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Effects of feed withdrawal duration on animal behaviour, rumen microbiota and blood chemistry in feedlot cattle: implications for rumen acidosis

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*Feed withdrawal (**FW**) is a frequent issue in open outdoor feedlot systems, where unexpected circumstances can limit the animals' access to food. The relationship among fasting period, animal behaviour during feed reintroduction (**FR**) and acidosis occurrence has not been completely elucidated. Twenty steers fitted with rumen catheters were fed a high-concentrate diet (concentrate : forage ratio 85 : 15) and were challenged by a protocol of FW followed by FR. The animals were randomly assigned to one of the four treatments: FW for 12 h (**T12**), 24 h (**T24**), 36 h (**T36**) or no FW (control group) followed by FR. The steers' behaviour, ruminal chemistry, structure of the ruminal microbial community, blood enzymes and metabolites and ruminal acidosis status were assessed. Animal behaviour was affected by the FW–FR challenge (*$P < 0.05$*). Steers from the T12, T24 and T36 treatments showed a higher ingestion rate and a lower frequency of rumination. Although all animals were suspected to have sub-acute ruminal acidosis (**SARA**) prior to treatment, a severe case of transient SARA arose after FR in the T12, T24 and T36 groups. The ruminal pH remained below the threshold adopted for SARA diagnosis (pH value = 5.6) for more than three consecutive hours (24, 7 and 19 h in the T12, T24 and T36 treatments, respectively). The FW–FR challenge did not induce clinical acute ruminal acidosis even though steers from the T36 treatment presented ruminal pH values that were consistent with this metabolic disorder (pH threshold for acute acidosis = 5.2). Total mixed ration reintroduction after the withdrawal period reactivated ruminal fermentation as reflected by changes in the fermentation end-products. Ruminal lactic acid accumulation in steers from the T24 and T36 treatments probably led to the reduction of pH in these groups. Both the* FW *and the* FR *phases may have altered the structure of the ruminal microbiota community. Whereas fibrolytic bacterial groups decreased relative abundance in the restricted animals, both lactic acid producer and utiliser bacterial groups increased (*$P < 0.05$*). The results demonstrated a synchronisation between* Streptococcus *(lactate producer) and* Megasphaera *(lactate utiliser), as the relative abundance of both groups increased, suggesting that bacterial resilience may be central for preventing the onset of metabolic disturbances such as ruminal acidosis. A long-FW period (36 h) produced rumen pH reductions well below and lactic acid concentration increased well above the accepted thresholds for acute acidosis without any perceptible clinical signs.*

Keywords: sub-acute ruminal acidosis, ruminal environment, food restriction, livestock, fattening

Implications

In intensive livestock production systems, cattle are fed high-concentrate diets and exhibit high rates of fermentation. Combined with inadequate buffering, this can occasionally lead to the accumulation of acids and thus extended periods with a low ruminal pH. Feed withdrawal followed by feed reintroduction accentuated the acidotic condition. However, all clinical signs of acidosis were absent even in 36 h feed-withdrawn steers. The circumstances that limit the animals' access to feed should be avoided as a feed withdrawal *per se* could aggravate a sub-acute acidosis condition. The studies on repeated feed withdrawal events and production parameters are needed for a better understanding.

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Introduction

In cattle fed high grain-based diets, the accepted normal ruminal pH is approximately 5.8 (Plaizier *et al.*, 2012). In this scenario, intermittent daily drops in pH between 5.6 and 5.0 are expected (Schwartzkopf-Genswein *et al.*, 2004). There is no general consensus regarding the threshold for a diagnosis of acidosis. However, sub-acute ruminal acidosis (**SARA**) is considered to occur when episodes of rumen pH between 5.2 and 5.6 occur for more than three consecutive hours during a single day (Gozho *et al.*, 2005 and 2007). The clinical signs of acute acidosis are generally observed when ruminal pH drops below 5.2 (Mutsvangwa *et al.*, 2002). The analysis of ruminal and systemic changes related to these conditions has been performed using two general experimental induction models. One model involves a challenge with fermentable carbohydrates by oral or ruminal administration (Brown *et al.*, 2000). The second approach consists of a deliberate fluctuation in the amount of food resulting in daily or weekly variations in feed intake (Galyean *et al.*, 1992). To date, studies focussing on restriction periods as a prompt for acidosis are not available. In contrast, feed withdrawal (**FW**) has typically been used as a tool to stimulate and increase the amount and consumption rate of the acidosis-inducing diet (McCann *et al.*, 2016). However, some authors argue that access to a high-grain diet after a period of feed restriction, even in adapted animals, may precipitate ruminal acidosis (Garry, 2002).

Feed withdrawal periods could mimic a common situation in open outdoor feedlots where unexpected situations prevent the animals' access to food (e.g. irregular feed delivery and adverse climate conditions that create mud and moisture that limit the animals' movement). In addition, the coexistence of dominant and sub-ordinate animals in heterogeneous groups may interfere with regular food intake.

The hypothesis of this study is that a feed withdrawal–feed reintroduction (**FW–FR**) protocol may increase the risk of acidosis in grain-adapted cattle. No previous studies have closely examined the relationship among fasting periods and animal behaviour during FR and acidosis onset. In this work, the FW–FR model is explored to simulate typical field situations in which the access to the bunk feeder is limited.

Material and methods

Experimental design and acidosis challenge model

The experiment was performed at the farm research station of the Instituto Nacional de Investigación Agropecuaria (**INIA**, Colonia, Uruguay 34°S, 57°W). Twenty clinically healthy Hereford steers (aged 18 to 24 months and with an average BW of 379.4 ± 6.4 kg) fitted with permanent rumen catheters were used in this study. The catheters were surgically implanted in the dorsal sac of rumen 3 weeks prior to the start of this study. The catheters consisted of a silicone rubber tubing with a collar in the external flange. The animals were housed in individual pens and adapted to a total mixed ration (**TMR**) composed of 73% commercial pelleted fattening ration (ERRO P12®, Mercedes, Uruguay), 12% sunflower expeller and 15% *Setaria italica* hay. Overall, this TMR consisted of 85% concentrate and 15% forage, and its chemical composition was 89.7% DM, 12.9% CP, 24.9% ADF, 35.4% NDF and 56.6% non-fiber carbohydrates. This TMR was formulated based on the recommendations for a live weight gain of 1.6 kg/animal per day in the final fattening stages (National Research Council, NRC, 2000). The steers were adapted to the finishing diet during 45 days prior to this study, and a stable intake (11.9 ± 0.3 kg/day) was ensured. To calculate the feed intake, TMR refusals were collected and weighed daily, and this was subtracted from the amount of feed delivered. Intake was considered stable when no fluctuation in feed consumption was registered. The animals had *ad libitum* access to water.

Five animals were randomly assigned to each of four treatments using random numbers generated in an Excel sheet (Microsoft Corporation, Redmond, WA, USA). The treatments differed in the length of time that feed was withdrawn: 12 h (**T12**), 24 h (**T24**) and 36 h (**T36**), whereas the control group (**CON**) had no feeding restrictions. Once the FW period was concluded, the animals received unrestricted quantities of the same TMR. The trial included three different phases besides the control: no FW (regular feed delivery), FW (no access to feed) and FR (restoration of access to food). Time 0 h corresponded to the start of FW in each group (Figure 1). The entire trial ran from time −42 h to time 204 h, and several sampling procedures were performed (Figure 1).

The behaviour of the steers was examined to evaluate ingestive activity and intake rate and to detect any clinical signs of acidosis such as lack of coordination, lethargy, anorexia, ruminal motility cessation, diarrhoea or dehydration. Samples of ruminal liquid were collected from the ventral sac through the catheter using a connected syringe. Ruminal chemistry (pH, volatile fatty acids (**VFA**), lactic acid and ammonia nitrogen (**NH_3–N**)) and the structure of the microbial community were quantified using these samples. In addition, blood samples were collected from the jugular vein for the assessment of enzymes and metabolites.

Animal behaviour

The steers were continuously monitored by direct observation to identify signs of the onset of clinical acidosis. The feeding behaviour was monitored during the first 12 h after FR. For this assessment, two persons documented the behaviour of each steer every 10 min. Feeding activity was documented as follows: (i) eating (seeking, grasping or chewing TMR), (ii) drinking (consuming water) and (iii) ruminating (regurgitation, mastication of a regurgitated bolus or swallowing a bolus). Moreover, during FR, the individual intake rate was determined by recording the weight of the feed offered and rejected every 0.5 h (Abrahamse *et al.*, 2008).

Ruminal environment (pH, volatile fatty acids, lactic acid and ammonia nitrogen)

Ruminal pH was assessed at time −42 h and then every 6 h until FR (12 h for T12; 24 h for T24 and 36 h for T36). Ruminal pH was then assessed every 2 h for the first 12 h after FR, every 6 h up to 36 h and then every 12 h up to time 204 h, for every

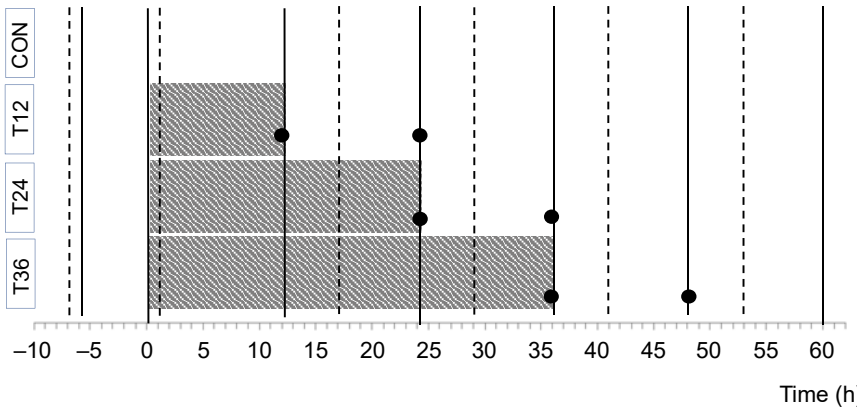

Figure 1 Experimental design and sampling schedule. Twenty feedlot steers were adapted to a high-concentrate diet (concentrate : forage = 85 : 15) prior to FW. The grey box shows the FW period that was applied (12 h in T12, 24 h in T24 and 36 h in T36); time 0 h represents the beginning of the withdrawal period. Animals in the control group had no FW. Black circles show the samples obtained for ruminal bacterial microbiota analysis. Vertical solid lines represent the ruminal fluid sampling for VFA, lactic acid and ammonia analysis. Dashed lines indicate the blood sampling. Sampling for pH is explained in the text. VFA=Volatile fatty acids; FW = feed withdrawal.

treatment group. Ruminal pH was timed and manually measured. The measurements were made using a digital pH metre calibrated with pH 4.0 and 7.0 standard solutions (model EW-05991-36 pH metre, Cole Parmer, Vernon Hills, IL, USA). For the major VFAs (acetic, propionic and butyric acids) and the lactic acid analysis, aliquots of fresh rumen liquid were obtained at −6, 0, 12, 24, 36, 48 and 60 h (Figure 1); mixed with perchloric acid 0.1 M (50 : 50, V/V) and immediately stored at −20°C until analysis. The VFA concentrations were measured by HPLC (Dionex Ultimate® 3000, Dionex Corporation, Sunnyvale, CA, USA) on centrifuged samples with a Rezex ROA-Organic acid column (300 mm × 7.8 mm; Phenomenex, Torrance, CA, USA) as described by Adams *et al*. (1984). To determine the NH_3–N concentration, ruminal liquid samples were obtained at −6, 0, 12, 24, 36 and 48 h (Figure 1) and were preserved at −20°C with sulfuric acid (50%) at a 1 : 50 dilution. Determinations were performed using the phenol-hypochlorite reaction (Weatherburn, 1967).

Ruminal bacterial microbiota analysis

Ruminal fluid samples were obtained during FR and 12 h after FR (Figure 1). Paired samples from animals in the control (**CON**) group were also obtained. Metagenomic DNA was extracted from 10 g of ruminal contents using the ZR Fecal DNA MiniPrep™ kit (ZYMO Research Corporation, Orange, CA, USA) following the instructions of the manufacturer. The DNA samples were sequenced using the Illumina MiSeq pyrosequencing PE250 (500) platform instrument targeting the V1–V2 region of the 16S rRNA gene (Microbiome Core Facility, Chapel Hill, NC, USA). The sequencing data were processed using the Quantitative Insights into Microbial Ecology software package (**QIIME** version 1.9.1, Caporaso *et al*., 2010). Chimeric sequences were checked using USEARCH and removed from further analysis. All clean high-quality sequences were allocated to the original samples based on barcodes. The classification into taxa and the generation of operational taxonomic units (**OTUs**) were performed by aligning the reads to the GreenGenes 16S rRNA database (DeSantis *et al*., 2006) through assignment to a template alignment using the PyNAST tool (Caporaso *et al*., 2010). UCLUST was selected for OTUs' clustering at 0.03 dissimilarity (Edgar, 2010). Quantitative Insights into Microbial Ecology bioinformatics pipelines were used to estimate the alpha diversity by calculating species richness (Chao index) and species diversity (Shannon index). Differences in microbial diversity were quantified with a non-parametric *t* test. A principal component analysis was performed to identify clustering among samples. The similarity was analysed by QIIME using Analysis of similarities (ANOSIM) and ADONIS.

Assessment of enzymes and metabolites

For the assessment of enzymes and metabolites, blood samples were collected at −7, 1, 5, 17, 25, 29, 41 and 53 h into evacuated tubes that were processed to obtain plasma or serum as needed (Figure 1). The metabolic profiles (glucose, insulin, urea, aspartate aminotransferase and non-esterified fatty acids) and oxidative-inflammatory profiles (haptoglobin, total protein, albumin and globulin) were assessed. Glucose and urea concentrations were quantified using enzymatic colorimetric kits (GLUCOSE, 11503 and UREA/BUN-COLOR, 11536, BioSystems, S.A. Costa Brava 30, Barcelona, Spain). The concentration of non-esterified fatty acids in the plasma was measured by colorimetric assay using a commercial kit (Wako Pure Chemical Industries Ltd, Osaka, Japan). Insulin was quantified using an immunoradiometric assay (DIA Source Immuno Assays S.A., Nivelles, Belgium). For the quantification of haptoglobin in the serum, an ELISA kit (Tridelta Development Ltd, Ireland) on a microplate reader (Thermo Electron Corporation, Multiscan EX) was used. The remainder of the blood parameters were measured with an automated biochemistry analyser (Automatic BT 3000 plus W®, Biotecnica Instruments, Italy). The globulin concentration was estimated to be the difference between total serum protein and albumin concentration. Further details regarding quality controls are presented in Supplementary Material S1.

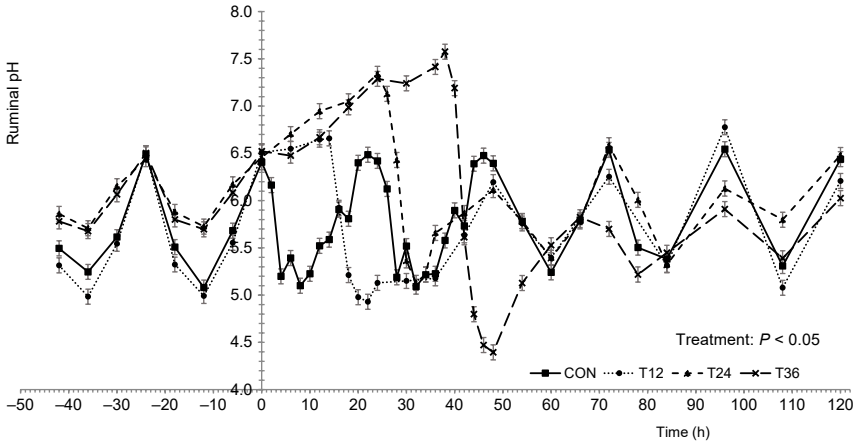

Figure 2 Pattern of ruminal pH in feedlot steers during the study. The FW–FR challenge was applied for 12 h in T12, for 24 h in T24 and for 36 h in T36, whereas the control group had no FW (CON). Time 0 h represents the beginning of the withdrawal period. The means from five animals in each treatment are presented. The SEM is represented by the bars. FW = feed withdrawal; FR = feed reintroduction.

Statistical analysis

To analyse differences between the main effects and the interactions, data were analysed using the MIXED procedure (SAS Inst. Inc., Cary, NC, USA) for a completely randomised design. For all statistical analyses, each steer was considered as the experimental unit. For feeding behaviour and intake rate, the model was $Yik = \mu + Si + eik$, where *Yik* is the dependent variable, μ is the general mean, *Si* is the fixed effect of the treatment (i = CON, T12, T24 or T36) in k animal replicate (n = 20 steers) and *eik* is the residual error. The measurements at different times on the same animal were considered as repeated measures in the model. The rest of the variables were analysed as according to the model $Yijk = \mu + Si + Tj + (S \times T)ij +$ steer-κ + *eijk*, where *Yijk* is the dependent variable, μ is the general mean, *Si* is the fixed effect of FW treatment (i = CON, T12, T24 or T36), *Tj* is the fixed effect of sample collection time, steer-k is the random effect of steer k (k = 20) and *eijk* is the residual error. A Bayesian fit criterion was used to determine the best variance–co-variance structure for the repeated measure analyses. A decreased Bayesian fit approach was used to choose a structure that provided the best fit to the data. Least squares means and the corresponding SEM were computed. The pre-FW measures were considered as co-variants for the variables of the ruminal environment (VFAs, lactic acid and NH_3–N) and the enzymes and metabolites. The ruminal pH data were co-variate adjusted with an average value calculated from the several measurements registered during the pre-FW phase. The differences were considered significant when $P < 0.05$.

Results

Animal behaviour

No clinical signs of acidosis were detected during the experiment in any animal. Feeding behaviour during FR was affected by FW ($P < 0.05$). Both, ingestive activity and intake rate, were increased in feed-withdrawn animals. Feed-withdrawn steers from the T12, T24 and T36 treatments showed a higher rate of intake than those in the CON group (T12 = 0.885, T24 = 0.829 and T36 = 0.906 *v.* CON = 0.737) (SEM = ±0.062; $P < 0.05$). The steers in the T36 treatment spent significantly more time ingesting food than the animals in the CON group and rumination was diminished. The proportion time spent ruminating was CON = 0.173 *v.* T12 = 0.082, T24 = 0.099 and T36 = 0.031 (SEM = ±0.023; $P < 0.05$). Steers from the T12, T24 and T36 treatment maintained high levels of food intake until 5 h after FR, whereas the voluntary intake of steers in the CON group was discouraged ($P < 0.05$).

Ruminal environment (pH, volatile fatty acids, lactic acid and ammonia nitrogen)

The FW–FR challenge resulted in significant changes in the ruminal pH (treatment (FW): $P < 0.05$, time (T): $P < 0.05$, treatment x time interaction (FW x T): $P < 0.05$). The ruminal pH values up to 120 h are presented in Figure 2 and pH values up to 204 h are presented in Supplementary Figure S1. The animals in the CON group showed a daily average pH of 5.8 ± 0.1, and a pattern characterised by a reduced pH after feed administration and a gradual return to higher values overnight. Before the FW period began, ruminal pH was similar in all steers ($P > 0.05$, Figure 2). During FW, ruminal pH was higher in the feed-withdrawn animals ($P < 0.05$), in which the ruminal pH increased systematically up to a maximum of approximately 7.5. Daily means during FW were as follows: T12 = 6.59 ± 0.11, T24 = 6.94 ± 0.10 and T36 = 7.02 ± 0.09 ($P < 0.05$) (Supplementary Table S1). After FR, daily means of pH values significantly decreased in these steers compared with animals in CON ($P < 0.05$), showing 5.06 in T12, 5.34 in T24 and 4.82 in T36. Immediately after FR, the pH plummeted in each group reaching the nadir value (T12 = 4.93, T24 = 5.13 and T36 = 4.39) observed between 8 and 10 h post-re-feed. The ruminal pH remained below 5.6 for 24 h, 7 h and 19 h for the T12, T24 and T36 treatments, respectively. Within the animals from the T36 treatment, four of five steers showed at least 2 h of pH values lower than 4.5. Five days after the beginning

Table 1 *Volatile fatty acid and NH_3–N concentrations during pre-FW, FW and FR in feedlot steers (concentrate : forage = 85 : 15) (n = 20)*

		Time[3]								*P*-value[4]		
Variables[1]	Treatment[2]	−6	0	12	24	36	48	60	SEM	FW	T	(FW x T)
Total VFA mM	CON	53.0	81.6^{a}	58.6^{a}	82.6^{a}	68.8^{a}	84.2^{a}	59.9^{a}	6.11	<0.0001	<0.0001	<0.0001
	T12	48.4	36.4^{b}	44.3^{a}	34.6^{b}	57.4^{a}	–	–	12.67			
	T24	39.0	36.0^{b}	11.4^{b}	20.3^{b}	34.4^{ab}	28.0^{c}	–	6.81			
	T36	44.7	33.7^{b}	11.3^{b}	14.8^{b}	11.1^{b}	42.4^{b}	49.4^{b}	4.06			
Acetic acid, mM	CON	31.7	44.4^{a}	35.7^{a}	39.6^{a}	23.7^{a}	44.3^{a}	31.8	4.67	<0.0001	<0.0001	<0.0001
	T12	25.6	20.0^{b}	26.9^{a}	16.7^{b}	35.5^{b}	–	–	7.03			
	T24	22.2	16.9^{b}	7.6^{b}	13.3^{b}	19.7^{a}	15.4^{c}	–	3.93			
	T36	23.6	22.0^{b}	5.7^{b}	9.8^{b}	8.3^{a}	27.5^{b}	29.5	3.62			
Propionic acid, mM	CON	15.9	26.7^{a}	19.9^{a}	30.3^{a}	21.8^{a}	28.2^{a}	23.0^{a}	3.40	<0.0001	<0.0001	<0.0001
	T12	15.9	13.1^{b}	13.3^{a}	14.3^{b}	21.0^{a}	–	–	4.72			
	T24	11.9	12.8^{b}	2.6^{b}	4.4^{c}	11.2^{b}	10.4^{b}	–	2.60			
	T36	12.8	8.13^{b}	4.3^{b}	2.9^{c}	1.9^{c}	9.7^{b}	14.9^{b}	1.63			
Butyric acid, mM	CON	5.2	10.4^{a}	2.9^{ab}	12.6^{a}	11.7^{a}	11.5^{a}	5.0	2.05	<0.0001	<0.0001	0.0001
	T12	6.8	3.2^{b}	4.1^{b}	3.4^{b}	12.2^{a}	–	–	2.27			
	T24	4.8	6.1^{b}	1.2^{a}	2.6^{b}	3.3^{b}	2.1^{b}	–	1.08			
	T36	8.2	3.5^{b}	1.3^{a}	2.0^{b}	0.8^{b}	5.1^{b}	4.9	1.21			
NH_3–N, mg/dl	CON	1.46^{ab}	1.58	1.73^{b}	0.67^{a}	1.81^{ab}	0.66^{a}	–	0.95	<0.0001	<0.0001	<0.0001
	T12	1.13^{a}	1.03	0.22^{c}	0.66^{a}	2.28^{ab}	1.05^{a}	–	1.59			
	T24	1.96^{b}	2.06	3.18^{a}	1.49^{b}	3.36^{b}	2.56^{b}	–	1.19			
	T36	1.48^{ab}	1.67	2.56^{ab}	2.02^{b}	0.11^{a}	0.70^{a}	–	1.42			

FW=feed withdrawal; FR=food reintroduction; VFA = volatile fatty acids;NH_3–N = ammonia Nitrogen.
[1] Data were co-variate adjusted with pre-FW measurements, effect of co-variant = $P < 0.05$.
[2] Treatment: CON = no FW; T12 = 12 h of FW; T24 = 24 h of FW; T36 = 36 h of FW.
[3] Time: expressed relative to FW; 0 h = beginning of FW.
[4] *P*-value = level of significance of the effect of FW = treatment (FW); T = time; FW x T = treatment x time.
[a,b,c] Values within a column with different superscripts differ significantly at $P < 0.05$.

of food deprivation, all groups exhibited similar ruminal pH profiles ($P > 0.05$).

The FW–FR challenge affected the VFA concentration ($P < 0.05$, Table 1). Feed-withdrawn animals showed lower total VFA concentrations throughout the entire study compared with those from the CON group ($P < 0.05$). The concentration of VFAs in steers from the T12, T24 and T36 treatments remained low throughout the FW period (24.4 mM overall). After FR, steers from the T24 and T36 groups showed significant increases in the post-prandial VFA concentration increasing from 20.3 mM to 34.3 mM and from 11.1 mM to 42.4 mM, respectively. Additionally, the VFA profile was affected by the FW–FR challenge (FW: $P < 0.05$, T: $P < 0.05$, FW x T: $P < 0.05$). After FR, propionic acid represented a greater proportion of the total VFA concentration in the feed-withdrawn animals (FW: $P < 0.05$, T: $P < 0.05$, FW x T: $P < 0.05$).

Furthermore, the FW–FR challenge noticeably affected lactic acid concentration of the rumen (FW: $P < 0.05$, T: $P < 0.05$, FW x T: $P < 0.05$, Figure 3). Although lactic acid was detected in all the groups, greater concentrations were observed at 12 to 13 h after TMR reintroduction in animals from the T24 (48.1 mM) and T36 (56.1 mM) treatments, and these increased levels of acid remained elevated for at least 12 h. Reduced levels of NH_3–N occurred for animals from the feed-withdrawn groups compared with the CON group (FW: $P < 0.05$, T: $P < 0.05$, FW x T: $P < 0.05$, Table 1).

Enzyme and metabolite assessments

The mean plasma concentrations of haptoglobin remained low in the CON steers (near 0.47 mg/ml) during the entire experimental period, and this biomarker was unaffected by the FW–FR challenge in steers from the T12 treatment (Figure 4). Conversely, animals in the T24 and T36 treatments showed an increase in haptoglobin concentration (FW: $P < 0.05$, T: $P > 0.05$, FW x T: $P < 0.05$). The haptoglobin response was as follows: three of five steers in the T24 treatment and four of five steers in the T36 treatment showed increases above baseline concentrations. The haptoglobin concentration increased by more than 0.5-fold in the T24 treatment and 1.6-fold in the T36 treatment.

In addition, both the non-esterified fatty acids and urea concentrations were increased by FW (FW: $P < 0.05$, T: $P < 0.05$, FW x T: $P < 0.05$), but neither the glucose (FW: $P > 0.05$, T: $P < 0.05$, FW x T: $P > 0.05$) nor insulin concentration (FW: $P > 0.05$, T: $P < 0.05$, FW x T: $P < 0.05$) was affected (Table 2). There was an increased concentration of serum albumin and elevated activity of the aspartate aminotransferase enzyme in feed-withdrawn steers compared with CON animals (FW: $P < 0.05$, T: $P < 0.05$, FW x T: $P < 0.05$), but total proteins and globulins were not affected (FW: $P > 0.05$, FW x T: $P < 0.05$, Table 2).

Ruminal bacterial microbiota

Feed withdrawal in treated animals caused substantial shifts in the structure of ruminal microbiota when compared with

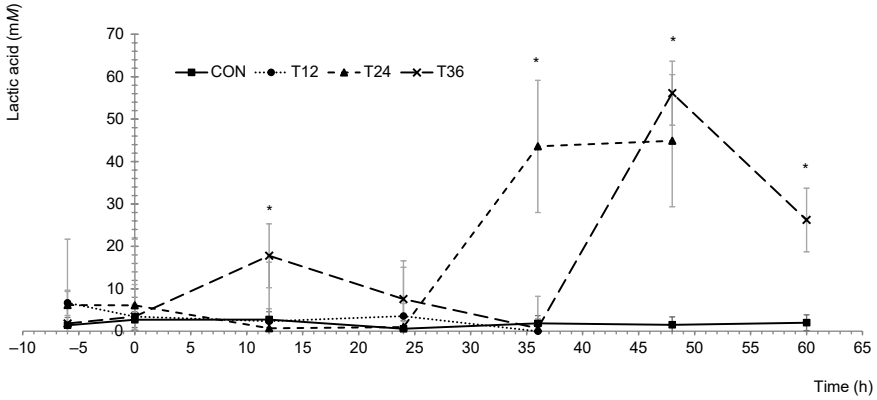

Figure 3 Pattern of ruminal lactic acid concentration in feedlot steers during the study. The FW–FR challenge was applied for 12 h in T12, for 24 h in T24 and for 36 h in T36, whereas the control group had no FW (CON). Time 0 h represents the beginning of the withdrawal period. The means from five animals in each treatment are presented. The SEM is represented by the bars. Significant differences ($P < 0.05$) between the treatments and control are marked by an asterisk. FW = feed withdrawal; FR = feed reintroduction.

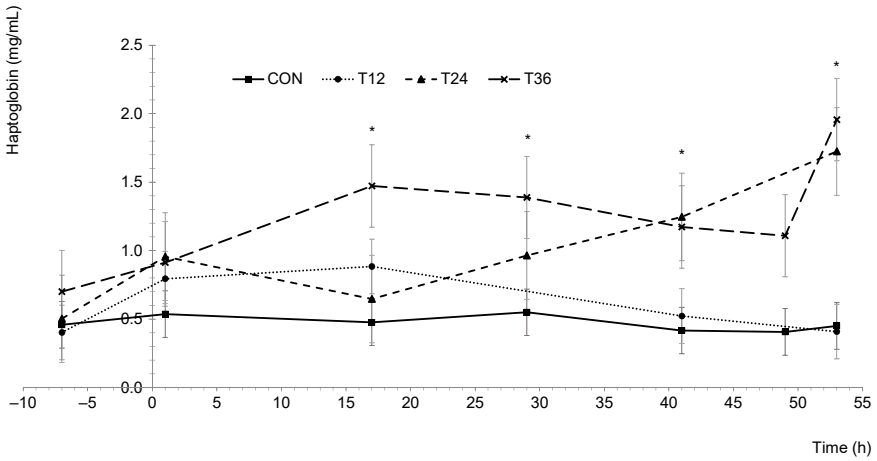

Figure 4 Serum haptoglobin concentrations (mg/ml) in feedlot steers during the study. The feed was withdrawn for 12 h in T12, for 24 h in T24 and for 36 h in T36, whereas the control group had no FW (CON). Time 0 h represents the beginning of the FW period. The means from five animals in each treatment are presented. The SEM is represented by the bars. Significant differences ($P < 0.05$) between the treatments and control are marked by an asterisk. FW = feed withdrawal.

the microbial composition of steers in the CON group ($P < 0.05$). On average, 2350 sequences per sample were studied. Bacteroidetes was the most represented phylum in all groups during FR with relative abundances ranging from 51% in CON steers to 44% in the T12 and T24 animals and 57% in animals from the T36 treatment. After 12 h of FR, the feed-withdrawn animals showed a significantly increased relative abundance of Firmicutes, whereas Bacteroidetes remained the dominant phylum in the CON steers ($P < 0.05$) (Figure 1 and Table 3). The Firmicutes to Bacteroidetes ratio increased by two- to five-fold at 12 h after FR compared to this ratio at FR (T12 = 1.47, T24 = 1.44, T36 = 1.83 *v.* CON = 0.63).

Information regarding the shifts in the relative abundance at the genera level and their significance is detailed in Supplementary Table S2. Although relative abundance of *Streptococcus* was less than 0.1% in all the treatments during FR, 12 h after food re-establishment, *Streptococcus* OTUs were assigned to more than 24% of the sequences in steers from the T24 animals and 36% in the T36 animals ($P < 0.05$). The relative abundance of *Lactobacillus* was similiar under all conditions. Within the family Veillonellaceae, *Megasphaera* was the dominant genus and showed a substantial increase reaching 1% of sequences in steers from the T24 treatment and 9% in steers from the T36 treatment at 12 h after FR ($P < 0.05$) (Supplementary Table S2). The relative abundance of *Selenomonas* was not affected by treatments. Fibrolytic bacteria genera such as *Ruminococcus*, *Butyrivibrio* and *Prevotella* diminished in challenged steers from the T24 and T36 treatments ($P < 0.05$). The relative abundance of other fibrolytic bacteria such as *Eubacterium* and *Fibrobacter* did not differ between groups. The relative abundance of *Ruminobacter* spp. increased in steers from the T36 treatment ($P < 0.05$).

Comparisons among the treatments revealed differences in the diversity indices. The alpha diversity statistics calculated for each treatment revealed lower diversity in the T24 and T36 steers at 12 h after FR ($P < 0.05$) (Shannon values of 4.91 and 4.35, respectively). The richness of the bacterial community was not affected by the FW–FR challenge,

Table 2 *Concentrations of glucose, insulin, non-esterified fatty acids, urea and hepatic metabolites in feedlot steers during pre-FW, FW and FR (concentrate : forage = 85 : 15) (n = 20)*

		Time[2]							*P*-value[3]		
Variables[4]	Treatment[1]	−7	1	17	29	41	53	SEM	FW	T	(FW x T)
Glucose, mg/dl	CON	122.6	115.3	127.7	112.6	123.7	121.5	34.73	0.236	<0.0001	0.147
	T12	136.0	118.2	155.9	–	154.3	153.7	18.67			
	T24	127.1	113.7	133.9	189.4	136.6	146.5	17.56			
	T36	128.8	115.1	132.7	138.4	176.5	133.6	17.49			
Insulin, µUI/ml	CON	33.8	27	35.5	24.2	34	26.7	3.13	0.250	<0.0001	<0.0001
	T12	44.5	26.4	55.4	–	31.9	29.3	8.27			
	T24	38.3	26.2	15.1	12.9	51.1	36.2	10,0			
	T36	38	26	21.7	14.5	29.9	22.3	5.37			
NEFA, mmol/l	CON	0.22	0.28	0.24^{a}	0.21^{a}	0.22^{a}	0.24^{a}	0.01	<0.0001	<0.0001	<0.0001
	T12	0.25	0.45	0.24^{a}	–	0.20^{a}	0.24^{a}	0.02			
	T24	0.23	0.55	0.64^{b}	0.78^{b}	0.21^{a}	0.22^{a}	0.03			
	T36	0.2	0.49	0.59^{b}	0.71^{b}	0.51^{b}	0.34^{b}	0.06			
Urea, mg/dl	CON	35.1	32.0	32.7^{ab}	24.4^{a}	24.1^{a}	31.7^{b}	2.55	0.047	<0.0001	<0.0001
	T12	33.7	28.5	46.3^{c}	–	26.0^{a}	32.2^{b}	3.71			
	T24	35.6	32.0	37.0^{b}	39.2^{b}	31.8^{b}	22.5^{a}	5.09			
	T36	33.4	29.7	29.7^{a}	32.7^{b}	51.6^{b}	28.0^{ab}	4.25			
Albumin, g/dl	CON	3.50	3.43^{a}	3.35^{a}	3.47^{a}	3.32^{a}	3.20^{a}	0.07	<0.0001	<0.0001	<0.0001
	T12	3.59	3.55^{b}	3.51^{ab}	–	3.55^{b}	3.44^{ab}	0.07			
	T24	3.46	3.61^{b}	3.62^{b}	3.80^{b}	3.52^{b}	3.54^{b}	0.05			
	T36	3.42	3.62^{b}	3.66^{b}	3.67^{b}	3.63^{b}	3.22^{a}	0.09			
Globulin, g/dl	CON	3.97	4.22	3.79	4.06	3.76	3.89	0.20	0.319	0.093	0.0005
	T12	3.69	3.88	3.64	–	3.82	3.52	0.20			
	T24	3.78	3.75	4.24	3.59	3.56	3.74	0.13			
	T36	4.00	3.78	3.79	4.35	4.10	3.22	0.17			
GOT-AST, U/l	CON	106.4^{a}	82.1^{bc}	87.1^{a}	77.4	72.2^{a}	72.6^{ab}	5.14	0.003	0.030	<0.0001
	T12	87.0^{b}	61.9^{a}	63.4^{b}	–	68.0^{a}	65.4^{a}	3.72			
	T24	67.8^{b}	94.0^{c}	–	–	89.4^{b}	54.7^{a}	10.22			
	T36	87.9^{b}	101.0^{c}	70.2^{ab}	76.4	102.8^{b}	90.2^{b}	11.47			
Total protein, g/dl	CON	7.47	7.65	7.15	7.53	7.08	7.09	0.23	0.849	0.001	0.0007
	T12	7.29	7.43	7.15	–	7.37	6.96	0.20			
	T24	7.24	7.36	7.87	7.39	7.08	7.28	0.15			
	T36	7.43	7.40	7.45	8.03	7.73	6.45	0.19			

NEFA = non-esterified fatty acids; GOT-AST = aspartate aminotransferase; FW = feed withdrawal.
[1] Treatment: CON = no FW; T12 = 12 h of FW; T24 = 24 h of FW; T36 = 36 h of FW.
[2] Time: expressed relative to FW; 0 h = beginning of FW.
[3] *P*-value = level of significance of the effect of FW = treatment (feed withdrawal); T = time; FW x T = treatment x time.
[4] Data were co-variate adjusted with pre-FW measurements, effect of co-variant = $P < 0.05$.
[a,b,c] Values within a column with different superscripts differ significantly at $P < 0.05$.

as the Chao richness index was similar among groups ($P > 0.05$). The FW–FR model altered the rumen microbial composition at the structural level. The principal component analysis plots assessed at FR and 12 h later showed similarities between the microbial communities within samples from each treatment. The first two components explained approximately 78.6% (at FR) and 71.2% (at 12 h after reintroduction) of the total variation. Animals in the CON and T12 groups appeared to cluster together, whereas steers from the T24 and T36 groups showed a similar microbiota population structure (Figure 5). The sample clusters for the four groups were revealed by a significant ADONIS ($R^2 = 0.123$; $P = 0.001$) and ANOSIM ($R = 0.299$; $P = 0.004$) at FR and by a significant ADONIS ($R^2 = 0.119$; $P = 0.003$) and ANOSIM ($R = 0.342$; $P = 0.002$) 12 h after feed was reintroduced.

Discussion

Although at the beginning of the experimental period the adapted animals had a low baseline ruminal pH, which is consistent with other observations on concentrate-fed beef cattle, the FW–FR protocol triggered SARA for extended periods. A baseline SARA condition was not unexpected because feedlot cattle receiving grain-based diets can show a low baseline ruminal pH (Schwartzkopf-Genswein *et al.*, 2004). After FR, the ruminal pH values as well as the periods with a pH below the 5.6 threshold corresponded to a proposed value for the SARA condition (Gozho *et al.*, 2005). Additionally, the ruminal pH dropped to less than 4.5 in steers from the T36 treatment, which is below even the acute acidosis threshold (Nagaraja and Titgemeyer, 2007).

Table 3 *Relative abundance expressed as proportional data of bacterial phyla in feedlot steers measured at FR and 12 h after FR (n = 20)*

	Treatment[1]									
	CON		T12		T24		T36		*Significance*	
Bacterial phyla	FR	12 h FR	FR	12 h FR	FR	12 h FR	FR	12 h FR	FW	T
Actinobacteria	0.006	0.005	0.006	$5.45\ 10^{-02}$	0.009	0.017	0.004	$2.36\ 10^{-02}$		
Armatimonadetes	0	0	0	$1.00\ 10^{-06}$	$3.97\ 10^{-06}$	$4.08\ 10^{-06}$	$9.54\ 10^{-07}$	0		
Bacteroidetes	0.541	0.528	0.438	0.357	0.436	0.367	0.573	0.318		
Chloroflexi	0	$1.81\ 10^{-06}$	0	$1.88\ 10^{-06}$	$2.06\ 10^{-05}$	$1.22\ 10^{-05}$	$1.74\ 10^{-05}$	$9.15\ 10^{-06}$		
Cyanobacteria	0.031	0.039	0.024	0.002	0.032	0.002	0.007	0.003		
Deferribacteres	$3.29\ 10^{-06}$	0	0	0	$1.59\ 10^{-06}$	0	$9.80\ 10^{-06}$	$5.58\ 10^{-06}$		
Elusimicrobia	0.001	0.002	0.001	$3.55\ 10^{-04}$	0.001	$8.33\ 10^{-04}$	0.006	$8.93\ 10^{-04}$		
Euryarchaeota	$1.92\ 10^{-06}$	0	0	$7.22\ 10^{-06}$	$7.94\ 10^{-07}$	0	0	0		
Fibrobacteres	0.073	0.018	0.120	0.001	0.013	0.009	0.084	0.021	*	+
Firmicutes	0.245	0.332	0.298	0.525	0.262	0.529	0.209	0.580	*	+
Fusobacteria	$2.03\ 10^{-05}$	$2.14\ 10^{-05}$	$3.53\ 10^{-05}$	$2.04\ 10^{-05}$	$7.54\ 10^{-05}$	$7.25\ 10^{-04}$	$1.83\ 10^{-04}$	0.002		
Gemmatimonadetes	0	0	0	$1.87\ 10^{-06}$	0	0	0	0		
Lentisphaerae	$1.13\ 10^{-04}$	$2.34\ 10^{-04}$	$8.69\ 10^{-04}$	$3.08\ 10^{-04}$	0.001	$4.87\ 10^{-04}$	0.002	$3.62\ 10^{-04}$		
Planctomycetes	$4.70\ 10^{-07}$	0	0	0	$9.63\ 10^{-07}$	0	$2.83\ 10^{-06}$	$7.39\ 10^{-06}$		
Proteobacteria	0.006	0.007	0.006	0.004	0.162	0.015	0.011	0.017	*	
Spirochaetes	0.027	0.017	0.040	0.014	0.021	0.018	0.030	0.009		
Synergistetes	$4.46\ 10^{-04}$	$2.94\ 10^{-04}$	$5.33\ 10^{-04}$	$2.91\ 10^{-04}$	0.001	$5.28\ 10^{-04}$	0.002	0.002		
Tenericutes	0.027	0.015	0.020	0.016	0.012	0.007	0.008	0.003		
Saccharibacteria	$5.52\ 10^{-04}$	$9.79\ 10^{-04}$	0.003	0.001	0.003	$1.92\ 10^{-04}$	0.004	$7.06\ 10^{-04}$		
Unassigned	0.040	0.030	0.041	0.022	0.037	0.026	0.049	0.017		
Verrucomicrobia	$6.36\ 10^{-04}$	0.005	0.002	$6.38\ 10^{-04}$	0.007	0.006	0.005	$1.70\ 10^{-03}$		
Candidate division WPS-2	0.001	$7.05\ 10^{-04}$	$6.27\ 10^{-05}$	5.07 10–^{05}	0.002	$4.64\ 10^{-04}$	0.003	$3.53\ 10^{-04}$		
Absconditabacteria	0	0	0	$2.41\ 10^{-06}$	$4.14\ 10^{-05}$	$3.42\ 10^{-06}$	$3.41\ 10^{-05}$	0		
Other	$2.28\ 10^{-04}$	$1.63\ 10^{-04}$	0.001	$1.83\ 10^{-05}$	0.001	$3.44\ 10^{-04}$	0.001	$2.20\ 10^{-05}$		
Firmicutes/Bacteroidetes ratio	0.45	0.63	0.68	1.47	0.60	1.44	0.37	1.83		

FR = feed reintroduction; FW = feed withdrawal.
[1] CON = no FW; T12 = 12 h of FW; T24 = 24 h of FW; T36 = 36 h of FW.12 h-FR: 12 h after feed reintroduction.
Symbols (* and +) indicate the statistical significance of the different effects at $P < 0.05$. FW (*) = effect of treatment (FW); T (+) = effect of time.

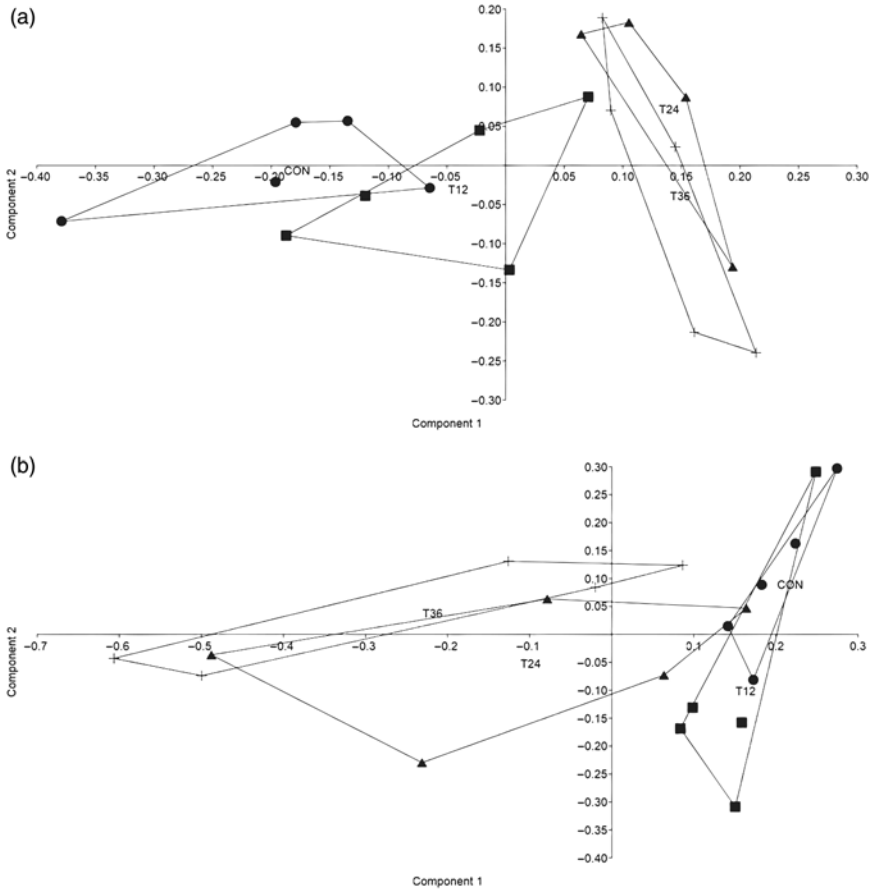

Figure 5 Two-dimensional principal component analyses of the bacterial community structures in feedlot steers at FR (a) and 12 h after FR (b). Animals were exposed to FW for 12 h (T12 = squares), for 24 h (T24 = triangles) or for 36 h (T36 = pluses), whereas steers from the control group had no feed withdrawal (CON = circles). Each point on the plot represents the bacterial community composition of a single sample. FR = feed reintroduction; FW = feed withdrawal.

However, no clinical manifestation of acidosis such as incoordination, lethargy and recumbency, anorexia, ruminal motility cessation, watery and foamy faeces or dehydration (Olson and Hollis, 2007) occurred.

The behavioural pattern observed in feed-withdrawn animals is consistent with a compensation strategy for the previous hours of feed restriction (Forbes and Mayes, 2002). As has been reported previously, fasted steers focussed primarily on ingestion when the feed was again available (Félix *et al.*, 2017). In addition, the higher ingestion rate was followed by a lower rumination frequency. The amount of saliva produced and mixed with the ingesta was most likely reduced given both the high intake rate and the type of diet delivered that was an 85% concentrate-TMR. This TMR, which is rich in easily fermentable carbohydrates, intrinsically produces poor stimulation of rumination and chewing due to its composition (Giger-Reverdin, 2018).

These latter characteristics could explain the critical drop in ruminal pH that was registered and the resulting occurrence of SARA after FR. The TMR availability after FW may have induced the reactivation of rumen fermentation activity, leading to VFA and lactic acid production and accumulation (Owens *et al.*, 1998; Golder *et al.*, 2012). However, the VFA concentration was always lower in feed-withdrawn animals compared with CON steers during the post-prandial period. As a result, and consistent with a previous study, the lactic acid accumulation in steers from the T24 and T36 groups appears to be the primary explanation for the drop in ruminal pH (Golder *et al.*, 2012). The levels of lactic acid in these two groups of animals were higher than 40 mM, which is the threshold concentration for acidosis (Owens *et al.*, 1998, Figure 3). The ruminal pH started to decrease immediately after FR and the lowest pH values were registered at approximately 30 to 32 h in the T24 group and at 46 to 48 h in the T36 group, most likely connected with the increase in lactic acid concentration recorded at 36 and 48 h, respectively. The increased levels of lactic acid in these groups remained elevated for at least 12 h. However, the sampling regime for lactic acid was not as frequent as for pH, which prevented the gathering of stronger evidence in support of this point.

A disruption of the ruminal microbiota composition during FW could have conditioned post-prandial VFA peaks in the feed-withdrawn animals. Additionally, once the ruminal pH dropped below 5.6, more un-dissociated VFAs (pKa ~4.9) would have enhanced the total VFA absorption rate. Conversely, at the same pH, lactic acid is less protonated (pKa 3.9), leading to its accumulation and the accentuation of sudden decreases in pH.

These ruminal biochemical variations were coupled with microbial changes during the acidosis disturbance. The microbial population shifted to gram-positive bacteria with a predominance of Firmicutes and a decrease in Bacteroidetes. The low ruminal pH is connected with gram-negative rumen bacterial lysis, which has been demonstrated by an increase in the bacterial endotoxin concentration (Gozho *et al.*, 2007; Nagaraja and Titgemeyer, 2007). Moreover, the ruminal bacterial diversity would be negatively affected by FW–FR episodes (Plaizier *et al.*, 2017). Current data indicate that the changes in bacteria may reasonably be linked to changes in ruminal parameters (Golder *et al.*, 2018). The microbiota shifts that followed the FW could have led to an increase in endotoxin concentrations in the rumen that were mirrored by activation of the acute phase response.

Most of the changes produced during the fermentation process can be connected to the disruption of the microbial structure. The relative abundance of potentially fibrolytic bacteria genera such as *Ruminococcus*, *Butyrivibrio* and *Prevotella* decreased in challenged steers from the T24 and T36 treatments. Reductions in the relative abundance of *Ruminococcus* in response to acidotic conditions have been reported elsewhere (Nagaraja and Titgemeyer, 2007), as ruminal acidotic conditions with pH values below 6 affect the growth of cellulolytic bacteria. *Prevotella* has also been shown to be sensitive to low-ruminal pH, and so the higher acidity after the FR most likely affected its relative abundance (Kim *et al.*, 2018).

The relative abundance of *Ruminobacter* spp. increased in the steers from the T36 treatment at 12 h after FR. Bacteria belonging to *Ruminobacter* genera are linked to starch digestion that readily metabolises carbohydrates. Once the challenged steers were re-feed, the proliferation of amylolytic genera may be explained by the high substrate availability (starch) from fermentable carbohydrates arriving at the rumen during this phase.

Both the lactate producer and utiliser bacterial groups increased in challenged animals. The proliferation of *Streptococcus* spp. and *Lactobacillus* spp. populations has been reported under severe acidosis conditions (Khafipour *et al.*, 2009). Within these groups, the greatest relative change in abundance was found for the *Streptococcus* spp. in steers from the T24 and T36 treatments. When ruminal pH decreases below 5.0, lactic acid producers such as *Streptococcus* spp. proliferate, and lactic acid accumulations exceed normal (10 mM) concentrations (Owens *et al.*, 1998; Gill *et al.*, 2000; Ghorbani *et al.*, 2002).

Changes in lactic acid producer bacteria were mirrored by shifts in the lactate-metabolising microbiota. The increased relative abundance of *Streptococcus* spp. was accompanied by an increase in *Megasphaera* spp. *Megasphaera*, which primarily uses lactic acid for its metabolism, played a relevant ecological role by processing the released lactic acid and relieving acidosis in the rumen. This bacterial proliferation may be a consequence of lactic acid accumulation, which can metabolise lactic acid into VFAs. These VFAs can be absorbed within the rumen as a preventive mechanism against acute acidosis, supporting the idea of a synchronised relationship between lactic acid producers and utilisers (Oetzel, 2003; McCann *et al.*, 2016). The capability for resilience in the microbiota may help to prevent the onset of overt signs of illness. Variations in the bacterial community structure may represent a mechanism to restore the ruminal fermentation process, assisting the animal's recovery and avoiding the consequent manifestation of symptomatology.

This FW–FR challenge generated transient but severe disturbances in ruminal fermentation. The feed-withdrawn steers required 5 days after the FW–FR challenge to exhibit pH values similar to animals from the CON group (Supplementary Figure S1), as previously observed (Patra *et al.*, 1996, Brown *et al.*, 2000).

Feed withdrawal influenced the activation of lipomobilisation mechanisms in an attempt to preserve energy homeostasis (Van Harmelen *et al.*, 1999), which was reflected by the high non-esterified fatty acid concentrations. The rise in urea concentration was similar to previous reports (Patra *et al.*, 1996). This may signal the increase in ruminal NH_3–N that was registered after the feed was reintroduced, as blood urea nitrogen values and ruminal NH_3–N concentrations are positively associated (DePeters and Ferguson, 1992). Similarly, this increase in urea may reflect the microbial nitrogen release following the death of ruminal bacteria, reflecting changes in the bacterial metabolism. Additionally, increased urea levels could indicate a compensation strategy against the energy deficit status.

Consistent with previous reports on grain-induced SARA, the challenge substantially increased haptoglobin concentrations in steers from the T24 and T36 treatments (Gozho *et al.*, 2007). The level of haptoglobin registered in the steers from the CON group could be viewed as a baseline that indicates a background inflammatory condition typically attributed to high-concentrate diets. Additionally, the surgical procedure required for the catheter insertion may have boosted this baseline level in all the steers, even though the animals were allowed 3 weeks to recover from the intervention. The increased haptoglobin level could indicate a response to tissue injury and inflammatory processes. The process of bacterial lysis releases endotoxins that may activate an acute phase response and result in a haptoglobin increase (Wassell, 2000; Nagaraja and Titgemeyer, 2007). Physiologically, haptoglobin binds free haemoglobin, capturing the iron and affecting bacterial proliferation (Wassell, 2000). The large and sudden fluctuations in ruminal pH produced by the FW–FR challenge could have affected the integrity of the ruminal microbiota, leading to an increase in haptoglobin.

The increase in albumin concentrations may suggest a level of dehydration that was not clinically evidenced. Lactic acid could cause sequestration of water from the blood system into the rumen leading to dehydration (Lean *et al.*, 2007). Enhanced aspartate aminotransferase levels after acidosis induction are linked with the mobilisation of fat reserves and may also reflect the hepatic and muscle tissue damage that occurs during SARA (Patra *et al.*, 1996).

This study demonstrates an approach to investigating the role of periods of involuntary FW in inducing SARA in feedlot cattle. The destabilisation of the ruminal microbiota during diet withdrawal together with a rapid TMR intake rate and depressed ruminating activity could have promoted a sharp decline in ruminal pH after FR. Our results question the postulate that claims lactic acid as the most critical determining factor of acidosis. These results indicate that rumen pH can fall well below and lactic acid concentrations can rise well above the previously defined thresholds for acute acidosis, without any detectable clinical effects. Although there was no clinical manifestation, the steers showed increased haptoglobin levels, which indicates an inflammatory response and suggests a deleterious effect on animal welfare. Indeed, these findings encourage further investigation in this field, such as the consequences on animal productivity and the effect of repeated FW episodes.

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Declaration of interest

The authors declare no recognised conflicts of interest linked with this paper, and there is no financial assistance for this study that could influence its outcome.

Ethics statement

The animal procedures were conducted following the regulations and guidelines set by the Bioethics Committee of the Facultad de Veterinaria, Uruguay (file number 0009/11) and the Ethics and Animal Welfare Commission of INIA, Uruguay (file number: 2014-38).

Software and data repository resources

None of the data were deposited in an official repository.

Supplementary material

To view supplementary material for this article, please visit https://doi.org/10.1017/S1751731119001538

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