Bovine Rumen Epithelium Undergoes Rapid Structural Adaptations During Grain-Induced Subacute Ruminal Acidosis

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Abstract

Alterations in rumen epithelial structure and function during grain-induced subacute ruminal acidosis (SARA) are largely undescribed. In this study, four mature non-lactating dairy cattle were transitioned from a high forage diet (HF; 0% grain) to a high grain diet (HG; 65% grain). After feeding the HG diet for three weeks, the cattle were transitioned back to the original HF diet, which was fed for an additional three weeks. Continuous ruminal pH was measured on a weekly basis and rumen papillae were biopsied during the baseline and the first and final weeks of each diet. The mean, minimum and maximum daily ruminal pH were depressed (P<0.01) in the HG period compared with the HF period. During the HG period, SARA was diagnosed only during week 1 indicating ruminal adaption to the HG diet. Microscopic examination of the papillae revealed a reduction (P<0.01) in stratum basale, spinosum and granulosum layers, and total depth of the epithelium during the HG period. The highest (P<0.05) papillae lesion scores were noted during week 1 when SARA occurred. Biopsied papillae exhibited a decline in cellular junctions, extensive sloughing of the stratum corneum and the appearance of undifferentiated cells near the stratum corneum. Differential mRNA expression of candidate genes, including Desmoglein 1 and IGF binding proteins 3, 5 and 6, was detected between diets using qRT-PCR. These results suggest that the structural integrity of the rumen epithelium is compromised during grain feeding and is associated with the differential expression of genes involved in epithelial growth and structure.

Key Words; rumen, acidosis, epithelium, structure, adaptation
Introduction

It has become common to feed diets rich in rapidly fermentable non-structural carbohydrates (high grain) to increase feed conversion in intensive ruminant agriculture systems (46, 49). When ruminants are fed excessive levels of grain, rumen fermentation of carbohydrates can exceed the rate of ruminal absorption and buffering, causing an accumulation of short chain fatty acids (SCFA). When ruminal pH is depressed below 5.6 for more than three hours, the disease state of subacute ruminal acidosis (SARA) begins to develop (1, 33, 46). SARA is associated with major changes in the populations of rumen microflora (30), ruminal SCFA fermentation patterns, altered gastrointestinal function, feed intake, milk production and composition, and liver abscesses (53). The economic consequences resulting from poor performance and animal health have made SARA one of the most prevalent animal welfare issues in intensive ruminant production systems (64).

The ruminant forestomach is essential for the fermentation of structural carbohydrates and plays a critical role in the absorption of SCFA across the stratified squamous rumen epithelium (SSE) (15). Papillae protruding from the rumen wall greatly increase the surface area for absorption of SCFA which accounts for 75% of the metabolizable energy supply (8). The ruminal SSE is composed of four distinct strata with multiple functions (21, 63). Adjacent to the basal lamina are the stratum basale and spinosum which have functional mitochondria contributing to the metabolic properties of the ruminal SSE, primarily the production of ketones from SCFA (35). The stratum granulosum is adjacent to the stratum spinosum and is characterized by tight gap junctions termed desmosomes acting as the permeability barrier of the SSE (5, 16, 21). Desmosomes are composed of multiple proteins (desmosglein, desmocollin, desmoplakin, plakoglobin and plakophilin) which link intermediate filaments to sites of
intercellular adhesions ultimately to add mechanical strength to the epithelium (22, 25). The stratum corneum is in direct contact with the rumen milieu and consists of cornified keratinocytes commonly referred to as horn cells (34). It acts as a physical protective barrier between the rumen milieu and the lower viable strata (63).

The rumen has been shown to adapt to increasing levels of dietary grain by increasing the size of the papillae, thus maximizing the surface area for SCFA absorption (17, 18, 47). In sheep and cattle, increasing dietary grain or SCFA concentrations can rapidly increase ruminal SSE proliferation (18, 59) and morphogenesis (62). Of the SCFAs, butyrate is thought to stimulate cellular proliferation events (54) and inhibit apoptosis (41). In cattle fed high grain diets, it has been suggested that the rate of cellular aging decreases and the SSE can become parakeratotic (64). Parakeratosis and hyperkeratosis are commonly associated with high grain feeding and severely compromise SCFA absorption (11, 24). Ruminal acidosis can also cause erosion of the SSE, enabling translocation of rumen microbes into the portal bloodstream, thus causing liver abscesses (44). It has recently been shown that grain-induced SARA is associated with increased translocation of microbial lipopolysaccharides (LPS) from the rumen and intestine into the blood, causing levels of pro-inflammatory cytokines and acute phase proteins to become elevated in dairy cattle (13, 20, 31, 32). Despite these documented consequences of compromised SSE function, it remains unknown how SSE structure and function adapt during SARA.

Little research has been conducted to uncover the molecular mechanisms governing ruminal SSE adaptation to high grain diets. Candidate growth factors which have been shown to increase rumen epithelial cell proliferation in-vitro include insulin-like growth factor (IGF) and epidermal growth factor (EGF) (4). Both IGF and EGF induce their cellular response by binding to their respective cellular membrane receptors, thereby initiating a signal transduction cascade.
that turns on genes responsible for proliferation (27). Rumen papillae proliferation in goats fed high energy diets has been associated with increased plasma IGF-1 and IGF-1 receptor expression in rumen tissue (59). However, IGF signalling is modulated by six known IGF binding proteins (IGFBP) which play a critical role in epithelial proliferation and differentiation in many intestinal epithelial cell models (14). Testing the expression of these candidate genes is a first step in elucidating the molecular based mechanisms of ruminal SSE adaptation to high grain diets.

To develop a full understanding of this syndrome, it is necessary to characterize how ruminal acidosis and increased SCFA affect SSE histology and ultrastructure. The primary objective of this study was to characterize the micro-architectural adaptations of the rumen epithelium during SARA using light and electron microscopy imaging techniques for histological and ultrastructural analysis. The secondary objective was to characterize the expression of candidate genes, and to begin identifying molecular markers with structural and morphoregulatory properties that may be related to functional changes in ruminal SSE during SARA. We hypothesized that the structural integrity of the rumen epithelium would be compromised during SARA and associated with differential expression of genes associated with growth (IGF and EGF gene families) and structure (desmosome gene family).

**Materials and Methods**

**Management of Animals and Experimental Design**

The outline of the experiment design and treatments has been described by Steele et al (61). Briefly, four mature (9.3 ± 2.2 years, Age ± SD) rumen-cannulated, non-pregnant and non-lactating Holstein dairy cows (760 ± 30 kg, BW ± SD) were used in this experiment, conducted in the dairy barn at the University of Guelph (Guelph, Ontario). All experimental procedures
were conducted within the guidelines of the Canadian Council on Animal Care and approved by
the University of Guelph Animal Care Committee. Prior to the experiment, the cattle were fitted
with rumen cannulae (12), acclimatized to the experimental environment and consumed a high
forage (HF) diet consisting exclusively of chopped hay (90.6% dry matter, 114 g crude
protein/kg dry matter, 600 g neutral detergent fibre/kg dry matter, 173 g non-fibre
carbohydrate/kg dry matter, 69 g starch/kg dry matter; 1.91 mCal/kg dry matter) for 10 months.

Throughout the experiment, cattle were housed in a tie-stall facility and provided with
free-choice access to water and supplemented with a commercial mineral and vitamin premix
each day (0.02% of body weight). The duration of the experiment was seven weeks, during
which time the cattle were switched from the HF diet to a high-grain (HG) diet (35% chopped
hay and 65% mixed grain; 88.9% dry matter, 117 g crude protein/kg dry matter, 307 g neutral
detergent fibre/kg dry matter, 504 g non-fibre carbohydrate/kg dry matter, 409 g starch/kg dry
matter; 2.60 mCal/kg dry matter). The mixed grain consisted of 40% ground wheat, 40% ground
barley and 20% ground corn and was pelleted. The chopped hay portion of the diet was fed in
equal allotments daily at 0800h and 1600h and the mixed grain was fed in equal amounts at
0800h, 1200h, and 1600h based upon nutritional models for inducing SARA developed in our
laboratory (29, 62). To minimize variation commonly found during ad libitum feeding (9, 50),
diets were fed at 1.4% of BW (HF) and 1.7% of BW (HG) to minimize feed refusals while
meeting the metabolizable energy and protein requirements (45) for non-lactating cattle. At the
end of week 0 a baseline measurement was taken before transition to the HG diet. The transition
to HG took four days (grain was increased by 0.28% of bodyweight per day and hay was
decreased by 0.20% of bodyweight per day) and the HG diet was fed until the end of
experimental week 3 (HG period). After the HG period, the cattle were switched back to the original HF diet and monitored for three additional weeks (HF period).

**Physiological Measurements**

Starting at 0700h, ruminal pH was recorded every minute during the last two days of each experimental week using a pH monitoring system and methodology previously described by Alzahal et al. (2). Ruminal fluid samples were collected via the cannula at 1900h on the last day of each experimental week when we suspected ruminal pH to be depressed based upon previous experimentation (29, 62) to temporally characterize how ruminal SCFA concentrations and osmolality respond to shifts in dietary grain. After collection, the ruminal fluid samples were immediately frozen at -80°C until analysis. Ruminal SCFA concentrations were quantified in duplicate by gas chromatography using methods previously described by Mutsvangwa et al. (42). The osmalality of the rumen fluid samples was determined by freezing point depression using an automatic osmometer (31).

**Rumen Papillae Biopsies**

Rumen papillae were biopsied from the ventral sac at the end of experimental weeks 0, 1, 3, 4 and 6 (0700h after 48 hours of continuous ruminal pH recording) as described by Kelly et al. (28). In brief, the reticulorumen contents were partially evacuated to facilitate the retraction of the ventral sac. Since the ventral sac has the highest capillary blood flow per unit weight mucosa of any location within the rumen, it was chosen as the biopsy site (66). Approximately 150 mg of rumen papillae were collected from exclusive sites in the ventral sac during each biopsy and washed in ice cold phosphate buffered saline (pH = 7.4, 1X) 20 times prior to immediate fixation or freezing.
Microscopy

Five papillae were prepared for light microscopy histomorphometric analysis using methods previously described by Odongo et al. (47). Formalin-fixed, paraffin-embedded papillae were sectioned at a four micron thickness, stained with hematoxylin and eosin, and mounted for analysis. The microscopist was blinded to treatment during the examination and histomorphometric analysis. Measurements of each stratum were made using the 20X objective lens and two images were captured per papillae for a total of four measurements per papillae and 20 for each animal biopsy. Two separate estimates were made for each pre-planned parameter on all layers of the SSE. Image Pro Plus software (Media Cybernetics, Bethesda, MD, USA) was used to measure pre-defined criteria (a review of the criteria for epithelial layer measurements has been summarized in the Supplementary Material 1).

In addition to measurements of cell layers, lesion scores on each papilla were also quantified using the Olympus BX60 light microscope and a 10X objective lens. A score of 1 indicated nil to minor lesions, a score of 5 indicated minor lesions with corneum sloughing, and a score of 9 indicated severe deep lesions with large amounts of corneum sloughing. Five papillae per cow per week were examined for lesion scoring (36).

Additional papillae were prepared for electron microscopy using methodology reported by Graham and Simmons (21). Washed papillae were immediately fixed in 2% glutaraldehyde for 24 hours, post-fixed for one hour in 1% osmium and dehydrated in a graded series of ethanol solutions. For scanning electron microscopy (SEM), the papillae were subjected to critical-point drying using liquid CO₂ as the medium, mounted and coated with gold. Samples were then examined using SEM (Hitachi S-570, Hitachi Technologies, Tokyo, Japan). For transmission electron microscopy (TEM), dehydrated samples were then placed in a mixture of Spurr resin
(60) and acetone (1:1) for 30 min, followed by 2 hr in 100% resin with changes every hour. Samples containing resin were placed in molds and polymerized at 70°C for 8 hours to 3 days. Semi-thin (0.25-0.5 um) sections were cut with glass knives and stained with 1% toluidine blue-O in 1% sodium borate. Ultrathin (70-90 nm) sections were cut with a diamond knife, stained with methanolic uranyl acetate followed by lead citrate, and examined using TEM (Philips Model EM 208S, Feico Company, Hillsboro, Orgeon, USA).

RNA Isolation and Quality Assessment

Rumen papillae that were immediately frozen in liquid nitrogen were then stored at -80°C until RNA isolation. Total RNA was isolated using an RNeasy midi kit (Qiagen, Missisauga, Canada), treated with DNase (Invitrogen, Burlington, Canada) and assessed for quality using a Agilent 2100 Bioanalyzer (Agilent Technologies Inc., Palo Alto, CA, USA) and the RNA 6000 Nano kit (Caliper Life Sciences, Mountain View, CA, USA). The RNA Integrity Number (RIN) of samples taken for microarray and quantitative real-time reverse-transcription polymerase chain reaction (qPCR) analysis were 9.88 ± 0.21 and 8.75 ± 0.20 respectively.

Microarray Screen

Microarrays were utilized to screen the expression patterns in rumen papillae from each cow (n = 4) for candidate genes associated with IGF, EGF and desmosomes during weeks 0, 1 and 3 (12 microarrays in total). Hybridization of microarrays and data acquisition was carried out in the London Regional Genomics Facility (University of Western Ontario, London, Canada) using the 24K Affymetrix GeneChip Bovine Genome Arrays (Affymetrix, Santa Clara, CA). A complete description of the microarray hybridization protocol can be found in the Supplementary Material 1 and the GEO repository (GSE17849). The effect of dietary treatment on gene expression (relative mRNA content) was evaluated after subjecting the data to the GC Robust
Multichip Analysis (GCRMA) preprocessor in Partek (Partek, St. Charles, MO, USA; http://www.partek.com/) at the University of Kentucky Microarray Core Facility. Week 0 was compared to weeks 1 and 3 by ANOVA and then a means separation test was used to uncover differentially-expressed genes (DEG) ($P<0.05$; fold change $>\pm 1.5$) using Partek.

Quantitative Real-Time Polymerase Chain Reaction (qRT-PCR)

Candidate genes from our selected gene families that met our criteria were validated using qRT-PCR on all biopsied rumen papillae. After RNA isolation and quality assessment, samples were reverse transcribed before iTaq SYBR Green (Bio-Rad Laboratories, Mississauga, Ontario, Canada) qRT-PCR analysis in triplicate, using an ABI Prism 7000 (Applied Biosystems, Streetsville, Ontario, Canada). When possible, exon-spanning primers were designed for target genes using Primer Express 3.0 (Applied Biosystems). Dissociation curves were generated at the end of amplification to verify the presence of a single product. The efficiency of qRT-PCR amplification for each gene was calculated using the standard curve method ($R^2$, standard curve slope, and primer efficiencies of primers are presented in Table 1). Three common bovine house-keeping genes were originally tested for all samples prior to qRT-PCR analysis, and glyceraldehyde-3-phosphate dehydrogenase (GAPD) and beta actin (ACTB) were determined to be suitable house-keeping genes for this study (standard deviation = 0.12, ± Ct; coefficient of variation = 0.64, %Ct) (52).

Calculations and Statistical Analyses

Values from the two days of ruminal pH recordings were averaged for each experimental week before analysis. A mixed model procedure with repeated measures (67) in Statistical Analysis System (56) was used to contrast ruminal pH, ruminal SCFAs and osmolality data between experimental weeks and diets. The model included the fixed effect of time, the random
effect of the cow, and the best fitting covariance structure for each variable was determined using the Akaike criterion. To analyze mRNA expression, normalized qRT-PCR data were transformed to obtain a perfect mean of 1.0 at week 0. The abundance of genes was calculated using the inverse of qRT-PCR efficiency raised to Delta Ct (10, 57). For the temporal gene expression analysis, normalized qRT-PCR data was presented as fold-change relative to the first baseline HF measurement (week 0). Gene expression data was also analyzed using the same mixed model procedure with repeated measures to determine significance. For all statistical analysis, the a priori significance for difference between means was $P<0.05$.

**Results**

*Continuous Ruminal pH, SCFA Concentrations and Osmolality*

As expected, the mean, maximum and minimum daily ruminal pH values were higher during the HF period compared to the HG period ($P<0.01$) (Fig. 1). Week 1 of the HG period was marked by the lowest daily ruminal pH mean (5.86 ± 0.05), minimum (5.26 ± 0.06) and maximum (6.40 ± 0.02; Fig. 1). Ruminal pH during weeks 2 and 3 of the HG diet increased to HF levels, and ultimately increased by week 4 to equal the mean and maximum ruminal pH baseline measure whilst on the baseline diet (Fig. 1). As presented by Steele et al. (61), SARA was diagnosed during week 1 of the HG period as ruminal pH was depressed below 5.6 for 4.6 ± 1.4 hours daily and below 6.0 for 14.9 ± 5.2 hours per day. During weeks 2 and 3 of the HG period, no time was spent below pH 5.6 and, therefore, SARA could not be diagnosed.

*Insert Figure 1*

The concentration of SCFAs and the osmolality in rumen fluid were higher ($P<0.01$) during the HG period compared to the HF period (Supplementary Material 1, Table 1). As presented by Steele et al. (61), ruminal fluid SCFA concentrations were highest during the first
week of the HG diet (99.89 mmol ± 5.56) but gradually declined \((P<0.01)\) during the next two
weeks. Ruminal butyrate had the largest increase (330%) from baseline to week 1 before
decreasing \((P<0.01)\), but remained elevated compared to baseline (210%; \(P<0.05)\)
(Supplementary Material 1, Table. 1). Ruminal fluid osmolality shared a similar pattern with the
ruminal SCFA results. Osmolality was also highest during week 1 (327 ± 11 mOsm/kg: \(P<0.05)\); however, it returned to levels not significantly different from baseline once the cattle
returned to the HF diet.

**Histology, Morphology and Ultrastructure of Rumen Papillae**

Representative light micrographs of rumen papillae cross sections from the HF and HG
diets are shown in Fig. 2A and B. Cross sections of rumen papillae viewed under the light
microscope revealed no significant difference in the width of stratum corneum between the HF
and HG diet, regardless of period. In contrast, the thickness of the strata granulosum, basale and
spinousum and the entire SSE thickness in the biopsied rumen papillae was reduced \((P<0.01)\)
during the HG diet. During the interval between the baseline week and week 1 of the HG diet,
the thickness of the stratum granulosum as well as the sum of the stratum basale and spinosum
were reduced from 29.5 \(\mu\)m ± 2.5 and 129.4 \(\mu\)m ± 8.0 to 26.5 \(\mu\)m ± 0.6 and 117.2 \(\mu\)m ± 3.3,
respectively. The re-introduction of the HF diet was marked by increased \((P<0.01)\) thickness of
all living strata to levels not significantly different from baseline. Lesion scoring was not
significantly different between treatments; however, week 1 of the HG period was marked by the
highest ruminal lesion score of 5 ± 0.5 compared to all other weeks. Lesion scores increased
\((P=0.02)\) from baseline to week 1 of the HG diet, yet declined to levels not significantly different
from baseline by week 3 of the HG diet.
SEM micrographs of rumen papillae from all cattle during the HF period revealed deep ridges and indentations on the surface (Fig. 2C). The deep ridges were rich in morphologically heterogeneous microflora, including bacteria and protozoa (Supplementary Material 1, Fig. 1A, B). During the first week of the HG diet the ridges became shallow (Fig. 2D), concomitant with a reduction in microbial colonization (Supplementary Material 1, Fig. 1F, G). At high magnification, individual granular keratinized squamous cells of the stratum corneum were evident. Desquamation of the dead keratinized cells was apparent throughout the surface of the papillae in both diets; however, it was most striking during the HG diet in all cattle, especially during week 1 when SARA occurred.

Transmission electron micrographs of rumen papillae cross sections during the HF diet displayed a higher degree of intracellular organization at all levels with few intracellular spaces (Fig. 3A). Cells were strongly anchored to one another with tight cellular junctions, especially the desmosomes. The stratum corneum was compact with few signs of delamination (Supplementary Micrographs). The living strata displayed very tight, well defined, zona occludens and desmosomes (Supplementary Material 1, Fig. 1C, D, E). Few transition cells were found between the stratum corneum and granulosum. The cells of the stratum basale and spinosum were cuboidal or short columnar with long axes perpendicular to the basal lamina. These cells possessed large ovoid nuclei and numerous mitochondria, ribosomes, ER and golgi as well as large vesicles, predominately at the apical part of the cell.

During the HG diet, the ultrastructure of the ruminal SSE, as viewed by TEM, was dramatically altered as large spaces between levels of strata containing large amounts of bacteria were evident. In addition, cells in all strata below the stratum corneum appeared to have an accelerated migratory state (Fig. 3B). Extensive sloughing of the stratum corneum was
observed throughout the epithelial surface, especially during the first week of the HG diet when SARA occurred. High magnification SEM revealed cytoplasmic protrusions with a microvilli-like appearance from the horn cells of the corneum (Fig. 4A). The horn cell cytoplasmic protrusions were longer and not as densely spaced during the HG diet compared to the HF diet. The demarcation of the different strata became diffuse as cells from the stratum basale migrated luminally at an increased rate. The adhesion between cells of the stratum corneum and stratum granulosum appeared to be compromised, as evidenced by large gaps between cells. Cell junctions, especially desmosomes, became indistinct during the HG period and there was little indication that granulosum cells were forming a tight barrier. Granular cells bordering the stratum corneum had a distinct flattened shape and contained large keratohyaline granules (Fig. 4B). Cells of the basale layer became more pyramidal in shape and displayed the largest intracellular spaces, some approaching 3μm (Fig. 4C, Supplementary Material 1, Fig. 1H, I, J).

**Insert Figures 2, 3, 4**

**Microarray Screen and qRT-PCR**

Based upon preliminary candidate gene selection by microarray (Supplementary Material 2), the cadherin DSG1 and IGFBP 3, 5 and 6 met our pre-determined criteria ($P<0.05$, fold change>$±1.5$) and were validated by qRT-PCR. DSG1 mRNA expression was highest ($P<0.01$) during the HF diet compared to the HG diet (Fig. 5A). The expression was down-regulated by $0.25 ± 0.03$ fold from baseline to week 3. Once the cattle were switched back to the HF diet, expression increased to $3.57 ± 0.55$ fold compared to baseline, and remained elevated compared to the HG diet for the remainder of the experiment.

Differential mRNA expression was observed for all three IGFBPs during the HG diet (Fig. 5B.). IGFBP5 mRNA expression was up-regulated during weeks 1 and 3 from $1.60 ± 0.16$
to 1.79 ± 0.32 fold, respectively. In contrast, IGFBP3 mRNA expression was down-regulated by 0.53 ± 0.05 fold during week 1 of the HG diet and 0.70 ± 0.03 fold by week 3. IGFBP6 mRNA expression followed a similar expression pattern to IGFBP3 as mRNA levels were depressed 0.70 ± 0.07 and 0.56 ± 0.05 fold during weeks 1 and 3 of the HG diet respectively. The mRNA expression of all three IGFBPs during weeks 4 and 6 of the HF diet was not significantly different from the baseline period (week 0, HF).

Insert Figure 5

Discussion

The objectives of this study were to characterize the structural adaptations of the ruminal SSE during SARA and to identify prospective molecular mechanisms orchestrating these changes. Hence, it was imperative that we characterize rumen papillae histology, ultrastructure and, concurrently, mRNA expression of key genes at different stages of adaptation to the HG diet. In this study, SARA was induced and naturally mitigated by the second and third weeks of HG feeding. These results agree with the time dependent increases in ruminal pH after prolonged adaptation to starch previously reported in dairy cattle (40). It has recently been demonstrated in sheep that the epithelial capacity for apical uptake of SCFAs is a key determinant for intraruminal pH and susceptibility to SARA (51). To date, enhanced absorption of SCFA is thought to be achieved by increasing the epithelial surface area coupled to increased activity of ion exchangers (15, 37). Our findings suggest that more factors are involved in ruminal SSE adaptation, both structural and functional, as evidenced by microscopic and qRT-PCR analyses. The cellular and molecular adaptations of the SSE involved in elevating ruminal pH during the final weeks of the HG diet in this study may be useful in the prevention and treatment of ruminal acidosis induced by rapid shifts in dietary carbohydrates.
Ruminal SSE proliferation (54) and metabolism (28) can dynamically change throughout the day, but it is unknown how quickly micro-architecture can be modified. As demonstrated in this study, a dramatic adaptation of the ruminal SSE was observed after only one week of feeding the HG diet. Histological and ultrastructural micrographs of the ruminal epithelium indicated a deterioration of cellular junctions and large spaces between cells, thus providing opportunity for microbial infiltration from rumen to blood. Stratum granulosum thickness was reduced and it was apparent from TEM images that the degree of cellular adhesion was decreased as the number of desmosomes diminished. Our results are in contrast to a study conducted in sheep which demonstrated that high energy diets have a positive effect on barrier function of the rumen epithelium (37). However, unlike many studies investigating ruminal SSE adaptation to high grain diets we found no accumulation of the stratum corneum which may be causing this effect in other experimental models. It is also important to note that alterations in the normal expression pattern of desmosomal cadherins can result in compromised epidermal structure and function (25). In this study, desmosomal cadherin DSG1 was down-regulated during high grain feeding. DSG1 is known to be highly expressed in most SSE, particularly in the stratum granulosum, unlike DSG2 which is expressed at lower levels predominantly in the basale layer (22). The combined results of diminished granular and epithelial thickness and disruption of tight junctions between cells suggest that increased permeability or paracellular transport may be one of the mechanisms by which SCFA are cleared from the rumen in response to the increased organic acid load imposed on the ruminal environment during SARA.

Nevertheless, excessive permeability throughout the ruminal epithelium caused by a deterioration of cellular junctions can be detrimental to the animal’s health. Microbes and LPS from the rumen milieu can bypass this barrier and translocate into portal circulation during
SARA (13, 20). This can lead to liver abscesses, laminitis and whole-animal inflammatory responses (53). In the present study, acute phase protein serum amyloid-A (SAA) level increased 230% \((P<0.05)\) from the baseline to the third week of the HG diet (Supplementary Material 1, Fig. 2). The concentration of plasma SAA remained elevated \((P<0.05)\) during the first week back on the HF diet (week 4) before decreasing to a concentration not significantly different from baseline during weeks 5 and 6 of the HF diet experimental period. It may be that the elevated acute phase proteins were due to an increase in microbial and LPS infiltration into the blood; however, it cannot be ruled out that this response was due to effects of HG diets on endocrine and immune components of the entire digestive system (31, 32).

During feeding of the HG diet, ruminal SSE components displayed signs of accelerated differentiation and cellular migration. During the first week of the HG diet, when SARA was diagnosed, the rumen papillae appeared to be parakeratotic as small sized keratohyaline granules and cytoplasmic keratin aggregates were abundant in the sub corneum strata. The results of the present study are in agreement with previous descriptions of parakeratosis in the ruminal SSE by Tamate and Kikuchi (65). These authors hypothesized that keratin synthesis in the SSE might be completed in the lower strata because the rate of post mitotic aging is much faster in parakeratotic tissue. In this study there was no evidence of hyperkeratosis; however, SEM and TEM images indicated accelerated sloughing and delamination of the corneum. This finding was in contrast to other results published on sheep (17) and beef cattle (24) which showed a numerical increase in the number of cell layers within the stratum corneum with increasing levels of concentrate feeds in the diet. The lack of hyperkeratosis in the present study may be due to the brief duration of the grain challenge compared to other studies (17), the
physical nature of the diet (58), or to the excessive sloughing of the corneum found during the HG diet.

It has been well documented that increasing the intake of rapidly fermentable carbohydrates or intraruminal infusion of SCFAs augments the rate of tissue growth in the SSE (54). Goodlad (18) suggested that the ruminal SSE possesses self-regulatory mechanisms which can increase or decrease the rate of mitosis and cellular differentiation according to ruminal SCFAs, yet the molecular basis of this putative cellular regulatory mechanism remains unknown. Growth promoters such as EGF and IGF have been shown to increase proliferation of rumen epithelial cells in-vitro (4) and their gene families may be regulating proliferation and morphogenesis in-vivo.

In this study, we found no evidence of differential expression of EGF or its binding protein in rumen papillae based upon microarray data (Supplementary Material 2). Most EGF is produced in the parotid gland and supplied to the rumen via saliva, while limited expression has been detected in rumen tissue (48). Since HG diets typically lack physically effective fiber which supports saliva production (7), it is highly unlikely that EGF plays an important role in the adaptive response of the ruminal SSE during a grain challenge.

In contrast to EGF, the IGF family has received considerable attention as IGF-1 plasma concentrations are correlated with rumen papillae growth when ruminants are fed higher levels of butyrate (19) and energy (59). IGFBPs have been shown to modulate IGF stimulated proliferative events in epithelial tissue (14) and have been proposed as playing a role in bovine mammary epithelial differentiation (39). Their expression can be independent of IGF-1 concentrations as SCFA concentrations can regulate the expression of IGFBPs in the intestinal epithelium (55). Furthermore, studies of colonic cell culture with varying SCFA concentrations
have shown that SCFA can alter the expression of IGFBPs, thereby triggering cellular proliferation (43).

In this study we found that the grain challenge down-regulated IGFBP3 and IGFBP6 and up-regulated IGFBP5. Of the IGFBPs, IGFBP3 and IGFBP5 have been extensively characterized and are thought to modulate IGF-1 cellular events in an opposing fashion (6). In the case of IGFBP5, it is known to potentiate IGF-1 effects which may encourage proliferation in the rumen epithelium when up-regulated (14). Since IGFBP3 is thought to block IGF-1 cellular events its down-regulation, as seen in this study, would encourage tissue growth (6). We also noted few transition cells were found between the stratum corneum and granulosum in our histological analysis, which suggests a slow apoptotic rate of granular cells. Interestingly, the SCFA, butyrate, down-regulates the expression of IGFBP3, blocking apoptosis in intestinal epithelial cells (55). This finding agrees with Mentschel et al (41) who reported that intraruminal butyrate infusions induced papillae growth by decreasing apoptosis in the SSE. Since ruminal butyrate concentrations were elevated during the HF diet in the present study (Supplementary Material 1, Fig. 2), it is plausible that butyrate can trigger the down-regulation of IGFBP3 in the ruminal SSE, thus blocking apoptosis and increasing growth. In contrast to IGFBP3 and IGFBP5, IGFBP6 binds IGF-2 preferentially over IGF-1. In cancer cells, IGFBP6 functions as an inhibitor of IGF-2 cellular events; therefore, it is possible that it has a similar role in rumen papillae as it is down-regulated during high grain feeding (3). Similarities in our expression profile compared to previous experiments in intestinal cells support the hypothesis that shifts in ruminal SCFA concentrations during grain feeding may regulate the expression of IGFBPs, thereby controlling ruminal SSE proliferation and differentiation.
Perspectives and Significance

Our findings provide the first detailed characterization of ruminal SSE adaptation during SARA. This study introduced new information to describe epithelial adaptation during grain feeding, including increased detail concerning the proliferation, differentiation and migration of the cells of the SSE in response to increased fermentable dietary carbohydrates. Our findings suggest that HG diets and SARA may be associated with increased permeability through reduced organization and thickness of the ruminal SSE and reduced intercellular adhesion between cells of stratum granulosum. Additionally, HG diets induce accelerated cellular migration and post-mitotic aging during SARA. Together, these changes are likely responsible for the cumulative effect commonly known as parakaratosis. Finally, we also identified molecular markers (DSG1, IGFBP3, IGFBP5 and IGFBP6) that may play a pivotal role in signalling the adaptive response of the SSE during a HG diet. To advance our knowledge it is imperative to study the expression of candidate genes at the protein level and at different time points relative to feeding. Further research is necessary to enhance our understanding of the cellular and molecular adaptive mechanisms of the SSE during grain challenges or SARA in order to develop technologies to attenuate the detrimental welfare and productive impact that acidosis imposes on ruminants.

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Figures and Tables

Fig. 1. Summary of daily continuous ruminal pH recordings taken during the last 2 days of every week. Data are presented as the mean (n = 4) for daily minimum, mean and maximum per day during each experimental week (weeks 1, 2 & 3; high-grain diet and weeks 4, 5 & 6; high-forage diet). * Different from week 0 baseline, P<0.05. Pooled SEM for minimum, mean and maximum ruminal pH are 0.12, 0.05 and 0.04, respectively.

Fig. 2. Comparison of rumen papillae histology during the high forage (week 0) and high grain (week 1) periods.

A) Light micrograph of papillae cross section during the high forage period (scale bar = 200 μm).

B) Light micrograph of papillae cross section during the high grain period (scale bar = 200 μm).

C) SEM of papillae surface during the high forage period (scale bar = 230 μm).

D) SEM of papillae surface during the high grain period (scale bar = 150 μm).

Fig. 3. Comparison of rumen papillae ultrastructure during the high forage (week 0) and high grain (week 1) periods.

A) TEM of papillae cross section during the high forage period (scale bar = 6 μm).

B) TEM of papillae cross section during the high grain period (scale bar = 6 μm).

Fig. 4. Ultrastructure of the rumen epithelium during high grain feeding using SEM and TEM.
A) SEM of rumen papillae surface. Note the extended cytoplasmic protrusions from the horn cell (CP) (scale bar = 3 µm).

B) TEM of stratum granulosum cell with intact nuclei (N) and keratohyaline-like granules (KG) bordering the stratum corneum (C) (scale bar = 3 µm).

C) TEM of stratum basale cell rich in mitochondria (M) with diffuse junctions with large extracellular space (ES) between neighbouring cells (scale bar = 3 µm).

**Fig. 5.** Rumen papillae mRNA expression of Desmoglein 1 (A) and IGF-Binding Protein 3, 5 and 6 (B) using quantitative real-time PCR (qRT-PCR) throughout the characterization experiment (Baseline, week 0; HG feeding, weeks 1 to 3; HF feeding, weeks 4 to 6). Values are means (n = 4) expressed as fold-change from the baseline measurement (week 0; HF) which was set to 1.0. (* indicates different from week 0 baseline; \( P < 0.05 \))  Pooled SEM: DSG1, 0.31; IGFBP3, 0.08; IGFBP5, 0.21; IGFBP6, 0.13.
Table 1. Primers for quantitative real time PCR (qRT-PCR)

<table>
<thead>
<tr>
<th>Gene Name</th>
<th>Gene ID</th>
<th>Primers*</th>
<th>Amplicon Size</th>
</tr>
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<tbody>
<tr>
<td>IGF Binding Protein 3 (IGFBP3)***</td>
<td>NM_001075549</td>
<td>F855-GCGACAAGAAGGGCTTTTACAA R936-TATCCACACACCAGCAGAAACC</td>
<td>81</td>
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<tr>
<td>IGF Binding Protein 5 (IGFBP5)</td>
<td>BC102850</td>
<td>F701-CTACAAGAGAAAGCAGGGAACACGACAGGAGTGCAGAACC R764-TCCACGCACCAGCAGATG</td>
<td>63</td>
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<td>IGF Binding Protein 6 (IGFBP6)</td>
<td>NM_001105613</td>
<td>F440-CGCAGAGACCAACAGGAGAAGTGCAGAACC R527-GGGACCCATCTCAGTGCTCTTG</td>
<td>88</td>
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<tr>
<td>Desmoglein 1 (DSG1)</td>
<td>NM_001013004</td>
<td>F775-AGACAGAGAACATATGCGCCAGT R863-TTCACACTCTCTGACATACCATCT</td>
<td>121</td>
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<tr>
<td>Glyceraldehyde-3-phosphate dehydrogenase (GAPD)****</td>
<td>NM_177497</td>
<td>F276-TGGAAGGCCATCACCATCT R335-CCCACCTTGATGGTGGCAG</td>
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<tr>
<td>Beta Actin (ACTB) *****</td>
<td>BF041965</td>
<td>F438-CGTGAGAAGATGACCCAGAAGTACCGACGATCA R563-TCACCGGAGTCCATCGAT</td>
<td>125</td>
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</table>

*Exon junctions underlined

** The PCR efficiency was calculated as follows: \( E = -1 + 10^{(-1/\text{slope})} \times 100 \); the slope was obtained using the 5 point standard curve, with a minimum \( R^2 \) of 0.99.

***IGFBP3 primers previously used by Loor (38)

****GAPD primers previously used as housekeeping genes in bovine liver (26)

*****ACTB primers previously used as housekeeping genes in bovine liver and muscle (23)


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