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Feed Restriction in Angus Steers Impacts Ruminal Bacteria, Its Metabolites, and Causes Epithelial Inflammation

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Simple Summary: Diet composition and intake level are important factors affecting microbial communities in the rumen. A low level of feed intake, common during the transition into lactation or periods of stress (e.g., transport, comingling), changes ruminal microbiome composition. The specific objectives of our study were to harvest ruminal digesta and epithelial biopsies from ruminally cannulated steers before, during, and after feed restriction to determine alterations in the relative abundance of 16 major ruminal bacteria, metabolite profiles, and mRNA abundance of inflammatory gene markers. Short-term feed restriction increased the abundance of *Succinivibrio dextrinosolvens*, *Streptococcus bovis*, and *Bifidobacteria* spp. (N124), highlighting their role in degrading rapidly fermentable carbohydrates to intermediates such as lactate and succinate. The significant decrease in the levels of most metabolites during feed restriction was likely due to both a lack of feed for microbes and changes in their digestive processes. Thus, short-term feed restriction affected the ruminal bacteria composition, lowered the levels of most ruminal metabolites, and caused inflammation.

Abstract: We identified alterations in the ruminal microbiome, metabolome, and epithelial inflammatory response due to moderate feed restriction (FR). Ruminal digesta and epithelial biopsies from seven ruminally cannulated Angus steers were initially collected during ad libitum access to feed (PRE). After a 10 day recovery, steers underwent a 3-day FR period (FRP) at 25% intake of PRE followed by a 15 day recovery (POST) phase with ad libitum access to feed. At the end of FRP and POST, ruminal digesta and epithelial biopsies were collected again for microbial DNA and tissue RNA extraction. RT-qPCR was applied for relative microbial abundance and RNA extraction. Metabolite profiling of digesta was performed via GC-MS. The abundance of *Succinivibrio dextrinosolvens*, *Streptococcus bovis*, and *Bifidobacteria* spp. (N124) was higher ($p < 0.05$) during FRP than PRE and POST, while *Lactobacillus* spp. *(C25)*, *Escherichia coli (EC42405)*, *Fibrobacter succinogenes*, and *Megaspheara elsdenii* abundances were lower in FRP than PRE ($p < 0.05$). The *TNF* and *TLR2* mRNA abundance was greater in FRP than PRE ($p < 0.05$). Among 15 detected amino acids, glutamine, isoleucine, lysine, phenylalanine, threonine, and valine were lower ($p < 0.05$) in FRP than PRE. Metabolite pathway analysis revealed alterations in amino acid, fatty acid, vitamin, and energy metabolism during FRP ($p < 0.05$). The mRNA of the proinflammatory genes *TNF* and *TLR2* in the epithelium peaked ($p < 0.05$) at FRP and remained higher at POST. Results indicated that a short FR influenced ruminal bacteria, reduced concentrations of most metabolites, and triggered an inflammatory response.

Keywords: beef cattle; immune response; microbiota; rumen function

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1. Introduction

Diet composition, e.g., forage and concentrate level, and rate of intake are two well-known effectors of changes in the numbers and profiles of microbial communities in the rumen [1,2]. The lack of access to feed, as it may occur normally during the transition into lactation, or during periods of stress (e.g., transport, comingling) also can impact the physiology and metabolism in ruminants. Feed restriction (FR) in Holstein cows induces a dramatic reduction in milk production and changes milk composition. A comprehensive review of the available literature [3] concluded that FR increases milk fat content and somatic cell count while reducing milk protein and lactose content. Additionally, this review discussed the impact of FR on the endocrine system, noting decreased concentrations of insulin, IGF-1, and leptin [3], which resulted in heightened fat and muscle mobilization, as well as a decrease in ileal villus height and crypt depth [4,5].

Angus–Holstein calves exposed to restricted concentrate feeding had greater intestinal permeability, demonstrated by higher lactulose concentrations in the bloodstream [6]. Employing a 40% reduction in feed intake to mimic decreased voluntary daily dry matter consumption in dairy cows had adverse effects on the structure of the ileum and resulted in increased levels of circulating lipopolysaccharide (LPS)-binding protein and serum amyloid A [4]. As determined by the paracellular transport marker Cr-EDTA, it is noteworthy that even a 40% FR induced a "leaky gut" [4].

Short-term FR in beef heifers resulted in elevated plasma cortisol, haptoglobin, and ceruloplasmin levels, indicating an inflammatory response [7]. In beef cattle, a 5-day 25% reduction in feed intake led to decreased ruminal total volatile fatty acid and propionate concentrations, which were linked to a reduced total absorption rate of volatile fatty acids [8,9]. Depending on the extent of FR, research has suggested that the overall barrier function of the digestive tract may be compromised, potentially allowing harmful molecules like LPS to pass into the bloodstream [10]. Additionally, at a molecular level, FR at 0.4 times the maintenance energy requirements increased the abundance of leptin receptor mRNA in both the duodenum and liver of steers [11].

Feed restriction triggered alterations not only in metabolic processes and physiology but also in the ruminal microbiome. For example, in Nellore bulls, a period of FR decreased the abundance of *Prevotella ruminicola* species and *Succinovibrio* genus [12]. In pregnant ewes, the *Lentisphaerae* and *Elusimicrobia* phylum abundance and the genus abundance of *Papillibacter*, *Comamonas*, and unclassified *Neisseriaceae* were greater during FR [13]. These animals also had a lower abundance of *Suttonella*, *Desulfobulbus*, and *Howardella*. Along with reported changes at the microbial level due to diet, the application of NMR (nuclear magnetic resonance) spectroscopy nearly 15 years ago allowed the first evaluation of changes in ruminal metabolites in lactating dairy cows as a function of the incremental feeding of cereal grain [14]. A simultaneous evaluation of metabolites and major bacteria in the rumen during FR could provide valuable information. For example, it may allow the identification of metabolites that can have toxic or inflammatory effects on the ruminal epithelium, as reported in dairy cows fed high-grain diets at parturition [15].

We hypothesized that a short period of FR dysregulates the abundance of major ruminal bacteria and metabolite profiles in the rumen and triggers an upregulation of inflammation-related markers in the epithelium. The specific objectives were to harvest ruminal digesta and epithelial biopsies from ruminally cannulated steers before, during, and after FR in order to assess changes in the relative abundance of 16 major ruminal bacteria along with metabolite profiles via GC-MS, and the mRNA abundance of inflammatory gene markers.

2. Materials and Methods

2.1. Animal Handling, Experimental Design, and Sample Collection

The Institutional Animal Care and Use Committee (IACUC) at the University of Illinois approved the procedures (#19182). Seven cannulated Angus steers (663 $\pm$ 73 kg BW) from the University of Illinois Beef Unit herd were used. Previous studies focused on the effect

of nutrition on the ruminal environment (including mRNA abundance) have used 6 to 8 [16–18] or even 5 animals [19] and demonstrated that this range is appropriate in terms of statistical power. Furthermore, the use of a repeated sampling protocol over time, as we reported in a previous study of the ruminal epithelium in the periparturient period of dairy cows [20], enhances the statistical power because each animal serves as its own control. Thus, the choice of 7 steers to address our objectives was deemed appropriate. Steers were free of clinical disease and were fed a typical finishing diet composed of 40% corn silage, 15% modified wet distiller grain, and 35% dry rolled corn as a total mixed ration (TMR). The first sample collection occurred at day 5 during a 15-day period where steers had ad libitum access to feed in group-fed conditions. At the end of PRE, all steers underwent a 3-day FR period (FRP) during which they were fed at 25% of the PRE period. An additional 15-day period was used as a post-FR period (POST) during which steers had ad libitum access to feed similar to PRE. Ruminal digesta was collected at the beginning of the experiment (day 0) and at the end of FRP and POST (days 13 and 28). Steers were initially housed in a pen of 4.88 × 4.88 m in dimension and with an individual waterer. Individual intakes were not recorded while steers remained in this pen. The pen was constructed of 5.08 cm galvanized steel tubing, had slatted concrete floors covered by interlocking rubber matting, and was in a barn constructed of a wood frame with a ribbed metal roof and with siding on the north, west, and east sides. The south side of the barn was covered with polyvinyl chloride-coated 1.27 by 1.27 cm wire mesh bird screen and equipped with retractable curtains for wind protection. After the first sampling, steers were moved to a metabolism barn with tie stalls. Stalls (2.3 × 1.3 m) were equipped with individual feed bunks and nonsiphoning automatic water bowls. The barn was equipped with a heating, ventilation, and air-conditioning system, providing a controlled environment set at 18.3 °C. Steers remained in their individual stalls until the end of FRP. The amount of TMR fed to each steer during the FRP period was calculated based on the group-fed estimates of dry matter intake. There were no TMR refusals during the FRP period. During POST, the steers were returned to the original barn and remained as a group. Four layers of gauze were used to filter ruminal contents, and the mixed digesta sample was immediately stored in liquid nitrogen and subsequently at −80 °C until analysis. Ruminal papillae were biopsied from the ventral sac using surgical scissors after pulling the tissue out of the rumen via the cannula in a chute. Biopsy samples were immediately stored in liquid nitrogen and eventually at −80 °C until analyses. Steers did not show signs of discomfort during the procedure, and no analgesics were administered.

2.2. DNA Extraction and RT-qPCR

The microbial DNA from 500 µL of ruminal digesta was extracted using a commercial kit (QIAamp® PowerFecal® DNA Kit, Qiagen, Hilden, Germany) according to the manufacturer's protocol. The DNA concentration was measured using a NanoDrop ND-1000 (NanoDrop Technologies, Wilmington, NC, USA), and the DNA was stored at −80 °C until analysis. The RT-qPCR protocol for bacterial abundance determination followed the exact steps as in one of the previous studies [21]. Details and primers are in the Supplementary File. The primer sequences were originally published in one of our previous studies [21].

The original standard was produced by mixing samples and subsequently applied to serial dilution to generate the standard curve. The relative 16S rRNA copy standard curve was calculated via the Ct value and log10 of the relative copies. The relative copy of the original standard was set to 100%. The slope and intercept were calculated from the relative copy standard curve for exponential amplification value (EAMP, amplicon doubling per cycle) calculation. The equation for determining relative 16S rRNA copies per run was:

$$E_{AMP} = 10^{(-\frac{1}{slope})};\ \text{Relative copies per run} = E_{AMP}{}^{(intercept-Ct)}$$

To obtain the relative 16S rRNA copies per nanogram (ng) of DNA, the relative 16S rRNA copies per run were divided by the DNA amount (ng). Subsequently, the 16S rRNA copies per microliter (μL) of ruminal digesta were derived by multiplying the relative 16S rRNA copies per ng of DNA by the DNA concentration (ng/μL) of the ruminal digesta sample.

2.3. RNA Extraction, cDNA Synthesis, and RT-qPCR

Approximately 50 mg of tissue was homogenized with 1 mL Qiazol (Qiagen, Hilden, Germany), and RNA was extracted according to our published protocols (Supplemental File). The average RNA Quality Number was 9.48. The cDNA and RT-qPCR were performed according to our previous protocols (Supplemental File).

2.4. Metabolomics Analysis

Samples of ruminal digesta were delivered to the Metabolomics Unit of the High-Throughput DNA Sequencing and Genotyping Unit of the W. M. Keck Biotechnology Center at the University of Illinois, Urbana-Champaign, for metabolomics analysis via Gas chromatography–mass spectrometry (GC-MS). Details are in the Supplementary File. Pathway enrichment analysis using the 124 detected metabolites was performed via MetOrigin (https://metorigin.met-bioinformatics.cn/home/; accessed on 17 April 2023) [22]. The match index was calculated by the number of matched metabolites with significant differences divided by the number of metabolites in a specific pathway.

2.5. Statistical Analysis

Data were analyzed using the MIXED procedure of SAS OnDemand for Academics (SAS Institute Inc., Cary, NC, USA; https://welcome.oda.sas.com/login). Experimentally, variations observed across periods were assumed to occur primarily due to the presence or absence of FR. The model included the fixed effect of the period (PRE, FRP, and POST) and the random effect of the steer. The bacterial abundance was normalized via $\log[p/(1-p)]$, where p represents the relative abundance of bacterial species. The real-time quantitative PCR data were log2 transformed prior to statistical analysis. The final least square means (LSM) were back-transformed based on the original LSM generated in SAS ($OLSM$): 2^{OLSM}. The standard error of the mean (SEM) was transformed as $\left(2^{OLSM}\right) \times (\log 2) \times OLSM$. The separation of least squares means was performed via the PDIFF procedure with Tukey adjustment.

3. Results and Discussion

3.1. Abundance of Specific Bacteria

The relative abundance of *Clostridium* spp. (*C122*) and *Selenomonas ruminantium* was greater than 100‰ (Table 1). The relative abundance of *Lactobacillus* spp. (*C25*) and *Escherichia coli (EC42405)* were lower ($p < 0.05$) in FRP than POST. The relative abundance of *Fibrobacter succinogenes* and *Megaspheara elsdenii* was lower ($p < 0.05$) in FRP compared with PRE and tended to be lower in FRP ($p = 0.06$, $p = 0.08$) compared with POST. Although no significant differences in *Butyrivibrio fibrisolvens* and *Succinimonas amylolytica* relative abundance were detected between PRE and FRP, their relative abundance was greater ($p < 0.05$) in POST compared with FRP. The *Succinivibrio dextrinosolvens* relative abundance was ~60-fold greater ($p < 0.05$) in FRP than PRE and POST. Similar to *Sucinivibrio dextrinosolvens*, *Streptococcus bovis* and *Bifidobacteria* spp. (N124) relative abundance was greater ($p < 0.05$) in FRP compared with PRE and POST. The *Prevotella bryantii* relative abundance was greater ($p < 0.05$) in POST compared with FRP. In contrast, the *Eubacterium ruminantium* relative abundance in PRE tended ($p = 0.06$) to be lower than FRP, and its relative abundance in FRP was greater ($p < 0.05$) than in POST. The 16S rRNA, the ribosomal RNA in eukaryotes, was used as an indicator of total bacterial numbers in our study. Two universal bacterial primers were selected from our previous study to calculate the relative 16S rRNA copies

relative to the original standard. Both universal 1 and 2 16S rRNA copies (Figure 1) were greater ($p < 0.05$) in FRP than PRE and POST.

Table 1. Abundance (relative units) of major bacterial species in ruminal digesta from beef steers ($n = 7$) before (PRE) and during a 3-day feed-restriction period (FRP) at 25% intake of PRE and after a 15-day recovery (POST).‰: per myriad (per ten thousand). Bacteria are arranged in descending order of relative abundance in PRE.

Species	Period			*p*-Value
	PRE	FRP	POST	
>100‰ in PRE				
Clostridium spp. *(C122)*	578.27 ^{b}	687.05 ^{ab}	989.57 ^{a}	0.03
Selenomonas ruminantium	140.65	89.01	119.34	0.10
<100‰, >0.1‰ in PRE				
Lactobacillus spp. *(C25)*	0.63 ^{a}	0.09 ^{b}	0.58 ^{a}	<0.01
Escherichia coli (EC42405)	1.43 ^{a}	0.19 ^{b}	1.14 ^{a}	<0.01
Fibrobacter succinogenes	0.79 ^{a}	0.29 ^{b}	0.72 ^{ab}	0.02
Megaspheara elsdenii	1.32 ^{a}	0.28 ^{b}	0.70 ^{ab}	<0.01
Butyrivibrio fibrisolvens	0.64 ^{a}	0.41 ^{a}	0.10 ^{b}	<0.01
Succinimonas amylolytica	0.25 ^{ab}	1.87 ^{a}	0.06 ^{b}	0.02
Bacteroides spp. *(BF25)*	1.39 ^{a}	0.46 ^{ab}	0.14 ^{b}	0.05
Succinivibrio dextrinosolvens	0.55 ^{b}	76.00 ^{a}	1.36 ^{b}	<0.01
Rumicoccus flavefaciens	1.80	0.63	0.87	0.31
Rumicoccus albus	0.31	0.05	0.21	0.17
<0.1‰ in PRE				
Streptococcus bovis	0.018 ^{b}	0.211 ^{a}	0.010 ^{b}	0.01
Bifidobacteria spp. (N124)	0.008 ^{b}	0.130 ^{a}	0.002 ^{b}	<0.01
Prevotella bryantii	0.035 ^{ab}	0.009 ^{b}	0.129 ^{a}	<0.01
Eubacterium ruminantium	3.29×10^{-6} ^{ab}	9.02×10^{-6} ^{a}	1.48×10^{-6} ^{b}	<0.01

^{a,b} Means in the same row with different superscripts differ ($p < 0.05$).

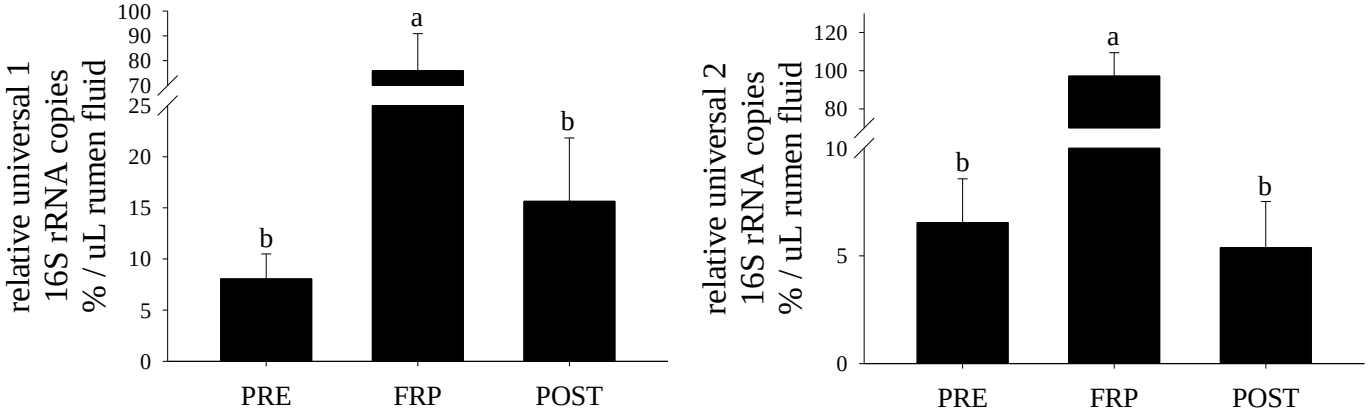

Figure 1. Relative universal 1 and 2 16S rRNA copies per µL of ruminal digesta from beef steers ($n = 7$) before (PRE) and during a 3-day feed-restriction period (FRP) at 25% intake of PRE and after a 15-day recovery (POST). ^{a,b} Means differ ($p \leq 0.05$).

Feed restriction clearly dysregulated the profiles of bacteria in the rumen, but many appeared to recover following the FR period. Although ruminal pH was not measured in the present study, and it is known that FR often decreases pH [8,15], the recovery can be attributed, in part, to the increase in pH within the rumen after animals are allowed free access to feed [8]. Among those bacteria that experienced increases during FRP and a return to values similar to PRE, the increase in *Succinivibrio dextrinosolvens* during FR could have been related to its ability to utilize ammonia and urea [23] during the shortage of feed. The utilization of non-protein nitrogen such as ammonia and urea increased the nitrogen

sources for the ruminants. The increase in *Streptococcus bovis* and *Bifidobacteria* spp. (N124), both acid-resistant bacteria, during FR agrees with the notion that a lack of access to feed likely decreases ruminal pH.

Furthermore, this response also suggested that lactic acid production in the rumen increased during FRP. The decrease in *Lactobacillus* spp. *(C25)* and *Escherichia coli (EC42405)* abundance, both lactic acid producers, during FRP was surprising and potentially correlated with the decrease in *Megasphaera elsdenii* abundance, which utilizes lactic acid and prevents drastic drops in pH [24]. This response agreed to some extent with the lack of statistical changes in lactate concentration. It is well known that lactate accumulation induces ruminal acidosis and could be fatal because the D-isomer of lactate is a neurotoxin [25]. Research with cattle [26] and sheep [27] identified a connection between lactate production and utilization, feed conversion efficiency, and methane production. These studies suggest that high-producing animals, which exhibit lower methane emissions, tend to have a ruminal microbiota with increased activity of lactate-producing and lactate-utilizing bacteria, particularly *Megasphaera elsdenii* and *Coprococcus catus (Lachnospiraceae)*. The proposed explanation is that compared with fermentation by hydrogen-producing microorganisms, carbohydrate fermentation involving lactate cross-feeding produces less hydrogen, and consequently less methane. Instead, the carbon is primarily directed towards propionate, which enhances the energy available from the diet for the host [28]. Thus, the decrease in lactate-producing and lactate-utilizing bacteria (*Lactobacillus* spp. *(C25), Escherichia coli (EC42405)*, and *Megasphaera elsdenii*) potentially could increase the methane production and consequently reduce energy availability.

Despite the dysregulation in the abundance of these major bacterial species, not all the bacteria altered during FRP returned to values detected in PRE even after the 15-day of ad libitum access to feed. In fact, the nearly two-fold increase in *Clostridium* spp. *(C122)* abundance in POST relative to PRE was surprising given that it was the most abundant species in the present study. Although several species of *Clostridium* are considered pathogens that cause disease to animals, e.g., *Clostridium botulinum*, *Clostridium tetani*, and *Clostridium perfringens* [29–31], species such as *Clostridium butyricum* can metabolize acetate and lactate to butyrate under normal and stressed conditions [32]. *Clostridium botulinum* produces neurotoxins and can grow in the cow's gastrointestinal tract [33]. *Clostridium perfringens* causes enterotoxemia in dairy cows [34,35]. The fact that *Clostridium* spp. *(C122)* increased linearly in spite of the return to full feed for a period of 15 days also raises the possibility that they exert positive effects within the ruminal ecosystem [36]. Whether such effects are due to their cellular components and metabolites, such as butyrate and indolic compounds, is unclear [37]. What seems evident judging from non-ruminant studies is that metabolites such as these can play a probiotic role directly by interacting with gut cells [36].

The decrease in the abundance of *Fibrobacter succinogenes* during FRP was not unexpected as it is an important bacteria for fiber digestion [1]. Although the abundance of other fibrolytic bacteria (*Selenomonas ruminantium*, *Butyrivibrio fibrisolvens*, *Rumicoccus flavefaciens*, *Rumicoccus albus*, *Prevotella bryantii*) did not differ between PRE and FRP, a decrease in cellulose digestion ability was reported during FR [38]. Thus, it appears that some fibrolytic bacteria are more susceptible to short periods of undersupply of nutrients.

Despite the fact that FR is often considered an unfavorable condition for the ruminal microbiota, i.e., due to a lack of nutrients, the increase in relative copies of 16S rRNA during FRP indicated a greater bacterial concentration in ruminal digesta. Whether there were differences in the numbers of live or dead bacterial cells could not be assessed. It is likely that the decrease in fermentable solid feed in the rumen during FR potentially caused bacteria to detach from the rumen solids. Consequently, while bacterial concentration appeared higher in ruminal digesta during FRP, it remains uncertain if this effect was a true reflection of the whole rumen. Furthermore, variations in bacterial composition may influence the number of 16S rRNA copies, as a single bacterial genome could contain several or even more than 10 copies of 16S rRNA [39]. Because of the substantial impact

of dominant bacteria and its composition on metabolite concentrations [40], we sought to investigate metabolite profiles in ruminal digesta.

3.2. Pro-Inflammatory mRNA Abundance

The mRNA abundance of tumor necrosis alpha (*TNF*, also known as *TNFA*) and toll-like receptor 2 (*TLR2*) in ruminal tissue was greater ($p < 0.05$) during FRP than PRE (Figure 2). Intermediate responses for *TNF* and *TLR2* were observed at POST relative to PRE and FRP.

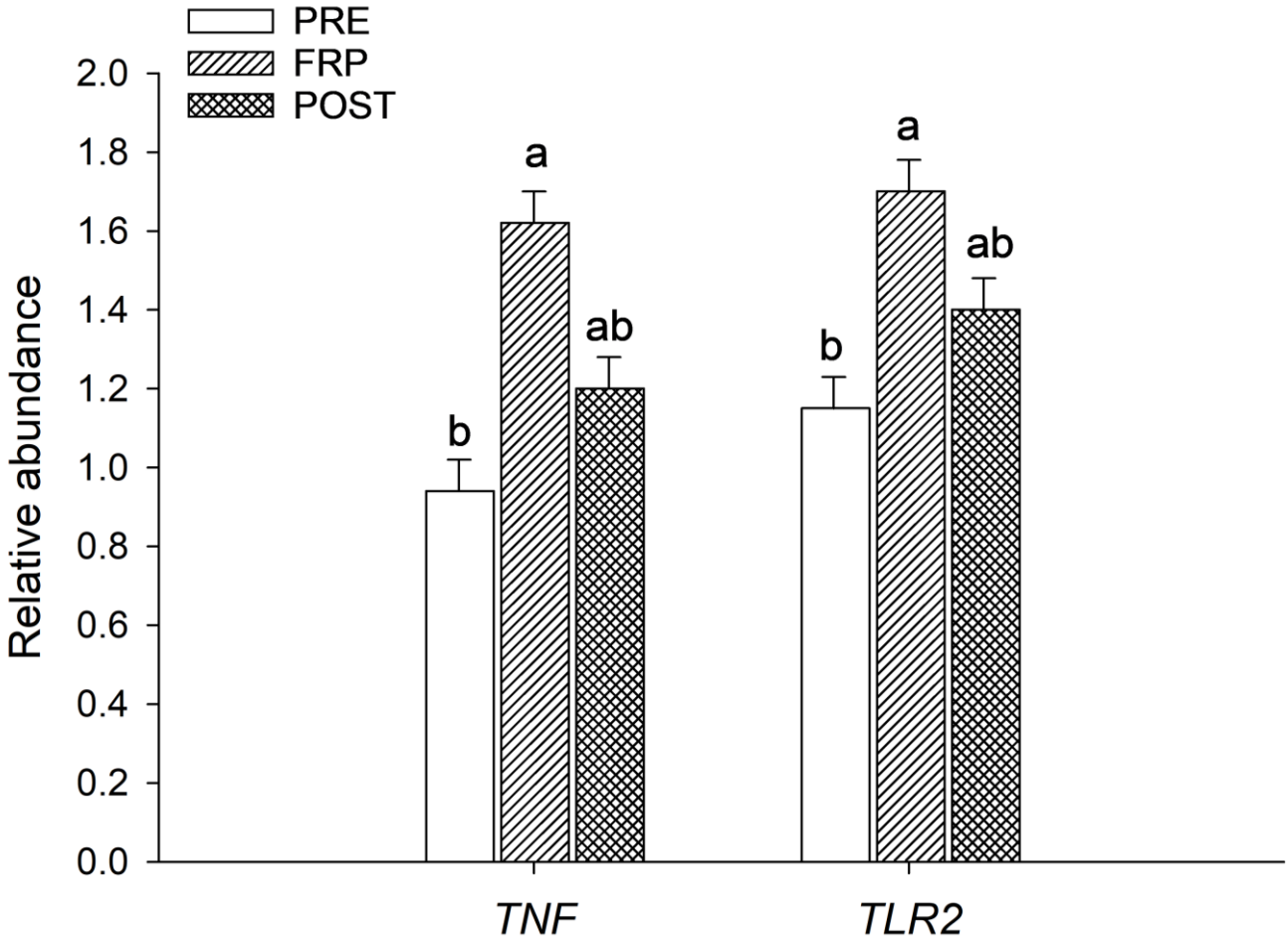

Figure 2. mRNA abundance (relative units) of the proinflammatory genes tumor necrosis factor (*TNF*) and toll-like receptor 2 (*TLR2*) in ruminal epithelium from beef steers during a pre-feed-restriction period (PRE), a 3-day 25% feed-restriction period (FRP), and a post-feed-restriction period (POST) ($n = 7$). ^{a,b} Means differ ($p \leq 0.05$).

The higher *TNF* and *TLR2* during FRP suggested the existence of an inflammatory state in the ruminal epithelium. In dairy heifers, a 24 h FR increased the plasma concentration of LPS-binding protein, which is considered a pro-inflammatory and gut permeability marker [7]. Although we did not assess gut permeability in the present study, if, in fact, the FRP caused the disruption of the ruminal barrier, it may have allowed for the passage of bacterial products capable of eliciting an inflammatory response. For example, the increased passage of bacterial-derived lipoteichoic acid and lipopolysaccharide across the gut epithelium could explain the upregulation of *TNF* and *TLR2* [41,42]. Furthermore, the alterations in bacterial species observed in this study and others that were not assessed could have resulted in the production of bioactive molecules that interacted with the ruminal epithelium and caused a pro-inflammatory response [15]. The sections below

discuss the possibility of some of the metabolites detected as potential triggers of immune responses in the ruminal epithelium.

3.3. Metabolites

A total of 124 metabolites were identified in ruminal fluid. Among these, 92 were consistently detected across all three periods (Tables 2–6). Additionally, 18 metabolites were exclusively found in the PRE and POST periods (Table 7), while 12 metabolites were uniquely identified in the PRE-FR period, including adenosine, b-sitosterol, docosanoic acid, gluconic acid, heptanoic acid, isomaltose, mannitol, mimosine, orotic acid, sebacic acid, tetracosanoic acid, and urocanic acid. Notably, alfa-Tocopherol was solely detected in the POST period, and tricarballylic acid was restricted to the FRP and POST periods. The detected metabolites were enriched in pathways outlined in Table 8. The predicted metabolic pathways were assumed to reflect major alterations in the microbial community.

Table 2. Relative concentrations (per 100 µL digesta), standard error of the mean (SEM), and *p*-value of metabolites associated with amino acids in ruminal digesta from beef steers ($n = 7$) before (PRE) and during a 3-day feed-restriction period (FRP) at 25% intake of PRE, and after a 15-day recovery (POST).

Metabolite	PRE	FRP	POST	SEM	*p*-Value
Amino acids					
Alanine	788 ^{a}	256 ^{b}	468 ^{b}	72.9	0.01
Aspartic acid	728	288	552	121	0.11
Beta-alanine	9.43 ^{a}	2.50 ^{b}	4.63 ^{b}	0.99	0.01
Glutamic acid	556 ^{a}	121 ^{b}	289 ^{b}	59.7	0.01
Glutamine	918 ^{a}	273 ^{b}	690 ^{a}	92.9	0.01
Glycine	504 ^{a}	117 ^{b}	314 ^{ab}	46.1	<0.01
Isoleucine	361 ^{a}	69.0 ^{b}	190 ^{ab}	55.9	0.03
Leucine	210	41.7	91.1	45.4	0.09
Lysine	1160 ^{a}	248 ^{b}	707 ^{ab}	132	0.01
Phenylalanine	157	48.7	108	30.3	0.11
Proline	122	19.4	84.6	39.3	0.25
Serine	314 ^{a}	73.4 ^{b}	178 ^{ab}	32.3	0.01
Threonine	394 ^{a}	89.6 ^{b}	189 ^{ab}	62.9	0.04
Tryptophan	8.50	1.73	3.03	2.25	0.16
Valine	362 ^{a}	80.4 ^{c}	226 ^{b}	28.1	<0.01
Amino acid-related metabolites					
Cadaverine	33.9 ^{a}	4.77 ^{b}	24.5 ^{a}	4.41	0.02
Fumaric acid	6.73 ^{a}	3.07 ^{b}	5.60 ^{a}	0.28	<0.01
N-Acetylglutamic acid	133 ^{a}	39.3 ^{c}	84.7 ^{b}	10.2	<0.01
Ornithine	127 ^{a}	15.0 ^{c}	49.3 ^{b}	5.01	<0.01
Putrescine	339 ^{a}	24.3 ^{b}	128 ^{ab}	68.2	0.04
γ-Aminobutyric acid	20.2 ^{a}	10.6 ^{b}	14.2 ^{ab}	1.41	0.02
1,3-diaminopropane	30.8 ^{a}	6.80 ^{b}	16.9 ^{b}	3.45	0.01
Glyoxylic acid	160	83.9	139	17.8	0.06
Succinic acid	109 ^{b}	128 ^{b}	205 ^{a}	14.4	0.01
p-hydroxyphenylacetic acid	10.9 ^{b}	12.9 ^{b}	22.8 ^{a}	1.83	0.01
Glyceric acid	70.2	42.6	45.2	31.3	0.80
Pyruvic acid	2.30	1.53	3.13	0.53	0.17
Spermidine	31.5	11.9	34.0	6.77	0.11
Urea	11.3	12.1	10.4	1.37	0.69
Amino acid derivatives					
N-methylalanine	11.3 ^{a}	1.43 ^{b}	13.2 ^{a}	1.87	0.01
L-methionine sulfoxide	18.4 ^{a}	3.27 ^{b}	9.97 ^{ab}	2.98	0.03
N-alpha-acetyl-l-lysine	86.0 ^{a}	28.5 ^{b}	50.8 ^{ab}	9.05	0.01
Pentanoic acid, 5-amino	26.0	10.8	12.1	5.65	0.19

^{a,b,c} Means in the same row with different superscripts differ ($p < 0.05$).

3.3.1. Amino Acids and Associated Metabolites

Among 15 detected amino acids (AAs), 11 (alanine, beta-alanine, glutamic acid, glutamine, glycine, isoleucine, lysine, phenylalanine, serine, threonine, and valine) were lower ($p < 0.05$) in FPR compared with PRE (Table 2). Similarly, aspartic acid and leucine concentrations were also lower ($p \leq 0.10$) in FRP compared with PRE. After FR, only glutamine and valine were returned to levels in PRE ($p < 0.05$), while glycine and lysine tended ($p \leq 0.10$) to increase. Among 27 predicted affected pathways (Table 8), 14 were related to AA metabolism or biosynthesis. Among these metabolites belonging to AA-related pathways, cadaverine, fumaric acid, n-acetylglutamic acid, and ornithine concentrations decreased ($p < 0.05$) in FRP compared with PRE and subsequently increased in POST. Similarly, putrescine, γ-aminobutyric acid, and 1,3-diaminopropane concentrations also decreased in FRP compared with PRE ($p < 0.05$) but did not recover in POST. The glyoxylic acid concentration also tended to be lower ($p < 0.10$) in FRP than PRE. Despite concentrations of succinic acid and p-hydroxyphenylacetic acid not changing in FRP, concentrations in POST were greater ($p < 0.05$) than in FRP. Regarding AA derivatives, the N-methylalanine, L-methionine sulfoxide, and N-alpha-acetyl-l-lysine concentrations were lower ($p < 0.05$) in FRP than PRE. N-methylalanine concentrations recovered ($p < 0.05$) during POST, while L-methionine sulfoxide and N-alpha-acetyl-l-lysine concentrations did not.

It is not surprising that concentrations of most AAs, their derivatives, and related metabolites were lower during FRP, indicating that fewer nitrogen sources were available to the animal and microbiome. The concentrations of several AAs and cadaverine in the rumen increased with a higher ratio of barley grain to barley silage in dairy cow diets [14,15]. Altering these dietary ratios resulted in elevated crude protein content [14], increasing AA and cadaverine concentrations. Cadaverine, originating from lysine, serves as an intermediate in various pathways and is involved in cell wall and siderophore biosynthesis [43]. Putrescine, another polyamine, is produced from arginine and ornithine or from the agmatinase pathway [44]. The production of gamma-aminobutyrate is derived from glutamate decarboxylation. In our study, the reduced concentrations of several AA-related metabolites (cadaverine, putrescine, and gamma-aminobutyrate) and AA derivatives (N-methylalanine, L-methionine sulfoxide, and N-alpha-acetyl-l-lysine) further demonstrated the close association between AA metabolism and protein intake. Thus, under a low dietary protein condition due to FR, microbes break down less protein into AAs and ammonia, a preferred nitrogen source [45].

3.3.2. Metabolites Associated with Ribose, Nitrogenous Bases, and Their Derivatives

Table 3 lists metabolites associated with ribose, nitrogenous bases, and their derivatives. The adenine, thymine, uracil, hypoxanthine, xanthine, 2-deoxy-D-ribose, erythronic acid, and ribose concentrations in FRP were greater ($p < 0.05$) than in PRE and POST. Similarly, the tyrosine and inosine concentrations were greater ($p < 0.05$) in FRP than PRE, while their concentrations were not significantly different in FRP and POST. Predicted alterations in metabolic pathways are discussed below.

The downregulation of metabolites associated with ribose, nitrogenous bases, and their derivatives indicated that FR dampened nucleoside and nucleotide metabolism. Purine and pyrimidine nucleotides serve as precursors for nucleic acids, exerting partial control over cell growth. In addition, they function as metabolic signals, energy providers, and essential components of coenzymes in various biological processes [46].

In the "purine metabolism" pathway, inosine, hypoxanthine, and xanthine are metabolites related to the degradation of guanine and adenine [47]. Ribose, purine, and pyrimidine are essential components of nucleotides [48], and erythronic acid is the product of ribose degradation. Under non-FR conditions, when compared with low-producing dairy cows, the downregulation of hypoxanthine, guanosine, and cytosine in ruminal digesta from high-producing dairy cows was associated with the overall downregulation of "purine production" and "pyrimidine metabolism" pathways [49]. Saleem et al. [15] proposed that a high-grain diet induced bacterial cell lysis, leading to greater levels of xanthine,

uracil, alanine, ornithine, and ethanolamine, all of which were suggestive of increased cell death. Such a response may partly account for the lower hypoxanthine concentration in high-producing cows. Thus, the fact that FR resulted in decreased concentrations of xanthine, uracil, alanine, ornithine, and ethanolamine in the present study suggested the possibility that during feed restriction, fewer microbial cells survived in the rumen, and most of the measured 16S rRNA copies originated from dead bacterial cells or from bacteria that entered a dormant state by forming endospores [50]. Further investigation is warranted to explore the viability of bacteria and the state of sporulation during FR.

Table 3. Relative concentrations (per 100 µL digesta), standard error of the mean (SEM), and *p*-value for metabolites associated with ribose, nitrogenous bases, and their derivatives in ruminal digesta from beef steers ($n = 7$) before (PRE) and during a 3-day feed-restriction period (FRP) at 25% intake of PRE and after a 15-day recovery (POST).

Metabolite	PRE	FRP	POST	SEM	*p*-Value
Adenine	3.87 ^{a}	0.93 ^{b}	3.30 ^{a}	0.49	0.02
Cytosine	5.93	10.3	7.30	2.43	0.48
Thymine	153 ^{a}	21.7 ^{b}	164 ^{a}	26.0	0.02
Tyrosine	217 ^{a}	50.0 ^{b}	130 ^{ab}	27.5	0.01
Uracil	772 ^{a}	165 ^{b}	672 ^{a}	29.2	<0.01
Inosine	108 ^{a}	28.1 ^{b}	35.2 ^{b}	9.69	0.01
Hypoxanthine	404 ^{a}	74.6 ^{b}	326 ^{a}	23.2	<0.01
Xanthine	464 ^{a}	89.5 ^{b}	377 ^{a}	26.1	<0.01
2-deoxy-D-ribose	289 ^{a}	14.2 ^{b}	185 ^{a}	37.9	0.01
Erythronic acid	22.0 ^{a}	2.43 ^{b}	22.5 ^{a}	4.40	0.03
Ribose	3535 ^{a}	748 ^{b}	3015 ^{a}	128	<0.01

^{a,b} Means in the same row with different superscripts differ ($p < 0.05$).

3.3.3. Fatty Acids and Associated Metabolites

Concentrations of several fatty acids (FA) including tetradecanoic acid, pentadecanoic acid, palmitic acid, stearic acid, oleic acid C18:1 (11), oleic acid C18:1 (9), and heptadecanoic acid decreased ($p < 0.05$) in FRP compared with PRE (Table 4). However, only the concentrations of tetradecanoic acid, palmitic acid, and stearic acid returned to the levels in PRE ($p < 0.05$) during POST. The "biosynthesis of unsaturated fatty acids" and "fatty acid biosynthesis" pathways were predicted to be altered by FR.

Table 4. Relative concentrations (per 100 µL digesta), standard error of the mean (SEM), and *p*-value for fatty acids and associated metabolites in ruminal digesta from beef steers ($n = 7$) before (PRE) and during a 3-day feed-restriction period (FRP) at 25% intake of PRE and after a 15-day recovery (POST).

Metabolite	PRE	FRP	POST	SEM	*p*-Value
Fatty acids					
Tetradecanoic acid [1] (C14:0)	86.2 ^{a}	39.5 ^{b}	84.4 ^{a}	5.42	<0.01
Pentadecanoic acid (C15:0)	26.4 ^{a}	6.77 ^{b}	17.4 ^{ab}	2.61	0.01
Palmitic acid (C16:0)	1275 ^{a}	394 ^{b}	688 ^{b}	79.3	<0.01
Stearic acid (C18:0)	3816 ^{a}	597 ^{b}	1166 ^{b}	311	<0.01
Oleic acid C18:1 (11)	129 ^{a}	12.0 ^{b}	59.9 ^{ab}	24.3	0.05
Oleic acid C18:1 (9)	80.1 ^{a}	14.1 ^{b}	49.9 ^{ab}	8.25	<0.01
Heptadecanoic acid [2] (C17:0)	16.7 ^{a}	2.77 ^{b}	9.50 ^{ab}	2.36	0.02
Fatty acid-related metabolites					
Hexadecanol	15.6 ^{a}	4.77 ^{b}	20.7 ^{a}	1.66	<0.01
Octadecanol [3]	6.63	3.37	5.07	1.56	0.39
Tetracosanol	9.13 ^{a}	1.67 ^{b}	5.40 ^{ab}	1.20	0.01
Stigmastan-3-ol	24.7 ^{ab}	14.6 ^{b}	37.2 ^{a}	3.29	0.01

^{a,b} Means in the same row with different superscripts differ ($p < 0.05$). [1] Also known as myristic acid; [2] also known as margaric acid; [3] also known as stearyl alcohol.

Unsurprisingly, the concentrations of FA were lower due to a lack of feed, which consequently downregulated the FA-related pathways in the microbiome. FA synthesis is highly energy-intensive, and it is crucial that the biophysical properties of the phospholipid membrane consistently maintain fluidity. Thus, the alterations in FA concentrations and related pathways potentially could alter the quantity and the composition of the FA in the phospholipids produced [51]. The "Biosynthesis of unsaturated fatty acids" and "Steroid biosynthesis" pathways were upregulated in ruminal digesta from high-producing compared with low-producing dairy cows [41], a response that was associated with greater dry matter intake in the former [52]. Many studies have already demonstrated that FR has a negative impact on production [3], and the observation in the present study of the downregulation of key metabolites in the "Biosynthesis of unsaturated fatty acids" and "Fatty acid biosynthesis" pathways induced by FR seems to underscore the importance of these pathways to the microbiota.

At high concentrations of unsaturated fatty acids (USFA), these are incorporated into bacterial cell membranes, disrupting membrane fluidity and function and causing toxicity [53,54]. To mitigate this effect, ruminal bacteria biohydrogenate USFA into saturated fatty acids (SFA), resulting in a higher concentration of SFA than USFA in the rumen [55]. Palmitic acid (C16:0) and stearic acid (C18:0) are major fatty acids of ruminal bacteria and protozoa [56]. Bacteria import and assimilate exogenous FA, with the FadL-FadD complex facilitating the uptake of long-chain FA for use as an energy source or for incorporation into complex lipids (e.g., phospholipids) [57]. It is likely that FR results in lower FA synthesis within cells as well as reduced FA uptake by the cells. Such effects likely depress microbial metabolism, aligning with predictions of alterations in the "Biosynthesis of unsaturated fatty acids" and "Fatty acid biosynthesis" pathways in the present study.

Hexadecanol, octadecanol, and tetracosanol are fatty alcohols with 16, 18, and 24 carbons, respectively. Some bacteria can produce fatty alcohols [58], and their production is increased by fatty acid starvation in *Escherichia coli* [59]. However, it is worth noting that long-chain fatty alcohols also exhibit antibacterial activity [60,61], although specific details regarding the types of fatty alcohols involved are not well-documented. Limited studies have addressed the relationship between long-chain fatty alcohols in the rumen. For example, ethanol, a short-chain fatty alcohol, has been shown to increase the digestion of cellulose, rumen fill, gas production in vitro, and the utilization of sulfate for AA and glutathione synthesis. However, it had little effect on the VFA concentrations in the rumen [62,63].

3.3.4. Metabolites Associated with Energy Sources

The glucose and N-acetyl glucosamine concentrations were lowest ($p < 0.05$) in FRP, and their concentrations in POST were lower than in FRP (Table 5). Similarly, the glycerol and glycerol-3-phosphate concentrations were lower ($p < 0.05$) in FRP than PRE and POST. The fructose concentration was greater ($p < 0.05$) in PRE than FRP and POST. The arabinose concentration in POST was greater ($p < 0.05$) than in FRP. The Arabitol concentration in FRP tended to be lower ($p < 0.10$) than in PRE and POST.

Starch is the most important storage form of energy in cereal grains fed to cattle, while cellulose is a crucial component of the cell wall in forages [64]. Glucose in the rumen is derived from the hydrolysis of starch and cellulose [65,66]. Cellulose and lignin content in feed can be accessed via the acid detergent fiber analysis [67], whereas neutral detergent fiber allows the measurement of cellulose, lignin, and hemicellulose [68]. The microbiota hydrolyzes cellulose into glucose and cellobiose [65,69]. Hemicellulose, another crucial polysaccharide in the cell wall, is hydrolyzed by bacteria into glucose, xylose, and arabinose, all of which serve as energy sources [65,70,71]. Fructan is also a significant non-fiber carbohydrate in ruminant feed, primarily composed of fructose [72] that is degraded by the ruminal microbiota [72–74], with the resulting fructose transported into bacteria for fermentation [75–77]. Monosaccharides in the rumen are typically products hydrolyzed from polysaccharides by the rumen microbiome [78]. Consequently, the lower

concentrations of most monosaccharides detected during FR may have been due to a lack of available polysaccharides from the feed for the ruminal microbiome and also the host.

Table 5. Relative concentrations (per 100 µL digesta), standard error of the mean (SEM), and *p*-value for metabolites associated with energy sources for microbes in ruminal digesta from beef steers ($n = 7$) before (PRE) and during a 3-day feed-restriction period (FRP) at 25% intake of PRE and after a 15-day recovery (POST).

Metabolite	PRE	FRP	POST	SEM	*p*-Value
Disaccharides					
Cellobiose	5050	884	1978	1129	0.09
Maltose	1444	220	411	318	0.07
Monoglycerides and derivatives					
Arabinose	142 ^{ab}	88.1 ^{b}	178 ^{a}	21.0	0.04
Arabitol	5.03	2.73	4.87	0.59	0.06
Fructose	90.0 ^{a}	4.83 ^{b}	30.1 ^{b}	7.66	<0.01
Glucose	13,810 ^{a}	1576 ^{c}	5872 ^{b}	323	<0.01
N-acetyl glucosamine	106 ^{a}	9.23 ^{c}	57.3 ^{b}	6.66	<0.01
Mannose	21.6	2.50	32.6	13.9	0.38
Other					
Glycerol	763 ^{a}	243 ^{b}	840 ^{a}	93.9	<0.01
Glycerol-3-phosphate	8.27 ^{a}	3.37 ^{b}	8.27 ^{a}	0.79	0.01

^{a,b,c} Means in the same row with different superscripts differ ($p < 0.05$).

3.3.5. Other Metabolites

Regarding other metabolites, '2-quinolinecarboxylic acid, 4,8-dihydroxy', hydrocinnamic acid, malic acid, nicotinic acid, and O-phosphoethanolamine concentrations in FRP were lower ($p < 0.05$) than in PRE and POST (Table 6). The azelaic acid and inositol concentrations were greater ($p < 0.05$) in PRE than FRP. The inositol concentration tended to be greater ($p < 0.10$) in POST than FRP. Interestingly, the pyrrole-2-carboxylic acid concentration was lower ($p < 0.05$) in PRE than FRP and POST. The glycolic acid concentration was greater ($p < 0.05$) POST than FRP, and its concentration tended to be greater ($p < 0.10$) in PRE than FRP. A list of metabolites that were only present in PRE and POST is in Table 7. The eicosanoic acid and pantothenic acid concentrations were greater ($p < 0.05$) in PRE than POST.

The fact that pantothenic acid (known as vitamin B5) was only detectable in PRE and POST and nicotinic acid (known as niacin and vitamin B3) exhibited markedly low concentrations during FR seems to underscore a precarious condition during FRP that rendered the animals deficient (at least transiently) in these two important B vitamins [79]. The fact that ruminal microbiota are important sources of B vitamins to the host is well known [80,81]. However, just as for several metabolites, the dynamics among the B vitamin synthesis, degradation, passage out of the rumen, and possibly ruminal absorption occur simultaneously and render the interpretation of concentrations challenging. In general terms, it seems safe to assume that differences in the supply of readily fermentable carbohydrates and fiber can impact vitamin B production by the microbiota [82,83]. In the context of the present study, the fact that species such as *Streptococcus bovis* and *Megaspheara elsdenii* require pantothenic acid and likely use an excess synthesized by other bacteria [84,85] could account for the increase in abundance of the former and the inability to detect pantothenic acid during FRP. In the case of nicotinic acid, the fact that the intake and ruminal digestibility of starch in lactating dairy cows are highly positively correlated with its ruminal synthesis [84] likely explains the ~80% decrease in concentration during FRP.

Table 6. Relative concentrations (per 100 µL digesta), standard error of the mean (SEM), and *p*-value for metabolites in ruminal digesta from beef steers (n = 7) before (PRE) and during a 3-day feed-restriction period (FRP) at 25% intake of PRE and after a 15-day recovery (POST).

Metabolite	PRE	FRP	POST	SEM	*p*-Value
1,3-dihydroxyacetone	44.4	8.60	47.0	14.9	0.21
2-quinolinecarboxylic acid, 4,8-dihydroxy	4.40 ^{a}	1.07 ^{b}	3.40 ^{a}	0.36	<0.01
3-(3-hydroxyphenyl)propionic acid	12.53	4.00	17.1	5.83	0.34
3-hydroxybutanoic acid	4.10	1.67	1.27	0.72	0.08
Aminomalonic acid	47.0	18.6	34.4	8.60	0.14
Azelaic acid	241 ^{a}	96.0 ^{b}	149 ^{ab}	21.9	0.01
Benzeneacetic acid	210	210	130	29.1	0.18
Benzoic acid	9.17	7.63	7.00	1.74	0.68
Benzoic acid, 3-hydroxy	2.43	1.80	3.00	1.12	0.76
Dodecanedioic acid	15.4	10.1	16.1	5.32	0.70
Ethanolamine	106	67.2	77.4	18.4	0.23
Glutaric acid	8.10	8.17	13.5	1.87	0.14
Glycolic acid	32.6 ^{ab}	11.4 ^{b}	36.8 ^{a}	6.08	0.04
Hydrocinnamic acid	466 ^{a}	174 ^{b}	416 ^{a}	29.9	<0.01
Indole-3-acetic acid	1.87	1.70	2.47	0.36	0.36
Inositol	34.3 ^{a}	1.77 ^{b}	31.7 ^{ab}	8.34	0.04
Inositol-2-phosphate	8.93	1.83	8.70	2.17	0.10
Lactic acid	140	98.7	193	30.0	0.17
Lactose	5.30	0.33	3.90	1.27	0.08
Malic acid	170 ^{a}	26.9 ^{c}	108 ^{b}	12.7	<0.01
Nicotinic acid [1]	30.6 ^{a}	5.80 ^{b}	18.7 ^{a}	2.38	<0.01
O-phosphoethanolamine	42.9 ^{a}	9.57 ^{b}	47.0 ^{a}	5.22	<0.01
Propan-1,2-diol	20.7	18.1	33.4	5.88	0.23
Pyrrole-2-carboxylic acid	4.97 ^{b}	8.07 ^{a}	7.43 ^{ab}	0.67	0.04
Suberic acid	9.17 ^{a}	4.37 ^{b}	7.20 ^{a}	0.58	<0.01
Valeramide	3.47	3.10	3.03	0.80	0.88

^{a,b,c} Means in the same row with different superscripts differ ($p < 0.05$). [1] Also known as niacin and vitamin B3.

The sole metabolite that increased during the FRP, pyrrole-2-carboxylic acid, is derived from fungi, plants, and bacteria [86]. Considering that FR decreased plant biomass in the rumen, the presence of pyrrole-2-carboxylic acid likely originated from fungi and bacteria. Although fungi constitute less than 10% of the total microbial biomass of the rumen in normal conditions, they produce a number of important biopolymer-degrading enzymes, e.g., xylanases, cellulases, chitinases, pectinases, and esterases [87]. Some evidence suggests that dietary fiber is the main driver of fungal growth in the rumen [88]. Thus, a period of FR, as in the present study, would be expected to reduce the fungal population. The sole study reporting concentrations of pyrrole-2-carboxylic acid in the rumen pertains to a comparison of ruminal microbiota and metabolome between lactating Montbéliarde × Holstein and Holstein cows, with the former having greater concentrations of this compound when fed the same corn silage-based diet [89]. Although the relative abundance of fungal species was not assessed, the genus *Succiniclasticum* sp. was the only statistically significant genus and was two-fold more abundant in the Montbéliarde × Holstein cows. The genus *Succiniclasticum* was first identified and characterized in 1995 [90] from the rumen of a dairy cow fed a silage-based diet. This organism had an optimal growth rate with succinate and did not produce propionate. It could be possible that in the present study, the marked increase in *Succinivibrio dextrinosolvens* abundance during FR not only explains the decrease in glucose concentration (Table 5) but also indirectly explains the increase in pyrrole-2-carboxylic acid. Because *Succinivibrio dextrinosolvens* produces succinate as a major endproduct [91], it is possible that *Succiniclasticum* sp. also thrived in those conditions. Dietary protein in the rumen also could alter pyrrole-2-carboxylic acid, as indicated by a study in which Tibetan sheep fed with 12.1% protein compared to 10.1% protein had greater ruminal concentrations of this molecule [92]. A positive correlation between Rikenellaceae_RC9_gut_group and pyrrole-2-carboxylic acid was also revealed in another study [92]. Further investigations are warranted to better understand the origin of pyrrole-2-carboxylic acid in the rumen and its relationship with the microbiota. This is particularly important given the potential physiological activities that this and chemically related carboxaldehydes can have in the body [86].

Table 7. Relative concentrations (per 100 µL digesta), standard error of the mean (SEM), and *p*-value for metabolites in ruminal digesta from beef steers (n = 7) before (PRE) and during a 3-day feed-restriction period (FRP) at 25% intake of PRE and after a 15-day recovery (POST).

Metabolite	PRE	POST	SEM	*p*-Value
1-Pentadecanol	2.91	4.54	2.09	0.64
2,8-Quinolinediol	18.5	18.6	2.09	0.96
2-Methylsuccinic acid	6.82	3.13	0.86	0.09
3-Hydroxyphenylacetic acid	42.2	33.0	5.25	0.27
3-Hydroxypyruvic acid	1.48	1.17	0.25	0.48
Adipic acid	9.75	8.23	2.23	0.68
Dodecanoic acid (C12:0)	11.0	10.9	1.03	0.93
Eicosanoic acid	25.0 [a]	6.18 [b]	2.39	0.02
Galactose	36.4	16.3	8.25	0.23
Glyceraldehyde	0.83	1.03	0.29	0.66
Glycylproline	7.32	5.42	0.92	0.28
Guanine	1.55	1.06	0.55	0.37
Linoleic acid	25.7	13.9	7.54	0.35
N-acetyl mannosamine	15.1	10.4	3.03	0.38
Panthotenic acid [1]	15.9 [a]	6.96 [b]	0.81	0.02
Pseudo uridine	18.5	10.9	2.21	0.12
Sedoheptulose	39.2	38.7	19.4	0.99
Sorbitol	5.92	8.56	1.28	0.28

[a,b] Means in the same row with different superscripts differ ($p < 0.05$). [1] Also known as vitamin B5.

Table 8. Match index, *p*-value, and matched metabolites of predicted pathways in ruminal digesta from steers that were affected during feed restriction. Metabolites in **bold** font had statistically significant differences. Metabolites in **green** were only present before (PRE) and after (POST) feed restriction. Metabolites in **blue** were only present before feed restriction (PRE). The match index was calculated with the number of significantly different matched metabolites divided by the number of metabolites in a specific pathway.

Pathway ID	Name	Match index	*p*-Value	Matched Metabolites
Pathways associated with amino acids				
KO00220	Arginine biosynthesis	21.74%	<0.0001	**Fumaric acid; L-Glutamic acid; Ornithine; L-Glutamine; N-Acetylglutamic acid; L-Aspartic acid**; Urea
KO00250	Alanine, aspartate, and glutamate metabolism	21.43%	<0.0001	**Gamma-Aminobutyric acid; Fumaric acid; L-Glutamic acid;** L-Alanine; Succinic acid; L-Glutamine; L-Aspartic acid; Pyruvic acid
KO00410	beta-Alanine metabolism	20.00%	<0.0001	**1,3-Diaminopropane; Beta-Alanine; Gamma-Aminobutyric acid; Pantothenic acid; Uracil**; L-Aspartic acid; Spermidine
KO00970	Aminoacyl-tRNA biosynthesis	17.31%	<0.0001	**Glycine; L-Glutamic acid; L-Tyrosine; L-Alanine; L-Threonine; L-Isoleucine; L-Lysine; L-Glutamine; L-Valine**; L-Phenylalanine; L-Proline; L-Aspartic acid; L-Leucine; L-Tryptophan
KO00470	D-Amino acid metabolism	16.07%	<0.0001	**Glycine; L-Glutamic acid; L-Alanine; L-Lysine; Ornithine; L-Glutamine; N-Acetylglutamic acid; Putrescine; Cadaverine**; L-Proline; L-Aspartic acid; Pyruvic acid
KO00480	Glutathione metabolism	15.63%	0.0001	**Glycine; L-Glutamic acid; Ornithine; Putrescine; Cadaverine**; Spermidine
KO00290	Valine, leucine, and isoleucine biosynthesis	13.04%	0.0039	**L-Threonine; L-Isoleucine; L-Valine**; Pyruvic acid; L-Leucine
KO00910	Nitrogen metabolism	10.53%	0.0287	**L-Glutamic acid; L-Glutamine**
KO00430	Taurine and hypotaurine metabolism	9.09%	0.0377	**L-Glutamic acid; L-Alanine;** Pyruvic acid
KO00260	Glycine, serine, and threonine metabolism	8.51%	0.0040	**1,3-Diaminopropane; Glycine; L-Threonine; Hydroxypyruvic acid;** Glyoxylic acid; Glyceric acid; L-Aspartic acid; Pyruvic acid; L-Tryptophan
KO00330	Arginine and proline metabolism	7.94%	0.0018	**1,3-Diaminopropane; Gamma-Aminobutyric acid; L-Glutamic acid; Ornithine; Putrescine;** Glyoxylic acid; L-Proline; Pyruvic acid; Urea; Spermidine
KO00350	Tyrosine metabolism	7.14%	0.0076	**p-Hydroxyphenylacetic acid; Fumaric acid; L-Tyrosine; Succinic acid;** Pyruvic acid
KO00360	Phenylalanine metabolism	6.98%	0.0207	**Fumaric acid;** L-Tyrosine; Succinic acid; L-Phenylalanine; Phenylacetic acid; Pyruvic acid
KO00310	Lysine degradation	5.77%	0.0361	**L-Lysine; Succinic acid; Cadaverine**
Pathways associated with nitrogenous bases and derivatives				
KO00230	Purine metabolism	9.30%	<0.0001	**Adenine; Adenosine; Glycine; Guanine; Hypoxanthine; Inosine; Xanthine; L-Glutamine;** Glyoxylic acid; Urea
KO00030	Pentose phosphate pathway	8.33%	0.0137	**D-Glucose; D-Ribose; Gluconic acid;** Glyceric acid; Pyruvic acid
KO00240	Pyrimidine metabolism	8.20%	0.0015	**Beta-Alanine; Orotic acid; Thymine; Uracil; L-Glutamine;** Urea
Pathways associated with fatty acids				
KO01040	Biosynthesis of unsaturated fatty acids	11.11%	0.0015	**Palmitic acid; Linoleic acid; Stearic acid; Arachidic acid**
KO00650	Butanoate metabolism	9.09%	0.0032	**Gamma-Aminobutyric acid; Fumaric acid; L-Glutamic acid; Succinic acid;** Pyruvic acid; 3-Hydroxybutyric acid
KO00061	Fatty acid biosynthesis	5.66%	0.0379	**Palmitic acid; Myristic acid (also called Tetradecanoic acid);** Dodecanoic acid
Pathways associated with energy sources				
KO00052	Galactose metabolism	10.87%	0.0004	**Glycerol; D-Galactose; myo-Inositol; Sorbitol; Isomaltose**
KO00020	Citrate cycle (TCA cycle)	10.00%	0.0316	**Fumaric acid; Succinic acid;** Pyruvic acid
KO00630	Glyoxylate and dicarboxylate metabolism	8.93%	0.0010	**Glycine; L-Glutamic acid; Succinic acid; L-Glutamine; Hydroxypyruvic acid;** Glyoxylic acid; Glyceric acid; Pyruvic acid
KO00520	Amino sugar and nucleotide sugar metabolism	4.50%	0.0192	**D-Xylose; D-Galactose; N-Acetyl-D-glucosamine; D-Fructose; N-Acetyl mannosamine**
Pathways associated with vitamins				
KO00770	Pantothenate and CoA biosynthesis	14.81%	0.0005	**Beta-Alanine; Pantothenic acid; Uracil; L-Valine;** L-Aspartic acid; Pyruvic acid
KO00760	Nicotinate and nicotinamide metabolism	7.84%	0.0054	**Gamma-Aminobutyric acid; Fumaric acid; Succinic acid; Nicotinic acid;** L-Aspartic acid; Pyruvic acid
Other predicted pathways				
KO00997	Biosynthesis of various other secondary metabolites	8.89%	0.0034	**L-Tyrosine; L-Alanine; L-Lysine; Ornithine;** L-Phenylalanine; L-Tryptophan

4. Conclusions

The short-term feed restriction clearly impacted a number of bacterial species studied, with the increased abundance of *Succinivibrio dextrinosolvens*, *Streptococcus bovis*, and *Bifidobacteria* spp. (N124) underscoring their unique niche in the degradation of rapidly-fermentable carbohydrates to intermediates such as lactate and succinate that could serve as substrates for other microorganisms. The marked decrease in concentrations during feed restriction for most metabolites detected was not surprising and likely reflected a combination of the reduction in substrates for the microorganisms and the alterations in the biochemical pathways of the microbiota. In that context, the dramatic decrease in vitamins B3 and B5 during feed restriction highlighted a potential transient deficiency in the supply of these vitamins to the animal. The origin of pyrrole-2-carboxylic acid in the rumen (the only metabolite with a marked increase during feed restriction), its relationship with the microbiota, and its physiological activities in the animal's tissues merit further study.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/ruminants4030028/s1, Supplemental methods: DNA microbe RT-qPCR; Metabolomic analysis; RNA extraction; RNA RT-qPCR; Relative mRNA abundance; Table S1: Species-specific primers for the quantification of target ruminal bacterial species; Table S2: GenBank accession number and sequence of primers for Bos taurus used to analyze gene expression; Table S3: Median Ct, Median ΔCt Slope, coefficient of determination of the standard curve (R^2), and efficiency of amplification.

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