Adaptive responses in short-chain fatty acid absorption, gene expression, and bacterial community of the bovine rumen epithelium recovered from a continuous or transient high-grain feeding

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ABSTRACT

The feeding of high-grain diets to dairy cows commonly results in lowered pH and ruminal dysbiosis, characterized by changes in absorption dynamics of short-chain fatty acids (SCFA) across the reticuloruminal wall, epithelial function, and the epithelial bacteria community structure. Therefore, the present study evaluated the effect of high-grain feeding persistence on the absorption kinetics of reticuloruminal SCFA, gene expression in the rumen epithelium, and the associated shifts in the epithelial bacteria in cows recovering from either a long-term continuous high-grain feeding model or a long-term transient high-grain feeding model. In a crossover study design, 8 nonlactating Holstein cows were fed 60% concentrate either continuously for 4 wk (continuous) or with a 1-wk break in the second week of the high-grain feeding (transient). After the high-grain feeding, all animals were fed a diet of 100% forage (recovery) for an additional 8 wk. Rumen papilla biopsies and SCFA absorption measurements were taken at the start of the trial (baseline), after the 4-wk high-grain feeding (49 d), after 2-wk recovery forage feeding (63 d), and after 8-wk recovery forage (105 d). Absorption of SCFA was determined in vivo using the washed and isolated reticulorumen technique. Rumen papillae biopsies were used for adherent bacterial DNA and host RNA extraction. The epithelial bacteria were determined using Illumina MiSeq (Microsynth AG, Balgach, Switzerland) sequencing of the 16S rRNA gene. No significant effects of the high-grain feeding model were seen for bacterial diversity. However, bacterial diversity increased with time spent in the recovery forage feeding period regardless of feeding model. The relative abundance of *Acidobacteria* phyla and *Acetivibrio* spp. increased when animals were fed a transient

high-grain feeding model. A trend toward increased *CLDN4* expression was observed in the continuous model. Furthermore, there were interactions between feeding model and sampling day for gene targets *CD14*, *DRA*, *NHE2*, *NHE3*, and *MCT2*. When comparing length of recovery, in the continuous model increased relative absorption of SCFA was sustained at 63 d but dropped to baseline measurements at 105 d. A similar pattern was found with the transient model but it did not reach significance. The only gene target that was found to significantly correlate to relative absorption of SCFA was *DRA* (correlation coefficient ≤ -0.41). Whereas, genera *Alkalibaculum*, *Anaerorhabdus*, *Coproccoccus*, and *Dethiobacter* all showed positive correlations to gene targets for pH regulation (*NHE2* and *NHE3*) and SCFA uptake (*MCT1*) but negative correlations to SCFA absorption. We conclude that while the rumen absorption and epithelial bacteria were able to recover to baseline levels after 8 wk of forage feeding, the time needed for re-establishment of homeostasis in host gene expression is longer, especially when high-grain feeding is interrupted.

Key words: gene expression, ruminal absorption, rumen epithelium, bacteria

INTRODUCTION

Modern dairy production uses rapidly fermentable nutrient-dense diets to support consistent improvements in milk yield (Plaizier et al., 2008). These diets replace forage with high proportions of grain resulting in a rapid accumulation of short-chain fatty acids (SCFA). The rapid accumulation of acids and resulting decrease in pH for extended periods (4–6 h; Nocek et al., 2002) result in a rumen dysbiosis referred to as SARA (Khafipour et al., 2009b). The effects of SARA extend beyond ruminal pH to include rumen microbial changes (Petri et al. 2013a,b), epithelial damage (Steele et al., 2011), inflammation (Khafipour et al., 2009b), decreased DMI (Kleen et al., 2003), and liver abscesses

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(Dirksen, 1985). However, the severity of SARA and the subsequent effects on animal health and production are based on feeding conditions. Previous research has often focused on a permanent exposure to SARA, mostly conducted over a short period of time lasting (1 d or 2 wk; Dirksen et al., 1984; Dohme et al., 2008; Khafipour et al., 2009a). However, in a dairy herd cows often experience SARA conditions that go far beyond 1 to 2 wk. Under farm conditions, SARA might also be transient as animals temporally alter their intake pattern to restore ruminal balances (Gao and Oba, 2016). Therefore, our group conducted a long-term SARA model to compare both continuous and transient feeding in nonlactating dairy cows. We previously showed that reticulorumen SCFA absorption is increased with prolonged high-grain feeding (Qumar et al., 2016), and that rumen pH changes in SARA were more severe in transient feed cows (Pourazad et al., 2016). These changes in rumen environment affected the attachment of rumen microbes to forages incubated in situ (Petri et al., 2017). Additionally, the rumen epithelial (epimural) bacteria were shown to be less stable due to transient feeding (Wetzels et al., 2016), and that long-term continuously challenged epithelial microbes are significantly reduced in diversity (Wetzels et al., 2017). There are indications that the rumen epithelium elicits an adaptive response to high-grain feeding at the gene expression level by increasing barrier function (McCann et al., 2016), changing the expression of SCFA transporters and rumen epithelial cell metabolism enzymes (Laarman et al., 2013; Zhao et al., 2019), as well as inflammation-related gene expression (Zhang et al., 2016). The sensitivity of the epithelial bacterial community to changes in the diet (Petri et al., 2013a) as well as an effect of the host on the rumen microbial community (Weimer et al., 2010) might be associated with the involvement in barrier function, localized immune response, as well as SCFA transport.

Whereas rumen adaptation to high-grain diets is time dependent (Dirksen, 1985), damage to the rumen epithelium may occur under low pH conditions (Penner et al., 2011) and would depend on the duration and severity of decreased pH (Steele et al., 2011). Therefore, it is plausible that high-grain feeding could have a long-lasting effect on the reticulorumen. However, there is a lack of information on the effect of the SARA feeding model on the recovery of the rumen ecosystem with specific attention to the interface where rumen bacteria and SCFA are in direct contact with the host. Understanding such recovery processes is especially important for dairy cows undergoing repeated cycles of dietary change and may explain why the severity of SARA could change after repeated high-grain feeding periods (Dohme et al., 2008). Therefore, the aim of

this study was to assess the effect of high-grain feeding (continuous vs. transient) on the recovery (return to baseline) of rumen SCFA absorption capacity (washed reticulorumen technique, gene expression for SCFA transport, pH regulation, rumen barrier function, epithelial inflammation, and the epimural bacteria community in the rumen). We hypothesized that cattle fed a transient high-grain diet would show differences from cattle fed a continuous high-grain diet in gene expression related to SCFA absorption, barrier function, and inflammation, which would correspond to changes in the reticulorumen absorption rates and rumen epimural bacterial community composition in the short-term recovery but that these feeding-model-related changes would not be seen after an 8-wk recovery period in which only forage was fed.

MATERIALS AND METHODS

Animals and Experimental Design

The effects of different feeding models during a high-grain challenge have been previously described in the associate publications (Pourazad et al., 2016; Qumar et al., 2016; Wetzels et al., 2016, 2017). In this paper we only focus on the data of the end of high-grain feeding and recovery from high-grain feeding in different feeding models. The samples taken at the baseline sampling time point were the only samples re-analyzed from previous data for use in the current study. Using 2 different previously described high-grain feeding models, transient (Wetzels et al., 2016) and continuous (Wetzels et al., 2017), 8 rumen-cannulated (100-mm inner diameter; Bar Diamond, Parma, ID), nonlactating Holstein cows (initial BW: 710 ± 118 kg, mean $\pm$ SD) were randomly allocated in a longitudinal crossover design feeding study. The experiment was conducted in 2 separate runs, each 15 wk in length, with 4 cows per model per run. Animals were kept together in a loose housing barn at the research farm of the University of Veterinary Medicine Vienna (Pottenstein, Austria). The animal experimental protocol of this research was reviewed and approved by the institutional ethics committee of the University of Veterinary Medicine Vienna, and all procedures were carried out in accordance with Good Scientific Practice guidelines and the national authority according to section 26 of Law for Animal Experiments (2012; GZ 68.205/0093-II/3b/2013).

Feed and Feeding Models

Ingredient and chemical composition of the experimental diets as well as the dietary adaptation has been previously reported by Pourazad et al. (2016). In brief,

the continuous high-grain feeding model was as follows: 2 wk of 100% forage feeding, followed by 1 wk of gradual adaptation to a 60% concentrate diet, followed by 4 wk of continuous feeding with 60% concentrate (Figure 1). The transient high-grain feeding model was similar to the continuous with the exception that, after the 1-wk adaptation to a 60% concentrate diet, the high-grain diet was given for 1 wk followed by 1 wk of 100% forage feeding and then a second 2-wk high-grain feeding period with a brief 2-d stepwise adaptation to 60% grain feeding (Figure 1). Cows that did not completely consume their concentrate allowance during the high-grain feeding had theorts supplied via the cannula. At the end of the high-grain feeding for both models, animals were returned to a 100% forage diet for an 8-wk period (recovery). Access to fresh feed and water was continuously provided. During the grain feeding periods, cows had daily access to forage at 0800 h and concentrate at 1000 h.

Reticulorumen SCFA Absorption and Calculations

The SCFA absorption across the reticuloruminal epithelium of all cows was determined by the temporarily isolated and washed reticuloruminal procedure (Qumar et al., 2016). Briefly, the washed reticuloruminal procedure was conducted 4 times in each feeding model: 14 d as a baseline, at the end of the high-grain feeding at 49 d, and at 63 d and 105 d during the forage feeding recovery phase (Figure 1). For each analysis, the reticulorumen content was manually evacuated and then the reticulorumen was isolated from the rest of the gastrointestinal tract by occluding the esophagus with a nasogastric tube for saliva collection (University of Leipzig, Leipzig, Germany), and the omasal orifice with a Foley balloon catheter (Rusch Gold Foley Catheter 30–50 mL, Teleflex Medical, Athlone, Ireland; Qumar et al., 2016). After a washing procedure, 20 L of experimental buffer solution [CaCl_2 (2 mM), MgCl_2 (2 mM),

Figure 1. Visualization of the continuous and transient high-grain feeding models with feeding phases and sampling times. Arrows pointed downward represent the sampling for short-chain fatty acid (SCFA) absorption, and the arrows pointed upward represent the sampling times for papillae biopsies.

NaCl (10 mM), sodium acetate (60 mM), K₂HPO₄ (5 mM), sodium propionate (30 mM), butyrate (10 mM), and NaHCO₃ (25 mM)] was infused into the reticulorumen and the rumen cannula was sealed with a foam lid. The buffer was continuously gassed with CO₂ during the procedure. The buffer samples were collected at 0 (before infusion), 35, and 65 min and stored at -20°C for further SCFA analyses. After the procedure saliva was poured back into the reticulorumen, ruminal digesta was restored, and the cannula was closed.

The concentration of the test SCFA (acetate, propionate, and butyrate) was determined by GC and quantified by known quantities of standards (internal standard, 4-methylvaleric acid, and external standard, acetate:propionate:butyrate at 3:2:1 vol/vol/vol; Sigma-Aldrich Co. LLC, St. Louis, MO), following the protocol previously described in detail by Kumar et al. (2016). The absolute absorption rates (mmol/h) at 0 to 35 min and 35 to 65 min were estimated as follows:

$$\text{rate (mmol/h)} = \frac{(S1 - S2)}{(T2 - T1)} \times 60,$$

where S1 and S2 are the initial and final amounts of analyte in 20 L of experimental buffer solution respectively, T1 is the initial time point, and T2 is the final time point (min). Subsequently, changes in the SCFA absorption rate for each sampling (49, 63, and 105 d) were calculated in relation to the baseline value for each cow before subjecting to statistical analysis as follows:

$$\text{relative change (\% of baseline)} = \frac{(\text{phase} - \text{base})}{\text{base}} \times 100.$$

Rumen Papillae Sampling

Before performing the washed reticuloruminal procedure, rumen papillae biopsies (whole papillae of ~1 cm³, 0.5 g in triplicate) were excised from the ventral rumen wall with aseptic scissors and tweezers after a partial evacuation of the rumen. Rumen papillae biopsies were taken from the rumen wall of the ventral sac about 40 to 50 cm below the bottom edge of the rumen cannula located in the left *fossa paralumbalis*, using the method described previously by Wetzels et al. (2016). Papillae were rinsed with sterile 1 × PBS, cut in small pieces, snap frozen in liquid nitrogen, and stored at -80°C for further analysis. Sampling occurred at 14 d as a forage baseline, after 4 wk of high-grain feeding (49 d) and then at 63 d as short (recovery 1) and 105 d as long-term recovery (recovery 2) samples in both models (Figure 1).

DNA Extraction, Sequencing, and Analysis

Rumen papillae biopsies were thawed on ice and genomic DNA was extracted from 0.25 g of rumen papillae using the PowerSoil DNA Isolation Kit (Mo Bio Laboratories Inc., Carlsbad, CA) as described by Wetzels et al., (2016). Amplicon sequencing was performed using the Illumina MiSeq sequencing platform (Microsynth AG, Balgach, Switzerland). The V3–V5 hypervariable region of bacterial 16S rRNA genes was amplified using the primer set 341F (5'-CCTACGGGGRSGCAGCAG-3'; Zakrzewski et al., 2012) and 909R (5'-TTTCAGYCTTGCGRCCGTAC-3'; Tamaki et al., 2011) to generate paired-end reads of 300 bp. The 16S rRNA gene PCR, library preparation, and sequencing were performed by Microsynth as described by Wetzels et al. (2016). Briefly, libraries were constructed by ligating sequencing adapters and indices onto purified PCR products. The Nextera XT Sample Preparation Kit (Illumina) was used according to the manufacturer's recommendations. After sequencing, corresponding overlapping paired-end reads were stitched to get an approximate amplicon size of 568 bp. Sequence data were analyzed with the software package Mothur (<http://www.mothur.org/>), according to the Illumina MiSeq procedure described by Kozich et al. (2013). Barcode sequences, primer, and low-quality sequences were trimmed using a minimum average quality score of 35 with a window size of 50 bp. Sequences were subsampled to 40,000 sequences per sample, and a total 2,093,194 sequences (96.6%) passed the chimera check and quality control. These sequences were clustered into operational taxonomic units (OTU) with a 97% similarity cutoff (= 0.03 distance). The Silva SSU reference database v119 was used for alignment and the RDP trainset (trainset9_032012.rdp.tax) were used for taxonomic classification. All OTU with less than 10 sequences were removed (272,462 sequences). A total of 8,031 OTU (1,820,732 sequences) were used for downstream analysis. The nonparametric diversity estimates of coverage, Chao 1, the observed number of OTU, and Shannon diversity indices, were calculated using Mothur. Sequencing data are available in the National Center for Biotechnology Information short-read archive database under the accession number SRP158246.

RNA Extraction, Reverse Transcription-PCR, and Host Gene Expression

Rumen epithelium samples, stored on ice, were weighed and approximately 20 mg was combined with lysis buffer (RNeasy Mini QIAcube kit, Qiagen, Hilden, Germany), according to the manufacturer's protocols,

and autoclaved ceramic beads (0.6 g; 1.4 mm; VWR, Radnor, PA). Samples were homogenized at a 6.5 ms^{-1} for 30 s using the FastPrep-24 (MP Biomedicals, Santa Ana, CA). The remainder of the RNA extraction protocol was completed using the automated QIAcube robotic workstation (Qiagen). After extraction, all samples were treated with Turbo DNA kit (Ambion, Foster City, CA) to remove genomic DNA and then quantification of RNA was determined with the Qubit 2.0 Fluorometer (Life Technologies, Carlsbad, CA) applying the Qubit RNA Assay kit (Life Technologies). The quality of extracted RNA was evaluated using the Agilent Bioanalyzer 2100 (Agilent RNA 6000 Nano Assay, Agilent Technologies, Santa Clara, CA) with an average RNA integrity number (RIN) = 7.2. The majority of samples had a RIN value between 7 and 9; however, 5 samples had a RIN value range between 4.4 and 6.7. Complementary DNA was synthesized using 2 μg of RNA diluted in 20 μL of water combined with 20 μL of master mix (High Capacity cDNA RT kit, Applied Biosystems, Waltham, MA) composed of 4 μL of $10 \times$ reaction buffer, 1.6 μL of 100 mM of $25 \times$ deoxynucleotide triphosphate mix, 4 μL of $10 \times$ random primers, 2 μL of reverse transcriptase, and 7.4 μL of DNase/RNase-free water (diethylpyrocarbonate treated, G-Biosciences, St. Louis, MO), and 1 μL of RNase inhibitor (Biozym, Hessisch Oldendorf, Germany) was added. The 2-step PCR was performed by a Mastercycler nexus (Eppendorf, Hamburg, Germany) using the following temperature program: incubation at 25°C for 10 min, reverse transcription at 37°C for 120 min, and a final heating step at 85°C for 5 min. Reverse-transcription controls were completed as a control for residual DNA contamination.

Twenty-five gene targets were analyzed for the physiological parameters of tight junction complex, membrane transport, cellular pH regulation, cellular metabolism, and pattern recognition receptors. Primers used for rumen epithelium quantitative reverse-transcription PCR are listed in Supplemental Table S1 (<https://doi.org/10.3168/jds.2018-15691>). Target genes for barrier function (desmoglein1, *DSG1*; corneodesmosin, *CSDN*; zona occludens, *TJP1*; occludin, *OCN*; claudin 1, 2, 4, and 7, *CLDN*), cell transport (monocarboxylate co-transporter, isoform 1, 2, and 4, *MCT*; downregulated in adenoma, *DRA*; anion exchange transporter, *SLC26A6*; proton/amino acid transporter 1, *SLC36A1*), cell metabolism (3-hydroxy-3-methylglutarylcoenzyme A synthase, isoform 1 and 2, *HMGCS*; β -hydroxybutyrate dehydrogenase, isoform 1 and 2, *BDH*), cellular pH regulation (anion exchanger isoform 2, *AE2*; sodium/proton exchanger isoform 1, 2 and 3, *NHE*; sodium/potassium ATPase transporting subunit alpha 1,

ATP1A1), pattern recognition receptors (toll-like receptor 4, *TLR4*; cluster of differentiation 14, *CD14*), and housekeeping genes (glyceraldehyde-3-phosphate dehydrogenase, *GAPDH*; β actin, *ACTB*; hypoxanthine phosphoribosyltransferase 1, *HPRT1*; ornithine decarboxylase antizyme 1, *OAZ1*; tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein, zeta, *YWHAZ*) were analyzed. The primers were designed and verified with PrimerBLAST using the *Bos taurus* genome (<https://www.ncbi.nlm.nih.gov/tools/primer-blast/>) and tested for efficiencies and specificity using melting curves (Supplemental Table S1, <https://doi.org/10.3168/jds.2018-15691>; Ye et al., 2012). Each 20- μL reaction consisted of 50 ng in 1 μL of sample cDNA, 10 μL of $1 \times$ Fast Plus Eva Green master mix with low ROX (Biotium, Hayward, CA), 1 μL each of 200 nM forward and reverse primers, and 7 μL of DNase/RNase-free water in a 96-well plate (Agilent Technologies). Analysis was performed according to Petri et al. (2018). Briefly, all reactions were performed on a real-time PCR MX3000P (Agilent Technologies) thermocycler using the following conditions: 95°C for 5 min, followed by 60°C for 30 s and 72°C for 30 s for 40 cycles. The presence of a single PCR product was verified with an additional dissociation stage. Analysis of 5 reference genes was performed and the 3 best-fitting reference genes (*OAZ1*, *HPRT1*, and *YWHAZ*) were selected using NormFinder (Andersen et al., 2004) and BestKeeper (Pfaffl, 2001). The mRNA abundance of target genes was calculated using the cycle threshold (Cq) method, as previously described (Gui and Shen, 2016; Petri et al., 2018). The relative expression values were calculated by using the formula: relative expression = $2^{-\Delta\Delta\text{Cq}}$ (Pfaffl, 2001). Relative expression was then calculated as a fold change relative to the baseline sample for each animal and each run to account for variation between the baselines in the crossover design and provide normalized data.

Statistical Analysis

All data were checked for the normality by means of the Shapiro-Wilk test to ensure that the data fit a normal distribution before hypothesis testing. Diversity indices, relative abundances, and epithelial gene expression data calculated as fold change were analyzed using the MIXED procedure of SAS 9.4 (SAS Institute Inc., Cary, NC). Terms in the model included the sequence of crossover, feeding model, and sample day with cow as random within run. However, due to significantly differing baselines between the feeding models all microbial and gene expression data were normalized to the baseline value for the corresponding experimental

Table 1. Changes in relative ruminal absorption rate (% increase or decrease in relation to baseline) of cows following high-grain feeding, and after 2 wk (recovery 1) and 8 wk (recovery 2) of forage-only feeding

Item	14 (baseline; mmol/h $\pm$ SD)	Sampling day ¹			SEM	<i>P</i> -level ²		
		49	63	105		Day (D)	Time (T)	D $\times$ T
Acetate					10.92	0.01	<0.001	0.01
0–35 min	475 $\pm$ 110	64.0 ^a	49.1 ^a	0.1 ^b				
35–65 min	420 $\pm$ 92	–10.9	–35.7	–21.5				
Propionate					10.17	0.01	<0.001	0.05
0–35 min	273 $\pm$ 60	61.3 ^a	42.5 ^{ab}	7.6 ^b				
35–65 min	236 $\pm$ 53	–6	–30.4	–18.3				
Butyrate					9.76	0.002	<0.001	0.05
0–35 min	93 $\pm$ 19	57.4 ^a	36.1 ^{ab}	4.1 ^b				
35–65 min	75 $\pm$ 18	–5.2	–27.4	–19.1				
Total					10.33	0.004	<0.001	0.02
0–35 min	841 $\pm$ 188	62.3 ^a	45.4 ^a	2.8 ^b				
35–65 min	731 $\pm$ 163	–8.7	–33.1	–20.2				

^{a,b}Means with different superscripts differ at $P < 0.05$ according to Tukey's method.

¹Sampling day based on sample protocol in Figure 1. Data in baseline are expressed as absolute absorption determined at the beginning of the experiment when the cows were based on a forage-only diet.

²Mean values of both continuous and transient feeding models are presented because no significant effects of feeding model, model $\times$ incubation time, or model $\times$ phase were found. However, a tendency ($P < 0.10$) was observed for model $\times$ phase $\times$ incubation time in the case of acetate, butyrate, and total short-chain fatty acids (see Figure 2).

period before statistical analysis. Animal was considered the experimental unit and sampling day was a repeated measure with a first-order autoregressive covariance structure. Absorption data were analyzed with fixed effects of run, sequence of crossover, feeding model, sample day, incubation time (0–35 and 35–65 min of incubation), and 2-way and 3-way interactions. Animal was considered the experimental unit. Group means were calculated using the LSMEANS option, and data are expressed with standard error of the mean. Degrees of freedom were estimated using the Satterthwaite method. Means were separated using PDIF. Significance was declared at a P -value of ≤ 0.05 , and trends were indicated at $0.05 < P \leq 0.10$. Boxplots were created with the corrplot and ggplot2 packages (Wickham, 2009) in R Studio (version 1.0.136). To illustrate microbial and gene expression changes, linear canonical discriminant analysis was done in JMP Pro (version 14, 2018; SAS Institute Inc.) with genera or gene expression as covariates and sampling period as the categorical variable. Correlation of various parameters was performed using the CORR procedure of SAS. According to Hinkle et al. (2003) the correlation coefficient was interpreted as follows: 0.00 to 0.30 as negligible, 0.30 to 0.50 as low, 0.50 to 0.70 as moderate, 0.70 to 0.90 as high, and 0.90 to 1.00 as substantial.

RESULTS

SCFA Absorption

Table 1 shows the absorption rates of SCFA at base and the absorption changes in relation to base with

respect to sampling day and incubation time as mean values of the 2 feeding models. There was an effect of sampling day, incubation time, and day $\times$ time interaction on all SCFA tested ($P \leq 0.05$). As expected, the absorption rates of SCFA increased during high-grain feeding. The relative change in absorption (% of baseline) at 0 to 35 min of incubation ranged from 57.4 to 64% for the individual SCFA and was 62.3% for the total SCFA. The changes were sustained at 63 d in the first part of the recovery from high-grain feeding at 49 d. Specifically, the relative changes in absorption of acetate and total SCFA on d 63 were similar to that of 49 d, whereas the relative changes of propionate and butyrate were intermediate of 49 and 105 d. The relative change at 105 d was a decrease to 0.1 to 7.6% of the baseline for the individual SCFA and to 2.8% for total SCFA, being significantly lower than high-grain feeding ($P < 0.05$).

A tendency was observed for a feeding model $\times$ sampling day $\times$ time interaction for acetate, butyrate, and total SCFA ($P < 0.10$). The relative changes in absorption with respect to the challenge model are depicted in Figure 2. The data show that the differences among feeding phases were most obvious in the continuous feeding model and at 0 to 35 min of incubation only. Specifically, high-grain feeding increased the absorption of all SCFA, and the levels, especially of acetate, were sustained up to 2 wk (63 d) after high-grain feeding ended. The absorption dropped to baseline levels at 8 wk of recovery forage feeding. Less dramatic changes were found for the transient feeding model at the 0 to 35 min incubation time. The relative absorption at 35 to 65 min of incubation showed decreased absorption

Figure 2. Short-chain fatty acid (SCFA) absorption relative to the baseline at 0 to 35 min and 35 to 65 min for both the transient and continuous high-grain feeding models. Error bars represent the SE of absorption within an SCFA, within a time point. Means within the challenge model sharing no common letters (a,b) differ ($P < 0.05$) according to Tukey's method.

(minus values), especially during the recovery forage feeding sampling days (Table 1 and Figure 2). Despite this variation, no differences were observed among feeding phases at this incubation time.

Epimural Bacteria Diversity and Community Composition

Diversity indices for nonparametric analysis showed an effect of sampling day for coverage ($P < 0.001$), ob-

served species ($P < 0.001$), Shannon index ($P = 0.02$), and Chao 1 ($P = 0.004$; Figures 3A–3D, respectively). No effect of crossover or interaction between sampling and sequence was seen.

In total, 19 phyla were found. An average of 94.7% of the sequences identified were affiliated with *Proteobacteria* (44.1% relative abundance), *Firmicutes* (35.0% relative abundance), and *Bacteroidetes* (15.6% relative abundance; Figure 4A). *Synergistetes* reached a relative abundance of 1.6%, *Elusimicrobia* a relative

abundance of 1.3%, and the remaining phyla showed less than 1% relative abundance. Despite the relatively low abundance within the epithelial bacteria, 8 of the 14 phyla showed a significant effect of sampling day (Figure 4B). A significant effect of the feeding model was only found for *Acidobacteria* ($P = 0.02$), which was increased in relative abundance in the transient feeding model (Figure 4). A total of 288 genera were detected with *Campylobacter*, *Kingella*, and *Brachymonas* being the most abundant (17.6, 9.0, and 5.1% relative abundance, respectively). However, only *Brachymonas* showed a tendency toward being lower during high-grain feeding ($P = 0.08$). A list of the 100 most abundant genera is shown in Supplemental Table S2 (<https://doi.org/10.3168/jds.2018-15691>), and of these only *Acetivibrio* spp. showed a significant effect of model ($P = 0.03$) with a relative abundance of 0.3% more in the transient feeding model. Of the 288 genera analyzed, 70 showed an effect of sampling day, and of those 70 only 17 belonged to a phyla that was also significantly affected by sampling day when compared between the feeding models (Figure 5). Genus *Anerophaga* from the phylum *Bacteroidetes* (2.67% relative abundance) and

Bifidobacterium (0.8% relative abundance) from the *Actinobacteria* phylum decreased in relative abundance with time in the recovery forage feeding. In contrast, *Gordonibacter* (0.16% relative abundance; phyla *Actinobacteria*), *Anaerorhabdus* (0.68% relative abundance; phyla *Bacteroidetes*), and *Ornithobacterium* (1.13% relative abundance; phyla *Bacteroidetes*) all increased in relative abundance with increased time on forage feeding.

Linear canonical discriminant analysis of the significantly affected genera is shown in Figure 6, with the first 2 eigenvectors representing 96% of the variation between samples. Sampling days for both models tended to cluster together with a clear differentiation between the high-grain feeding and the recovery forage feeding samplings. The largest variability was seen in the ratios between *Gordonibacter* spp. and *Coprococcus* spp. with all other genera. These 2 genera also showed the longest link and therefore had the strongest reciprocal ratio relationship. *Alkalibaculum* spp. and *Ornithobacterium* spp. had a similar reciprocal relationship that matched to the separation between high-grain feeding and the second recovery forage sampling at 105 d.

Figure 3. Whisker boxplots of nonparametric (alpha) diversity metrics coverage (A), observed number of species (B), Shannon index (C), and Chao 1 index (D) for samples in the continuous (white) feeding model or the transient (gray) feeding model during the high-grain feeding period, and in both the short (63 d) and long (105 d) recovery forage feeding. Boxplots represent the middle 50% of sample variation, with the median value indicated with a dark line and total variation indicated by whiskers. OTU = operational taxonomic units.

Relative Gene Expression

There was no significant effect of the sequence of feeding model application. Gene targets *CD14*, *DRA*, *NHE2*, *NHE3*, and *MCT2* showed variation between the sampling days with relation to the feeding model (Figure 7). The *CLDN4* was the only tight junction protein that tended to be higher in the continuous model 49 and 63 d ($P = 0.10$). The *MCT2* showed a decrease throughout both feeding models in relation to the baseline. Between the recovery 1 and recovery 2 sampling times, for both the continuous (0.3-fold; $P = 0.08$) and transient (0.4-fold; $P = 0.02$) feeding models, a decrease in *MCT2* expression was found. The *NHE2* was similar in fold change for the high-grain and the recovery 1 sampling regardless of feeding model ($P > 0.71$). However, in the transient feeding model, *NHE2* increased by 46% in the second recovery sampling compared with the first recovery sampling ($P = 0.04$). Similarly, *NHE3*

also increased between the high-grain feeding and the long-term recovery ($P < 0.05$) in both the continuous (0.44-fold) and transient (0.67-fold) models. Relative to the baseline, *DRA* expression increased by 1.6-fold in the transient model when sampled at 105 d in the forage recovery feeding ($P = 0.01$). Whereas standard variation in *DRA* gene expression was higher ($SEM = 0.27$), the variation between sampling days was not due to any outliers. The *CD14* showed a quadratic change in gene expression relative to the baseline with an increase from the high-grain feeding to the short-term recovery 1 ($P = 0.04$) and then a decrease at 105-d long-term recovery 2 sampling ($P = 0.01$; Figure 7).

Linear canonical discriminant analysis of all genes was performed to determine relationships between sampling days and gene targets (Figure 8), with the first 2 eigenvectors representing 73.7% of the variation between samples. Unlike for the analysis of genera, canonical analysis of gene targets showed that sam-

Figure 4. Relative abundance of the major (A) and minor phyla in the continuous (C) feeding model and the transient (T) feeding model (B) relative to sampling time point. *Phyla with an asterisk are significantly different ($P \leq 0.05$) based on sampling day.

Figure 5. Relative abundance of the genera with an effect of sampling day ($P \leq 0.05$) from significantly affected phyla with a relative abundance $>0.09\%$ sampled during the high-grain feeding, recovery 1 (63 d), and recovery 2 (105 d). The inner ring is for the continuous model, and the outer ring is for the transient model. Additional genera with a significant effect of sampling day but relative abundance $<0.09\%$ included *Aldercruzia*, *Corynebacterium*, and *Enterorhabdus* from the phyla *Actinobacteria*, *Fontibacter*, *Leadbetterella*, *Persicitalea*, and *Siphonobacter* from the phyla *Bacteroidetes*; as well as *Akkermansia* and *Coralimargarita* from the *Verrucomicrobia* phyla.

Figure 6. Canonical component analysis of genera affected by sampling day in relation to the feeding model (transient vs. continuous) and sampling time [high-grain, 42 d (continuous) or 49 d (transient); recovery_1, 63 d; recovery_2, 105 d].

pling days for both models did not cluster together. The recovery phase for the continuous and transient models clustered independently. The high-grain feeding for the transient model clustered apart from the other samplings. The largest variability was seen in the ratios between *MCT1* and *CDSN* and therefore these genes have the strongest reciprocal ratio relationship. Groups of genes (barrier function, pH regulation, and SCFA uptake) did not cluster together despite clustering of samples together.

Correlation Analysis

Pearson correlation analysis of significantly affected bacterial genera, relative absorption parameters, and gene expression targets is shown in Table 2. Most correlations tended to be low to moderate. The strongest correlation was found between *NHE3* and *Coprococcus*

spp. ($r = 0.67$). *Coprococcus* had positive correlations with *DRA*, *MCT1*, and *NHE2* as well as negative correlations to absorption parameters. This trend was also seen for *Alkalibaculum*, *Anaerorhabdus*, and *Dethiobacter* spp. Interestingly, *MCT2* had no correlations to any uptake parameters or genera. The inverse of this was only seen with *Desulfobulbus* spp. where absorption parameters were negatively correlated and *NHE2* and *NHE3* were positively correlated. The only gene target with a significant correlation to the absorption parameters was *DRA*. Correlation between genera and *DRA* was positive and the correlation between absorption and *DRA* was negative (Table 2).

DISCUSSION

The present study aimed at determining the effect of either a continuous or transient feeding model on the

Figure 7. Fold change in significantly affected genes relative to sampling day in the continuous feeding model (A) and the transient feeding model (B). Gene targets analyzed with quantitative real-time PCR method (qPCR). *Significant effect ($P < 0.05$) of sampling day on gene expression within a specific feeding model. **Trend toward significant effect of sampling day ($0.10 < P > 0.05$).

reticuloruminal absorption capacity, epithelial bacteria, and host gene expression during and after high-grain feeding. We previously reported that the transient grain feeding induced more severe SARA conditions. Specifically, the daily mean ruminal pH at the end of high-grain feeding of cows in the continuous model was 6.15, whereas it was 5.93 for the transient group (Pourazad et al., 2016). Furthermore, the duration of pH suppression below 5.8 in the transient feeding model

(500 min) was twice as long as in the continuous feeding model (220 min; Pourazad et al., 2016). The lower severity of SARA found with the continuous feeding model was linked to elevated SCFA absorption (Qumar et al., 2016), although no clear effect of grain feeding model was seen for endotoxin production or metabolic responses (Qumar et al., 2017). The persistence in high-grain feeding also promoted restoration of ruminal fibrolytic capability during the grain feeding (Pourazad

Figure 8. Canonical component analysis of all gene targets in relation to the feeding model (transient vs. continuous) and sampling time [high-grain, 42 d (continuous) or 49 d (transient); recovery_1, 63 d; recovery_2, 105 d].

et al., 2016). Additionally, the rumen epithelial bacteria were shown to be less stable after a week of forage feeding in the transient feeding (Wetzels et al., 2016), but overall less diverse after a 4-wk continuous high-grain feeding compared with the start of high-grain feeding (Wetzels et al., 2017). Certainly, rumen adaptation to high-grain diets is time dependent (Dirksen, 1985) and damage to the rumen epithelium would depend on the duration and severity of decreased pH (Steele et al., 2011). It was not known whether the high-grain diet and the feeding persistency have a long-lasting effect on the reticulorumen epithelial adaptation and the associated bacteria during time of recovery from high-grain feeding. As previously reported (Schurmann et al., 2014; Schwaiger et al., 2013), we found that the high-grain feeding promoted the SCFA absorption and the absorption declined during forage feeding. Because morphological adaptation requires 6 to 8 wk of diet adaptation to reach the maximum changes (Penner et al., 2010; Steele et al., 2011; Zhang et al., 2013a), it is also likely that the associated changes for recovery to forage feeding could also require several weeks. Since at the 63 d (2-wk recovery) a sustained capacity to absorb SCFA was found, but not seen at 105 d (8-wk recovery), the results of the present study would indicate that a full adaptation to forage diet occurs between 3 and 8 wk of continuous forage feeding.

Epithelial Bacterial Community

In agreement with previous studies, the diversity of the rumen epithelial bacterial community increased

in diversity with forage feeding (Petri et al., 2013a). The increase in diversity showed a linear pattern from the last high grain sampling to 8-wk forage feeding in both feeding models. Similar to the SCFA absorption, a forage-only interruption in the transient model did not allow a stable adaptation of the ruminal epithelium as well as the bacteria at the 49-d sampling. Consistent with our results, Dohme et al., (2008) demonstrated that the severity of low pH resulting from a grain challenge worsens with repeated challenges, and hypothesized that the unstable ruminal milieu due to grain challenges caused instability of the ruminal microflora, and potentially decreased the absorptive capacity of the ruminal epithelium. However, previous studies looking at microbiome and epithelial recovery used shorter recovery periods (Petri et al., 2013a; Zhang et al., 2013b).

Host Gene Expression

Compared with high-grain feeding, forage feeding lowers DMI, slows down the release and production of SCFA, and shifts the composition of SCFA, resulting in increased pH and decreased osmolarity of the ruminal fluid (Aschenbach et al., 2011). Previous research shows epithelial adaptation to increased SCFA production is due to increases in transporter expression (Andersen et al., 1999), which would imply that decreased SCFA production associated with a forage diet would result in decreased transporter expression. One such transporter is *MCT1*, which is involved in protein-mediated flux of SCFA (Graham et al., 2007; Laarman et al., 2016) and intracellular pH homeostasis (Müller et al., 2000). In

Table 2. Significant Pearson correlation coefficients for genera, gene expression, and absorption parameters

Genera	Relative absorption				<i>DRA</i> ¹	<i>MCT1</i> ²	<i>NHE2</i> ³	<i>NHE3</i>	<i>TJP1</i> ⁴
	Acetate	Propionate	Butyrate	Total SCFA ⁵					
<i>Alkalibaculum</i>	−0.42	−0.43	−0.45	−0.43	0.43	0.42	0.40	0.47	NS ⁶
<i>Anaerorhabdus</i>	−0.56	−0.54	−0.54	−0.56	0.59	0.30	0.45	0.52	NS
<i>Coprobacillus</i>	−0.49	−0.45	−0.45	−0.48	0.50	NS	0.40	0.45	NS
<i>Coprococcus</i>	−0.32	−0.33	−0.33	−0.33	0.56	0.51	0.62	0.67	NS
<i>Desulfobulbus</i>	0.35	0.32	0.38	0.35	NS	−0.41	−0.33	NS	0.30
<i>Dethiobacter</i>	−0.41	−0.39	−0.37	−0.40	0.44	0.34	0.35	0.34	NS
<i>Ethanoligenens</i>	−0.49	−0.49	−0.47	−0.49	0.44	NS	NS	NS	NS
<i>Gelria</i>	−0.51	−0.50	−0.46	−0.51	0.44	NS	0.37	0.35	NS
<i>Ornithobacterium</i>	−0.52	−0.51	−0.50	−0.52	0.43	0.13	NS	0.40	NS
<i>Petrimonas</i>	NS	NS	NS	NS	NS	NS	NS	NS	0.52
<i>DRA</i>	−0.49	−0.42	−0.41	−0.46	NS	NS	NS	NS	NS

¹*DRA* = downregulated in adenoma.

²*MCT1* = monocarboxylate transport 1.

³*NHE2/NHE3* = sodium/hydrogen exchanger isoforms 2 and 3.

⁴*TJP1* = zona occludens 1.

⁵Total SCFA = short-chain fatty acids in percentage.

⁶NS = Pearson correlation coefficient is statistically not significant according to Tukey's honestly significant difference method.

the current study, we found the decreases in expression of *MCT1*, as well as *MCT2* and *MCT4*, relative to the baseline in all sampling days even during the high-grain feeding. These data correspond to previously published data from Laarman et al. (2016), but bring into question the specific role of *MCT1* in both the short and long term under high-grain feeding (Dohme et al., 2008; Laarman et al., 2016). Despite similarities to previously published data, it is critical to assess that changes in nutrient and ion transporters in our study are relative to the location of tissue sampled and the variation that occurs with multiple samplings. Furthermore, our data show that despite 8 wk of forage feeding, which is thought to be a sufficient amount of time for rumen adaptation to diet (Bevans et al., 2005), alterations in the gene expression profiles compared with baseline could still be seen. It is important to note that the relative expression of mRNA is used to indicate the molecular response of the host to dietary changes but does not necessarily reflect the amount of functional protein present in the cell (Steele et al., 2012). Therefore, further study is required to elucidate the role of the functional protein and molecular gene adaptation of the rumen epithelium with relation to increased SCFA.

Of the 3 bicarbonate exchangers analyzed in this study (*DRA*, *AE2*, and *PAT1*), significant upregulation in the expression of the *DRA* (Yan et al., 2014) was seen in response to forage feeding phase and more predominantly in the transient feeding model. Yan et al. (2014) reported an upregulation of the gene expression for bicarbonate exchangers including *DRA* under reduced pH and highly correlated with SCFA produced in the rumen. However, the expression of *DRA* in our study continued to increase with the highest expression levels occurring in the recovery phase at 105 d of sampling. As a sulfate anion transporter, *DRA* mediates chloride and bicarbonate exchange and previous research has shown a potential binding between the gene product of *DRA* and *NHE3*, linking bicarbonate and sodium/hydrogen exchange in the intestine (Lamprecht et al., 2002). This is supported by a similar trend between the 2 gene targets with regard to patterns of expression.

The rumen epithelial barrier is a complex, functional syncytium of cells with intercellular tight junctions that regulate the permeability (Graham and Simmons, 2005) and prevent the translocation of LPS and other toxins (Minuti et al., 2015). Compromised barrier function has been associated with the release of free endotoxin (LPS; Emmanuel et al., 2007), which could have both local and systemic effects on inflammation (Emmanuel et al., 2008). However, in the current study, the relative gene expression in rumen papillae showed no significant changes in the expression of tight junction

proteins after 4 wk of high-grain feeding as well as in both short- and long-term recovery forage feeding. However, numerical increases in the fold change of gene expression for *TJP1* with more than 2 times the expression in the transient feeding model compared with the continuous would indicate that changes are occurring in the rumen papillae due to the feeding of high grain that may not have been captured with the current sampling program. The current data for gene expression of tight junction proteins are supported by the companion study (Qumar et al., 2017) showing that no systemic inflammation was developed despite the heavy load of LPS in both models. This might be related to the level of pH decrease induced by our high-grain feeding, which was less severe than the pH capable of triggering hyperpermeability of rumen tissues in response to LPS (Emmanuel et al., 2008). In line with that, the relative expression of *CD14* was found to decrease with the onset of high-grain feeding, but this decrease continued throughout the recovery period with the lowest expression occurring at 105 d of sampling regardless of feeding model. This was not true for *TLR4*, which increased in expression in the continuous model at 49 d and then decreased during the recovery phases. The alterations in the expression of LPS ligating proteins such as *TLR4* and *CD14* could indicate an adaptive response of the animal to extended exposure to increased levels of LPS.

Correlations Between Host, Epithelial Bacteria, and SCFA Absorption

Interestingly, the linear canonical discriminant analysis shows a clear separation between both models for gene expression targets in the recovery phases even though cows in both models were fed identically after the different models of grain feeding. This would indicate that the high-grain feeding model has an effect on the ability of the gene expression to recover. In addition, the high-grain feeding for the transient model clustered apart from the other samplings, indicating that the gene expression at 49 d in the transient model was affected differently than in the continuous model. Interestingly, from those bacteria that showed a significant effect of sampling day, many that were increased (*Alkalibaculum*, *Coprobacillus*, *Coprococcus*, *Gelria*, *Ethanoligenes*, and *Dethiobacter*) in the forage feeding period were gram-positive bacteria and not the LPS-producing gram-negative bacteria. Therefore, the role of the rumen epithelial bacteria in host gene expression with regard to commensal versus opportunistic pathogens requires further research and the development of reliable assays for other *TLR* targets such as *TLR2*. Of those genera found to be significantly different based

on sampling day *Alkalibaculum*, *Coprococcus*, and *De-thiobacter* had low to moderate Pearson correlations ($34 < r > 67$) to *DRA*, *MCT1*, *NHE2*, and *NHE3*, and all correlated negatively to SCFA absorption parameters. The increase in relative abundance, combined with a decrease in SCFA absorption with increased time on forage feeding in the recovery phase, would indicate that the *DRA* and *NHE3* are responsible for rumen cellular pH balance under normal rumen pH conditions.

Additionally, looking at the changes in other proteins within the inflammation cascade in the rumen epithelium may give more insight into the role changes that the epithelial bacterial community has on host cells.

CONCLUSIONS

The present study demonstrates that high-grain feeding regimen can affect the adaptation of the reticulo-ruminal epithelium to absorb SCFA, and that changes in the diet affect the rumen epithelial bacteria and the host gene expression independently but simultaneously. Patterns of gene expression differed between feeding models but the epithelial bacterial community structure was more closely associated with sampling day after the challenge than the feeding model used. Despite high-grain feeding, no significant changes in barrier function or LPS receptors were found that would indicate minimal ruminal dysbiosis. However, the use of a transient feeding model had more variable effects on host gene expression. Further research looking at the associated epimural community in correlation with the host protein expression will provide better insight into the host-microbe interaction in relation to high-grain feeding and long-term recovery.

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