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BUTYRATE-MEDIATED GENOMIC CHANGES INVOLVED IN NON-SPECIFIC HOST DEFENSES, MATRIX REMODELING AND THE IMMUNE RESPONSE IN THE RUMEN EPITHELIUM OF COWS AFFLICTED WITH SUBACUTE RUMINAL ACIDOSIS

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ABSTRACT

Subacute Ruminal Acidosis (SARA) is a disorder in cattle which can lead to chronic inflammation in the rumen epithelium, known as rumenitis. Butyrate has been shown to attenuate some of the detrimental effects of inflammatory gastroenteral disorders but the molecular mechanisms mediated by butyrate have not been defined. The objective of this study was to define the inflammatory-related genomic changes responsible for the beneficial effects of butyrate. Experimentally, 16 fistulated dairy cows at mid-lactation were fed a SARA-inducing (45% non-fiber carbohydrate) diet beginning 2 days before the beginning of treatment and continuing throughout the experiment. Cows were then evenly divided into treatment groups where a carrier with (n = 8) or without (n = 8) supplemental butyrate (2.5% initial DM intake) was deposited into the rumen daily for 7 days. The minimum rumen pH was higher in cows with supplemental butyrate (4.96±0.09 to 5.20±0.05, p = 0.040), but mean pH, maximum pH and the duration for which rumen pH was below 5.6 was unaffected. Free plasma Lipopolysaccharide (LPS) concentration was unaffected by treatment as was the concentration of Serum Amyloid A (SAA), although the LPS Binding Protein (LBP) concentration was increased by the addition of butyrate to the rumen (6.91±0.29 to 7.93±0.29 μg mL^{-1}, p = 0.024). Of the rumen Short Chain Fatty Acids (SCFA) tested, only butyrate showed a pronounced treatment effect, rising from 8.60±0.94 to 21.60±0.94 mM (p≤0.0001). Plasma Beta-Hydroxybutyrate (BHBA) concentration also increased (799.50±265.24 to 3261.63±265.24 μM, p≤0.001). Butyrate infusion did not affect milk parameters (total fat, lactose, total protein and LOS); however, when related to dry matter intake, milk production efficiency was increased (p = 0.035). Microarray and qRT-PCR analyses of rumen papillae biopsies collected on day 7 found that butyrate administration affected (p≤0.05) the expression of genes involved in Non-Specific Host Defense (NSHD), Remodeling or adaptation (RM) and Immune Response (IR). Of the 49 genes tested by qRT-PCR, 9 (LCN2, MMP1, MUC16, GPX2, CSTA, FUT1, SERPINE2, BCAM, RAC3) were upregulated, 20 (MTOR, AKIRIN2, NFKBIZ, NFKB2, ACVR2A, LAMB1, FRS2, PPARD, LBP, NEDD4L, SGK1, DEDD2, MAP3K8, PARD6B, PLIN2, ADA, HPGD, FMO5, BMP6, TCHH) were downregulated and 20 were unchanged due to butyrate administration in the proximal gastrointestinal tract.

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These results demonstrate the potential protective effect and molecular mechanisms involved in a novel butyrate treatment for inflammatory gastrointestinal conditions.

Keywords: Butyrate, Epithelium, SARA, Inflammation, Rumen, Wound Healing, Gene Expression

1. INTRODUCTION

SARA is a chronic pathological inflammatory condition of the rumen, affecting approximately 20% of all dairy cattle in North America (Garrett *et al*., 1999). Although attempts to define the etiology of SARA have gained headway in the past 10 years, its precise mechanisms remain uncertain. Due primarily to the microbial fermentation to SCFA of soluble starch of high grain diets, SARA can lead to liver abscesses, laminitis and acute inflammation of the rumen epithelium (Kleen *et al*., 2003; Plaizier *et al*., 2008). SARA does this by causing a sloughing of the rumen epithelium, compromising epithelial integrity (Steele *et al*., 2011a). Despite the fact that the transmigration of microbes across the rumen wall has been documented in cases of SARA, we have shown that this effect involves the immune system, although the extent of immune system stimulation is limited to local events and is not detectable systemically (Dionissopoulos *et al*., 2012a; 2012b). Experimentally, we have shown that adaptation to a SARA diet takes place within three weeks (Steele *et al*., 2011a) and previous experiments have shown that one of the principle methods by which the rumen adapts to an acidotic diet is by increasing papillae size and thus the total absorptive surface area of the rumen (Gabel *et al*., 2002), by increasing cellular turnover and an overall increase in total epithelial cell number (Goodlad, 1981).

There is strong evidence to support the involvement of butyrate in both normal and pathological conditions of the Gastrointestinal Tract (GIT) in both humans and experimental animals (Guilloteau *et al*., 2010). Roediger (1990), found a direct correlation between the severity of ulcerative colitis and the levels of butyrate. Since butyrate has been previously shown to be an important energy source for the GIT (Ahmad *et al*., 2000), we can speculate that low butyrate levels likely lead to an energy-deficient state (low ATP) and low metabolic rate in the rumen. These effects may culminate in the promotion of apoptosis and hence the degradation of the epithelial barrier. Indeed, butyrate can directly stimulate epithelial cell proliferation and differentiation (Aoyama *et al*., 2010; Zhang *et al*., 2010), both events which are hallmarks of the healing and restoration of normal function following an immunological insult. The ability to reduce or modulate the severity of the immune response is critical to our understanding of how sub-acute inflammatory conditions such as SARA exert their negative effects. A reduction in the duration and the extent of the immune response can have beneficial effects since nutrient partitioning favouring immune cell recruitment and growth can be limited (Dionissopoulos *et al*., 2006). In addition, reductions in the magnitude and severity of the immune response have been shown to favour wound healing and remodelling following injury (Eming *et al*., 2007). Since the reduction of pro-inflammatory cytokines such as IL-1β, IL-6 and TNF-α can reduce the length and severity of the immune response (Johnson, 1997), it follows that limiting such a response with naturally occurring substances in sub-clinical disease can have beneficial effects.

An understanding of the molecular mechanisms involved in rumen epithelial remodelling in sub-clinical disease would be incomplete without an exploration of inflammatory events at the gene expression level. The question that invariably arises centers around the principle effectors or transcription factors that mediate the immune cascade, which are known to be nuclear factor κB (NF-κB/p50/p65) and Peroxisome Proliferator Activated Receptor types (PPARs) in such a setting (Hoffmann and Baltimore, 2006; Calder, 2008). NF-κB, originally studied as a promoter of lymphocyte maturation (Kumar *et al*., 2004), has been found to have a much broader scope of action. NF-κB is found to be present in promoter sequences of pro-inflammatory cytokines, the inflammatory enzyme COX-2 and in leukocyte adhesion molecules (Sigal, 2006; Perkins 2007). Because of its importance, it can be seen how NF-κB and its cofactors will likely be targets for future immunomodulatory agents such as butyrate (Jobin and Sartor, 2000a; 2000b; Kinoshita *et al*., 2002; Zhang *et al*., 2006). Briefly, stimulation by bacterial or viral antigens, cytokines and Reactive Oxygen Species (ROS), IKK (an NF-κB kinase) phosphorylates the inhibitory subunit of the NF-κB complex, IκBα, prompting its dissociation from the p50/p65 NF-κB complex. Once free of its inhibitory complex, NF-κB traverses the nuclear envelope and begins the inflammatory cascade (Hoffmann and Baltimore, 2006).

PPARs are also important transcription factors in gastrointestinal inflammation. However, their effects are thought to act in an opposing manner to that of NF-κB as anti-inflammatory agents (Guri *et al*., 2010). Most evidence of the anti-inflammatory effect of PPARs comes from studies where subjects with advanced inflammatory bowel diseases generally show decreased levels of PPAR-γ mRNA expression (Desreumaux *et al*., 1999). More strikingly, in experimental mouse models, treatment with a PPAR-γ agonist reduced symptoms of

colitis (Desreumaux *et al*., 2001). Moreover, butyrate has been shown to stimulate the production of PPAR-γ in cell lines (Kinoshita *et al*., 2002). Perhaps most importantly is the suggestion that PPAR-γ inhibits NF-κB activation by interfering with the phosphorylation of IκB, preventing its degradation and the subsequent translocation of the NF-κB complex to the nucleus (Ross *et al*., 1999; Haegeman, 2003; Berghe *et al*., 2003).

We hypothesize that the addition of exogenous butyrate in cows afflicted with SARA will lessen the extent of immune system stimulation, will help the rumen adapt to a high grain/high energy diet and through adaptation, support changes to an epithelial barrier that is more resistant to insult or injury by pathogenic organisms to meet new metabolic demands. The goal of the experimentation described in this article is to characterize the mechanisms involved in rumen epithelial remodelling and, through butyrate supplementation, lessen the severity and extent of the negative effects of SARA. Here we outline the data relating the Immune Response (IR), rumen epithelial Remodelling (RM) and Non-Specific Host Defence (NSHD) in SARA cows supplemented with butyrate.

2. MATERIALS AND METHODS

2.1. Animals, Treatments and Diet

Sixteen mid-lactation, rumen cannulated (Duffield, 1999) primiparous and multiparous Holstein cows on a mid-lactation Total Mixed Ration (TMR) diet were selected and blocked by Days In Milk (DIM). Prior to the experiment, total Dry Matter Intake (DMI) and milk production were recorded for 7 days. Two days before the start of the study, a concentrate mix was added to the TMR to increase the Non-Fiber Carbohydrate (NFC) to 45.0% in 2 equal increments. According to previously published results, a NFC of 45% would be sufficient to induce SARA in this study (Steele *et al*., 2012). Each increment was 50% of the total amount of concentrate needed to increase the NFC to 45.0%. The composition of the diet is presented in **Table 1**.

At the start of the 7-day experiment, each cow was randomly assigned to one of two treatments. Butyrate cows (n = 8) received a ruminal dosing of butyrate (Proformix™, Probiotech Inc., Saint-Hyacinthe, QC) while Control cows received no supplemental butyrate but did receive carrier. The butyrate powder (50% butyric acid, sodium bicarbonate, lime) was dosed at a rate of 2.5% of pre-trial DMI, at 10.00 and 13.30 h daily, to coincide with regular daily feeding times.

Table 1. Diet fed to lactating Holstein dairy cows with or without supplemental butyrate

Ingredient	%DM
Corn silage	22.9
Haylage, 1st cut	22.9
Straw	5.1
High moisture corn	16.7
Protein supplement	15.8
Grain supplement	16.4
Barley grain	60.0
Corn grain	20.0
Wheat grain	20.0
Formulated (Calculated) Analysis	
DM, %	52.6
CP, % of DM	15.9
EE, % of DM	3.4
Starch, % of DM	24.5
NDF, % of DM	33.7
Forage NDF, %NDF	75.2
NFC, % of DM	44.0

2.2. Rumen SCFA, LPS and pH

Rumen fluid was collected approximately 3 h after the afternoon feeding from the ventral sac and squeezed through 4 layers of cheesecloth and frozen for analysis of total SCFA by gas chromatography (Steele *et al*., 2012). Another sample of rumen fluid was analyzed fresh for total free LPS using the chromogenic Limulus amoebocyte lysate end-point assay (Lonza Group LTD., Basel, Switzerland) as previously described (Gozho *et al*., 2005; Dionissopoulos, 2012b). Rumen pH was recorded continuously for the last two days of the trial using a pH recording system and protocols established by our laboratory (AlZahal *et al*., 2007).

2.3. Plasma BHBA, LBP and SAA

On days 1 and 7, blood was sampled through the tailhead at 16.30 h. After collection, the blood was stored on ice and subsequently spun at 3000 x *g*, then stored at -20°C until further processing. Plasma LBP was assayed according to established protocols (Khafipour *et al*., 2009b). Plasma BHBA was determined by the Animal Health Laboratory (AHL, University of Guelph, Guelph, Ontario) using the method of Williamson *et al*. (1962). Serum Amyloid A (SAA) protein levels were determined by enzyme-linked immuno assay using a multi-species SAA kit (TriDelta Development, Ltd, Maynooth, County Kildare, Ireland).

2.4. Dry Matter Intake (DMI), Daily Milk Production and Milk Component Analysis

DMI and milk production was calculated daily for each cow on the study. All cows were milked at 05.30 h

and 16.00h daily and their milk production numbers were pooled to establish a total daily milk production value. Milk protein and fat were determined spectroscopically by the CanWest DHI Laboratory (Guelph, Ontario). These values were compiled to yield an average weekly DMI, production value and milk component analysis.

2.5. Rumen Papillae Biopsies for Microarray and qRT-PCR

Rumen papillae were harvested from the rumen ventral sac at the end of experimental day 7 (Steele *et al*., 2012). Briefly, the rumen contents were partially evacuated to help access and retraction of the rumen ventral sac.

Table 2. Primer design for qRT-PCR analysis. Primer efficiency was calculated using the formula E = -1 + 10(-1/slope) ×100; the slope was derived from the PCR 5-point standard curve ($R^2 \geq 0.99$)

Accession #	Gene Name		5'→3'	Efficiency (%)
NM_174227	ACVR2A	F	GGTTGTTGGCTGGATGAT	86
		R	GGCTTAGGAGTTACTGGATT	
NM_173887	ADA	F	ACCAGATGACCAAGAATGAA	88
		R	CACCAGAGGAGGAGGAAT	
NM_001110087	AKIRIN2	F	CCTTGTTCCTTGTTGAGTTG	82
		R	GGTTGCTGCCTAAGAGTG	
NM_174741	BCAM	F	TCTTGAGGGGAACTTGACGC	94
		R	GGTTGTGCTGTTGTGCAGAG	
XM_002697620	BMP6	F	GCCAGCGACACCACAAAGA	91
		R	CGCCTCACCCTCAGGAATC	
NM_174008	CD14	F	GAGGCTCTGAGAATCTACTG	85
		R	CGGCAACCATACACTGAA	
NM_001166511	COL4A1	F	ACGCGAACGCTTACAGCTTT	94
		R	CGTGGGCTTCTTGAACATCTC	
NM_001167824	CSTA	F	CTCAAGTGGTTGCTGGAA	81
		R	TAGGAACACGCTGCTAGA	
NM_001076017	DEDD2	F	CAGACCCTCCCACAGGATGT	54
		R	GGAGGCTGGGAAGGAAACTC	
NM_001193131	FGF7	F	GAAGGAGGAGATATAAGAGTGA	98
		R	AGGTTATTGCCATAGGAAGA	
NM_001101304	FMO5	F	TCGTGTAGGAGACTATGGAT	84
		R	TATTGTTGGATGCTGACTGA	
NM_001163778	FN1	F	GAAGGCAGCGGACGTATCAC	100
		R	TCCCCCGGTTGTCCTTCT	
ENSBTAT00000025495	FRS2	F	CCCCATCCCTACTTCCTCGT	93
		R	AATGTGGCACACTGGTGACT	
NM_177499	FUT1	F	AGACCGTAGCCGGGTGAGAT	104
		R	TTCATCGGTGTGCCTGACA	
NM_001163139	GPX2	F	TGCCAAGTCCTTCTACGA	74
		R	CTCATTCTGACCATTCACATC	
NM_001034419	HPGD	F	ACCTCATTCTGCCTGCTA	88
		R	CCTCATATTCAATCCACTCCT	
NM_001102300	IGFBP7	F	GCGAGCAAGGTCCTTCCA	100
		R	GGGCACCAGTGACATTCCA	
NM_174088	IL10	F	GGCGGTGGAGAAGGTGAA	100
		R	GGCTTTGTAGACACCCCTCTCTT	
XM_002691608	LAMB1	F	CCGGCTCAACACGTTTGG	100
		R	GAAATCCGACACGGCGTAGTA	
NM_001038674	LBP	F	TGACGTGATTCCGCCTGAT	95
		R	AAGGCGCGGAAGGACTTG	
XM_002691670	LCN2	F	GATGACCTCTGTGACTACTG	83
		R	TGGCGAAGTTGATGAAGTT	
NM_001046517	LY96	F	CGTGGAATACTCTATCTCTACT	86
		R	CCTCGATGGCTTCTGTAAT	
NM_001099071	MAP3K8	F	GCCGACCTCAAGAATCTG	91
		R	TCCTTCACCTGCTTCCAT	

Tabe 2. Continued

NM_001033608	MIF	F	GGATCTACATCAACTTCTGCGACAT	88
		R	GAAGGTGGAGCCGTTCCA	
NM_001035411	MLST8	F	AGCGAATCTTCCAGGTGAA	86
		R	GCGGTGACGATGTATTGC	
NM_174112	MMP1	F	GACGTGGCTCCGTTTGTTCT	96
		R	TCCTGTAGGTCAGGTTTGTGTTCTC	
ENSBTAT00000020386	MTOR	F	CATTGAGCAGATTGTGGTAG	93
		R	AGAGGCATAGTCCGTGAA	
FN600737	MUC16	F	TGGACAGTAACAGCCTCTA	86
		R	GTTGGTGATGGTGAAGTTG	
ENSBTAT00000018334	NEDD4L	F	GCTGATGGACGCCGAGAA	89
		R	GGACGTCCCTGTGACAAACTG	
NM_001076409	NFKB1	F	CGAATGACAGATGCCTGTATACG	89
		R	CTGCAAATAGGCAAGATCAGGAT	
NM_001102101	NFKB2	F	CCAAGGAACTGAAGAAGGT	83
		R	CCAGAGGATAATAGGTGAACT	
NM_174726	NFKBIZ	F	TGGACTTGGAGGCAACTAACTATG	98
		R	TATGGGCCAAGACTGCACAGT	
NM_001098104	PARD6B	F	CGCAGGTAGAGCTCAGTTTCG	92
		R	CTTCGTTGGCAGGGATGAA	
NM_001017953	PDGFB	F	AGTGGTCAGACAGGAGTAA	68
		R	GCAGAAGAAGGTGGATAGG	
NM_173980	PLIN2	F	GTGGTCAGAGCCTGTCCAGAA	98
		R	GCAAAAGCATGAGGCCATAAA	
NM_001083636	PPARD	F	AATGTGAGCGGATCTGCAAAA	97
		R	GCGGCAGTACTGGCACTTG	
NM_001105323	PTGS1	F	CCCCAAATGAGACCCTGGAT	96
		R	CAACCATTGGCCTGGAGAA	
NM_174445	PTGS2	F	AATCTTCCAGTCGCAGTAG	103
		R	TTGAGGCAGTGTTGATGAT	
NM_001099179	RAC3	F	GCGAATGTGATGGTGGAT	83
		R	GGAGAAGCAGATCAGGAAG	
NM_174669	SERPINE2	F	CCCCCAGTGGCCTATGGT	96
		R	TGATGCTTTCGCCGTGGTA	
NM_001102033	SGK1	F	TTCTCCTGGCAAGACACAA	83
		R	ACATTCCGCTCCGACATAA	
NM_001076223	SMAD1	F	ATTGGAATGCTGCGAGTT	85
		R	GCTGTGCTGAGGATTGTAT	
ENSBTAT00000008311	STAT6	F	GAGCCTGATGGAACCTTC	82
		R	AAGTGAGCGAATGGACAG	
BC151676	TCHH	F	ATGTCTGCTGTAGTCTGTAG	81
		R	ATGTCTCCAAGGTAGTATCAG	
NM_001035313	TGFB1I1	F	TTCCGTGTCCAGAACCAT	84
		R	AGGCAGTAACCATCTTGTG	
NM_174198	TLR4	F	CTAAGGAGCAAGAACTACAGA	70
		R	CAAGAAGCATCAGGTGGAA	
NM_001101306	TNFRSF6B	F	GCACCCTTCCTGGTATTTATTCA	106
		R	TTCCGAAGCCTCCTTTGGT	

Approximately 150 mg of papillae was cut from the rumen and washed 20 times in ice-cold PBS, then placed in a stabilization agent (RNA*later*™, Qiagen, Hilden, GmbH) until the RNA could be isolated. Total RNA was isolated as previously described by Steele *et al*. (2012) using an RNeasy midi kit (Qiagen, Mississauga, Ontario, Canada). The concentration of RNA was determined using a NanoDrop (ND-1000, NanoDrop Technologies, Wilmington DE). To enhance the purity of the RNA, it was treated with DNase (Invitrogen, Burlington, Ontario, Canada) and its quality was determined using an Agilent 2100 Bioanalyzer (Agilent Technologies Inc., Palo Alto, CA and the RNA 6000 Nano kit (Caliper Life Sciences, Mountain View, CA). The RNA was then prepared for either microarray analysis, to determine the global expression pattern of genes, or for qRT-PCR, to confirm the relative expression of any genes (Steele *et al*., 2011b) involved in epithelial RM, the IR, or the NSHD. Where possible, primers were designed to span exon-exon junctions using NCBI/PrimerBLAST Primer Express (http://www.ncbi.nlm.nih.gov/tools/primer-blast/) and were verified as specific to the bovine genome using GenBank (National Center for Biotechnology Information, Bethesda, MD). All primer amplicons were confirmed by BLASTN in NCBI, as well as through the generation of dissociation curves to support the fidelity of single product amplification (Steele *et al*., 2011a), using GAPDH as the housekeeping gene. The stability of GAPDH amplicons were confirmed by low variance (mean Ct = 20.85; variance = 0.10). Primer information including sequences can be found in **Table 2**. Any treatment imposed differences in mRNA expression was determined using the inverse of qPCR efficiency raised to Delta Ct (Pfaffl *et al*., 2004). The expression values of the Control cows were pooled and expression differences were determined through the comparison of individual Butyrate group values to this index as described previously using a Student's t-test (Xue *et al*., 2010). Finally, all differentially expressed genes were subjected to analysis with the Ingenuity Pathway Analysis (IPA) tool (Ingenuity, Inc., Redwood City, CA) in cooperation with the University of Kentucky (Lexington, KY). The methodologies employed herein have been previously published (Steele *et al*., 2011b). It is important to note however that to determine the significance of differentially expressed genes in this study, the data derived from the microarray analysis was subjected to a pre-screen of 95% confidence and a false discovery rate of 0.1 according to the methods of Benjamini (Reiner *et al*., 2003).

2.6. Statistical Analysis

Measurements unrelated to the microarray or qRT-PCR analysis were analyzed using the MIXED procedure of SAS (2004) using a previously described model (Steele *et al*., 2012):

$$Y_{ij} = \mu + D_i + T_j + (D \times T)_{ij} + \varepsilon_{ij},$$

where, Y_{ij} is the dependent variable, μ is the variable mean, D_i denotes the fixed effect of diet (i = 1, 2), T_j is the fixed effect of time or day (j = 1,.., 7), $(D \times T)_{ij}$ is the diet by time interaction (ij = 1,...,14) and ε_{ij} represents the random residual error. Both diet and time were analyzed as fixed effects and day was analyzed as a repeated measurement with cow as the subject. The subject cow was treated to covariance structure measurements; the covariance structure resulting in the smallest Bayesian information criterion was used in this analysis.

3. RESULTS

3.1. Physiological Parameters

Table 1 represents the analysis of the diet which was formulated for this study. Both the Control and Butyrate groups received the same diet with the exception of supplemental butyrate in the treatment group.

Total rumen SCFA were analyzed for all animals and the data is presented in **Table 3**. Total SCFA levels differed significantly only on day 1 of the study and were higher in the Butyrate group (92.76 Vs 78.87 mM ±4.51; p = 0.04). However, as expected, total volatile butyrate levels were dramatically higher in the Butyrate group both on day 1 (22.60 Vs 9.88 mM ±0.94, p≤0.0001) and day 7 (21.60 Vs 8.60 mM ±0.94, p≤0.0001).

Total plasma LBP and free rumen LPS levels are also presented in **Table 3**. Plasma LBP levels were significantly higher in the Butyrate vs. the Control group on day 7 of the study (7.93 Vs 6.91 μg mL^{-1} ± 0.29; P = 0.024). Free LPS did not differ significantly for either treatment day between groups.

Also as expected, plasma BHBA levels were increased significantly due to treatment (**Table 3**). On day 1, Control Vs Butyrate levels were 909.50 vs. 4201.13 μM ±265.24 (p≤0.001) and on day 7 of the study, levels of BHBA rose significantly from 799.50 to 3261.63 μM ±265.24 (p≤0.001). Data for the serum acute phase protein SAA is presented in **Table 3**. No treatment differences were seen (p>0.05).

Table 3. Rumen SCFA, plasma BHBA, plasma LBP, rumen LPS, and SAA concentrations with (Butyrate group) or without (Control group) 2.5% butyrate, DMI. Values are expressed as means ± SEM; n = 8 per Control or Butyrate group. P values represent comparison between treatments on individual days

	Day 1		Day 7			P value	
	Control	Butyrate	Control	Butyrate	SEM	Day 1	Day 7
Total SCFA (mM)	78.87	92.76	81.82	87.59	4.51	0.0400	0.3700
Acetate	38.20	40.40	39.20	36.40	1.90	0.4100	0.3100
Propionate	25.80	23.90	27.70	24.40	2.70	0.6200	0.4000
Isobutyrate	1.70	2.20	2.50	1.90	0.29	0.2300	0.1800
Butyrate	9.88	22.60	8.60	21.60	0.94	≤0.0001	≤0.0001
Isovalerate	1.02	1.21	1.12	1.06	0.08	0.1100	0.6200
Valerate	2.27	2.51	2.67	2.27	0.24	0.5000	0.2600
BHBA (μM)	909.50	4201.13	799.50	3261.63	265.24	≤0.0010	≤0.0010
LBP (μg/mL)	6.68	6.80	6.91	7.93	0.29	0.7660	0.0240
LPS (EU/mL)	52723.00	28892.00	14425.00	7517.25	8485.84	0.0570	0.5700
SAA (ng/mL)	305.12	322.28	358.41	384.55	27.87	0.6700	0.5100

Table 4. Effect of butyrate on rumen pH in Holstein cows with (Butyrate group) or without (Control group) butyrate supplementation at 2.5% DMI. Time in min/day; AUC = area under the curve in pH ≤5.6× min/day

Treatment	Butyrate	Control	P
Min	5.20±0.05	4.96±0.09	0.04
Mean	5.67±0.04	5.66±0.06	0.89
Max	6.36±0.11	6.55±0.16	0.27
Time pH ≤ 5.6	536±89	598±97	0.65
AUC	87±26	168±40	0.11

Table 5. Dry matter intake, daily milk production, and daily milk production ratio. *The milk production ratio is adjusted for variations in dry matter intake related to treatment effects and indicates that the butyrate group was more efficient in the production of milk than was the control group

	Control	Butyrate	SEM	P-value
Dry Matter Intake, DMI (kg/day)	24.40	17.41	0.77	≤0.0001
Daily Milk, DMP Production (kg/day)	41.93	34.74	2.50	0.0617
Daily Milk Production Ratio, MPR (DMP/DMI)*	1.67	1.96	0.09	0.0354

As indicated previously, the effect of butyrate supplementation was examined on pH parameters in the rumen (**Table 4**). The minimum rumen pH reached during the time course of this study was lower in the Control group than the Butyrate group (4.96±0.09 Vs 5.20±0.05; p = 0.04). However, the addition of exogenous butyrate had no effect on the mean pH, max pH, the duration at which pH was below 5.6, or the area under the pH curve (p>0.05). DMI, milk production and production efficiency are presented in **Table 5**. Addition of exogenous dietary butyrate significantly lowered the DMI of the animals (17.41 Vs 24.40 kg day^{-1} ±0.77; p≤0.0001). Daily milk production was unaffected by treatment: 41.93 kg day^{-1} ±2.50 in the Control group to 34.74 kg day^{-1} ±2.50 in the Butyrate group (p>0.05). Interestingly, the daily milk production ratio, defined as the daily milk production Vs DMI, was significantly higher in the Butyrate group than in the Control group (1.96 Vs 1.67±0.09; p = 0.035).

3.2. Microarray Screen, qRT-PCR and Pathway Analysis

Gene expression results along with fold changes are presented in **Table 6**. Microarray data indicated the significant differential expression of 1191 genes (data not shown). These genes were screened in turn for involvement in NSHD, RM and IR pathways. Of the 49 genes selected for further analysis, 29 were confirmed significantly differentially expressed by qRT-PCR. Nine genes were found to be upregulated, 20 were downregulated and 20 were unaffected by butyrate supplementation. In turn, these genes were analyzed and placed into context-specific NSHD, RM and IR pathways, **Fig. 1 and Table 7**.

Table 6. Relative gene expression in rumen papillae from cows treated with or without butyrate

Symbol	Entrez Gene Name	Fold change	*P*-value
LCN2	lipocalin 2	27.0	0.000
MMP1	matrix metallopeptidase 1	21.6	0.002
MUC16	mucin 16, cell surface associated	14.9	0.001
GPX2	glutathione peroxidase 2 (gastrointestinal)	10.4	0.000
CSTA	cystatin A (stefin A)	7.1	0.000
FUT1	fucosyltransferase 1	4.3	0.000
SERPINE2	serpin peptidase inhibitor, clade E member 2	3.7	0.000
BCAM	basal cell adhesion molecule	1.5	0.006
RAC3	ras-related C3 botulinum toxin substrate 3	1.4	0.044
MTOR	mechanistic target of rapamycin	-1.4	0.027
AKIRIN2	akirin 2	-1.7	0.000
NFKBIZ	nuclear factor of kappa light polypeptide gene enhancer inhibitor zeta	-1.7	0.001
ACVR2A	activin A receptor, type IIA	-1.7	0.001
LAMB1	laminin, beta 1	-1.7	0.020
FRS2	fibroblast growth factor receptor substrate 2	-1.8	0.001
PPARD	peroxisome proliferator-activated receptor delta	-1.8	0.000
NFKB2	nuclear factor of kappa b light polypeptide gene enhancer 2	-2.0	0.001
LBP	lipopolysaccharide binding protein	-2.0	0.006
NEDD4L	E3 ubiquitin protein ligase	-2.1	0.000
SGK1	serum/glucocorticoid regulated kinase 1	-2.3	0.001
DEDD2	death effector domain containing 2	-2.6	0.000
MAP3K8	mitogen-activated protein kinase kinase kinase 8	-2.6	0.001
PARD6B	par-6 partitioning defective 6 homolog beta	-2.6	0.000
PLIN2	perilipin 2	-2.9	0.000
ADA	adenosine deaminase	-3.3	0.000
HPGD	hydroxyprostaglandin dehydrogenase 15-(NAD)	-3.3	0.000
FMO5	flavin containing monooxygenase 5	-3.4	0.000
BMP6	bone morphogenetic protein 6	-3.7	0.000
TCHH	Trichohyalin	-5.0	0.000
CD14/TLR4/LY96	TLR4 Receptor Complex	unchanged	ns
EGF	epidermal growth factor	unchanged	ns
EGFR	epidermal growth factor receptor	unchanged	ns
ERK1/2	extracellular signal related kinase 1/2	unchanged	ns
Fgf	fibroblast growth factor	unchanged	ns
Fgfr	fibroblast growth factor receptor	unchanged	ns
FN1	fibronectin 1	unchanged	ns
IGFBP7	IGF binding protein 7	unchanged	ns
IL10	interleukin 10	unchanged	ns
LY96	lymphocyte antigen 96	unchanged	ns
MIF	macrophage migration inhibitory factor	unchanged	ns
MLST8	MTOR associated protein	unchanged	ns
NFKB1	nuclear factor of kappa light polypeptide gene enhancer 1	unchanged	ns
PTGS1	prostaglandin-endoperoxide synthase 1	unchanged	ns
PTGS2	prostaglandin-endoperoxide synthase 2	unchanged	ns
SMAD1	mothers against decapentaplegic homolog 1	unchanged	ns
STAT6	signal transducer and activator of transcription 6, interleukin-4 induced	unchanged	ns
TGFB1I1	transforming growth factor beta-1-induced transcript 1	unchanged	ns
TLR4	toll-like receptor 4	unchanged	ns
TNFRSF6B	Tumor necrosis factor receptor superfamily member 6B	unchanged	ns

Table 7. Gene expression results and interpretation. *Biological interpretation in lieu of experimental model. Abbreviations: NSHD, non-specific host defense; Remodel, remodeling of cellular architecture; Other, non-categorized; IR, immune response; ECM, extracellular matrix; ROS, reactive oxygen species; PG, prostaglandins; NF-κB, nuclear factor kappa B; TGF-β, transforming growth factor type beta; TLR4, toll-like receptor 4

Category	Symbol	Function	Fold Change	Contextual Meaning*	Ref.
NSHD	LCN2	Iron sequestration from bacteria	27	↓ bacterial growth	(Flo *et al*., 2004; Schmidt-Ott *et al*., 2007)
RM	MMP1	ECM remodeling; degradation of ECM	21.6	Adaptation	(Pendas *et al*., 1996)
NSHD	MUC16	Mucous production and protection of epithelium	14.9	↓ bacterial attachment	(Perez and Gipson, 2008)
NSHD	GPX2	Protection against bacterial production of ROS in rumen	10.4	↓ bacterial attachment	(Brigelius-Flohe and Kipp, 2012)
NSHD	CSTA	Promotes cell-cell adhesion	7.1	↓ bacterial pathogenicity	(Blaydon *et al*., 2011)
NSHD	FUT1	Regulates pathogen attachment to epithelium	4.3	↓ bacterial attachment	(Yan *et al*., 2003; Wang *et al*., 2012)
NSHD	SERPINE2	Serine protease providing resistance to bacterial colonization	3.7	↓bacterial attachment	(Luo *et al*., 2011)
RM	BCAM	ECM receptor	1.5	Adaptation	(Eyler and Telen, 2006)
RM	RAC3	Cell growth, reorganization of the cytoskeleton	1.4	Adaptation	(Haataja *et al*., 2002)
RM	MTOR	Various; reorganization of the cytoskeleton	-1.4	Adaptation	(Sarbassov *et al*., 2004)
IR	AKIRIN2	Downstream TLR4 effector; regulates NF-κB	-1.7	Adaptation ↓IR	(Goto *et al*., 2008)
IR	NFKBIZ	NF-κB regulator	-1.7	Adaptation ↓IR	(Cowland *et al*., 2006; Totzke *et al*., 2006)
RM	ACVR2A	Reorganization of the cytoskeleton; downregulates immune response	-1.7	Adaptation ↓IR	(Lebrun *et al*., 1999; Tsuchida *et al*., 2004)
RM	LAMB1	ECM protein necessary for signaling and structural integrity	-1.7	Adatpation	(Taniguchi *et al*., 2009)
RM	FRS2	ECM reorganization; FGF receptor sigalling	-1.8	Adaptation	(Ong *et al*., 2000)
RM	PPARD	ECM reorganization	-1.8	Adaptation	(Tan *et al*., 2007)
IR	NFKB2	NF-κB regulator	-2.0	Adaptation ↓IR	(Al-Sadi *et al*., 2010)
IR	LBP	Mediator of LPS activity	-2.0	Adaptation ↓IR	(Gray *et al*., 1993)
IR	NEDD4L	Negative regulator of TGF-β signaling	-2.1	Adaptation ↓IR	(Kuratomi *et al*., 2005)
Other	SGK1	Na^+ channel regulatior	-2.3	Unknown	(Grahammer *et al*., 2006)
RM	DEDD2	ECM/cellular remodeling; low levels decrease apoptosis	-2.6	Adaptation	(Lee *et al*., 2002)
IR	MAP3K8	Various; low levels decrease immune response	-2.6	Adaptation ↓IR	(Miyoshi *et al*., 1991; Hatziapostolou *et al*., 2011)
NSHD	PARD6B	Cell polarization; low levels may increase tight junction integrity	-2.6	Adaptation	(Yamanaka *et al*., 2003)
IR	PLIN2	Maintenance of adipose tissue; may decrease macrophage recruitment	-2.9	Unknown ↓IR	(Hao *et al*., 2011)
NSHD	ADA	Maintenance of the immune system	-3.3	Adaptation ↓IR	(Wilson *et al*., 1991)
NSHD	HPGD	Metabolism of PG; low levels maintain protective PG	-3.3	Adaptation	(Cho *et al*., 2006)
Other	FMO5	Metabolism of drugs, pesticides, xenobiotics	-3.4	Unknown	(Janmohamed *et al*., 2001)
RM	BMP6	Matrix growth factor; low levels may aid reepithelialization	-3.7	Adaptation	(Kaiser *et al*., 1998)
NSHD	TCHH	Keratin filament-associated protein; low levels prevent rigidity of ECM	-5.0	Adaptation	(Steinert *et al*., 2003)

4. DISCUSSION

It has been shown that adaptation to energy-dense, high carbohydrate diets in dairy cows is facilitated by structural changes in the rumen favouring extracellular matrix remodelling (Steele *et al*., 2011a). This effect does not always follow a profound inflammatory phase and it was determined that favourable adaptive changes in the rumen take place beginning 1 week after a high grain challenge. The aim of the current study was to determine the efficacy of butyrate supplementation in ameliorating the negative ruminal effects induced by feeding a high carbohydrate to lactating dairy cows. In addition, since the effects of SARA on rumen physiological dynamics have been reported (Keunen *et al*., 2002; Penner *et al*., 2007), we sought to determine the effects of butyrate treatment on milk production, milk components, pH, SCFA, SCFA metabolism, the effects on NSHD and evidence for an IR.

4.1 Physiological Parameters

We previously reported pH results in cows fed a high grain diet that is in agreement with the current study (Steele *et al*., 2012). Of note however, is the effect of supplemental butyrate on minimum pH reported; the rumen of cows in the Control group reached a lower pH than did the Butyrate group. Since butyrate itself is a weak acid, it is unlikely that buffering by its conjugate base would be responsible for this effect. However, it is possible that butyrate altered the metabolic flux of the rumen, favouring the neutralization of H^+ through the efflux of bicarbonate. Similar effects have been reported (Kristensen *et al*., 1998; Penner *et al*., 2009a; Aschenbach *et al*., 2010).

A high dietary carbohydrate load such as that seen in this study is expected to favour ketogenesis. In the rumen, SCFA are created as the result of fermentation by resident microbes and are metabolized within the site of absorption in the epithelia of the rumen, prior to making their appearance as substrates in the general tissue beds (Bergman, 1990; Kristensen *et al*., 1998). The ensuing hyperketonemia and ketosis can be confirmed through plasma analysis of BHBA levels (Penner *et al*., 2009b). Indeed, plasma BHBA levels were raised considerably in the Butyrate group as expected, which confirmed experimental ketosis. This had a direct effect on DMI, which was substantially lower in the Butyrate group; the effects of high SCFA levels on increasing satiety and decreasing appetite have been documented previously (Arora *et al*., 2011; Lin *et al*., 2012). And although supplemental butyrate had no effect on daily milk production, milk fat, or milk protein, the efficiency of milk production was higher in the Butyrate cows. These results appear to be a function of a decreased DMI. The fact that the milk parameters differed in grain content from previous studies is likely due to the lower energy density of the current SARA diet (Lykos *et al*., 1997; Keunen *et al*., 2002).

In such a carbohydrate-rich diet, both commensal and pathogenic microbes thrive. This is because such microbes can use the more readily available sugars as a preferential metabolic substrate (Motoi *et al*., 1993; Gozho *et al*., 2005; Thibault *et al*., 2010). Although the grain level in our study was not as high as that previously reported, the 45% NFC is sufficient to promote the growth of gram negative bacteria, which is correlated with the levels of rumen LPS (Zebeli and Ametaj, 2009). Although the LPS levels in this study are in relative agreement with those published earlier (Khafipour *et al*., 2009a; 2009b; Li *et al*., 2012), it is not clear whether they are in sufficient concentration to favour transmigration and to trigger an immune response. In order for such a response to exist, free LPS must be transported to effector cells of the immune system by the soluble acute phase protein, LBP (Muta and Takeshige, 2001). Here we report that butyrate supplementation increases plasma levels of LBP. However, this effect may be muted by the addition of butyrate, as several groups have reported reductions in the downstream effects of LPS-mediated induction of the immune system (Chakravortty *et al*., 2000; Huuskonen *et al*., 2004; Morikawa *et al*., 2004; Ni *et al*., 2010) under similar conditions. In addition, SAA, also an acute phase protein, was unaffected by the addition of butyrate. Together, the data for LPS, LBP and SAA contradict earlier findings using similar models (Dionissopoulos *et al*., 2012a; 2012b), that although SARA can have profound effects on the physiology of the rumen, the bacterial overgrowth that typically occurs is not sufficient enough to result in a systemic inflammatory response. The tissue signals generated as a result of SARA at the site of tissue damage (Steele *et al*., 2011a) likely act locally in an autocrine or paracrine manner.

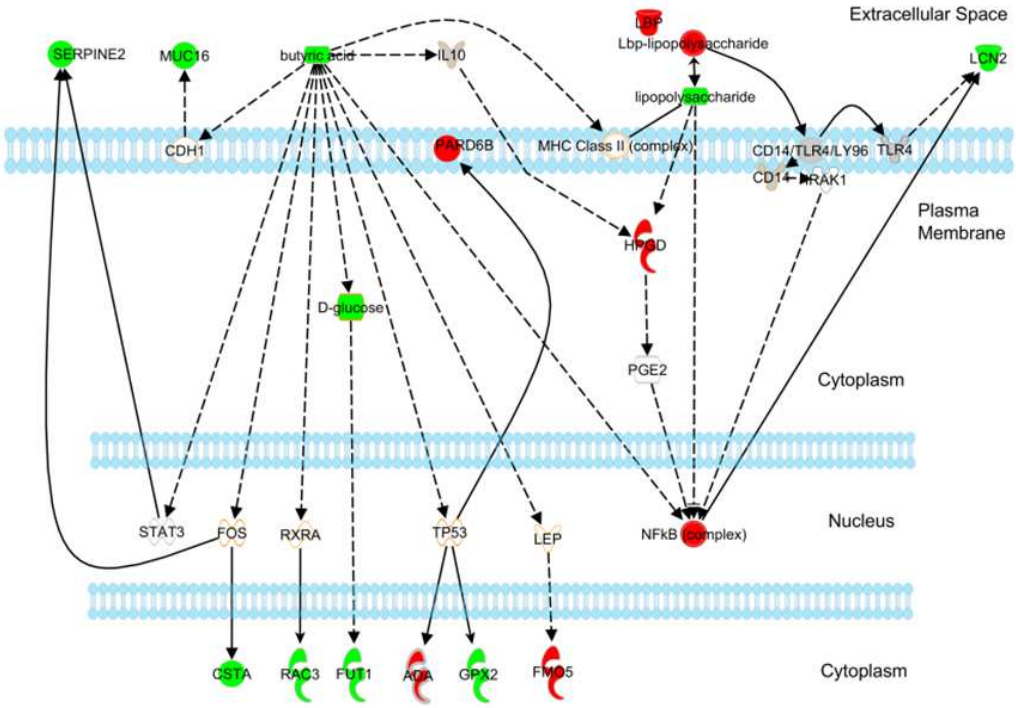
4.2. Gene Expression and Pathway Analysis

The rumen epithelium is part of a remarkable system that is meant to facilitate nutrient absorption and to function as a barrier to disease-causing organisms (Henrikson and Stacy, 1971). The molecular mechanisms responsible for the effects of rumen recovery or adaptation following SARA-mediated epithelial damage have not been determined. In

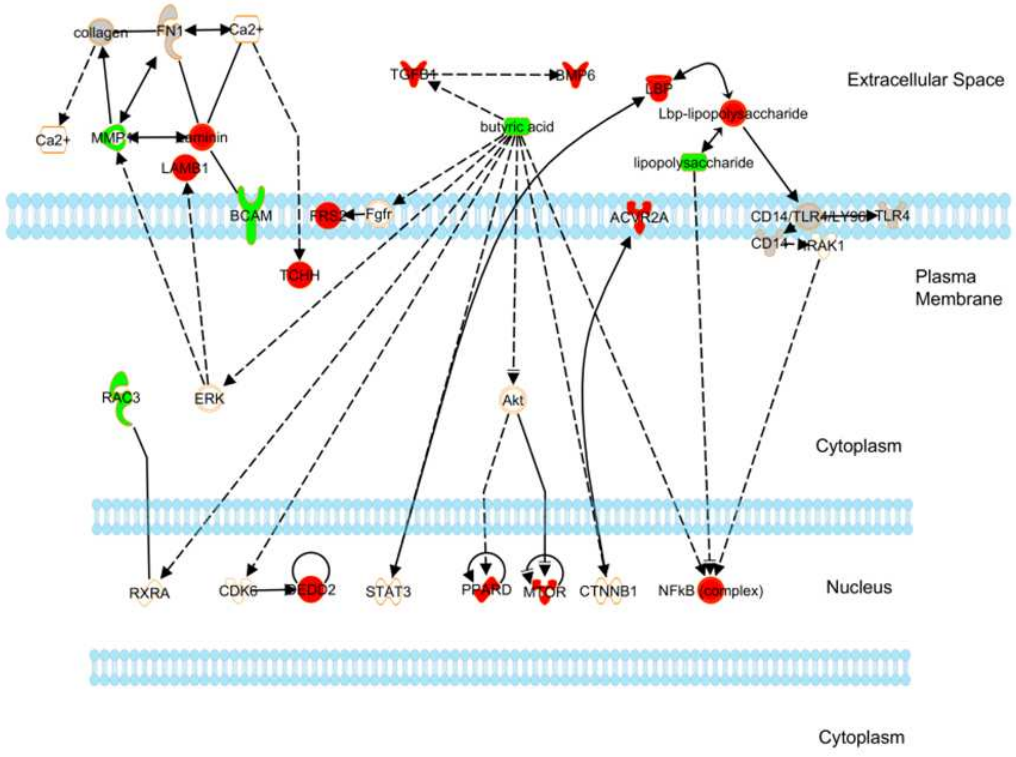
addition, since butyrate has been shown to be beneficial in models of gastrointestinal disease in a variety of species (Segain *et al*., 2000; Huuskonen *et al*., 2004; Borthakur *et al*., 2008; Hamer *et al*., 2008; Thibault *et al*., 2010), we sought to also determine the efficacy of exogenous butyrate administration by studying changes in rumen epithelial gene expression. To this end, a microarray analysis, followed by qRT-PCR confirmation was completed based on information from existing databases to determine the importance of any affected metabolic pathways at the molecular level. In this study it was found that the observed changes in gene expression could be grouped into three categories: 1. NSHD genes (LCN2, MMP1, MUC16, GPX2, CSTA, FUT1, SERPINE2, PARD6B, ADA, HPGD and TCHH), 2. RM Genes (BCAM, RAC3, MTOR, ACVR2A, LAMB1, FRS2, PPARD, DEDD2 and BMP6) and 3. Genes involved in the IR (NFKBIZ, NFKB2, LBP, NEDD4L, MAP3K8 and PLIN2). The individual genes, gene expression, role and contextual meaning are presented in **Table 7**.

NSHD genes are involved in conferring general protection to the organism and can take the form of increased barrier integrity, mucin production and reduced pathogen viability or attachment (Canonica, 2005). For example, by acting to sequester soluble Fe^{3+} from microbial siderophores, increased LCN2 expression can reduce the rate of microbial growth (Flo *et al*., 2004; Schmidt-Ott *et al*., 2007). Increased CSTA and GPX2 expression promote the maintenance and integrity of epithelial tight junctions (Blaydon *et al*., 2011) and limit the damaging effects of reactive oxygen species secreted by microorganisms (Brigelius-Flohe and Kipp, 2012). As such, they prevent the infiltration of potentially pathogenic bacteria into the deeper epithelial layers. Mucous layers are known lubricating barriers in epithelial cells and are a necessary component in the prevention of pathogen attachment (Perez and Gipson, 2008). FUT1 is an epithelial cell-surface receptor for pathogenic strains of E. coli and changes in its expression are thought to modulate pathogen attachment to epithelial cells (Yan *et al*., 2003; Wang *et al*., 2012) as is the expression of SERPINE2 (Luo *et al*., 2011). MUC16 which was highly elevated in this study is a glycosylated matrix protein known to provide a protective role in epithelial tissues (Perez and Gipson, 2008). PARD6B is one of a group of genes responsible for attachment and polarity of epithelial cells to basolateral membranes (Suzuki *et al*., 2001). In this study, PARD6B was downregulated which is in agreement with Gao *et al*. (2002) who found an inverse correlation between PARD6B expression and tight junction integrity. ADA is a ubiquitous enzyme which is needed for the development and maintenance of the immune system (Wilson *et al*., 1991). In studies where ADA expression was repressed or suppressed entirely, the immune response was limited and under full suppression, forms the biological basis for Severe Combined Immunodeficiency (SCID) mice (Martin Jr. and Gelfand, 1981; Wilson *et al*., 1991). ADA was reduced in our study helping to reduce the extent and severity of the immune response. HPGD, an enzyme responsible for prostaglandin metabolism, was reduced in our study. Prostaglandins can have diverse roles depending on the presence of disease. For example, Cyclooxygenase-1 (COX1) is constitutively expressed in a normal gastrointestinal tract and is responsible for the expression of protective prostaglandins, namely PGE2. COX2 is inducible and its expression rapidly increases in response to pathogenic stimuli (Cho *et al*., 2006; Wallace, 2008). It follows then that the lower expression of HPGD seen in this study can lead to protective effects in the rumen by controlling inflammation and helping to maintain levels of COX1 metabolites.

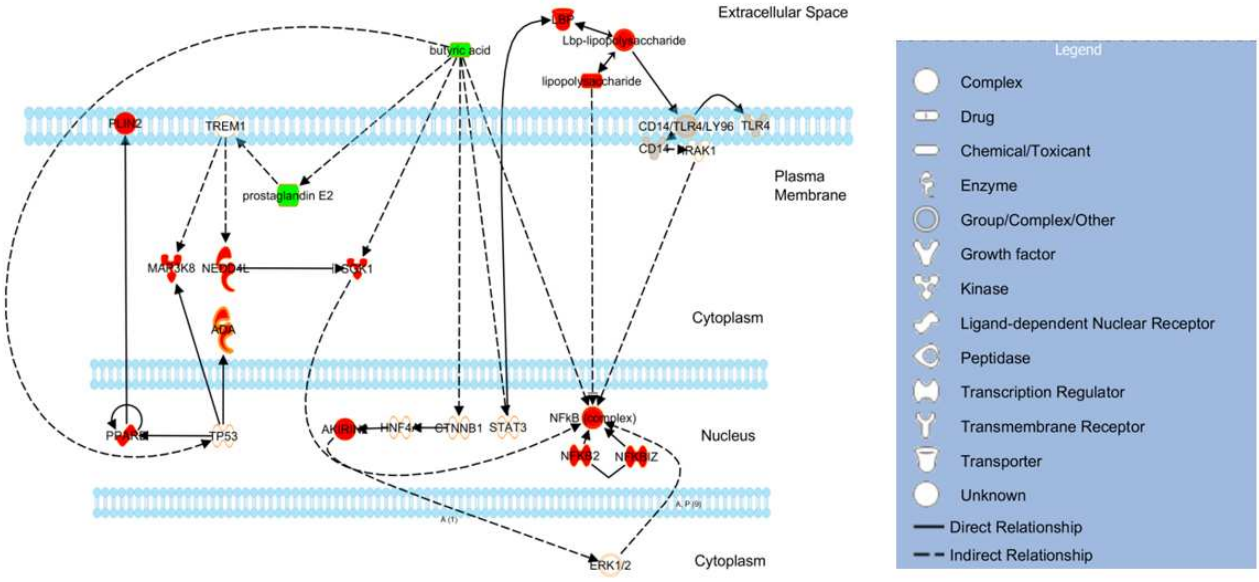
Genes involved in remodelling or adaptation is those that are responsible for the degradation of the extracellular matrix, its reorganization, cellular movement, signalling and adaptation to new metabolic requirements. The particulars of ECM reorganization with respect to wound healing have been reviewed extensively (Schreml *et al*., 2010). Briefly, ECM reorganization follows a tightly choreographed series of events initiated by the degradation of existing ECM proteins, followed by clearance, the deposition of a new matrix milieu and proliferation and differentiation of epithelial cells favouring the new conditions. MMP1 (+21.6) is a matrix metallopeptidase secreted by sub-epithelial fibroblasts in response to the need for a rearrangement of extracellular architecture in response to injury, growth, or adaptation (Birkedal-Hansen *et al*., 1993; Pendas *et al*., 1996; 1997). These events involve a wide variety of genes. In this study, BCAM (a receptor for matrix proteins) was elevated in addition to RAC3, which together aid in the remodelling process (Haataja *et al*., 2002; Eyler and Telen, 2006). It is unknown how long the early phases of remodeling occur in rumen epithelium. However, evidence from this study suggests that on the day the biopsies were taken for analysis, the animals were still in the early phase of remodeling. This can be seen in the downregulation of LAMB1 (Taniguchi *et al*., 2009), BMP6 (Kaiser *et al*., 1998), ACVR2A (Lebrun *et al*., 1999; Tsuchida *et al*., 2004), PPARD (Tan *et al*., 2007), TCHH (Steinert *et al*., 2003) and in the downregulation of MTOR, which has been shown to be necessary for ECM degradation (Sarbassov *et al*., 2004; Ong *et al*., 2007).


Extracellular Space
LBP
Lbp-lipopolysaccharide
SERPINE2
MUC16
butyric acid
IL10
LCN2
lipopolysaccharide
CDH1
PARD6B
MHC Class II (complex)
CD14/TLR4/LY96
TLR4
CD14
IRAK1
Plasma Membrane
HPGD
D-glucose
PGE2
Cytoplasm
STAT3
FOS
RXRA
TP53
LEP
NFkB (complex)
Nucleus
GSTA
RAC3
FUT1
ACTA
GPX2
FMO5
Cytoplasm

(A)


collagen
FN1
Ca2+
TGFB1
BMP6
Extracellular Space
LBP
Lbp-lipopolysaccharide
Ca2+
MMP
butyric acid
laminin
lipopolysaccharide
LAMB1
BCAM
IRS2
Fgfr
ACVR2A
CD14/TLR4/LY96
TLR4
CD14
IRAK1
TCR
Plasma Membrane
RAC3
ERK
Akt
Cytoplasm
RXRA
CDK6
CCND2
STAT3
PPARD
MTOR
CTNNB1
NFkB (complex)
Nucleus
Cytoplasm

(B)



(C)

Fig. 1. Context-specific pathway analysis of genes upregulated (green) or downregulated (red) due to the addition of exogenous butyrate supplementation at 2.5% DMI. Panels A, B, and C represent genes involved in Non-Specific Host Defense (NSHD), remodeling, or the Immune Response (IR) respectively. Genes coloured gray indicate no change while those in white are present as proposed pathway intermediates where necessary, but were not represented in the analysis

In a similar fashion, FRS2, which plays an integral role in cellular adhesion and growth (Ong *et al*., 2000; Norambuena and Schwartz, 2011), was downregulated and thus likely contributed to the degradation and reorganization of the ECM. Finally, during the adaptive process, recovering healthy epithelium is induced to proliferate. An anti-proliferative factor, DEDD2 was downregulated in this study. Apoptosis is usually seen in polymorphonuclear cells following the inflammatory phase of wound healing in skin models (Lee *et al*., 2002; Eming *et al*., 2007).

IR genes are general indicators of immune system status and stimulation. Indeed, a continued and prolonged immune response such as that seen in SARA, can be detrimental to the growth and maintenance of an organism. In fact, the partitioning of nutrients for the execution of an immune response is well known (Dionissopoulos *et al*., 2001; Meazza *et al*., 2004; Li *et al*., 2006). One of the most important ways in which tissues regulate the immune response is through the activation or suppression of NF-κB signalling pathways (Gilmore, 2006). In this study, all genes in the IR category were downregulated by the addition of exogenous butyrate. Most of these are upstream effectors of NF-κB (AKIRIN2, NFKBIZ, NFKB2) (Cowland *et al*., 2006; Totzke *et al*., 2006; Goto *et al*., 2008; Al-Sadi *et al*., 2010). Others such as LBP (- 2.0-fold), require binding of free LPS to its receptor, TLR4 for downstream signalling events to take place (Gray *et al*., 1993). In addition, Li *et al*. (2011) were able to determine that NEDD4L is a positive regulator of NF-κB which is in agreement with our results. Similarly, the ability of MAP3K8 and PLIN2 to activate the NF-κB pathway has been documented (Chan and Reed, 2005; Cismasiu *et al*., 2009; Mattos *et al*., 2010). These direct relationships are supported by results presented here.

All expression data was compiled and analyzed for pathway interactions using the Ingenuity Systems ™ KEGG database (Tanabe and Kanehisa, 2012; Zhou, 2013). As in **Table 7**, the PCR data was placed in the three functional categories of NSHD, RM and IR and hypothetical pathway diagrams were constructed for each based on experimental findings of the response to LPS and exogenous butyrate following a grain challenge. These figures clearly demonstrate the central role of butyrate in our experimental model. **Figure 1A** indicates the beneficial effects of Butyrate on NHSD. **Figure 1B** represents the effect of butyrate on the modulation of the rumen Epithelial Extracellular Matrix (ECM) and **Fig. 1C** shows the downregulation of the immune response to LPS following butyrate administration. Butyrate has been well known as a Histone Deacetylase (HDAC) inhibitor and its effects in attenuating inflammatory

conditions have been hypothesized to be the result of modulation of the NF-κB signalling cascade at multiple levels (Miyoshi *et al*., 2011). TLR4/NF-κB controls the expression of many inflammatory genes (**Fig. 1C**) and so it makes sense that it can serve as a control point for limiting the IR (Suuronen *et al*., 2003). Indeed, several studies have reported a decrease in the TLR4/NF-κB response to LPS following butyrate administration (Huuskonen *et al*., 2004; Wu *et al*., 2012). In addition to reductions in NF-κB family genes, we propose that butyrate limits the extent of the immune response to LPS by reducing the expression of additional genes shown to be effectors of the IR. Although we have placed the effects of butyrate in three different categories, it was our intent to demonstrate that butyrate mediates its beneficial effects through the differential expression of multiple genes related to NSHD, remodelling and the IR within epithelial cells exposed to LPS.

To our knowledge, this type of analysis has not been conducted on rumen epithelium adapting to an acidotic diet in response to exogenous butyrate. As with any new therapeutic intervention, it is not known if its administration will have negative effects on the well-being on the host. This type of approach allows the quantification of such findings and demonstrates the viability of butyrate supplementation in the treatment of SARA in response to a high grain/high energy diet.

5. CONCLUSION

Although the data presented here indicated that butyrate supplementation had little effect on milk parameters and pH, it can be seen that the extent of the immune response in SARA is further limited by reductions in the expression of the acute phase protein, LPS. In addition, gene expression data clearly shows the enhancement of preparative and compensatory mechanisms in SARA facilitated by butyrate. The advanced pathways explored here represent potential biomarkers and indicate the pervasive and yet benign nature of butyrate supplementation. It can be seen that exogenous butyrate reduces the expression of key inflammatory markers, enhances non-specific cellular defences to microorganisms and the remodelling of the ECM to favour adaptation to a high grain/high energy environment.

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