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Postmortem observations on rumen wall histology and gene expression and ruminal and caecal content of beef cattle fattened on barley-based rations

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*Sub-acute ruminal acidosis (**SARA**) can reduce the production efficiency and impair the welfare of cattle, potentially in all production systems. The aim of this study was to characterise measurable* postmortem *observations from divergently managed intensive beef finishing farms with high rates of concentrate feeding. At the time of slaughter, we obtained samples from 19 to 20 animals on each of 6 beef finishing units (119 animals in total) with diverse feeding practices, which had been subjectively classified as being high risk (three farms) or low risk (three farms) for SARA on the basis of the proportions of barley, silage and straw in the ration. We measured the concentrations of histamine, lipopolysaccharide (**LPS**), lactate and other short-chain fatty acids (**SCFAs**) in ruminal fluid, LPS and SCFA in caecal fluid. We also took samples of the ventral blind sac of the rumen for histopathology, immunohistopathology and gene expression. Subjective assessments were made of the presence of lesions on the ruminal wall, the colour of the lining of the ruminal wall and the shape of the ruminal papillae. Almost all variables differed significantly and substantially among farms. Very few pathological changes were detected in any of the rumens examined. The animals on the high-risk diets had lower concentrations of SCFA and higher concentrations of lactate and LPS in the ruminal fluid. Higher LPS concentrations were found in the caecum than the rumen but were not related to the risk status of the farm. The diameters of the stratum granulosum, stratum corneum and of the vasculature of the papillae, and the expression of the gene* TLR4 *in the ruminal epithelium were all increased on the high-risk farms. The expression of* IFN-γ *and* IL-1β *and the counts of cluster of differentiation 3 positive and major histocompatibility complex class two positive cells were lower on the high-risk farms. High among-farm variation and the unbalanced design inherent in this type of study in the field prevented confident assignment of variation in the dependent variables to individual dietary components; however, the CP percentage of the total mixed ration DM was the factor that was most consistently associated with the variables of interest. Despite the strong effect of farm on the measured variables, there was wide inter-animal variation.*

Keywords: acidosis, pathology, pH, diet, papillae

Implications

This study demonstrates a great capacity for adaptation by cattle to very high levels of rapidly fermentable carbohydrates in the diet. There was almost no recognisable pathology in the ruminal wall of animals in the study and none of the variables we measured was consistently elevated in animals on the farms with concentrate proportions ranging up to 90% of the total diet dry matter. Hence, we were unable to identify a useful *postmortem* indicator of the risk of acidosis, other than slightly raised ruminal lipopolysaccharide and caecal and ruminal lactate concentrations.

Introduction

Sub-acute ruminal acidosis (**SARA**) is a common inflammatory syndrome of ruminants arising from high dietary challenge with rapidly fermentable carbohydrates (Titgemeyer

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and Nagaraja, 2006; Kleen *et al.*, 2009; Kleen and Cannizzo, 2012). Ruminal acidosis is characterised by impaired fibre degradation, lower feed intake, laminitis, diarrhoea and displaced abomasum, with consequent increased culling rates in dairy herds (Plaizier *et al.*, 2008). Kleen and Cannizzo (2012) summarised reports relating to the occurrence of acidosis in dairy herds, providing examples to suggest the incidence of SARA to be 14% in Friesland (Kleen *et al.*, 2009), 19% to 40% in the USA (Garrett *et al.*, 1997) and 33% in some Italian herds (Morgante *et al.*, 2007). The condition also occurs in beef cattle (Nagaraja and Titgemeyer, 2007). The diagnosis and management of SARA is difficult, as there is little consensus on diagnostic criteria and it is largely a chronic predisposition to other specific diseases, rather than being a single disease entity itself (Kleen *et al.*, 2009; Denwood *et al.*, 2018).

The starch in cereal-rich diets is fermented quickly, leading to the rapid production of fermentation acids, principally short-chain fatty acids (**SCFAs**), mainly acetate, propionate and butyrate. The SCFA is absorbed through the reticuloruminal wall, providing energy to the host animal, and a moderate concentration remains in ruminal digesta (Aschenbach *et al.*, 2011). In most cases, the pH of the reticulorumen is held within a functional physiological band by a complex combination of endogenous buffers, dietary buffers, active transport by reticuloruminal epithelial cells and physical removal of protons from the reticulorumen into the distal digestive tract (Aschenbach *et al.*, 2011). When the rate of ingestion of the substrate for the production of SCFAs exceeds the buffering capacity of the reticulorumen, lactic acid accumulates, pH begins to fall and there are dramatic changes in the microbiome, with dominance of acid-tolerant genera such as *Streptococcus* and *Lactobacillus* (Slyter, 1976; Dawson *et al.*, 1997; Penner *et al.*, 2009).

From studies with dairy cattle, it is known that some farms are more susceptible to acidosis than others, and that within herds some animals are more prone to acidosis than others (Garrett, 1996; Morgante *et al.*, 2007; Kleen *et al.*, 2009; Penner *et al.*, 2009; Denwood *et al.*, 2018). Many of the experimentally determined interactions among feeding methods, feed and animal behaviour in feedlots have been reviewed elsewhere (e.g. Gonzalez *et al.*, 2012), but a need exists to determine how observations in experimentally induced acidosis in cattle relate to on-farm, commercial conditions. The aim of this study was to characterise measurable *postmortem* observations on variables that have previously been shown to be associated with acidosis on animals from divergently managed intensive beef finishing farms with high rates of concentrate feeding with a view to identifying indicators of prior exposure to diets with a high risk of acidosis.

Material and methods

Farms and animals

Ten beef farms situated in Aberdeenshire completed a questionnaire on dietary and farm management in the year prior to the study. Six were selected based on dietary composition and likely risk of acidosis (three high risk and three low risk) based on the questionnaire and on-farm observations. In total, 119 steers and heifers with an average age of 700 days and carcase weight of 371 kg were sampled at slaughter. Breeds included Aberdeen Angus, Blonde d'Aquitaine, Charolais, Limousin, Saler, British Blue, Shorthorn and Simmental, and crosses of these breeds. All animals were finished indoors in the Grampian region close to Aberdeen (mean annual temperature 7.5°C, rainfall 1130 mm) between January and July 2013.

Feed analysis

A representative sample of the total mixed ration (**TMR**) was assessed for each of the farms, once during the feeding period for each cohort of animals. Samples were taken for measurement of CP, crude fibre, starch and NDF on a DM basis using near infrared spectrophotometry (NIRS). Near infrared spectrophotometry was conducted using a FossNIRSystems 5000+ machine, using calibrations provided by Trouw Nutrition GB. Particle size of TMR was measured by Trouw Nutrition GB using a Penn State particle separator, with a 1.18 mm screen to enable quantification of fine particles. All cattle had *ad libitum* access to feed, and were estimated to consume 11 to 13.5 kg DM feed per day. Cattle at farm BH6 also had *ad libitum* access to barley straw.

Postmortem *sampling*

In the abattoir, carcasses of the animals involved in the study were followed from the kill point to evisceration where the rumens were marked with a labelled cable tie to allow identification of animals as they moved through to the gut room of the abattoir, where the hind gut was separated from the rumen by abattoir staff. Once the rumen was separated from the hindgut and incised, a sample of its contents was taken and strained through four layers of gauze muslin into 2×50 ml plastic centrifuge tubes.

A sample was taken from the middle of the ventral sac, washed quickly in water to remove most digesta (to reduce difficulties with subsequent sectioning with the microtome) and two samples were taken: one placed in RNAlater (for studies on gene expression) and one in 10% formalin for histology and immunohistochemistry (**IHC**). For the sample placed in RNAlater, the muscle layer was removed. For both samples, fat was trimmed as much as possible before placing it in the fixing solution. Samples of caecum wall were taken and caecum contents collected and strained through muslin into Falcon tubes. The caecum wall was washed in water to remove any excess digesta and then two samples taken and placed in bijoux, in either RNAlater or formalin, as with the rumen samples. The samples in RNAlater were then stored at −20°C for gene expression studies to be carried out at a later date. The samples that were fixed in 10% formalin for 2 days were then removed, rinsed with and stored in phosphate-buffered saline (**PBS**) prior to embedding in paraffin.

Samples were kept on ice in a passive cool-box for transportation back to the laboratory for further processing. Two 2 ml aliquots were taken of the strained rumen fluid and caecum contents, and then stored at −20°C for future SCFA determination, according to the method of Richardson *et al.* (1989). For lipopolysaccharide (**LPS**) and histamine determination, 1 ml of strained rumen fluid was thoroughly mixed with 1 ml PBS, which was then centrifuged at 16 000 × **g** for 45 min at 4°C. Sterile syringe and needle were then used to aspirate the supernatant, with minimal disruption to the pellet and filtering the supernatant through a sterile 0.2 μm filter (Elkay Laboratory Products (UK) Ltd, Basingstoke, UK), with the resultant filtrate collected in a pyrogen-free glass tube (Cat. # N207, Lonza Group Ltd, Basel, Switzerland). The sample was then heated at 100°C for 30 min, allowed to cool to room temperature and then stored at −20°C. The remaining strained content was stored raw at −20°C.

Gross assessment of rumen wall

The opened and washed wall of the ventral sac of the rumen was examined and photographed. Papillae were subjectively assessed for size and shape and the complete, open surface of the sac was characterised according to the prevalence of lesions and colour. The scoring system is shown in Supplementary Material Table S1.

Histamine measurement in ruminal fluid

Histamine concentration in the rumen fluid was measured using the Abnova Histamine ELISA Kit (Abnova, Catalogue Number KA1888), designed for analysis of histamine in human faeces. Duplicate measurements were used for all standards, controls and samples. Before use on research samples, several dilutions were tested to optimise the assay for ruminal fluid. A dilution of 1 : 300 provided the most consistent results and was subsequently used on all samples. The absorbance of the solution in the wells was read using a microplate reader set to 450 nm. Standard curves were plotted for ruminal and plasma samples, and unknown concentrations were calculated from the regression equation. Each sample was tested in duplicate, and the average within-sample CV was 20.56%.

Lipopolysaccharides in ruminal and caecal fluid

For measurement of LPS, a modified, scaled-down method of Li *et al.* (2012) was created. This was carried out immediately upon return from the abattoir, in order to minimise lysis of the gram-negative bacteria and further release of LPS into the extracellular fluid. Briefly, 1 ml of strained rumen fluid was thoroughly mixed with 1 ml PBS, which was then centrifuged at 16 000 × **g** for 45 min at 4°C. A syringe and needle were then used to immediately take up the supernatant, with minimal disruption to the pellet. The needle was then replaced with a sterile 0.2 μm disposable filter (Elkay Laboratory Products (UK) Ltd), with the resultant filtrate collected in a pyrogen-free glass tube (Cat. # N207, Lonza Group Ltd). The sample was then heated at 100°C for 30 min, allowed to cool to room temperature and then stored at −20°C. LPS was measured in the processed rumen and caecum fluid by the *Limulus* Amebocyte Lysate Assay (Kinetic-QCL, Lonza Group Ltd) according to the manufacturer's instructions. The kinetic assay was run in a 96-well microplate on an incubating spectrophotometer at an absorbance of 405 nm (SpectraMax 190 Microplate Reader with SoftMax Pro 6 Microplate Data Acquisition and Analysis Software; Molecular Devices, Sunnyvale, CA, USA). Appropriate dilutions of the samples were elucidated by following the method of Gozho *et al.* (2005). Briefly, dilutions ranged from around 1 : 160 000 up to 1 : 3 000 000, with the final dilution made up with 1 : 1 β-Glucan Blocker (Cat. # N190, Lonza Group Ltd). An inhibitory test, involving spiking samples as a positive control, was also conducted to ensure the dilutions were adequate. Results were expressed as endotoxin units per ml (EU/ml). Coefficients of variation (%) were obtained for both the standard curve and the individual samples in each run. The mean (± SD) within-sample between-assay CV was 2.31% ± 4.31%.

Short-chain fatty acids in ruminal fluid

Short-chain fatty acids were measured on centrifuged rumen fluid using gas chromatography (Richardson *et al.*, 1989). Samples were shipped to the laboratory on dry ice and analysed for acetate, propionate, butyrate, isobutyrate, valerate, isovalerate, succinate, formate and lactate. Two standards were tested in duplicate for each run. The CVs (%) for each were as follows: acetate, 1.96%; propionate, 0.84%; butyrate, 1.56%; isobutyrate, 1.40%; valerate, 1.13%; isovalerate, 1.62%; succinate, 13.98%; formate, 3.61% and lactate, 1.12%.

Histology

The rumen samples collected at the abattoir and fixed in 10% formalin for 48 h and stored in PBS were cut into 1 to 3 segments, depending on their size, removing any rough edges and ensuring that all layers of the rumen wall were present in each segment. Fixation of the segments continued with a fresh aliquot of 10% formalin. They were then rinsed in PBS and stored in PBS or 70% ethanol, before paraffin-embedding and micro-sectioning on a microtome. Sections of 3 μm thickness were cut, placed onto slides and baked in an oven at 60°C for 1 to 2 h, until excess wax had melted and the tissues adhered to the slides. Haematoxylin and eosin staining and Elastin Martius Scarlet Blue staining were then carried out by hand (this last stain is a Martius Scarlet Blue protocol modified to include a Miller's Elastin stain).

Immunohistochemistry

The IHC staining was performed by Veterinary Diagnostic Services at the University of Glasgow's School of Veterinary Medicine. Five slides were stained per sample for all samples: major histocompatibility complex class 2 (**MHCII**), myeloperoxidase, cluster of differentiation 3 (**CD3**), a negative rabbit and a negative mouse immunoglobulin control. The IHC was carried out at room temperature with Tris buffer pH

7.5 with Tween used for all buffer requirements. Antigen retrieval was carried out for all IHC and corresponding negative controls. The MHCII IHC used heat-induced epitope retrieval citrate buffer pH 6 (Dako #S1700) and mouse monoclonal anti-human MHCII (Dako #M0746) at a dilution of 1 : 20. Myeloperoxidase IHC used Dako Ready-to-Use (**RTU**) proteinase K and rabbit polyclonal anti-human (Dako #A0398) at a dilution of 1 : 1000. Cluster of differentiation 3 IHC used Dako heat-induced epitope retrieval sodium nitrate pH 6 and rabbit polyclonal anti-human CD3 (Dako #A0452) at a dilution of 1 : 100. All staining was carried out in a Dako Autostainer. Secondary antibody from Dako with conjugated horseradish peroxidase was used for detection of primary antibodies. Sections of 2.5 μm thickness were placed on charged slides and baked for 1 h at 56°C. All sections were rehydrated, followed by heat-induced epitope retrieval and sodium citrate buffer pH 6 for 1 min 40 s at 125°C and full pressure. Following that, enzymatic antigen retrieval using Proteinase K RTU was used for the specified time as per manufacturer's instructions. Next the slides were loaded onto the Dako Autostainer and rinsed with buffer for 5 min. Endogenous peroxidase activity was blocked using Dako Real TM Peroxidase blocking solution for 5 min, followed by a 5-min buffer rinse. The primary antibody was used at the recommended dilution, with Dako universal diluent. This was followed by 2 × 5 min buffer rinses. Next the appropriate secondary antibody was used according to the species in which the primary antibody was produced. This was followed again by 2 × 5 min buffer rinses. Detection was carried out using Dako K5007 DAB for two 5-min periods. The slides were then rinsed three times in water. Next the slides were counterstained for 27 s with Gill's haematoxylin before being washed in water. Finally, the slides were blued in Scott's tap water substitute, dehydrated and mounted in synthetic resin ready for scoring.

Examination, image capture, storage and analysis

All slides were initially scanned under low and high power using an Olympus CX41 microscope. Images of typical and atypical examples of all features of interest were captured using GXCam software, calibrated as recommended by the manufacturer, using a ×4, ×10 and ×40 graticule. The stratum corneum thickness was determined by taking the mean of 5 measurements in μm across the stratum corneum over two fields, using 40× magnification and haematoxylin and eosin stain. The same approach was taken for the stratum granulosum thickness. The vascular diameter was taken as the mean diameter of the single largest vessel in each of two papillae using 40× magnification and haematoxylin and eosin stain. Cluster of differentiation 3+ and MHCII+ cells were estimated as the count of the total number of CD3+ or MHCII+ cells in a single image taken at 40× magnification. All histological measurements and counts in this study were undertaken manually by one operator (HJF).

Gene expression

Ribonucleic acid extraction from rumen tissue was carried out using the miRNeasy® mini kit (Qiagen) and DNase® kit (Qiagen). The concentrations and quality of the RNA were assessed using the A260/280 and A260/230 ratios measured on a NanoDrop ND1000 spectrophotometer (Thermo Fisher, Glasgow, UK). If the RNA concentration was high (1000 μg/μl), RNase-free water was added to the eluant and it was measured again. Ribonucleic acid was stored at −80°C until further use. First strand cDNA was obtained using TaqMan reverse transcription (RT) reagents (Life Technologies #N8080234, Glasgow, UK) on 1 μg of total RNA in 20 μl reaction volume, following the manufacturer's instructions. Following RT reaction, 30 μl of RNase free water was added to each sample to a final volume of 50 μl. All cDNA products were stored at −20°C until further use. TaqMan real-time quantitative PCR was used to quantify gene expression levels from the ruminal cDNA using TaqMan Gene Expression Master Mix assay kit (Life Technologies, Glasgow, UK) and the respective probes as shown in Supplementary Material Table S2. The assay was carried out using a 384-well microplate (Thermo Fisher, Glasgow, UK) with three technical replicates for each sample to reduce measurement error. Each reaction consisted of 1× assay probes, 1× TaqMan® Gene Expression Master Mix (Applied Biosystems, Glasgow, UK) and RNase-free water to a final volume of 5 μl. The assays were carried out in singleplex or duplex, depending on the assay efficiency pre-determined in an optimisation assay prior. A pooled cDNA sample from all cDNA samples was used to generate a series of 3-fold serial dilution used as standard curve in every assay. Two microlitres of each sample was transferred into corresponding wells containing 3 μl of the reaction mix. The plates were sealed with Optical Adhesive Cover (Thermo Fisher, Glasgow, UK) and the thermal cycling was carried out using the 7900HT Fast Real-Time PCR System (Applied Biosystems, Glasgow, UK) with thermal cycling conditions set out in the kit protocol.

Statistical analysis

Data were initially managed in an Excel spreadsheet and then analysed in R (R core Team , 2015). All dependent variables were initially tested by application of the Shapiro–Wilk test of normality and most were found not to be normally distributed. Variables were then natural log-transformed, the Shapiro–Wilk test was re-applied and histograms of the transformed data were examined. In several cases, the Shapiro–Wilk statistic indicated that the data were not a good fit to a normal distribution, but in almost all cases (with the exception of *IL-2* gene expression), visual appraisal suggested a distribution that would be amenable to parametric analysis. Nonetheless, when the effects of farm and risk were tested, both parametric and non-parametric analyses were applied and the results were consistent. The effects of *a priori* risk classification and the farm of origin were tested using one-way ANOVA, after natural log-transformation in most cases. No correction factor for multiple tests was applied because the purpose of the analysis was primarily descriptive and it is not clear how families of variables should be defined. Calculated estimates of *P*-values are presented to enable readers to draw

their own conclusions regarding statistical significance with multiple comparisons. To assist in this interpretation, suggested threshold *P*-values derived using the Bonferroni method ($\alpha' = \alpha/m$, where α' is corrected *P*-value, α is the original *P*-value and *m* is the number of comparisons) have been applied. Input variables other than farm of origin and risk classification were completely or partially confounded with farm of origin. The relationships between selected dependent variables and six continuous independent variables (proportion of TMR composed of barley, straw, silage, fibre, CP and fine particles (<1.18 mm)) were individually examined using linear regression on natural log-transformed (if not normally distributed) or untransformed variables (if normally distributed). The potential predictor variables were selected because they are considered likely to be important drivers of acidosis in ruminants and because our dataset was almost complete for each. For each dependent variable, the predictor for which the model provided the highest *F*-statistic and adjusted *R*-squared value was chosen for presentation. Multiple regression or more complex mixed models, although necessary to identify interactions among potential predictive variables, were not possible because of singularity (each farm had fixed combinations of variable values). The subjective *postmortem* categories describing the appearance of the rumen wall were tabulated by farm and by risk classification and associations were tested using Fisher's exact chi-squared test. Pearson bivariate product moment correlations were estimated for selected variables.

Results

Farm and dietary assessment

The initial questionnaire-led assessment of 10 beef farms in the Grampian region of Scotland led to the identification of 6 farms that were quite similar in feeding and management practices but which might be identified as high risk (BH1, BH6 and BH7) or low risk (BL2, BL3 and BL7) according to the percentage of the diet that was provided by cereal grains, straw and silage. The main ingredient of all the diets on the six farms was barley, which was grown and processed on each of the farms. The diets were diverse in other respects. Sugar beet pulp, soya hulls and a buffer against acidosis were only used on one farm each and yeast was used on all farms. Rumitech is an essential oils (Harbro Ltd, Turriff, UK), included to modulate ruminal microbiome, depress methane production and increase feed conversion efficiency Santos *et al.*, 2010), and was included only on two of the high concentrate, high-risk farms. Full dietary records were not retrieved from two farms: BL7 and BH1. From BL7, we were unable to independently verify the statements provided by the farmer regarding the ration that was fed to the cattle, but we were able to measure the particle size of the TMR and obtain a measure of CP and crude fibre in the TMR. In this case, the unverified data were not used in statistical analyses but are presented in Table 1. Supplementary Material Figure S1 illustrates the diversity of the combinations of dietary components in each of the farm's rations.

Gross appearance and pathology of rumen

Supplementary Material Table S3 summarises the results of normality testing of all the dependent variables. There was relatively little variation in the gross appearance of the ruminal wall, as shown in Supplementary Material Tables S4 to S6. There was a weak association of the colour of the ruminal papillae and the *a priori* risk classification ($\chi^2 = 13.441$; df = 5; $P = 0.020$) such that animals from the low-risk farms were more likely to have black or brown papillae and those from the high-risk farms were more likely to have grey or pink-grey papillae. Only six of the animals from high-risk farms and eight from low-risk farms had any detectable abnormality on the ruminal wall. Eight of the 14 animals with detectable abnormalities showed small areas bare of papillae. Only one animal showed any signs of active inflammation, excoriation or scarring of the ruminal wall (Figure 1).

Farm of origin and a priori *risk classification*

The categorical factors that were assessed statistically were the farm of origin and the initial *a priori* risk classification. Table 2 lists the median and interquartile ranges for each of the dependent variables for each of the low-risk and high-risk farms, together with the Kruskal–Wallis *P*-value for the effects of farm and *a priori* risk classification. The table shows a very high degree of among-farm variation. Breed, sex, age and weight at slaughter were all confounded with farm of origin (see Table 1, Supplementary Material Figure S1). Rumen fluid histamine concentration did not differ significantly between risk categories ($P = 0.23$) (Figure 1). Ruminal LPS concentration was higher in the cattle on the high-risk farms ($P = 0.0050$, Figure 1). Caecal LPS concentrations were higher (up to 27-fold) than corresponding ruminal LPS concentrations, although caecal LPS concentration was lower in the high-risk group ($P = 0.0037$, Figure 1). Ruminal fluid lactate concentration was higher in cattle from the high-risk farms ($P = 2.03 \times 10^{-3}$, Figure 2). Caecal lactate concentration was higher on the high-risk farms ($P = 7.76 \times 10^{-5}$). Total SCFA concentration in ruminal fluid was lower on the high-risk farms ($P = 3.21 \times 10^{-7}$, Figure 2), as were each of acetate, propionate and butyrate (1.47×10^{-7}, 1.17×10^{-5}, and 1.09×10^{-6}, respectively, Figure 2). The CD3+ and MHCII+ cell counts in ruminal epithelial sections were lower on high-risk farms ($P = 3.67 \times 10^{-5}$ and 1.38×10^{-5}, respectively, Figure 3). *TLR4*, *IL-1β* and *IFN-γ* relative expression were all lower in rumen tissue from cows on high-risk farms ($P = 0.00014$, 0.0029 and 2.44×10^{-7}, Figure 3). The stratum corneum and stratum granulosum were thicker on the animals from the high-risk farms ($P = 1.80 \times 10^{-7}$ and 0.0035, respectively, Figure 4). Supplementary Material Figures S2 to S5 show box and whisker plots of ruminal and caecal SCFA by

Table 1 *Assessment of diets on six beef farms. Composition, chemical analysis and particle size distribution (%)*

	Farm					
	Low risk			High risk		
	BL2	BL3	BL7	BH1	BH6	BH7
Sex						
Male	11	1	10	0	18	20
Female	9	19	9	20	2	0
Age at slaughter (mean ± SD days)	646 ± 149	689 ± 50	671 ± 65	703 ± 19	702 ± 68	788 ± 85
Cold weight at slaughter (mean ± SD kg)	297 ± 27	383 ± 34	356 ± 24	384 ± 30	400 ± 10	403 ± 15
Breed						
Continental	16	19	12	20	20	18
British	4	1	7	0	0	2
Ingredients (%DM)						
Barley	48.8	43.5	50*	57.3	67.9	70
Straw	18.3	6.5	20*	10.7	–	–
Grass silage	24.4	50	25*	–	–	–
Pot ale syrup	8.5	–	5*	31.5	10.7	15
Dark grains	–	–	–	–	10.7	5
Sugar beet pulp	–	–	–	–	10.7	0
Soya hulls	–	–	–	–	–	10
Additives						
Minerals	Yes	Yes	Yes	Yes	Yes	Yes
Rumitech	No	No	No	No	Yes	Yes
Yeast	Yes	Yes	Yes	No	Yes	Yes
Buffer	No	Yes	No	No	No	No
TMR composition (%DM)						
CP	12.54	11.98	13.28	11.06	9.94	12.50
Crude fibre	8.23	5.43	11.48	13.23	5.35	4.84
Starch	24.73	33.26	na	37.13	36.93	39.63
NDF	25.85	21.40	na	13.81	16.75	19.51
Milled barley composition (%DM)						
CP	10.24	10.41	na	11.92	11.06	10.51
Crude fibre	5.51	3.63	na	4.83	5.15	3.50
Starch	56.86	57.44	na	56.26	55.62	46.98
NDF	25.95	15.62	na	19.56	21.83	14.13
TMR particle size (mm)						
19.0	75.6	30.7	55.1	23.6	0.1	0.0
19 × 8	7.1	4.2	12.6	7.5	8.2	3.0
8 × 1.18	16.5	46.9	31.6	65.1	69.5	74.2
<1.18	0.4	17.3	0.2	3.5	21.3	22.5
Milled barley particle size (mm)						
19.0	0.3	0	na	na	1.3	2.2
19 × 8	1.8	0	na	na	37.7	42.3
8 × 1.2	95.7	92.1	na	na	58.0	60.6
<1.18	2.7	7.6	na	na	7.6	1.5

BL2, BL3, BL7 = denominations of beef farms considered *a priori* to be at low risk of acidosis-related problems; BH1, BH6, BH7 = denominations of beef farms considered to be at high risk of acidosis-related problems; TMR = total mixed ration; na = not available.
*Ingredients values for farm BL7 were not verified by observation of ration sheets and are considered to be estimates only. They were not used in the statistical analysis but are shown here for consideration as they constituted a component of the *a priori* classification of RISK.

farm and by a priori risk classification. Supplementary Material Figure S6 shows gene expression, and Supplementary Material Figure S7 shows histological observations.

Dietary components

Supplementary Material Figure S1 shows the values for the predictive (dietary input) variables used in linear regression for each of the farms. It clearly shows the potential for complex interactions among the predictive variables at the farm level. Although most variables are not completely confounded, there is substantial overlap and the lack of replication of combinations of high, medium and low values for each of the variables means the study design does not allow for the examination of multiple predictors in a single model or the examination of any dietary interactions. Another problem with modelling the effects of potentially important predictor

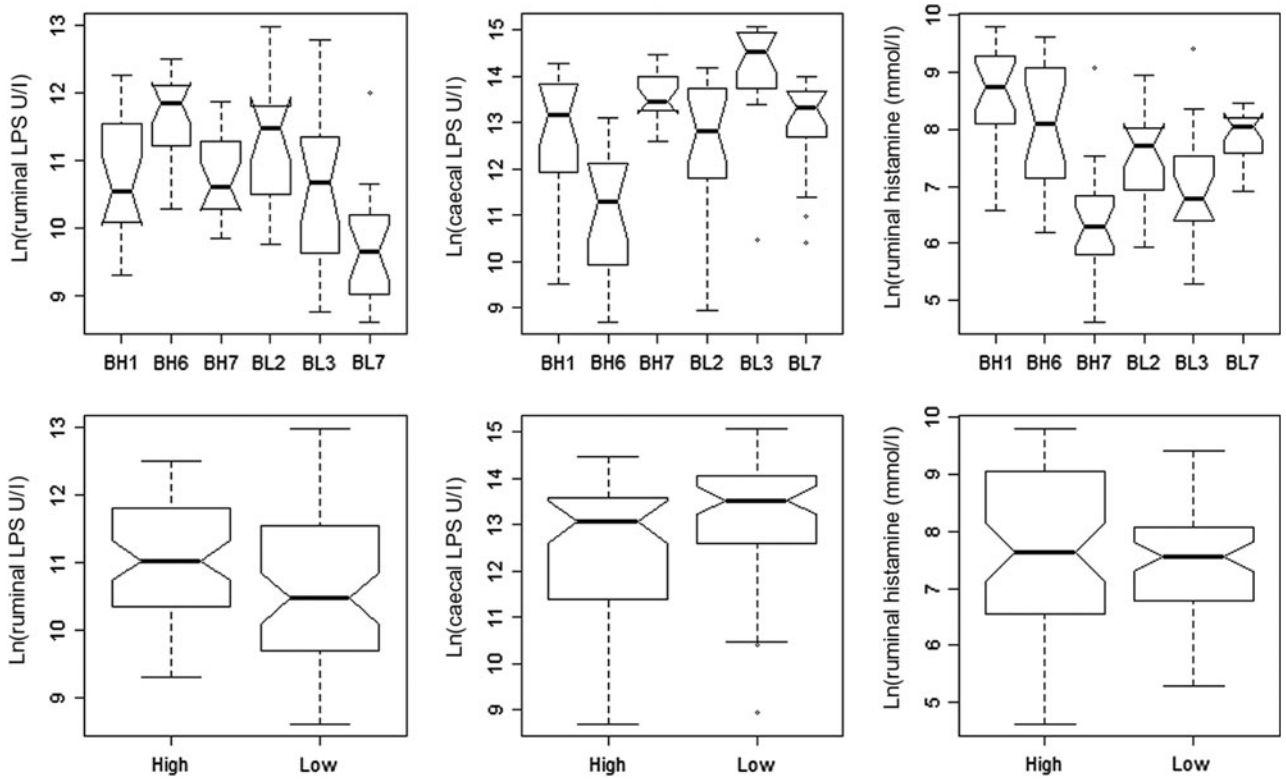

Figure 1 Boxplots showing concentrations of LPS and histamine in ruminal fluid and LPS in caecal fluid at slaughter of the cattle on six beef finishing units (above) and by risk category of farm of origin (below). Farms labelled as BH1, BH6 and BH7 were classed *a priori* as high risk and those labelled BL2, BL3 and BL7 were low risk. The central band in the box represents the median, and the box spans the second and third quartile, while the whiskers extend to 1.5 times the interquartile range above the third and below the second quartile. Stars mark data points that are located outside 1.5 times the interquartile range. LPS = lipopolysaccharide.

variables in this study arises from the non-uniform spacing among a relatively small number of levels. Hence, the linear regressions presented in this report must be treated with caution. Linear regression analysis (summarised in Table 3) suggests that at least one of the six factors (CP, crude fibre, barley, straw or silage as percentage of DM in diet, percentage of particles in TMR < 1.18 mm) was a significant predictor for 14 of 18 selected dependent variables at the Bonferroni-adjusted *P*-value threshold of 0.0005 and for all variables at the unadjusted *P*-value threshold of 0.05. This was expected because each of the predictors was aliased with farm of origin, by which all of the 18 variables differed. The CP content in the TMR explained the most variation for 13 of 18 variables, the proportions of fines and silage in the diet each explained two variables and proportion of fibre explained one. Figure 5 shows variation in six animal-level variables when herds were grouped in rank order of the strongest putative herd-level explanatory variable. Similar information is presented for the remaining 20 variables in Supplementary Material Figures S8 to S11.

Correlations among selected measures of ruminal fluid, caecal fluid and ruminal wall (gene expression and histological)

Supplementary Material Table S7 shows a correlation matrix of selected dependent variables. The expression of *TLR4* was weakly and positively associated with ruminal LPS concentration ($r = 0.16$, $P = 0.084$), not correlated with ruminal histamine concentration ($r = 0.026$, $P = 0.78$), but was moderately correlated with ruminal lactate concentration ($r = 0.37$, $P = 3.009 \times 10^{-5}$). The levels of expression of the genes in the epithelium correlated variably with each other, ranging from $r = -0.04$ for *NHE3* with *CCL11* up to $r = 0.57$ for *CCL11* with *IFN-γ*. They were not strongly correlated with the thickness of the stratum granulosum, nor with the density of CD3+ nor MHCII+ cells, nor lactate concentration in ruminal or caecal fluid (data not shown). Cold weight of carcase after slaughter was most closely associated with the ruminal fluid lactate concentration ($r = 0.50$, $P = 5.37 \times 10^{-7}$).

Discussion

All but 4 of the 31 dependent variables that we measured differed significantly ($P < 0.0008$) according to the farm of origin. Every farm was unique in its combination of input variables, which resulted in a level of confounding and lack of replication that made it impossible to test the effects of many of them. Potential confounding factors at the level of the farm were the inclusion of specific nutritional components, mixing and feeding practices, stocking density, faecal or bedding management, the source of cattle, variation in their genotype and enteric microbiome, health and nutritional management

Table 2 *List of the main continuous dependent variables investigated in the study on beef cattle with the median, first and third quartiles,* P*-values derived from ANOVA (on transformed data where non-normal) for the effects of farm, with median values for each risk classification. Estimated* P*-values are presented, derived from one-way ANOVA for each of the two factors (FARM and RISK)**

Variable	Normal	Q1	Median	Q3	FARM *P*-value	High risk median	Low risk median	RISK *P*-value
Rumen fluid								
Histamine (mmol/l)	No	801.9	1913	4281	9.81×10^{-12}	2098	1912	0.23
LPS (EU/ml)	No	25 676	43 420	113 003	1.98×10^{-9}	62 038	35 416	0.0050
Acetate (mmol/l)	No	40.34	55.27	73.17	$<2.00 \times 10^{-16}$	44.00	67.23	4.94×10^{-8}
Propionate (mmol/l)	No	13.82	18.96	25.86	1.13×10^{-13}	15.40	22.40	2.74×10^{-6}
Isobutyrate (mmol/l)	No	0.7	0.87	1.09	1.68×10^{-5}	0.91	0.84	0.26
Butyrate (mmol/l)	No	4.83	7.75	12.5	$<2.00 \times 10^{-16}$	5.47	9.67	1.33×10^{-6}
Isovalerate (mmol/l)	No	0.49	0.63	0.81	3.72×10^{-6}	0.67	0.57	0.0137
Valerate (mmol/l)	No	0.81	1.3	1.97	1.41×10^{-12}	0.95	1.57	0.00073
Lactate (mmol/l)	No	0.79	1.89	3.03	9.76×10^{-13}	2.51	0.80	2.03×10^{-10}
Acetate : Propionate	Yes	2.56	3.02	3.79	6.29×10^{-8}	3.00	3.08	0.453
Total SCFA (mmol/l)	No	67.44	91.73	118.7	$<2.00 \times 10^{-16}$	71.50	110.17	3.5×10^{-8}
Caecal fluid								
LPS (EU/ml)	No	170 267	606 309	1 090 939	6.32×10^{-11}	470 383	747 655	0.0044
Acetate (mmol/l)	Yes	41.18	49.26	55.87	0.0104	47.76	51.14	0.307
Propionate (mmol/l)	Yes	11.43	13.23	15.45	2.54×10^{-5}	13.53	13.04	0.063
Isobutyrate (mmol/l)	Yes	0.58	0.76	0.92	1.5×10^{-7}	0.79	0.72	0.0208
Butyrate (mmol/l)	No	2.99	3.62	4.75	0.21	3.38	3.71	0.643
Isovalerate (mmol/l)	No	0.45	0.60	0.77	6.77×10^{-9}	0.67	0.56	0.00518
Lactate (mmol/l)	No	0.60	1.05	1.63	5.7×10^{-8}	1.21	0.67	9.56×10^{-5}
Acetate : Propionate	No	3.32	3.69	4.03	7.08×10^{-9}	3.36	3.90	5.28×10^{-6}
Total SCFA (mmol/l)	Yes	60.86	70.48	81.5	0.00821	71.00	69.56	0.844
Gene expression								
TLR4 relative Exp.	No	0.036	0.056	0.078	5.59×10^{-5}	0.064	0.044	0.000126
IL1B relative Exp.	No	0.00079	0.0019	0.0041	0.000441	0.0014	0.0027	0.00209
CCL11 relative Exp.	No	0.019	0.042	0.083	0.0224	0.042	0.042	0.46
NHE3 relative Exp.	No	0.24	0.32	0.47	0.000263	0.32	0.31	0.764
IL2 relative Exp.	No	0.00015	0.00029	0.00059	0.000555	0.00028	0.00031	0.294
IFNG relative Exp.	No	0.00054	0.00089	0.0015	1.37×10^{-6}	0.00058	0.0013	5.52×10^{-7}
Histology and IHC								
Stratum corneum (μm)	No	5.84	6.89	10.87	$<2.00 \times 10^{-16}$	8.95	6.15	2.58×10^{-8}
Stratum granulosum (μm)	No	30.91	36.67	44.95	$<2.00 \times 10^{-16}$	40.34	36.18	0.000122
Vascular diameter (μm)	No	16.91	21.48	28.09	1.72×10^{-15}	23.03	20.59	0.0257
CD3+ cells/hpf	No	28.00	40.50	55	1.37×10^{-10}	32.00	47.00	1.34×10^{-5}
MHCII+ Cells/hpf	No	34.00	44.00	60	6.86×10^{-9}	39.00	56.5	1.38×10^{-5}

Q1 = first quartile; Q2 = second quartile; LPS = lipopolysaccharide; SCFA = short-chain fatty acids; CD3+ = cluster of differentiation 3; Exp. = expression; IHC = immunohistochemistry; hpf = high-powered field; MHCII = major histocompatibility complex class 2.
*A conservative approach to interpretation of the *P*-values using the Bonferroni correction would suggest that a *P*-value of 0.0008 would be an appropriate level to ensure that the null hypothesis was rejected at the same level as $P = 0.05$ for a single test.

before arrival at the farm of origin. Another important potential confounder is the length of the period from last feeding until slaughter, which was not able to be controlled in this study due to the limitations on commercial abattoir operations. This variable would be expected to result in variation in the concentrations of metabolites in the reticulorumen away from the normal diurnal pattern seen in animals during periods of *ad libitum* feed availability. This variation would be expected to be greater in animals that had the most extreme adaptations to high levels of feed.

By definition, animals on the high-risk farms were fed diets with higher proportions of barley and lower proportions of straw than animals on the low-risk farms, each of which included grass silage as a component of the diet. None of the high-risk farms included grass silage in the rations. The *a priori* risk classification can therefore be seen as a qualitative indicator of the proportion of forage in the diet (see Table 1), and was a significant factor ($P < 0.0008$) for 14 of 31 continuous dependent variables (Table 2). The animals on high-risk farms had significantly ($P < 0.0008$) higher concentrations of lactate in the rumen, as well as thicker epithelial strata cornea and strata granulosa, with higher levels of expression of *TLR4*. This was accompanied by lower ruminal SCFA concentrations. The expression of *IFN-γ* and the counts of MHCII+ and CD3+ cells in the epithelium were significantly lower in samples from cattle on the high-risk farms ($P < 0.0008$).

All the rations fed in this study had concentrate to forage ratios of at least 40% and the samples were taken after 90 to 100 days of concentrate feeding. Most of the studies that have reported on the response of SCFA, LPS, histamine and other markers of rumen function have focused on the acute period immediately after a challenge or at most, after a period of a couple of weeks of adaptation. The variables in our study are end-point observations after a prolonged period of adaptation, so it should not be expected that the relationships between input and dependent variables should be the same as those reported in acute response studies.

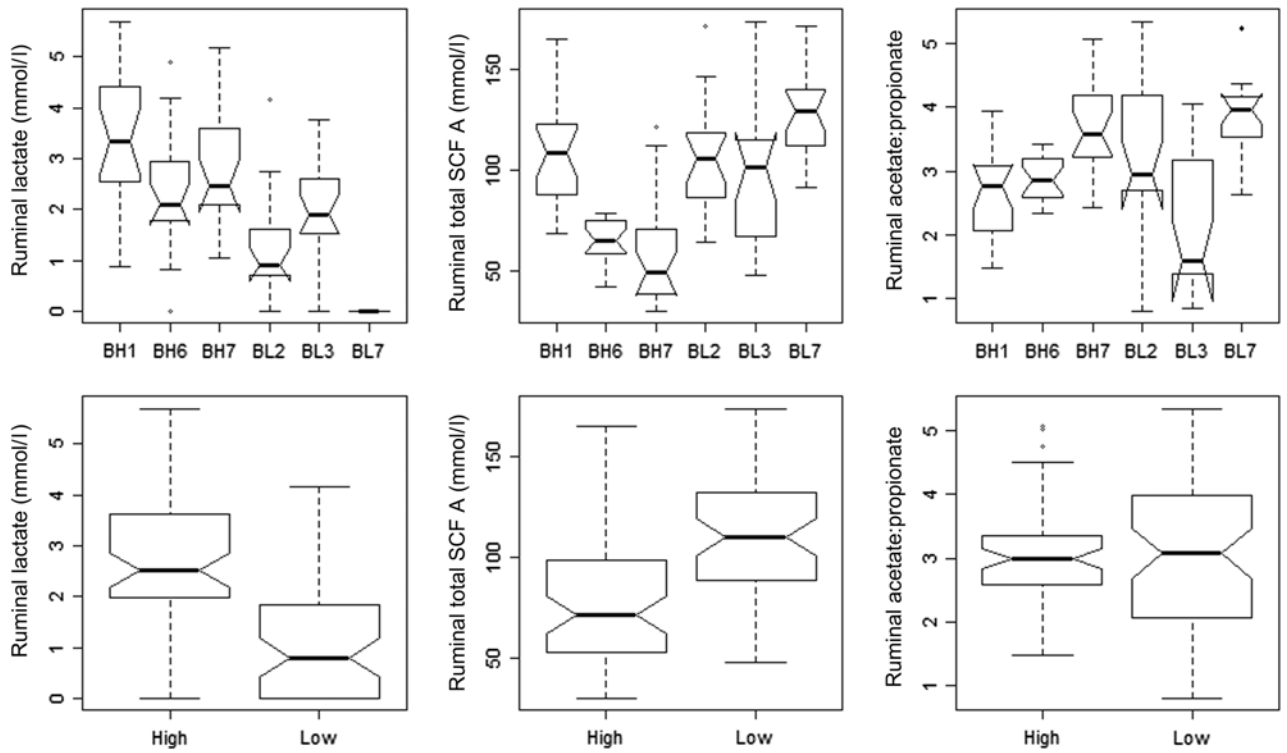

Figure 2 Box and whisker plots showing lactate and total SCFA concentration, and acetate to propionate ratio in ruminal fluid of the cattle on six beef finishing units (above), and by risk category of farm of origin (below). Farms labelled as BH1, BH6 and BH7 were classed *a priori* as high risk and those labelled BL2, BL3 and BL7 were low risk. The central band in the box represents the median, and the box spans the second and third quartile, while the whiskers extend to 1.5 times the interquartile range above the third and below the second quartile. Stars mark data points that are located outside 1.5 times the interquartile range. SCFA = short-chain fatty acid.

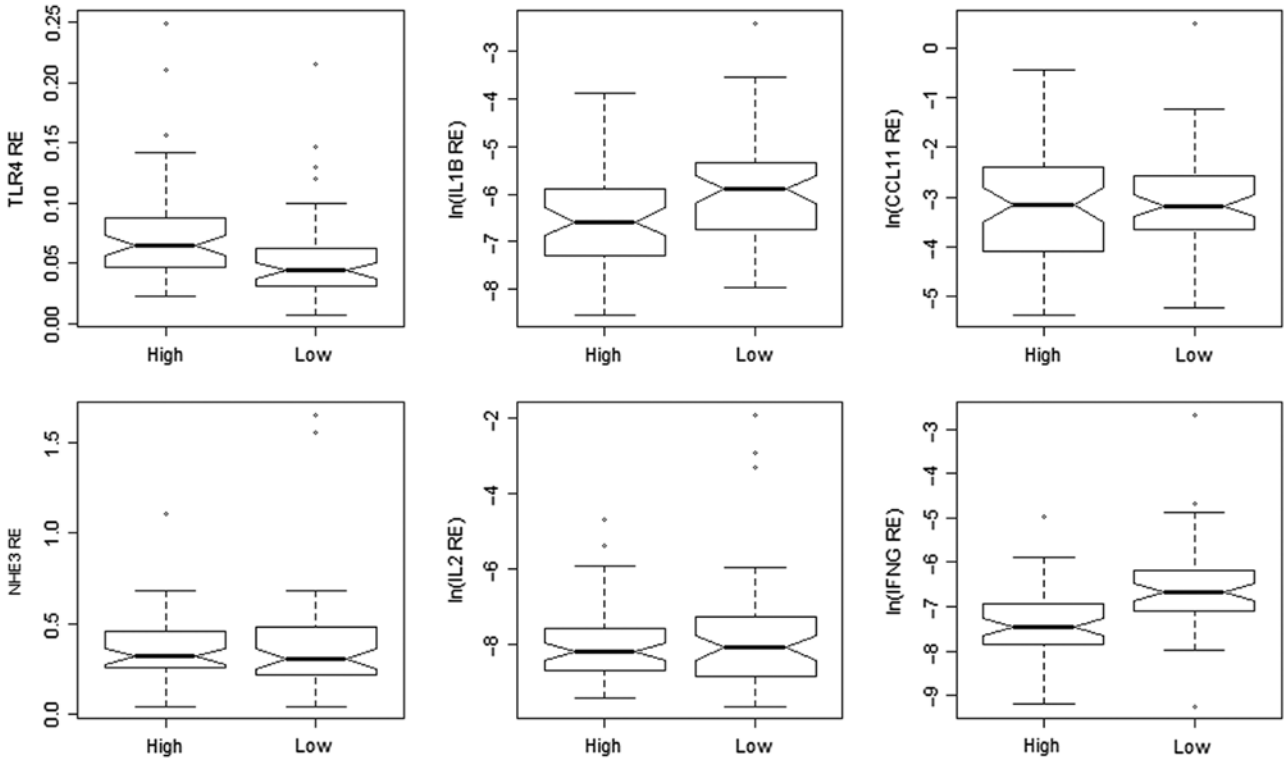

Figure 3 Box and whisker plots showing the relative gene expression of *TLR4*, *IL1B*, *CCL11*, *NHE3*, *IL2* and *IFNG* in the ruminal wall of cattle from high- and low-risk farms. Farms labelled as BH1, BH6 and BH7 were classed *a priori* as high risk and those labelled BL2, BL3 and BL7 were low risk. Note that *IL1B*, *CCL11*, *IL2* and *IFNG* relative expression values have been natural log-transformed for ease of visualisation. The central band in the box represents the median, and the box spans the second and third quartile, while the whiskers extend to 1.5 times the interquartile range above the third and below the second quartile. Stars mark data points that are located outside 1.5 times the interquartile range.

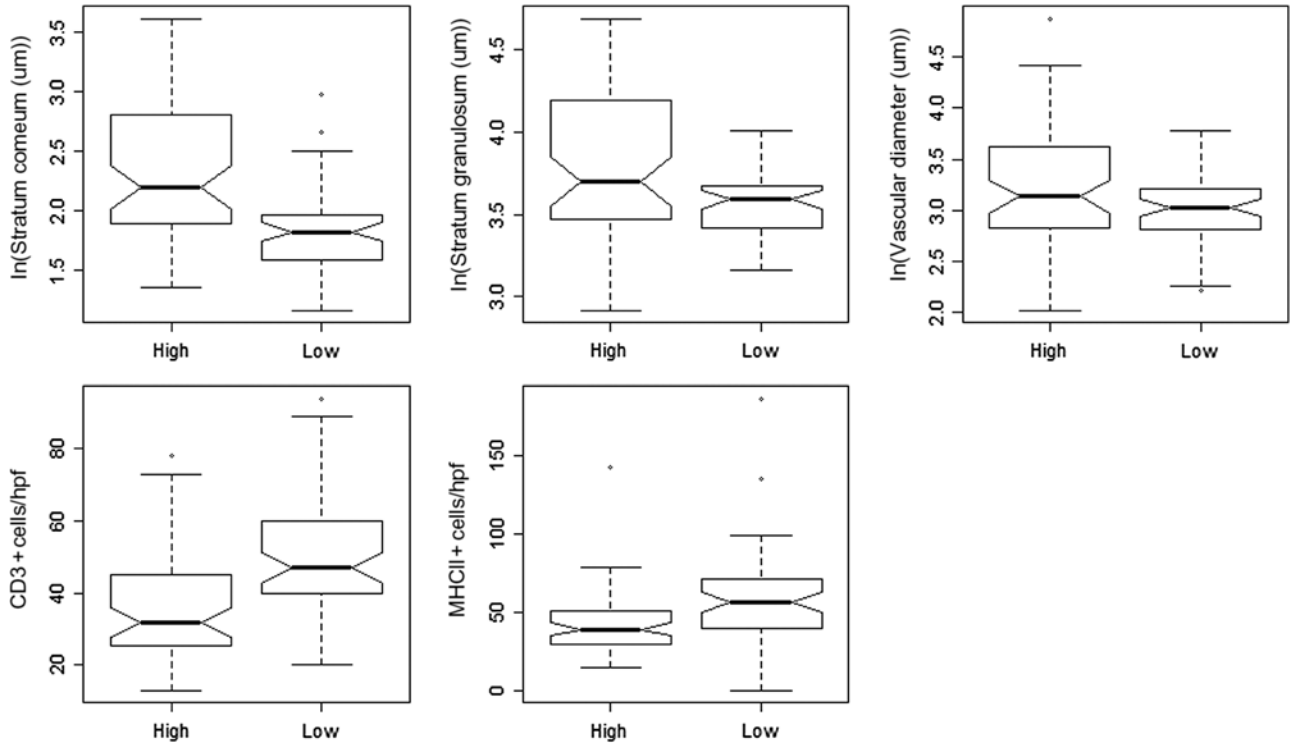

Figure 4 Box and whisker plots showing the histological observations on the ruminal wall of cattle from high- and low-risk farms. The central band in the box represents the median, and the box spans the second and third quartile, while the whiskers extend to 1.5 times the interquartile range above the third and below the second quartile. Stars mark data points that are located outside 1.5 times the interquartile range. Counts of MHCII+ and CD3+ cells are presented as cells per hpf. hpf = high-power field.

Table 3 *Summary of linear regression analysis of selected dependent variables from beef cattle against six selected dietary components (proportion of DM in the TMR as straw, silage, barley, CP or fine particles (<1.18 mm)). In each case, the best model was selected according to adjusted* R^2 *value and F-statistic*. Supplementary Material Table S8 shows the P-values and adjusted* R^2 *for the non-selected models*

Variable	Best predictor	Effect estimate	F-stat	df	Adjusted R^2 (%)	*P*-value*
Ln(RF histamine)	Fibre	0.17	32.02	117	20.81	1.11×10^{-7}
Ln(RF LPS)	Protein	−0.41	27.05	117	18.08	8.54×10^{-7}
Ln(CF LPS)	Silage	0.032	18.7	94	15.71	3.80×10^{-5}
Ln(RF lactate)	Protein	−0.66	47.31	117	28.19	3.17×10^{-10}
Ln(CF lactate)	Silage	−0.027	32.03	93	24.82	1.67×10^{-7}
RF total SCFA	TMR fine	−2.28	76.07	117	38.88	2.18×10^{-14}
CF total SCFA	Protein	3.66	6.67	111	4.83	0.011
Ln(*TLR4* RE)	Protein	−0.22	24.33	117	16.51	7.71×10^{-6}
Ln(*IL1B* RE)	TMR fine	−0.039	15.65	116	11.13	0.00013
Ln(*CCL11* RE)	Protein	0.16	3.612	117	2.17	0.060
Ln(*NHE3* RE)	Protein	−0.16	12.55	117	8.92	0.00057
Ln(*IL2* RE)	Protein	0.34	11.23	116	8.04	0.0011
Ln(*IFNG* RE)	Protein	0.39	26.46	116	17.87	1.11×10^{-6}
Ln(SC thickness)	Protein	−0.29	70.44	110	38.48	1.81×10^{-13}
Ln(SG thickness)	Protein	−0.20	57.35	110	33.67	1.21×10^{-11}
Ln(vascular diameter)	Protein	−0.21	28.42	110	19.81	5.26×10^{-7}
Ln(CD3+ cells/hpf)	Protein	0.24	60.53	110	34.91	4.23×10^{-12}
Ln(MHCII+ cells/hpf)	Protein	0.23	19.09	111	13.92	2.82×10^{-5}

RF = rumen fluid; LPS = lipopolysaccharide; CF = caecal fluid; SCFA = short-chain fatty acid; RE = relative expression; SC = stratum corneum; SG = stratum granulosum; Ln = natural logarithm; hpf = high power field; MHCII = major histocompatibility complex class 2.
*A very conservative approach to interpretation of the *P*-values using the Bonferroni correction would suggest that a *P*-value of 0.0005 would be an appropriate level to ensure that the null hypothesis was rejected at the same level as $P = 0.05$ for a single test.

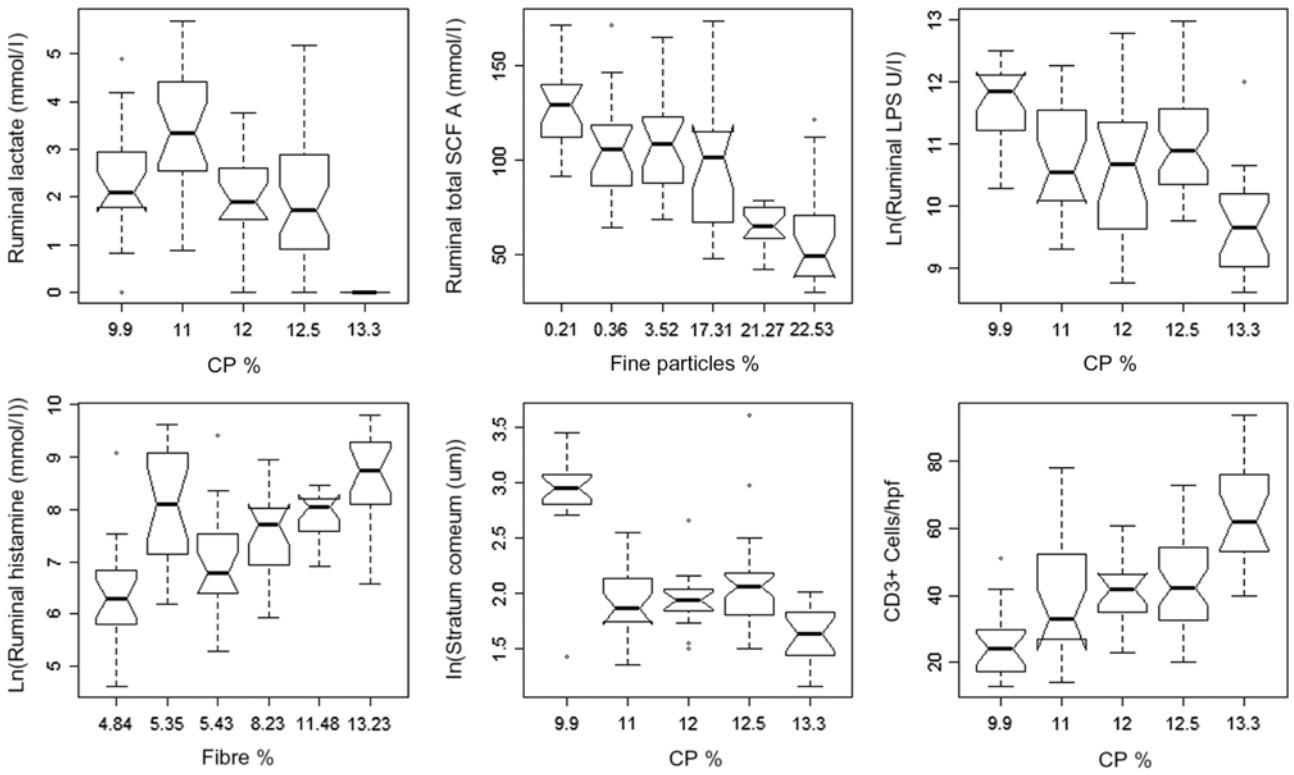

Figure 5 Box and whisker plots showing variation in six animal-level variables of cattle, when herds are grouped in rank order of the strongest putative herd-level explanatory variable. Note that two herds had the same CP percentage in the diet (farms BL2 and BH7 – both 12.5%). Farms labelled as BH1, BH6 and BH7 were classed *a priori* as high risk and those labelled BL2, BL3 and BL7 were low risk. The central band in the box represents the median, and the box spans the second and third quartile, while the whiskers extend to 1.5 times the interquartile range above the third and below the second quartile. Stars mark data points that are located outside 1.5 times the interquartile range. SCFA = short-chain fatty acids; LPS = lipopolysaccharides; hpf = count of cells per high-power field.

Additionally, during the period between each animal's last feed and its slaughter, it would be expected that absorption of metabolites and clearance from the reticulorumen would continue. The lactate concentrations in ruminal samples from high-risk farms were an order of magnitude lower than those that are expected with acute lactic acidosis, but were higher than those found on low-risk farms. The highest ruminal lactate concentration found here was 6 mmol/l, whereas concentrations over 100 mmol/l can occur with acute acidosis (Harmon *et al.*, 1985). This low concentration would have relatively little influence on ruminal pH, but it could indicate an imbalance between lactate-producing ruminal microbes and those that utilise lactate (Slyter, 1976; Russell and Hino, 1985; Nocek, 1997). There was a moderate correlation between carcase weight and the lactate concentration, suggesting that the animals with the higher lactate concentrations grew more rapidly. The highest ruminal LPS concentrations were similar to those observed in experiments in which SARA was induced experimentally (reviewed by Plaizier *et al.*, 2012), thus suggesting that the experimental induction of SARA leads to similar soluble LPS concentrations as occurs in animals under farm conditions.

The concentrations of metabolites or other compounds in the reticuloruminal fluid at a single time point reflect the net effects of production and clearance processes, so are intrinsically difficult to interpret. This is further complicated by variation in the interval during lairage between last feeding and sampling. The previously reported acute effects of increased proportions of barley in rations are to increase SCFA concentrations in reticuloruminal fluid or to increase the rate of appearance in the portal circulation (Loncke *et al.*, 2009; Aschenbach *et al.*, 2011), and acute studies suggest that the stratum corneum and stratum granulosum should become thinner rather than thicker when exposed to high levels of rapidly fermentable carbohydrates (Steele *et al.*, 2011). However, the duration of the period that the animals were fed on the rations in our study provided more than sufficient time for the adaptations described by Schwaiger *et al.* (2013) to take effect. Increased flux from the lumen, due to upregulation of protein-dependent mechanisms, proliferation of metabolically active cells in the stratum granulosum, together with the increased surface area from papillary development would explain the inverse relationship between rapidly fermentable carbohydrates with reticuloruminal SCFA concentrations in the present study. Given that in our study access to feed would have been restricted during the time that cattle were on lairage (i.e. after arrival at the abattoir) for 12 to 24 h before slaughter, it would be expected that the animals with the greatest reticuloruminal SCFA flux should have the most rapid reduction during lairage, possibly resulting in the lowest concentrations in the rumen immediately post-slaughter. Given that the absorption of lactate by the reticuloruminal mucosa is generally slow (Aschenbach *et al.*, 2011) and not driven to the same extent by active,

protein-dependant processes, it is not surprising that the direction of change of lactate concentrations in response to barley or proportion of fines was different from the other SCFA, such that higher concentrations of *postmortem* reflect a high level of production. The issue of the amount of time that animals were on lairage deserves further comment, as it was not a factor that we were able to control for. Given the standard procedures at the cooperating abattoirs, all animals were killed within 24 h of unloading into lairage and it is likely that all would have been killed between 12 and 18 h after unloading. Hence, the variables measured in the ruminal and caecal fluids could have been affected by a variation in fasting time of about 6 h. Whereas this might explain some of the among-farm variation in SCFA concentrations, LPS and histamine, it is not likely to have affected histological measurements. The extent to which it might have affected relative gene expression is unclear.

Univariate linear regression analysis suggested that of the 6 main dietary factors that we considered as potential predictors for 18 dependent variables, CP percentage was the strongest predictor for 13. As CP percentage in the ration increased, ruminal lactate, LPS, histamine, *NHE3* relative expression, *TLR4* relative expression and the thickness of stratum corneum and stratum granulosum decreased, while the count of MHCII+ cells and CD3+ cells increased. Almost all of the effects were in the opposite direction to those of the proportion of fine particles and proportion of barley in the ration, which tended to have similar effects on the dependent variables. In contrast to the potential role of starch and sugar supplementation, there is relatively little in the literature regarding dietary protein and the risk of acidosis. Golder *et al.* (2014) showed that a diet with excess metabolisable protein, when fed to dairy cattle, reduced the tendency to reticuloruminal acidosis. Pilachai *et al.* (2012) showed that high levels of rumen-degradable protein and rumen-undegradable protein in the diet had contrasting effects on SCFA production and the generation of histamine in the rumen, and that high levels of rumen-degradable protein resulted in a more rapid and greater increase in SCFA and histamine production. We did not differentiate between rumen-degradable protein and rumen-undegradable protein in our study, so it is not possible to infer much about the mechanisms by which the apparent effects of protein are mediated. Golder *et al.* (2012 and 2013) showed that supplementation with histidine (the precursor for histamine) caused elevated histamine concentrations in the rumen but did not elevate LPS. It seems likely that the effects of protein on the variables that we measured were mediated via complex interactions with other dietary components and the reticuloruminal microbial communities.

Much of the literature relating to the effect of particle size has considered concentrate and forage components separately. In our study, we were interested in the particle size in the TMR rather than the milled cereal grain because the particle size of the grain can be further reduced by mixing with the forage component. The effect of larger particle size of forage components relates primarily to increased eating time, saliva production, wetting of feed with saliva and time spent ruminating (e.g. Beauchemin and Yang, 2005). Large particle size in processed cereal grains however is primarily considered to be associated with fewer breaks in the integrity of the pericarp and to reduce the ratio of surface area to volume of starch (Gimeno *et al.*, 2016). De Nardi *et al.* (2014) showed that finely ground maize particles, less than 0.5 mm diameter, resulted in lower reticuloruminal pH in cattle than a diet with particles of 1.0 mm, when fed at a rate of 30% of DM. Gimeno *et al.* (2016) showed that reticuloruminal pH was higher in finishing beef calves when a 60% cereal (maize and barley) diet was fed in rolled form rather than ground, in which the particle size was smaller. We did not consider the chop length of the forage as a separate input variable, but in some feeding systems, long chop length forage can facilitate sorting and lead to acidosis (Nasrollahia *et al.*, 2017).

Over the last decade, questions relating to reticuloruminal physiology have increasingly been addressed by the quantification of gene and protein expression in ruminal tissue, using both *in vivo* and *in vitro* systems. Penner *et al.* (2011) provide a good review of the subject. Chen *et al.* (2012) quantified the expression of the toll-like receptor genes *TLR2* and *TLR4* in ruminal epithelium and suggested that *TLR4* expression should be a good indicator of exposure to high challenge with LPS. Penner *et al.* (2009) examined the expression of 21 different genes that were expected to be involved in fatty acid metabolism in the rumen epithelium. We selected genes primarily as indicators of immune response and inflammation (*IL-2, TLR4, IFN-γ, IL-1β, CCL11*) but also to indicate the level of metabolic activity associated with proton exchange (*NHE3*) and the level of challenge with LPS (*TLR4*). The expression of *TLR4*, which has previously been associated with resistance to the effects of soluble carbohydrate overload (Chen *et al.*, 2012), was weakly and positively associated with ruminal LPS concentration, not related with histamine, but was moderately correlated with ruminal lactate concentration.

A surprising result from this study was the lack of obvious pathology in the rumens that were examined. We used a gross scoring system with 5 categories but only 15 of 119 animals in total scored anything other than a baseline normal score ('A') for the variable 'Pre-Score'. We therefore combined the non-'A' categories for analysis and there was no strong relationship between the pre-score of the ruminal mucosa with any of the factors considered in the study. This should not be taken as definitive evidence that there was no pathology in the rumens that we examined, but that very little was detected using our matrix. The lack of clear-cut histological signs of pathology was consistent with this observation.

As a consequence of the lack of any distinct ruminal pathology, one of the objectives of the study, to characterise the possible pathological features of the reticulorumen in cattle from divergently managed intensive beef finishing farms with high rates of concentrate feeding, was not able to be achieved. Nonetheless, elevated concentrations of LPS and

lactate were seen in the reticuloruminal fluid of cattle on the high-risk farms and both were relatively strongly associated with increasing levels of barley supplementation and proportion of fine particles in the diet. Without any independent marker for performance or health (e.g. daily liveweight gain, feed intake, clear evidence of systemic illness) it was not possible to determine *de novo* thresholds at which LPS or lactate concentrations indicate pathology.

Despite the strong effect of farm on the measured variables, there was wide inter-animal variation. This was expected as previous studies have shown a wide range in reticuloruminal pH and other responses in animals fed on the same diet (Mohammed *et al.*, 2012; Gao and Oba, 2014). Some of this variation is likely due to variation in feeding behaviour, particularly the predisposition to sorting fine, concentrate particles from long fibre or to rapid eating (Gao and Oba, 2014). In addition to intrinsic natural variation among animals, the animals on any one farm would have been sourced from a variety of other farms, each contributing its own nutrition-induced and management-induced variation. Finally, variation in time of sampling in relation to the time of last feeding is expected to have introduced some among-farm and among-animal variation.

Conclusions

There are four main conclusions to be drawn from this study, the first two being of a more general nature. Firstly, despite a reasonable expectation of some degree of equivalence among farms due to their similar location, management and broad nutritional inputs, farm to farm variation in rations and practices was wide and the effects of individual farm factors on animal physiology dominated any other factors that were measured. This observation has implications for the design and interpretation of future studies on the impact of interventions in the field, in which a higher level of replication is required than for conventional, controlled experimental studies. It also implies a need to consider the effects of any nutritional intervention as part of a complex, interactive network of input factors. Secondly, the study provides an insight into the adaptive capacity of cattle. The animals in this study were those that made it to slaughter with the rest of their cohort and over the 3 months or so that they were on feed they had all successfully adapted to a highly challenging diet with little or no evidence of pathology. Future studies on the effects of high levels of soluble carbohydrate supplementation or seeking to describe pathology associated with SARA would be more effective if they were to focus on the animals that failed to meet performance targets. The third message from the study is that protein percentage and the proportion of fines in concentrate-based total-mixed rations are strong and opposing contributors to variation in the traits that have been used as indicators of exposure to high acidosis risk.

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Author contributions

WT, RJW, IA, CM and NNJ instigated the work. EMS, CDH and WT assessed the farms and feeds. CAMcC, RCC, HHCK-T, HF, EMS, TJS, CDH, NNJ and RJW collected and processed samples at the abattoir and the laboratory. NNJ undertook statistical analyses and drafted the manuscript, which all authors reviewed.

Declaration of interest

None of the authors has any potential financial interest arising from the outcomes of the work described in this study.

Ethics statement

Ethical approval was not sought for the work described in this manuscript. No research was conducted on live animals. All materials were obtained *postmortem* from licensed abattoirs in the UK.

Software and data repository resources

The data that were obtained as described in this paper are publicly available from the University of Glasgow data repository https://doi.org/10.5525/gla.researchdata.710

Supplementary material

To view supplementary material for this article, please visit https://doi.org/10.1017/S1751731119002878

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