial for the SCFA-mediated regulation of Na^+ influx (Lu et al., 2016). Once it is transported from the luminal side of the rumen to the cytoplasm of the ruminal cells, Na^+ is transferred to the blood circulation by the Na^+/K^+-ATPase, which is localized mostly in the basal layers of the RE (Graham & Simmons,

2005). In light of this, it is possible to speculate that a similar dysfunction of the Na^+/K^+-ATPase and NHE, as previously reported by Orlando et al. (1984), in the esophagus of the mouse, may also occur in high H^+-stressed ruminal epithelial cells of ruminant animals. Such a mechanism would potentially lead to an increase in volume of the ruminal cells and finally death. However, as it is possible to see from the immunocytochemistry pictures in the First Publication, the ruminal cells of epithelia incubated with different SCFA at different pH show a size comparable with the size of the ruminal cells of control epithelia. However, as we did not systematically measure the volumes of the cells, further speculation cannot be formulated.

Another mechanism that could play a role in the TJ disruption upon SCFA challenge at low pH may be the activation of specific intracellular signaling pathways. In human bronchial epithelial cells, it has been demonstrated that extracellular acidic stress may trigger TRPV (transient receptor potential vanilloid) 1, a non-selective cation channel-dependent signaling pathway, which leads to a rapid increment of intracellular Ca^{2+}, causing downregulation of claudin-4, but not claudin-1 and -7 (Xu et al., 2013). TRP channels have been proposed as channels for NH_4^+, K^+, Na^+, and Ca^{2+} in the rumen epithelium (Rosendahl et al., 2016; Schrapers et al., 2018), but not much is known about the role of these channels in the case of low ruminal pH. In the esophageal mucosa of patients suffering with gastrointestinal reflux disease, in which increased paracellular permeability is often present, TRP channels have been found upregulated (Blevins et al., 2018). In epithelial cells of the rat, it was found that extracellular acidosis may activate TNF-α, but also decrease the expression of MCP-1, iNOS, and COX-2 (Riemann et al., 2020). TNF-α is a pro–inflammatory cytokine which is able to induce EB failure and TJ disruption (Wang et al., 2005). Esophageal adaptations could be considered as a model for the rumen epithelium because of the embryologic relationship between the two organs. Different cell cultures incubated at low pH have been shown to slow down growth until a complete stop of all cellular activity (Taylor 1962). Comparable effects have been demonstrated in lung epithelial cells under acid stress. In these cells the reduction of viability was associated with an increase in the MAPKs signaling pathway and an increase in cell death rate (Chen et al., 2013). TRP channels are not the unique proteins sensing the extracellular pH. Other proteins with a comparable function are the acid sensing ion channels (ASICs) and a family of proton-sensing G protein-coupled receptors (GPCRs) (Sanderlin et al., 2015). The ASICs are mostly studied in the nervous system and no information is currently available in the RE. The proton-sensing GPCRs have been detected in RE and there they were also able to activate the MAPKs signaling pathway (Kim et al., 2013; Lu et al., 2015). It is known that the activation of the MAPKs signaling pathways may lead to epithelial barrier failure (Wang et al., 2004), whereas its inhibition has been shown to improve TJ expression in MDCK cells (Chen et al., 2000). However, before

speculating about it, an involvement of the MAPKs in the regulation of the EB in the RE still needs to be proven. Moreover, the MAPK signaling pathway may affect the TJ of different epithelia differently and in some cases, as previously reported, it may also improve the TJ protein expression (Yu et al., 2014; Kim & Breton, 2016). In human proximal tubular cell line, HK2, and in human umbilical vein endothelial cells (HUVECs), it was demonstrated that acidosis activates the G protein-coupled receptor 4 (GPR4) (Dong et al., 2018). The activation of GPR4 under low pH condition induces cAMP accumulation in kidney cells. The intracellular cAMP accumulation, in turn, influences the acid-basic transporters of the cells leading to decreased extrusion of intracellular H^+ (Dong et al., 2018). In the rumen epithelium of goat, GPR4 and GPR41 were detected and both were responsive to the SCFA concentration (Lu et al., 2015). The experiments of the First Publication did not explain the intracellular steps leading to the downregulation of TJ proteins in RE incubated with SCFA at low pH. Further studies may be useful to determine which mechanisms are involved. The understanding of the intracellular signaling pathways mediating the results may explain whether the TJ protein depletion in the epithelia results from a posttranslational mechanism, from a protease induced cleavage (Nava et al., 2013), or from a combination of these.

4.6. Conclusion

Different mechanisms and pathways have been taken into consideration in order to explain the results of the present study. A better understanding of the mechanisms regulating the synthesis, regulation and disassembly of the TJ forming the EB of the RE may offer the basis for new efficient nutritional and treatment strategies to protect the RE and improve livestock production. The results in the First Publication showed that the combination of low pH and one of the three main SCFA impaired the EB and depleted the TJ proteins to a comparable degree, even though the cellular transport and metabolisms of acetate, propionate, and butyrate in ruminal cells, as previously reported in the Introduction chapter, are dissimilar (Bergman, 1990). As reported in this study, this suggests that the EB failure in epithelia treated with single SCFA at 30 mM concentration, was most probably a consequence of the high H^+ load introduced with the SCFA inside the cells. The increased damage caused by increasing the concentration of acetate from 30 to 100 mM supports this interpretation. Therefore, the conclusion is that individual SCFA similarly damage the epithelial barrier at low pH environment. On the other hand, their molar proportion and the pH environment influence the harmful effect of the SCFA on the EB. In contrast to the results for the treatment groups at pH 5.1, rumen epithelia incubated at pH 6.1 showed different mRNA and protein expression after incubation with single SCFA. Different mechanisms have been taken into account for the different results in the epithelia incubated at pH 6.1. However, these

changes did not affect the tightness of the EB in these experiments. The Second Publication did not show functional changes of the EB. However, it was the first investigation into the role of GLP-1, GLP-2, and EGF on the EB of the RE and it demonstrated a potentially protective function of GLP-2 and EGF on the EB. Further *in vivo* studies are necessary to confirm the results. This work offered several new insights into the physiology of the EB of the RE. It suggested new potential candidates for the regulation of the EB in the RE. It highlighted the importance of the relation between the TJ forming the paracellular pathway and the transporting mechanisms controlling the transcellular pathway in the RE. It showed for the first time an indication of the role of EGF and GLP-2 in the regulation of the EB of the RE.

5. Summary

Regulation of the epithelial barrier in the rumen epithelium of sheep by incubation with single short-chain fatty acid at different pH, with glucagon-like peptide 1, glucagon-like peptide 2, and epidermal growth factor

The rumen epithelium (RE) functions as an absorbing layer for short-chain fatty acids (SCFA) and at the same time as a protective interface between the blood circulation and the ruminal environment. These functions are strongly interconnected by the epithelial barrier (EB). A failure of this barrier compromises both absorbing and protective abilities and may lead to sickness of the animal and economic loss for the farmer. One of the main causes of EB failure is nutritional oversupply of easily fermentable carbohydrates, which leads to acute or subacute ruminal acidosis. Under these conditions, several changes occur in the rumen environment. One of these changes is the increase of SCFA, which is associated with a decrease of pH. In a previous study, it was demonstrated that the combination of low pH and high SCFA is necessary for impairing the EB of the RE and low pH alone did not impair the EB. In this study the three main SCFA were tested all together in a mix. The present study investigated whether the effect of individual SCFA at physiological and low pH lead to different changes of the EB of the RE. Rumen epithelia for Ussing chamber experiments were obtained from seven sheep. Epithelia were incubated 7 h with 30 mM of either acetate, propionate, or butyrate on the mucosal side at mucosal pH of 6.1 or at pH 5.1. Some extra epithelia were incubated with 100 mM acetate and tested under both pH conditions. Electrophysiological parameters and fluorescein flux rates were monitored during the incubation. Following incubation, mRNA and protein abundance of the TJ proteins claudin-1, -4, -7, and occludin were investigated, and TJ proteins were visualized using immunofluorescence staining. The results showed that SCFA incubation at physiological mucosal pH did not functionally affect the epithelial barrier. However, the results showed increased mRNA expression of some TJ proteins suggesting some protective effects of SCFA on the EB, but also a reduction of claudin-7 at protein level after incubation with 100 mM acetate and 30 mM butyrate. At pH of 5.1 all individual SCFA impaired to a comparable degree the EB and downregulated the TJ proteins. The group of epithelia incubated with 100 mM acetate caused more intense barrier failure. Epithelia incubated without SCFA at pH 5.1 showed no signs or very little evidence of barrier failure. This data suggests that the RE is extremely resistant to low pH environment. However, the combination of low pH with SCFA impairs the EB. Each SCFA caused a similar detrimental effect on the EB if incubated at the same concentration, while increasing their concentration augmented the harmful effect on the EB. The second study focused on molecules that may potentially strengthen the EB. According to literature, GLP-1, -2, and EGF usually improve the EB of other different

epithelia. Therefore, their effects on RE were studied in *ex vivo* experiments. RE were obtained from six sheep and incubated with two different concentrations of GLP-1, GLP-2, and EGF in Ussing chambers. GLP-1 and GLP-2 were incubated on the serosal side. EGF was tested at a higher concentration on the serosal side and on the mucosal side, while a lower concentration of EGF was also tested on the mucosal side. The results of this second study did not show functional effects on the EB from any of the molecules tested. However, some potentially beneficial effects were seen at the mRNA level after incubation with high concentration of GLP-2 and high concentration of EGF on the serosal side. An interesting result was also the downregulation of claudin-7 at mRNA level in all epithelial treated with GLP-1, -2, and EGF. The result suggests that GLP-1 may have no, or a minor, role in EB regulation of RE, while EGF and GLP-2 may potentially regulate and protect the EB of the RE. Further studies *in vivo* are needed to confirm these results.

6. Zusammenfassung

Die Regulation der epithelialen Barriere im Pansenepithel von Schafen durch die Inkubation mit kurzkettigen Fettsäuren bei verschiedenen pH-Werten, sowie mit Glucagon-like Peptide 1, Glucagon-like Peptide 2, und dem Epidermale Wachstumsfaktor

Das Pansenepithel (PE) resorbiert eine große Menge an kurzkettigen Fettsäuren (SCFA) und wirkt gleichzeitig als schützende Schnittstelle zwischen dem Blutkreislauf und dem Pansenmilieu. Diese Funktionen sind durch die epitheliale Barriere (EB) stark miteinander verknüpft. Ein Versagen dieser Barriere beeinträchtigt sowohl die Absorptions- als auch die Schutzeigenschaften und kann zur Erkrankung des Tieres und zu wirtschaftlichen Verlusten für den Landwirt führen. Eine der Hauptursachen für das Versagen der EB ist die ernährungsbedingte Überversorgung mit leicht vergärbaren Kohlenhydraten, die zu einer akuten oder subakuten Pansenazidose führt. Unter diesen Bedingungen treten mehrere Veränderungen in dem Pansenmilieu auf. Eine dieser Veränderungen ist die Zunahme von SCFA, die mit einer Senkung des pH-Wertes verbunden ist. In einer früheren Studie zeigte sich, dass das Zusammenwirken von niedrigem pH-Wert und kurzkettigen Fettsäuren entscheidend für den Beginn des Versagens der EB ist. In dieser vorigen Studie wurde eine Kombination der drei wichtigsten kurzkettigen Fettsäuren verwendet. Um herauszufinden, ob einzelne Säuren eine unterschiedliche Wirkung auf die Epithelbarriere haben, wurden in der vorliegenden Arbeit Pansenepithelien von sieben Schafen in Ussing-Kammern auf der mukosalen Seite mit Azetat, Propionat oder Butyrat bei einem pH-Wert von 6,1 oder 5,1 inkubiert. Während der Inkubation wurden die elektrophysiologischen Parameter und die

Fluorescein-Flussraten erfasst. Nach der Inkubation in der Ussing-Kammer wurden die mRNA- und Proteinexpression der tight-junction-(TJ)-Proteine untersucht und deren Lokalisation durch Immunfluoreszenzfärbung nachgewiesen. Die Ergebnisse ergaben, dass die SCFA-Inkubation bei einem pH-Wert von 6.1 keine funktionelle Wirkung auf die Epithelbarriere hatte. Als potenziell positiver Effekt wurden einige TJ-Proteine auf mRNA-Ebene hochreguliert. Allerdings erfolgte auch eine Reduktion von Claudin-7 auf Proteinebene nach Inkubation mit 100 mM Acetat und 30 mM Butyrat. Bei dem pH-Wert von 5,1 beeinträchtigten alle einzelnen inkubierten SCFA in ähnlicher Weise die EB und regulierten die Proteinexpression der untersuchten TJ-Proteine herunter. Bei Epithelien, die mit 100 mM Acetat inkubiert wurden, trat ein intensiveres Versagen der Barriere auf. Die Epithelien, die ohne SCFA bei pH 5,1 inkubiert wurden, zeigten keine oder nur sehr wenige Anzeichen einer Schädigung. Diese Daten deuten darauf hin, dass das PE sehr resistent gegenüber niedrigem pH-Wert ist. Die Kombination von niedrigem pH-Wert mit SCFA beeinträchtigt jedoch die EB. Einzelne SCFA bei derselben Konzentration verursachten ähnlich schädliche Effekte auf die EB, während eine Erhöhung ihrer Konzentration den schädlichen Effekt auf die EB verstärkte. In der zweiten Studie wurden Moleküle untersucht, die möglicherweise die EB verbessern könnten. Der Fachliteratur zufolge verbessern GLP-1, -2 und EGF in der Regel die EB verschiedener anderer Epithelien. Daher wurden deren Wirkung auf das PE untersucht. Pansenepithelien von sechs Schafen wurden in Ussing-Kammern mit GLP-1, GLP-2 und EGF bei zwei verschiedenen Konzentrationen inkubiert. GLP-1 und GLP-2 wurden auf der Serosalseite zugesetzt. EGF wurde bei höherer Konzentration auf der serosalen Seite und auf der mukosalen Seite getestet, wobei auf der mukosalen Seite auch eine niedrigere EGF-Konzentration getestet wurde. Die Ergebnisse dieser Studie zeigten bei keinem der getesteten Moleküle funktionelle Auswirkungen auf die EB. Allerdings wurde nach der Inkubation mit hoher Konzentration von GLP-2 und hoher Konzentration von EGF auf der serosalen Seite ein potenziell wirksamer Effekt auf der mRNA-Ebene beobachtet. Interessantes Ergebnis war auch die Herunterregulierung von Claudin-7 auf mRNA-Ebene in allen mit GLP-1, -2 und EGF behandelten Epithelien. Diese Ergebnisse deuten darauf hin, dass GLP-1 nicht oder kaum an der EB-Regulierung im PE beteiligt ist, während EGF und GLP-2 möglicherweise die EB des PE regulieren und schützen könnten. Weitere Studien *in vivo* sind notwendig, um diese Ergebnisse zu bestätigen.

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Selbstständigkeitserklärung / Declaration of independence

Hiermit bestätige ich, dass ich die vorliegende Arbeit selbstständig angefertigt habe. Ich versichere, dass ich ausschließlich die angegebenen Quellen und Hilfen in Anspruch genommen habe.

I hereby confirm that I have written this thesis independently. I certify that I have used only the sources and aids indicated.

Berlin 10.03.2021 Gabriele Greco

Publication list

Peer reviewed publications

- **Greco, G.**, Amasheh, S., Shen, Z.; Lu, Z. & Aschenbach, J.R. (2019). Effects of glucagon-like peptides 1 and 2 and epidermal growth factor on the epithelial barrier of the rumen of adult sheep. Journal of Animal Physiology and Animal nutrition. 103(6): 1727–1738. Doi: 10.1111/jpn.13200

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- **Greco, G.**, Deiner, C., Amasheh, S. & Aschenbach, J.R. (2016). Influence of GLP-1 and GLP-2 on the epithelial barrier of the ovine rumen.
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- **Greco, G.,** Deiner, C., Amasheh, S. & Aschenbach, J.R. (2016). Modulation of the expression of tight junction proteins in ovine ruminal epithelia after stimulation with different concentrations of insulin.
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- **Greco, G.**, Deiner, C., Amasheh, S. & Aschenbach, J.R. (2016):
  Effect of IGF-1 on the epithelial barrier of sheep rumen.
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Author contribution

1) Greco, G., Hagen, F., Meißner, S., Shen, Z., Lu, Z., Amasheh, S. & Aschenbach, J.R. (2018). Effect of individual SCFA on the epithelial barrier of sheep rumen under physiological and acidotic luminal pH conditions. Journal of Animal Science. 96: 126-142. Doi: 10.1093/jas/skx017

I declare that I performed the data analysis and statistical assessment for all experiments. I personally performed Western blot analyses and immunofluorescence investigations. I wrote the first draft of the whole article and was involved in further editing.

2) Greco, G., Shen, Z., Lu, Z., Amasheh, S. & Aschenbach, J.R. (2019). Effects of glucagon-like peptides 1 and 2 and epidermal growth factor on the epithelial barrier of the rumen of adult sheep. Journal of Animal Physiology and Animal Nutrition. 103: 1727-1738. Doi: 10.1111/jpn.13200

I declare that I designed, planned, prepared and performed all parts of the work. I personally coordinated the purchase of all chemicals and antibodies necessary for the analyses. I observed the general health of the animals used for the study. I slaughtered the animals according to the local legislation and collected the tissues. I personally performed Ussing chamber experiments, mRNA extraction and evaluation, cDNA production, RT-qPCR analysis, protein extraction and Western blot analyses. I performed the analysis of all the data and the statistical assessment. I wrote the first draft of the whole article and was involved in further editing.

3) Additional data collected

The results of the present work are just one part of a series of investigations that were carried out by the author of this thesis. During these studies, IGF-1 and insulin were also tested on the EB of the RE and claudin-17 and claudin-18 expressions were investigated in the RE. From the results obtained testing IGF-1, a new investigation started on the role of the IGF-1-induced intracellular pathways on the regulation of the EB of the RE. In this study the inhibitors wortmannin, 10-DEBC, SB 202190, PD98059 were tested. This last investigation was also accomplished. The results of these other studies will be used for future publications.

Berlin 10.03.2021 Gabriele Greco

“..*fatti non foste a viver come bruti, ma per seguir virtute e canoscenza*..”

Odysseus, 12th Century BC

in vv. 119-120, Canto XXVI, Inferno, Divina Commedia, AD 1320, Dante Alighieri