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1 ***Post mortem* observations on rumen wall histology and gene expression and**
2 **ruminal and caecal content of beef cattle fattened on barley-based rations**

3 N.N. Jonsson [1,2], H.J. Ferguson [1,a], H.H.C. Koh-Tan [1], C.A. McCartney [3], R.C.
4 Cernat [3], E.M. Strachan [1], W. Thomson [2], T.J. Snelling [3], C.D. Harvey [2], I. Andonovic
5 [4], C. Michie [4], R.J. Wallace [3]
6
7 [1] *Institute of Biodiversity, Animal Health and Comparative Medicine, University of*
8 *Glasgow, Glasgow G61 1QH, UK*
9 [2] *Harbro Ltd., Turriff, Aberdeenshire AB53 4PA, UK*
10 [3] *Rowett Institute, University of Aberdeen, Aberdeen AB25 2ZD, UK*
11 [4] *Department of Electrical and Electronic Engineering, Strathclyde University,*
12 *Glasgow G1 1XW, UK*
13
14 [a] Present address: *SRUC Dairy Research and Innovation Centre, Hestan House,*
15 *The Crichton, DG1 4TA, UK*
16
17 Corresponding author: Nicholas N Jonsson. Email: nicholas.jonsson@glasgow.ac.uk
18
19 Short title: *Post mortem* ruminal findings in beef cattle
20
21 **Abstract**

22 Sub-acute ruminal acidosis (SARA) can reduce the production efficiency and impair
23 the welfare of cattle, potentially in all production systems. The aim of this study was
24 to characterise measurable *post mortem* observations from divergently managed
25 intensive beef finishing farms with high rates of concentrate feeding. At the time of

slaughter, we obtained samples from 19-20 animals on each of six 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 (SCFA) 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 distinction 3 positive (CD3+) and major histocompatibility complex type two positive (MHCII+) 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 crude protein percentage of the total mixed ration dry matter 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.

51

52 **Keywords**: Acidosis , Pathology , pH, Diet, Papillae

53

54 **Implications**

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

62

63 **Introduction**

64 Sub-acute ruminal acidosis (SARA) is a common inflammatory syndrome of
65 ruminants arising from high dietary challenge with rapidly fermentable carbohydrates
66 (Titgemeyer & Nagaraja, 2006; Kleen et al., 2009; Kleen & Cannizzo, 2012). Ruminal
67 acidosis is characterised by impaired fibre degradation, lower feed intake, laminitis,
68 diarrhoea and displaced abomasum, with consequent increased culling rates in dairy
69 herds (Plaizier et al., 2008). Kleen and Cannizzo (2012) summarised reports relating
70 to the occurrence of acidosis in dairy herds, providing examples to suggest the
71 incidence of SARA to be 14% in Friesland (Kleen et al., 2009), 19-40% in the USA
72 (Garrett et al., 1997); and 33% in some Italian herds (Morgante et al., 2007). The
73 condition also occurs in beef cattle (Nagaraja & Titgemeyer, 2007). The diagnosis
74 and management of SARA is difficult, as there is little consensus on diagnostic

75 criteria and it is largely a chronic predisposition to other specific diseases, rather
76 than being a single disease entity itself (Kleen et al., 2009, Denwood et al., 2018).
77
78 The starch in cereal-rich diets is fermented quickly, leading to the rapid production of
79 fermentation acids, principally short-chain fatty acids (SCFA), mainly acetate,
80 propionate and butyrate. The SCFA are absorbed through the reticuloruminal wall,
81 providing energy to the host animal, and a moderate concentration remains in
82 ruminal digesta (Aschenbach et al., 2011). In most cases the pH of the reticulorumen
83 is held within a functional physiological band by a complex combination of
84 endogenous buffers, dietary buffers, active transport by reticuloruminal epithelial
85 cells, and physical removal of protons from the reticulorumen into the distal digestive
86 tract (Aschenbach et al. 2011). When the rate of ingestion of the substrate for the
87 production of SCFAs exceeds the buffering capacity of the reticulorumen, lactic acid
88 accumulates, pH begins to fall, and there are dramatic changes in the microbiome,
89 with dominance of acid-tolerant genera such as *Streptococcus* and *Lactobacillus*
90 (Slyter, 1976; Dawson et al., 1997; Penner et al., 2009).
91
92 From studies with dairy cattle, it is known that some farms are more susceptible to
93 acidosis than others, and that within herds some animals are more prone to acidosis
94 than others (Garrett, 1996; Kleen et al., 2009; Morgante et al., 2007; Penner et al.,
95 2009; Denwood et al., 2018). Many of the experimentally determined interactions
96 among feeding methods, feed and animal behaviour in feedlots have been reviewed
97 elsewhere (e.g. Gonzalez et al., 2012), but a need exists to determine how
98 observations in experimentally induced acidosis in cattle relate to on-farm,
99 commercial conditions. The aim of this study was to characterise measurable *post-*

100 *mortem* observations on variables that have previously been shown to be associated
101 with acidosis on animals from divergently managed intensive beef finishing farms
102 with high rates of concentrate feeding with a view to identifying indicators of prior
103 exposure to diets with a high-risk of acidosis.
104
105 **Materials and Methods**
106 *Farms and animals*
107 Ten beef farms situated in Aberdeenshire completed a questionnaire on dietary and
108 farm management in the year prior to the study. Six were selected based on dietary
109 composition and likely risk of acidosis (three high-risk and three low-risk) based on
110 the questionnaire and on-farm observations. In total, 119 steers and heifers with an
111 average age of 700 days and carcase weight of 371 kg were sampled at slaughter.
112 Breeds included Aberdeen Angus, Blonde d'Aquitaine, Charolais, Limousin, Saler,
113 British Blue, Shorthorn and Simmental, and crosses of these breeds. All animals
114 were finished indoors in the Grampian region close to Aberdeen (mean annual
115 temperature 7.5 °C, rainfall 1130 mm) between January and July 2013.
116
117 *Feed analysis*
118 A representative sample of the total mixed ration (TMR) was assessed for each of
119 the farms, once during the feeding period for each cohort of animals. Samples were
120 taken for measurement of crude protein, crude fibre, starch, and neutral-detergent
121 fibre (NDF) on a dry matter basis using near infrared reflectance spectroscopy
122 (NIRS). NIRS was conducted using a FossNIRSystems 5000+ machine, using
123 calibrations provided by Trouw Nutrition GB. Particle size of TMR was measured by
124 Trouw Nutrition GB using a Penn State particle separator (PSPS), with a 1.18 mm

125 screen to enable quantification of fine particles. All cattle had *ad libitum* access to
126 feed, and were estimated to consume 11-13.5 kg dry matter feed per day. Cattle at
127 farm BH6 also had *ad libitum* access to barley straw.
128
129 *Post-mortem sampling*
130 In the abattoir, carcasses of the animals involved in the study were followed from the
131 kill point to evisceration where the rumens were marked with a labelled cable tie to
132 allow identification of animals as they moved through to the gut room of the abattoir,
133 where the hind gut was separated from the rumen by abattoir staff. Once the rumen
134 was separated from the hindgut and incised, a sample of its contents was taken and
135 strained through four layers of gauze muslin into 2 ×50 ml plastic centrifuge tubes.
136 A sample was taken from the middle of the ventral sac, washed quickly in water to
137 remove most digesta (to reduce difficulties with subsequent sectioning with the
138 microtome) and two samples were taken; one placed in RNAlater (for studies on
139 gene expression) and one in 10% formalin for histology and immunohistochemistry.
140 For the sample placed in RNAlater, the muscle layer was removed. For both
141 samples, fat was trimmed as much as possible before placing it in the fixing solution.
142 Samples of caecum wall were taken and caecum contents collected and strained
143 through muslin into Falcon tubes. The caecum wall was washed in water to remove
144 any excess digesta and then two samples taken and placed in bijoux, in either
145 RNAlater or formalin, as with the rumen samples. The samples in RNAlater were
146 then stored at -20°C for gene expression studies to be carried out at a later date.
147 The samples that were fixed in 10% formalin for 2 d were then removed, rinsed with
148 and stored in phosphate buffered saline (PBS) prior to embedding in paraffin.

149 Samples were kept on ice in a passive cool-box for transportation back to the
150 laboratory for further processing. Two 2 ml aliquots were taken of the strained rumen
151 fluid and caecum contents, and then stored at -20°C for future VFA determination,
152 according to the method of Richardson et al. (1989). For lipopolysaccharide(LPS)
153 and histamine determination, 1 ml of strained rumen fluid was thoroughly mixed with
154 1 ml PBS, which was then centrifuged at 16 000 ***g*** for 45 min at 4°C. Sterile syringe
155 and needle were then used to aspirate the supernatant, with minimal disruption to
156 the pellet and filtering the supernatant through a sterile 0.2 μm filter (Elkay
157 Laboratory Products (UK) Ltd., Basingstoke, UK), with the resultant filtrate collected
158 in a pyrogen-free glass tube (Cat. # N207, Lonza Group Ltd., Basel, Switzerland).
159 The sample was then heated at 100°C for 30 min, allowed to cool to room
160 temperature, and then stored at -20°C. The remaining strained content was stored
161 raw at -20°C.
162
163 *Gross assessment of rumen wall*
164 The opened and washed wall of the ventral sac of the rumen was examined and
165 photographed. Papillae were subjectively assessed for size and shape and the
166 complete, open surface of the sac was characterised according to the prevalence of
167 lesions and colour. The scoring system is shown in Table S1.
168
169 *Histamine measurement in ruminal fluid*
170 Histamine concentration in the rumen fluid was measured using the Abnova
171 Histamine ELISA Kit (Abnova, Catalogue Number KA1888), designed for analysis of
172 histamine in human faeces. Duplicate measurements were used for all standards,
173 controls and samples. Before use on research samples, several dilutions were tested

174 to optimise the assay for ruminal fluid. A dilution of 1:300 provided the most
175 consistent results and was subsequently used on all samples. The absorbance of the
176 solution in the wells was read using a microplate reader set to 450 nm. Standard
177 curves were plotted for ruminal and plasma samples and unknown concentrations
178 were calculated from the regression equation. Each sample was tested in duplicate
179 and the average within-sample CV was 20.56%.
180
181 *Lipopolysaccharides in ruminal and caecal fluid*
182 For measurement of LPS, a modified, scaled-down method of Li et al. (2012) was
183 created. This was carried out immediately upon return from the abattoir, in order to
184 minimise lysis of the Gram-negative bacteria and further release of LPS into the
185 extracellular fluid. Briefly, 1 ml of strained rumen fluid was thoroughly mixed with
186 1mL PBS, which was then centrifuged at 16 000 ***g*** for 45 min at 4°C. A syringe and
187 needle were then used to immediately take up the supernatant, with minimal
188 disruption to the pellet. The needle was then replaced with a sterile 0.2µm
189 disposable filter (Elkay Laboratory Products (UK) Ltd., Basingstoke, UK), with the
190 resultant filtrate collected in a pyrogen-free glass tube (Cat. # N207, Lonza Group
191 Ltd., Basel, Switzerland). The sample was then heated at 100°C for 30 min, allowed
192 to cool to room temperature, and then stored at -20°C. LPS was measured in the
193 processed rumen and caecum fluid by the *Limulus* Amebocyte Lysate Assay
194 (Kinetic-QCL, Lonza Group Ltd.) according to the manufacturer’s instructions. The
195 kinetic assay was run in a 96-well microplate on an incubating spectrophotometer at
196 an absorbance of 405 nm (SpectraMax 190 Microplate Reader with SoftMax Pro 6
197 Microplate Data Acquisition and Analysis Software, Molecular Devices, Sunnyvale,
198 CA, USA). Appropriate dilutions of the samples were elucidated by following the

199 method of Gozho et al. (2005). Briefly, dilutions ranged from around 1:160 000 up to
200 1:3 000 000, with the final dilution made up with 1:1 β-Glucan Blocker (Cat. # N190,
201 Lonza Group Ltd.). An inhibitory test, involving spiking samples as a positive control,
202 was also conducted to ensure the dilutions were adequate. Results were expressed
203 as endotoxin units per mL (EU/mL). Coefficients of variation (%) were obtained for
204 both the standard curve and the individual samples in each run. The mean (± SD)
205 within-sample between-assay CV was 2.31 ± 4.31%.
206
207 *Short-chain fatty acids (SCFA) in ruminal fluid*
208 SCFA were measured on centrifuged rumen fluid using gas chromatography
209 (Richardson et al. 1989). Samples were shipped to the laboratory on dry ice and
210 analysed for: acetate, propionate, butyrate, isobutyrate, valerate, isovalerate,
211 succinate, formate, lactate. Two standards were tested in duplicate for each run. The
212 CVs (%) for each were as follows: acetate, 1.96%; propionate, 0.84%; butyrate,
213 1.56%; isobutyrate, 1.40%; valerate, 1.13%; isovalerate, 1.62%; succinate, 13.98%;
214 formate, 3.61%; lactate, 1.12%.
215
216 *Histology*
217 The rumen samples collected at the abattoir and fixed in 10% formalin for 48 h and
218 stored in PBS were cut into 1-3 segments, depending on their size, removing any
219 rough edges and ensuring that all layers of the rumen wall were present in each
220 segment. Fixation of the segments continued with a fresh aliquot of 10% formalin.
221 They were then rinsed in PBS and stored in PBS or 70% ethanol, before paraffin-
222 embedding and micro-sectioning on a microtome. Sections of 3 μm thickness were
223 cut, placed onto slides and baked in an oven at 60°C for 1-2 h, until excess wax had

224 melted and the tissues adhered to the slides. Haematoxylin and Eosin staining
225 (H&E) and Elastin Martius Scarlet Blue staining (EMSB) were then carried out by
226 hand (EMSB stain is a Martius Scarlet Blue (MSB) protocol modified to include a
227 Miller's Elastin stain).
228
229 *Immunohistochemistry*
230 The immunohistochemistry (IHC) staining was performed by Veterinary Diagnostic
231 Services (VDS) at the University of Glasgow's School of Veterinary Medicine. Five
232 slides were stained per sample for all samples; major histocompatibility complex
233 class 2 (MHCll), myeloperoxidase, cluster of differentiation 3 (CD3), a negative rabbit
234 and a negative mouse immunoglobulin control. The immunohistochemistry was
235 carried out at room temp with Tris buffer pH 7.5 with Tween used for all buffer
236 requirements. Antigen retrieval was carried out for all IHC and corresponding
237 negative controls. The MHCll IHC used heat-induced epitope retrieval citrate buffer
238 pH 6 (Dako #S1700) and mouse monoclonal anti-human MHCII (Dako #M0746) at a
239 dilution of 1:20. Myeloperoxidase IHC used Dako Ready-to-Use (RTU) proteinase K
240 and rabbit polyclonal anti-human (Dako #A0398) at a dilution of 1:1 000. CD3 IHC
241 used Dako heat-induced epitope retrieval sodium nitrate pH 6 and rabbit polyclonal
242 anti-human CD3 (Dako #A0452) at a dilution of 1:100. All staining was carried out in
243 a Dako Autostainer. Secondary antibody from Dako with conjugated horseradish
244 peroxidase (HRP) was used for detection of primary antibodies. Sections of 2.5 μm
245 thickness were placed on charged slides and baked for 1 h at 56°C. All sections
246 were rehydrated, followed by heat-induced epitope retrieval and sodium citrate buffer
247 pH 6 for 1 min 40 s at 125°C and full pressure. Following that, enzymatic antigen
248 retrieval using Proteinase K RTU was used for the specified time as per

249 manufacturer's instructions. Next the slides were loaded onto the Dako Autostainer
250 and rinsed with buffer for 5 min. Endogenous peroxidase activity was blocked using
251 Dako Real TM Peroxidase blocking solution for 5 min, followed by a 5-min buffer
252 rinse. The primary antibody was used at the recommended dilution, with Dako
253 universal diluent. This was followed by 2 × 5 min buffer rinses. Next the appropriate
254 secondary antibody was used according to the species in which the primary antibody
255 was produced. This was followed again by 2 × 5 min buffer rinses. Detection was
256 carried out by using Dako K5007 DAB for two 5-min periods. The slides were then
257 rinsed three times in water. Next the slides were counterstained for 27 s with Gill's
258 haematoxylin before being washed in water. Finally, the slides were blued in Scott's
259 tap water substitute, dehydrated and mounted in synthetic resin ready for scoring.
260

261 *Examination, image capture, storage and analysis*
262 All slides were initially scanned under low and high power using an Olympus CX41
263 microscope. Images of typical and atypical examples of all features of interest were
264 captured using GXCam software, calibrated as recommended by the manufacturer,
265 using a ×4, ×10 and ×40 graticule. The stratum corneum (SC) thickness was
266 determined by taking the mean of 5 measurements in µm across the SC over 2
267 fields, using ×40 magnification and H&E stain. The same approach was taken for the
268 stratum granulosum (SG) thickness. The vascular diameter (VASCD) was taken as
269 the mean diameter of the single largest vessel in each of two papillae using ×40
270 magnification and H&E stain. CD3+ and MHCII+ cells were estimated as the count of
271 the total number of CD3+ or MHCII+ cells in a single image taken at ×40
272 magnification. All histological measurements and counts in this study were
273 undertaken manually by one operator (HJF).

274

275 *Gene Expression*

276 RNA extraction from rumen tissue was carried out using the miRNeasy® mini kit
277 (Qiagen) and DNase® kit (Qiagen). The concentrations and quality of the RNA were
278 assessed using the A260/280 and A260/230 ratios measured on a NanoDrop
279 ND1000 spectrophotometer (Thermo Fisher, UK). If the RNA concentration was high
280 (1 000 µg/µL), RNase-free water was added to the eluant and it was measured
281 again. RNA was stored at -80°C until further use. First strand cDNA was obtained
282 using TaqMan reverse transcription (RT) reagents (Life Technologies #N8080234)
283 on 1 µg of total RNA in 20 µl reaction volume, following the manufacturer's
284 instructions. Following RT reaction, 30 µl of RNase free water was added to each
285 sample to a final volume of 50 µl. All cDNA products were stored at -20°C until
286 further use. TaqMan real-time quantitative PCR (qPCR) was used to quantify gene
287 expression levels from the ruminal cDNA using TaqMan Gene Expression Master
288 Mix assay kit (Life Technologies, UK) and the respective probes as shown in Table
289 S2. The assay was carried out using a 384-well microplate (Thermo Fisher, UK) with
290 3 technical replicates for each sample to reduce measurement error. Each reaction
291 consisted of 1x assay probes, 1x TaqMan® Gene Expression Master Mix (Applied
292 Biosystems) and RNase-free water to a final volume of 5 µl. The assays were carried
293 out in singleplex or duplex, depending on the assay efficiency pre-determined in an
294 optimisation assay prior. A pooled cDNA sample from all cDNA samples was used to
295 generate a series of 3-fold serial dilution used as standard curve in every assay. Two
296 µl of each sample were transferred into corresponding wells containing 3 µl of the
297 reaction mix. The plates were sealed with Optical Adhesive Cover (Thermo Fisher,
298 UK) and the thermal cycling was carried out using the 7900HT Fast Real-Time PCR

299 System (Applied Biosystems) with thermal cycling conditions set out in the kit
300 protocol.
301
302 *Statistical analysis*
303 Data were initially managed in an Excel spreadsheet and then analysed in R (R core
304 Team 2015). All dependent variables were initially tested by application of the
305 Shapiro-Wilk test of normality and most were found not to be normally distributed.
306 Variables were then natural log-transformed, the Shapiro-Wilk test was re-applied,
307 and histograms of the transformed data were examined. In several cases the
308 Shapiro-Wilk statistic indicated that the data were not a good fit to a normal
309 distribution, but in almost all cases (with the exception of *IL-2* gene expression),
310 visual appraisal suggested a distribution that would be amenable to parametric
311 analysis. Nonetheless, when the effects of farm and risk were tested, both
312 parametric and non-parametric analyses were applied and the results were
313 consistent. The effects of *a priori* risk classification and the farm of origin were tested
314 using one-way ANOVA, after natural log transformation in most cases. No correction
315 factor for multiple tests was applied because the purpose of the analysis was
316 primarily descriptive and it is not clear how families of variables should be defined.
317 Calculated estimates of *p*-values are presented to enable readers to draw their own
318 conclusions regarding statistical significance with multiple comparisons. To assist in
319 this interpretation, suggested threshold p-values derived using the Bonferroni
320 method ($\alpha' = \alpha/m$, where α' is corrected *p*-value, α is the original *p*-value and m is the
321 number of comparisons) have been applied. Input variables other than farm of origin
322 and risk classification were completely or partially confounded with farm of origin.
323 The relationships between selected dependent variables and six continuous

324 independent variables (proportion of TMR composed of barley, straw, silage, fibre,
325 crude protein, and fine particles (< 1.18 mm)) were individually examined using linear
326 regression on natural log-transformed (if not normally distributed) or untransformed
327 variables (if normally distributed). The potential predictor variables were selected
328 because they are considered likely to be important drivers of acidosis in ruminants
329 and because our dataset was almost complete for each. For each dependent
330 variable, the predictor for which the model provided the highest *F*-statistic and
331 adjusted *R*-square value was chosen for presentation. Multiple regression or more
332 complex mixed models, although necessary to identify interactions among potential
333 predictive variables, were not possible because of singularity (each farm had fixed
334 combinations of variable values). The subjective *post mortem* categories describing
335 the appearance of the rumen wall were tabulated by farm and by risk classification
336 and associations were tested using Fisher's exact chi-sq test. Pearson bivariate
337 product moment correlations were estimated for selected variables.
338

339 **Results**

340 *Farm and dietary assessment*

341 The initial questionnaire-led assessment of 10 beef farms in the Grampian region of
342 Scotland led to the identification of six farms that were quite similar in feeding and
343 management practices but which might be identified as high-risk (BH1, BH6 and
344 BH7) or low-risk (BL2, BL3 and BL7) according to the percentage of the diet that was
345 provided by cereal grains, straw and silage. The main ingredient of all the diets on
346 the six farms was barley, which was grown and processed on each of the farms. The
347 diets were diverse in other respects. Sugar beet pulp, soya hulls and a buffer against
348 acidosis were only used on one farm each and yeast was used on all farms.

349 Rumitech is an essential oils (Harbro Limited), included to modulate ruminal
350 microbiome, depress methane production and increase feed conversion efficiency
351 Santos et al., 2010), and was included only on two of the high concentrate, high-risk
352 farms. Full dietary records were not retrieved from two farms: BL7 and BH1. From
353 BL7, we were unable to independently verify the statements provided by the farmer
354 regarding the ration that was fed to the cattle, but we were able to measure the
355 particle size of the TMR and obtain a measure of crude protein (CP) and crude fibre
356 (CF) in the TMR. In this case, the unverified data were not used in statistical
357 analyses but are presented in Table 1. Figure S1 illustrates the diversity of the
358 combinations of dietary components in each of the farm's rations.
359
360 *Gross appearance and pathology of rumen*
361 Table S3 summarises the results of normality testing of all the dependent variables.
362 There was relatively little variation in the gross appearance of the ruminal wall, as
363 shown in Tables S4-S6. There was a weak association of the colour of the ruminal
364 papillae and the *a priori* risk classification ($\chi^2 = 13.441$; df = 5; $p = 0.020$) such that
365 animals from the low-risk farms were more likely to have black or brown papillae and
366 those from the high-risk farms were more likely to have grey or pink-grey papillae.
367 Only six of the animals from high-risk farms and eight from low-risk farms had any
368 detectable abnormality on the ruminal wall. Eight of the 14 animals with detectable
369 abnormalities showed small areas bare of papillae. Only one animal showed any
370 signs of active inflammation, excoriation or scarring of the ruminal wall (Figure 1).
371
372 *Farm of origin and a priori risk classification*

373 The categorical factors that were assessed statistically were the farm of origin and
374 the initial *a priori* risk classification. Table 2 lists the median and interquartile ranges
375 for each of the dependent variables for each of the low-risk and high-risk farms,
376 together with the Kruskal-Wallis *p*-value for the effects of farm and *a priori* risk
377 classification. The table shows a very high degree of among-farm variation. Breed,
378 sex, age and weight at slaughter were all confounded with farm of origin (see Table
379 1, Figure S1). Rumen fluid histamine concentration did not differ significantly
380 between risk categories ($p = 0.23$) (Figure 1). Ruminal LPS concentration was higher
381 in the cattle on the high-risk farms ($p = 0.0050$, Figure 1). Caecal LPS concentrations
382 were higher (up to 27-fold) than corresponding ruminal LPS concentrations, although
383 caecal LPS concentration was lower in the high-risk group ($p = 0.0037$, Figure 1).
384 Ruminal fluid lactate concentration was higher in cattle from the high-risk farms ($p =$
385 2.03×10^{-3}, Figure 2). Caecal lactate concentration was higher on the high-risk farms
386 ($p = 7.76 \times 10^{-5}$). Total SCFA concentration in ruminal fluid was lower on the high-
387 risk farms ($p = 3.21 \times 10^{-7}$ Figure 2), as were each of acetate, propionate and
388 butyrate (1.47×10^{-7}, 1.17×10^{-5}, and 1.09×10^{-6} respectively Figure 2). The CD3+
389 and MHCII+ cell counts in ruminal epithelial sections were lower on high-risk farms
390 ($p =3.67 \times 10^{-5}$ and 1.38×10^{-5} respectively Figure 3). *TLR4*, *IL-1β* and *IFN-γ* relative
391 expression were all lower in rumen tissue from cows on high-risk farms ($p = 0.00014$,
392 0.0029 and 2.44×10^{-7}, Figure 3). The stratum corneum and stratum granulosum
393 were thicker on the animals from the high-risk farms ($p = 1.80 \times 10^{-7}$ and 0.0035
394 respectively, Figure 4). Figures S2-S5 show box and whisker plots of ruminal and
395 caecal SCFA by farm and by a priori risk classification. Figure S6 shows gene
396 expression, S7 shows histological observations.
397

398 *Dietary components*

399 Figure S1 shows the values for the predictive (dietary input) variables used in linear
400 regression for each of the farms. It clearly shows the potential for complex
401 interactions among the predictive variables at the farm level. Although most variables
402 are not completely confounded, there is substantial overlap and the lack of
403 replication of combinations of high, medium and low values for each of the variables
404 means the study design does not allow for the examination of multiple predictors in a
405 single model or the examination of any dietary interactions. Another problem with
406 modelling the effects of potentially important predictor variables in this study arises
407 from the non-uniform spacing among a relatively small number of levels. Hence, the
408 linear regressions presented in this report must be treated with caution. Linear
409 regression analysis (summarised in Table 3) suggests that at least one of the six
410 factors (crude protein, crude fibre, barley, straw or silage as percentage of dry matter
411 in diet, percentage of particles in TMR < 1.18 mm) was a significant predictor for 14
412 of 18 selected dependent variables at the Bonferroni-adjusted p-value threshold of
413 0.0005 and for all variables at the unadjusted p-value threshold of 0.05. This was
414 expected because each of the predictors was aliased with farm of origin, by which all
415 of the 18 variables differed. The crude protein content in the TMR explained the most
416 variation for 13 of 18 variables, the proportions of fines and silage in the diet each
417 explained two variables, and proportion of fibre explained one. Figure 5 shows
418 variation in six animal-level variables when herds were grouped in rank order of the
419 strongest putative herd-level explanatory variable. Similar information is presented
420 for the remaining 20 variables in Figures S8-S11.
421

422 *Correlations among selected measures of ruminal fluid, caecal fluid, and ruminal wall*
423 *(gene expression and histological)*
424 Table S7 shows a correlation matrix of selected dependent variables. The
425 expression of *TLR4* was weakly and positively associated with ruminal LPS
426 concentration ($r = 0.16$, $p = 0.084$), not correlated with ruminal histamine
427 concentration ($r = 0.026$, $p = 0.78$), but was moderately correlated with ruminal
428 lactate concentration ($r = 0.37$, $p = 3.009 \times 10^{-5}$). The levels of expression of the
429 genes in the epithelium correlated variably with each other, ranging from $r = -0.04$ for
430 *NHE3* with *CCL11* up to $r = 0.57$ for *CCL11* with *IFN-γ*. They were not strongly
431 correlated with the thickness of the stratum granulosum, nor with the density of
432 CD3+ nor MHCII+ cells, nor lactate concentration in ruminal or caecal fluid (data not
433 shown). Cold weight of carcase after slaughter was most closely associated with the
434 ruminal fluid lactate concentration ($r = 0.50$, $p = 5.37 \times 10^{-7}$).
435
436 **Discussion**
437 All but four of the 31 dependent variables that we measured differed significantly ($p <$
438 0.0008) according to the farm of origin. Every farm was unique in its combination of
439 input variables, which resulted in a level of confounding and lack of replication that
440 made it impossible to test the effects of many of them. Potential confounding factors
441 at the level of the farm were the inclusion of specific nutritional components, mixing
442 and feeding practices, stocking density, faecal or bedding management, the source
443 of cattle, variation in their genotype and enteric microbiome, health and nutritional
444 management before arrival at the farm of origin. Another important potential
445 confounder is the length of the period from last feeding until slaughter, which was not
446 able to be controlled in this study due to the limitations on commercial abattoir

447 operations. This variable would be expected to result in variation in the
448 concentrations of metabolites in the reticulorumen away from the normal diurnal
449 pattern seen in animals during periods of ad lib feed availability. This variation would
450 be expected to be greater in animals that had the most extreme adaptations to high
451 levels of feed.
452
453 By definition, animals on the high-risk farms were fed diets with higher proportions of
454 barley and lower proportions of straw than animals on the low-risk farms, each of
455 which included grass silage as a component of the diet. None of the high-risk farms
456 included grass silage in the rations. The *a priori* risk classification can therefore be
457 seen as a qualitative indicator of the proportion of forage in the diet (see Table 1),
458 and was a significant factor ($p < 0.0008$) for 14 of 31 continuous dependent
459 variables (Table 2). The animals on high-risk farms had significantly ($p < 0.0008$)
460 higher concentrations of lactate in the rumen, as well as thicker epithelial strata
461 cornea and strata granulosa, with higher levels of expression of *TLR4*. This was
462 accompanied by lower ruminal SCFA concentrations. The expression of *IFN-γ* and
463 the counts of MHCII+ and CD3+ cells in the epithelium were significantly lower in
464 samples from cattle on the high-risk farms ($p < 0.0008$).
465
466 All the rations fed in this study had concentrate to forage ratios of at least 40% and
467 the samples were taken after 90 to 100 d of concentrate feeding. Most of the studies
468 that have reported on the response of SCFA, LPS, histamine and other markers of
469 rumen function have focused on the acute period immediately after a challenge or at
470 most, after a period of a couple of weeks of adaptation. The variables in our study
471 are end-point observations after a prolonged period of adaptation, so it should not be

472 expected that the relationships between input and dependent variables should be the
473 same as those reported in acute response studies. Additionally, during the period
474 between each animal's last feed and its slaughter, it would be expected that
475 absorption of metabolites and clearance from the reticulorumen would continue. The
476 lactate concentrations in ruminal samples from high-risk farms were an order of
477 magnitude lower than those that are expected with acute lactic acidosis, but were
478 higher than those found on low-risk farms. The highest ruminal lactate concentration
479 found here was 6 mmol/l, whereas concentrations over 100 mmol/l can occur with
480 acute acidosis (Harmon et al., 1985). This low concentration would have relatively
481 little influence on ruminal pH, but it could indicate an imbalance between lactate-
482 producing ruminal microbes and those that utilise lactate (Slyter, 1976; Russell and
483 Hino, 1986; Nocek, 1997). There was a moderate correlation between carcase
484 weight and the lactate concentration, suggesting that the animals with the higher
485 lactate concentrations grew more rapidly. The highest ruminal LPS concentrations
486 were similar to those observed in experiments in which SARA was induced
487 experimentally (reviewed by Plaizier et al., 2012), thus suggesting that the
488 experimental induction of SARA leads to similar soluble LPS concentrations as
489 occurs in animals under farm conditions.
490
491 The concentrations of metabolites or other compounds in the reticuloruminal fluid at
492 a single time point reflect the net effects of production and clearance processes, so
493 are intrinsically difficult to interpret. This is further complicated by variation in the
494 interval during lairage between last feeding and sampling. The previously reported
495 acute effects of increased proportions of barley in rations are to increase SCFA
496 concentrations in reticuloruminal fluid or to increase the rate of appearance in the

497 portal circulation (Loncke et al., 2009; Aschenbach et al., 2011), and acute studies
498 suggest that the stratum corneum and stratum granulosum should become thinner
499 rather than thicker when exposed to high levels of rapidly fermentable carbohydrates
500 (Steele et al., 2011). However, the duration of the period that the animals were fed
501 on the rations in our study provided more than sufficient time for the adaptations
502 described by Schwaiger et al (2013) to take effect. Increased flux from the lumen,
503 due to upregulation of protein-dependent mechanisms, proliferation of metabolically
504 active cells in the stratum granulosum, together with the increased surface area from
505 papillary development would explain the inverse relationship between rapidly
506 fermentable carbohydrates with reticuloruminal SCFA concentrations in the present
507 study. Given that in our study access to feed would have been restricted during the
508 time that cattle were on lairage (ie after arrival at the abattoir) for 12 to 24 h before
509 slaughter, it would be expected that the animals with the greatest reticuloruminal
510 SCFA flux should have the most rapid reduction during lairage, possibly resulting in
511 the lowest concentrations in the rumen immediately post slaughter. Given that the
512 absorption of lactate by the reticuloruminal mucosa is generally slow (Aschenbach et
513 al., 2011) and not driven to the same extent by active, protein-dependant processes,
514 it is not surprising that the direction of change of lactate concentrations in response
515 to barley or proportion of fines was different from the other SCFA, such that higher
516 concentrations *post mortem* reflect a high level of production. The issue of the
517 amount of time that animals were on lairage deserves further comment, as it was not
518 a factor that we were able to control for. Given the standard procedures at the
519 cooperating abattoirs, all animals were killed within 24 h of unloading into lairage and
520 it is likely that all would have been killed between 12 and 18 h after unloading. Hence
521 the variables measured in the ruminal and caecal fluids could have been affected by

522 a variation in fasting time of about 6 h. Whereas this might explain some of the
523 among-farm variation in SCFA concentrations, LPS and histamine, it is not likely to
524 have affected histological measurements. The extent to which it might have affected
525 relative gene expression is unclear.
526
527 Univariate linear regression analysis suggested that of the six main dietary factors
528 that we considered as potential predictors for 18 dependent variables, crude protein
529 percentage was the strongest predictor for 13. As crude protein percentage in the
530 ration increased, ruminal lactate, LPS, histamine, *NHE3* relative expression, *TLR4*
531 relative expression, and the thickness of stratum corneum and stratum granulosum
532 decreased, while the count of MHCII+ cells and CD3+ cells increased. Almost all of
533 the effects were in the opposite direction to those of the proportion of fine particles
534 and proportion of barley in the ration, which tended to have similar effects on the
535 dependent variables. In contrast to the potential role of starch and sugar
536 supplementation, there is relatively little in the literature regarding dietary protein and
537 the risk of acidosis. Golder et al. (2014) showed that a diet with excess
538 metabolisable protein, when fed to dairy cattle, reduced the tendency to
539 reticuloruminal acidosis. Pilachai et al. (2012) showed that high levels of rumen
540 degradable (RDP) and rumen undegradable protein (RUP) in the diet had
541 contrasting effects on SCFA production and the generation of histamine in the
542 rumen, that high RDP resulted in a more rapid and greater increase in SCFA and
543 histamine production. We did not differentiate between RDP and RUP in our study,
544 so it is not possible to infer much about the mechanisms by which the apparent
545 effects of protein are mediated. Golder et al. (2012, 2013) showed that
546 supplementation with histidine (the precursor for histamine) caused elevated

547 histamine concentrations in the rumen but did not elevate LPS. It seems likely that
548 the effects of protein on the variables that we measured were mediated via complex
549 interactions with other dietary components and the reticuloruminal microbial
550 communities.
551
552 Much of the literature relating to the effect of particle size has considered
553 concentrate and forage components separately. In our study, we were interested in
554 the particle size in the TMR rather than the milled cereal grain because the particle
555 size of the grain can be further reduced by mixing with the forage component. The
556 effect of larger particle size of forage components relates primarily to increased
557 eating time, saliva production, wetting of feed with saliva, and time spent ruminating
558 (e.g. Beauchemin & Yang., 2005). Large particle size in processed cereal grains,
559 however is primarily considered to be associated with fewer breaks in the integrity of
560 the pericarp and to reduce the ratio of surface area to volume of starch (Gimeno et
561 al., 2016). De Nardi et al (2014) showed that finely ground maize particles, less than
562 0.5 mm diameter resulted in lower reticuloruminal pH in cattle than a diet with
563 particles of 1.0 mm, when fed at a rate of 30% of DM. Gimeno et al (2016) showed
564 that reticuloruminal pH was higher in finishing beef calves when a 60% cereal (maize
565 and barley) diet was fed in rolled form rather than ground, in which the particle size
566 was smaller. We did not consider the chop length of the forage as a separate input
567 variable, but in some feeding systems, long chop length forage can facilitate sorting
568 and lead to acidosis (Shani et al., 2017).
569
570 Over the last decade questions relating to reticuloruminal physiology have
571 increasingly been addressed by the quantification of gene and protein expression in

572 ruminal tissue, using both *in vivo* and *in vitro* systems. Penner et al (2011) provide a
573 good review of the subject. Chen et al (2012) quantified the expression of the toll-like
574 receptor genes *TLR2* and *TLR4* in ruminal epithelium and suggested that *TLR4*
575 expression should be a good indicator of exposure to high challenge with LPS.
576 Penner et al. (2009) examined the expression of 21 different genes that were
577 expected to be involved in fatty acid metabolism in the rumen epithelium. We
578 selected genes primarily as indicators of immune response and inflammation (*IL-2,*
579 *TLR4, IFN-γ, IL-1β, CCL11*) but also to indicate the level of metabolic activity
580 associated with proton exchange (*NHE3*) and the level of challenge with LPS
581 (*TLR4*). The expression of *TLR4*, which has previously been associated with
582 resistance to the effects of soluble carbohydrate overload (Chen et al., 2012), was
583 weakly and positively associated with ruminal LPS concentration, not related with
584 histamine, but was moderately correlated with ruminal lactate concentration.
585
586 A surprising result from this study was the lack of obvious pathology in the rumens
587 that were examined. We used a gross scoring system with 5 categories but only 15
588 of 119 animals in total scored anything other than a baseline normal score ("A") for
589 the variable "Pre-Score". We therefore combined the non-"A" categories for analysis
590 and there was no strong relationship between the pre score of the ruminal mucosa
591 with any of the factors considered in the study. This should not be taken as definitive
592 evidence that there was no pathology in the rumens that we examined, but that very
593 little was detected using our matrix. The lack of clear-cut histological signs of
594 pathology was consistent with this observation.
595

596 As a consequence of the lack of any distinct ruminal pathology, one of the objectives
597 of the study, to characterise the possible pathological features of the reticulorumen in
598 cattle from divergently managed intensive beef finishing farms with high rates of
599 concentrate feeding, was not able to be achieved. Nonetheless, elevated
600 concentrations of LPS and lactate were seen in the reticuloruminal fluid of cattle on
601 the high-risk farms and both were relatively strongly associated with increasing
602 levels of barley supplementation and proportion of fine particles in the diet. Without
603 any independent marker for performance or health (e.g. daily liveweight gain, feed
604 intake, clear evidence of systemic illness) it was not possible to determine *de novo*
605 thresholds at which LPS or lactate concentrations indicate pathology.
606
607 Despite the strong effect of farm on the measured variables, there was wide inter-
608 animal variation. This was expected as previous studies have shown a wide range in
609 reticuloruminal pH and other responses in animals fed on the same diet (Gao and
610 Oba, 2014; Mohammed, 2012). Some of this variation is likely due to variation in
611 feeding behaviour, particularly the predisposition to sorting fine, concentrate particles
612 from long fibre, or to rapid eating (Gao and Oba, 2014). In addition to intrinsic natural
613 variation among animals, the animals on any one farm would have been sourced
614 from a variety of other farms, each contributing its own nutrition-induced and
615 management-induced variation. Finally, variation in time of sampling in relation to the
616 time of last feeding is expected to have introduced some among-farm and among-
617 animal variation.
618
619 **Conclusions**

620 There are four main conclusions to be drawn from this study, the first two being of a
621 more general nature. Firstly, despite a reasonable expectation of some degree of
622 equivalence among farms due to their similar location, management and broad
623 nutritional inputs, farm to farm variation in rations and practices was wide and the
624 effects of individual farm factors on animal physiology dominated any other factors
625 that were measured. This observation has implications for the design and
626 interpretation of future studies on the impact of interventions in the field, in which a
627 higher level of replication is required than for conventional, controlled experimental
628 studies. It also implies a need to consider the effects of any nutritional intervention as
629 part of a complex, interactive network of input factors. Secondly, the study provides
630 an insight into the adaptive capacity of cattle. The animals in this study were those
631 that made it to slaughter with the rest of their cohort and over the three months or so
632 that they were on feed they had all successfully adapted to a highly challenging diet
633 with little or no evidence of pathology. Future studies on the effects of high levels of
634 soluble carbohydrate supplementation or seeking to describe pathology associated
635 with SARA would be more effective if they were to focus on the animals that failed to
636 meet performance targets. The third message from the study is that protein
637 percentage and the proportion of fines in concentrate-based total-mixed rations are
638 strong and opposing contributors to variation in the traits that have been used as
639 indicators of exposure to high acidosis risk.
640

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649
650 **Author Contributions**
651 WT, RJW, IA, CM, and NNJ instigated the work. EMS, CDH and WT assessed the
652 farms and feeds. CAMcC, RCC, HHCK-T, HF, EMS, TJS, CDH, NNJ and RJW
653 collected and processed samples at the abattoir and the laboratory. NNJ undertook
654 statistical analyses and drafted the manuscript, which all authors reviewed.
655
656 **Declaration of interest**
657 None of the authors has any potential financial interest arising from the outcomes of
658 the work described in this study.
659
660 **Ethics statement**
661 Ethical approval was not sought for the work described in this manuscript. No
662 research was conducted on live animals. All materials were obtained post mortem
663 from licensed abattoirs in the UK.
664
665 **Software and data repository**
666 The data that were obtained as described in this paper are publicly available from
667 the University of Glasgow data repository
668 http://dx.doi.org/10.5525/gla.researchdata.710
669

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782
783

Figure captions

Figure 1 Boxplots showing concentrations of lipopolysaccharide (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, BH7 were classed a priori as high-risk and those labelled BL2, BL3, 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.

Figure 2 Box and whisker plots showing lactate and total short-chain fatty acid (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, BH7 were classed a priori as high-risk and those labelled BL2, BL3, 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.

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, BH7 were classed a priori as high-risk and those labelled BL2, BL3, 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 high-power field (hpf).

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 crude protein percentage in the diet (farms BL2 and BH7 – both 12.5%). Farms labelled as BH1,BH6, BH7 were classed a priori as high-risk and those labelled BL2, BL3, 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.

***Table 1** Assessment of diets on six beef farms. Composition, chemical analysis, 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
Coldweight 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[1]	No	No	No	No	Yes	Yes
Yeast[2]	Yes	Yes	Yes	No	Yes	Yes
Buffer[3]	No	Yes	No	No	No	No
TMR[1] Composition (%DM)						
Crude Protein	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)						
Crude Protein	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 x 8	7.1	4.2	12.6	7.5	8.2	3.0
8 x 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 x 8	1.8	0	na	na	37.7	42.3
8 x 1.2	95.7	92.1	na	na	58.0	60.6
< 1.18	2.7	7.6	na	na	7.6	1.5

TMR = total mixed ration; na = not available; 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.

* 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

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}	2 098	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 & Immunohistochemistry								
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}

CD3+ = cluster of distinction 3, Exp = expression, hpf = high-powered field, LPS = lipopolysaccharide, Q1 = first quartile, Q2 = second quartile, MHCII = major histocompatibility complex class 2 ; SCFA = short-chain fatty acids

*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.

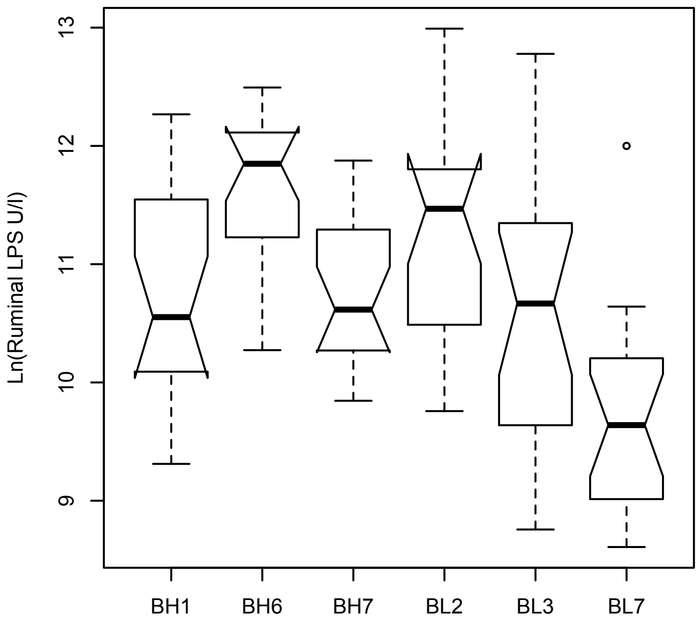
Table 3 *Summary of linear regression analysis of selected dependent variables from beef cattle against six selected dietary components (proportion of dry matter in the TMR as straw, silage, barley, crude protein or fine particles (< 1.18 mm)). In each case the best model was selected according to adjusted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

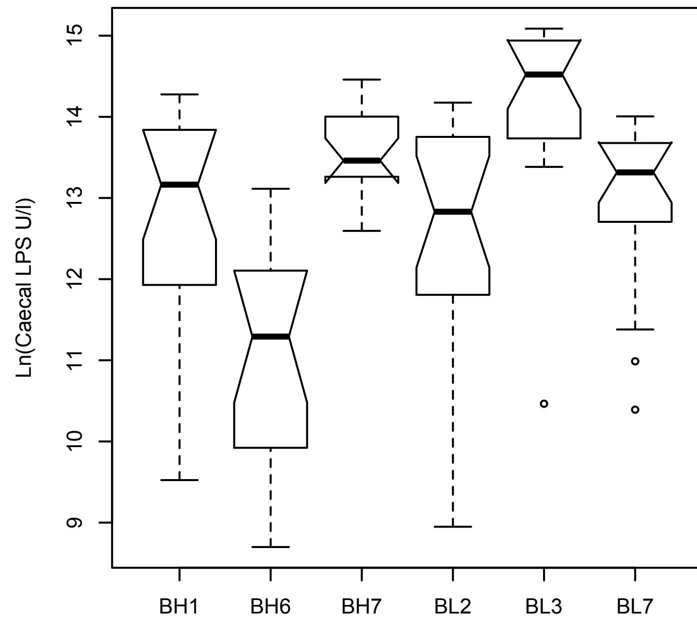
Variable	Best Predictor	Effect Estimate	F-stat	df	Adjusted R^2 (%)	*p*-value*
Ln(RF Histamine)	Fibre	0.17	32.02	117	20.81	1.11×10^{-7}
Ln(RF LPS)	Protein	-0.41	27.05	117	18.08	8.54×10^{-7}
Ln(CF LPS)	Silage	0.032	18.7	94	15.71	3.80×10^{-5}
Ln(RF Lactate)	Protein	-0.66	47.31	117	28.19	3.17×10^{-10}
Ln(CF 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}

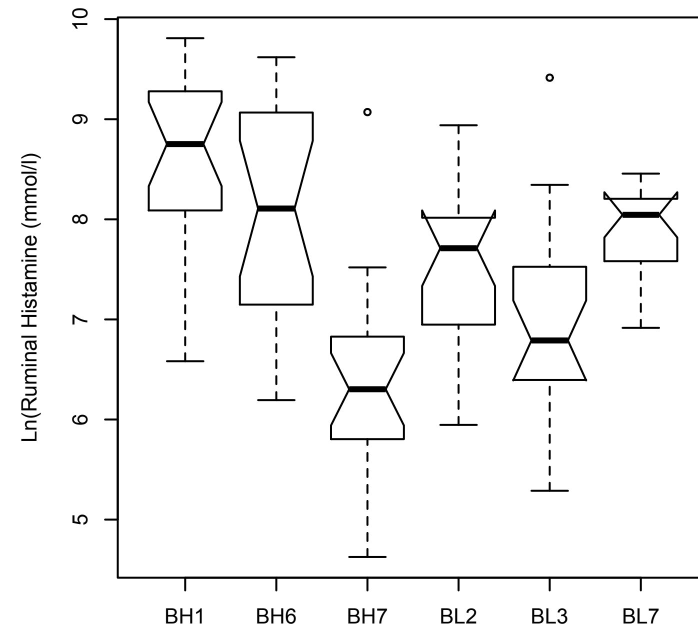
Ln(CD3+ Cells/hpf)	Protein	0.24	60.53	110	34.91	4.23×10^{-12}
Ln(MHCII+ Cells/hpf)	Protein	0.23	19.09	111	13.92	2.82×10^{-5}

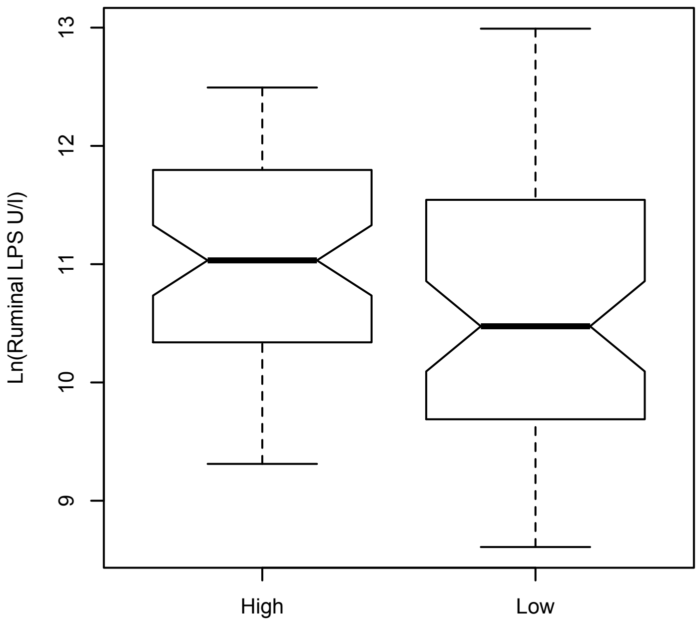
CF = cecal fluid, hpf = high power field, Ln = natural logarithm, LPS = lipopolysaccharide, RE = relative expression, RF = rumen fluid, SC = stratum corneum, SCFA = short-chain fatty acid, SG = stratum granulosum,

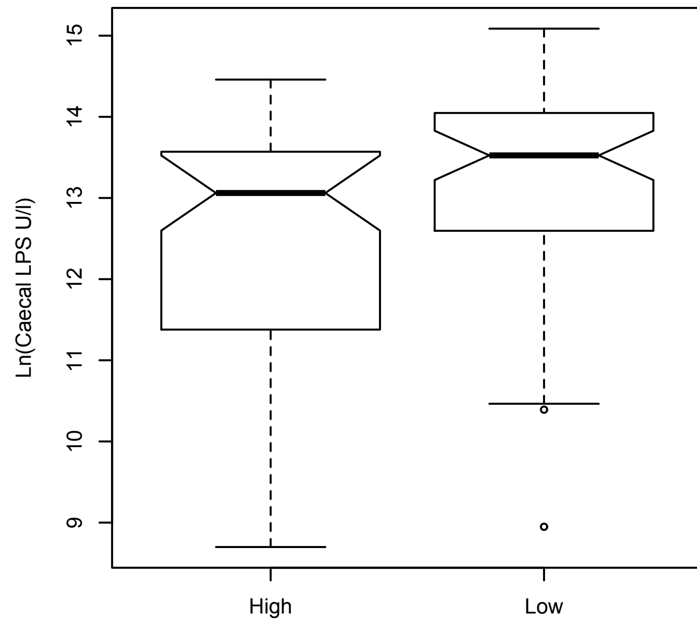
A very conservative approach to interpretation of the p-values using the Bonferroni correction would suggest that a p-value of 0.0005 would be an appropriate level to ensure that the null hypothesis was rejected at the same level as p = 0.05 for a single test.

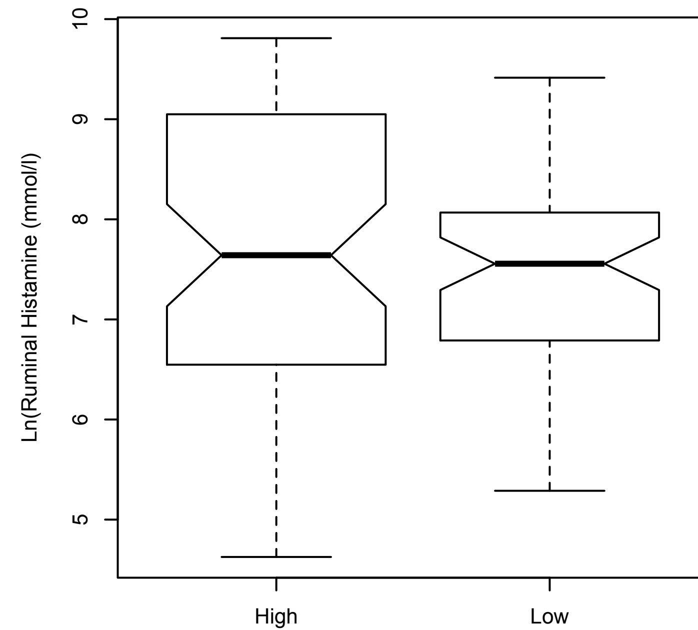

Ln(Ruminal LPS U/l)
9
10
11
12
13
BH1
BH6
BH7
BL2
BL3
BL7

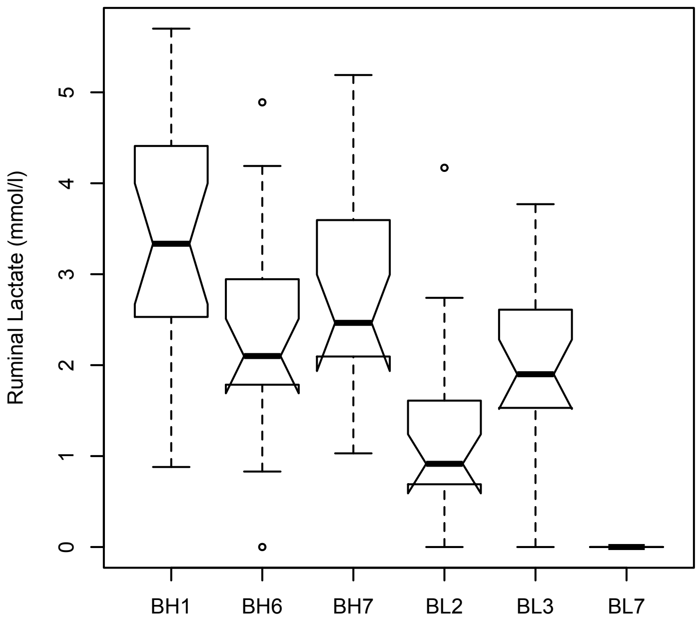

Ln(Caecal LPS U/l)
9
10
11
12
13
14
15
BH1
BH6
BH7
BL2
BL3
BL7

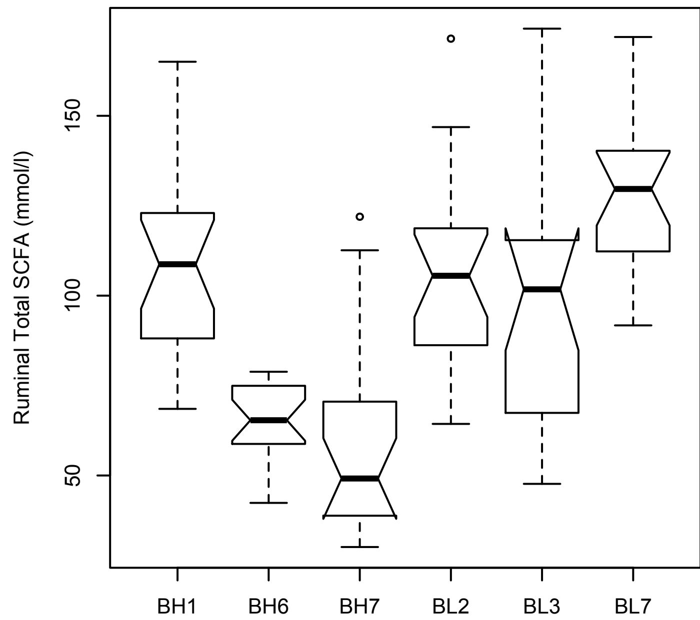

Ln(Ruminal Histamine (mmol/l))
5
6
7
8
9
10
BH1
BH6
BH7
BL2
BL3
BL7

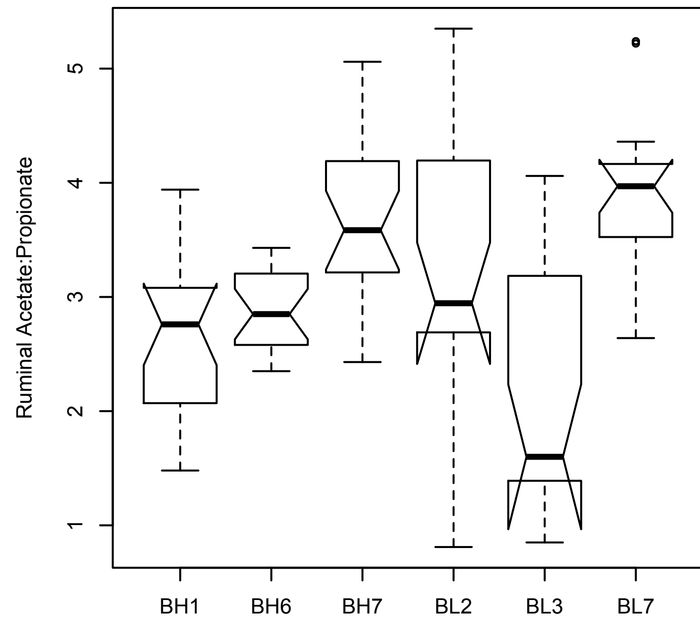

Ln(Ruminal LPS U/l)
9
10
11
12
13
High
Low

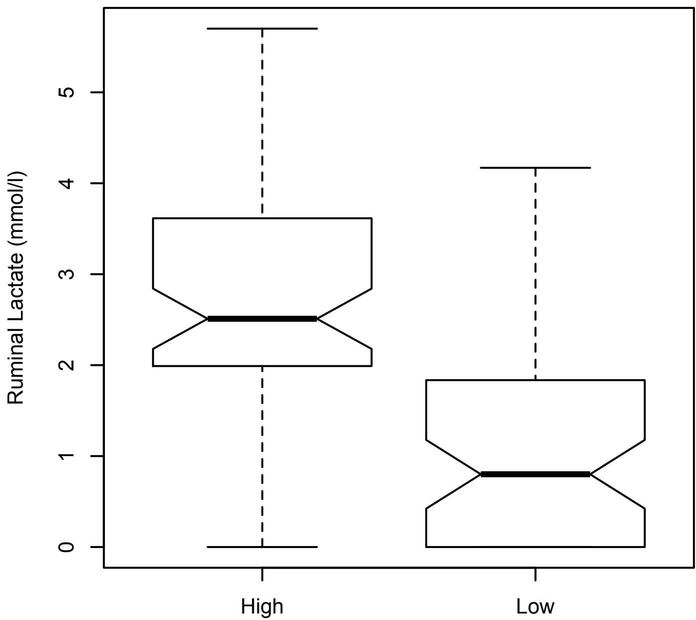

Ln(Caecal LPS U/l)
9
10
11
12
13
14
15
High
Low

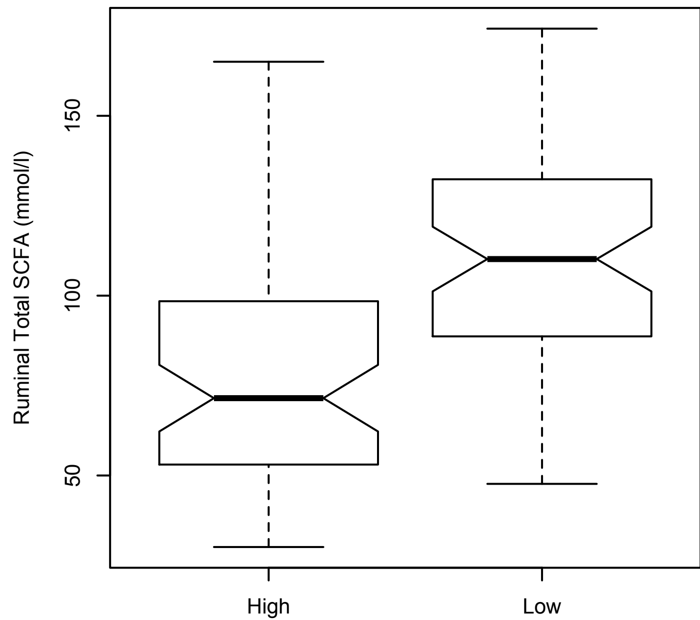

Ln(Ruminal Histamine (mmol/l))
5
6
7
8
9
10
High
Low

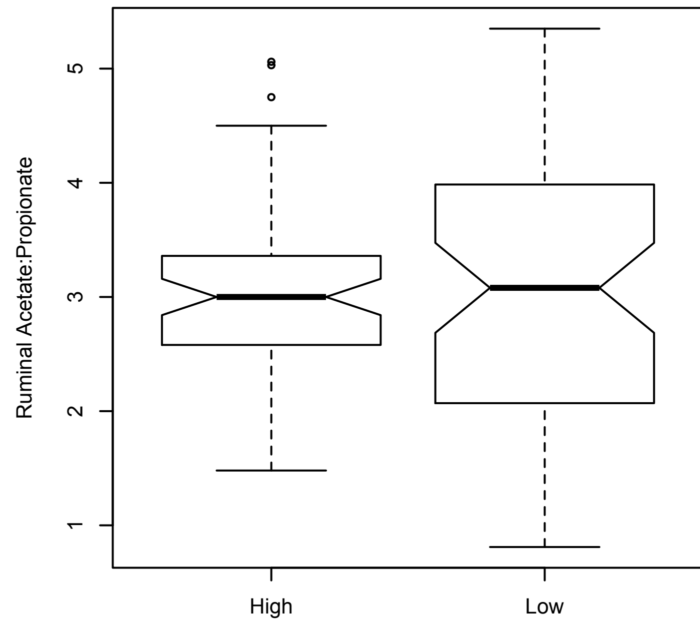

Ruminal Lactate (mmol/l)
0
1
2
3
4
5
BH1
BH6
BH7
BL2
BL3
BL7

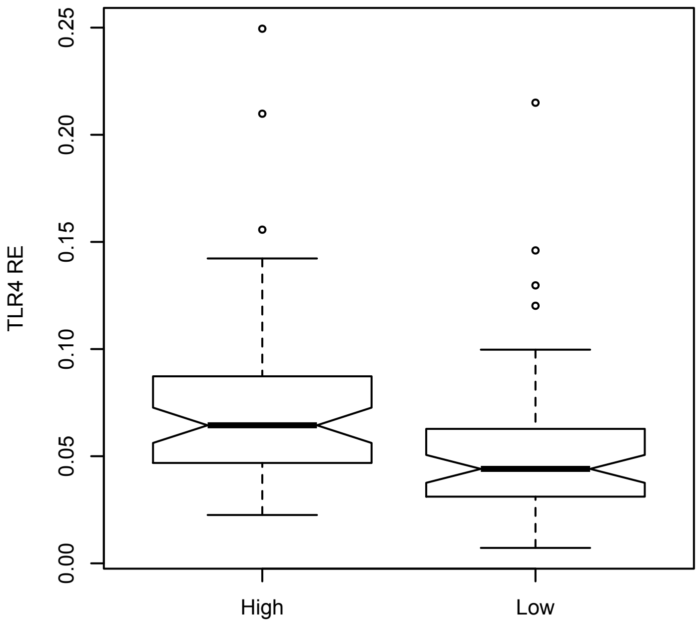

Ruminal Total SCFA (mmol/l)
50
100
150
BH1
BH6
BH7
BL2
BL3
BL7

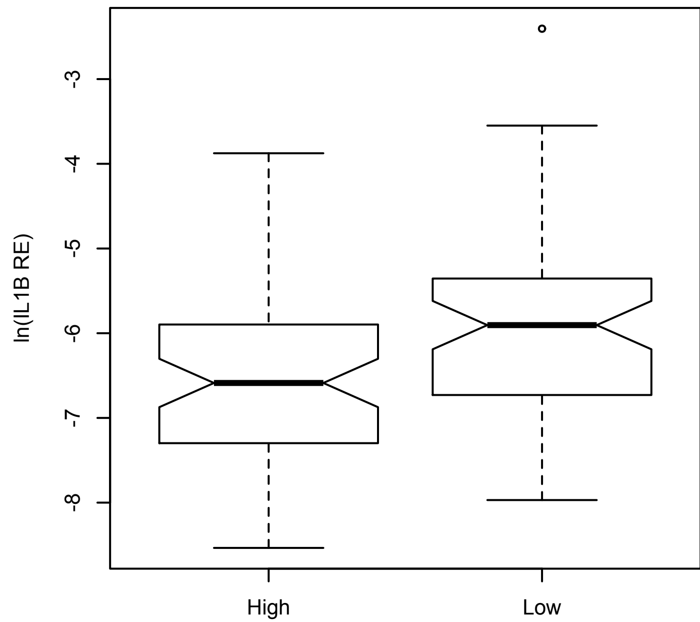

Ruminal Acetate:Propionate
1
2
3
4
5
BH1
BH6
BH7
BL2
BL3
BL7

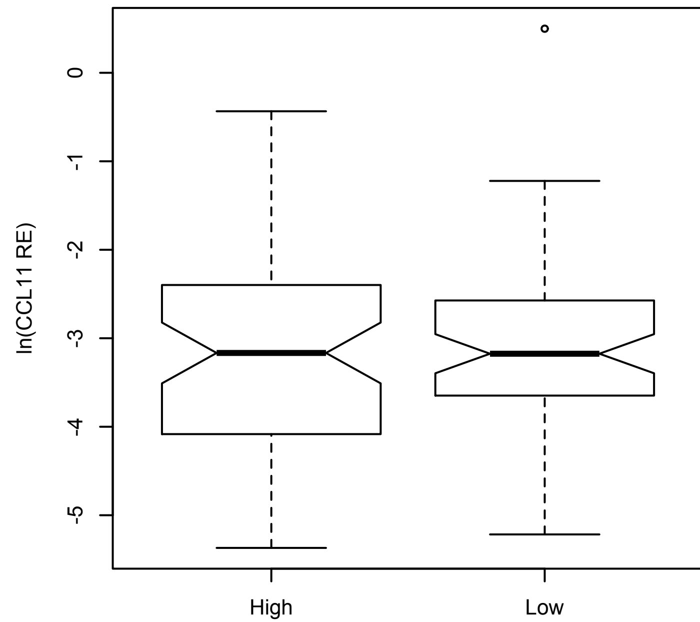

Ruminal Lactate (mmol/l)
0
1
2
3
4
5
High
Low

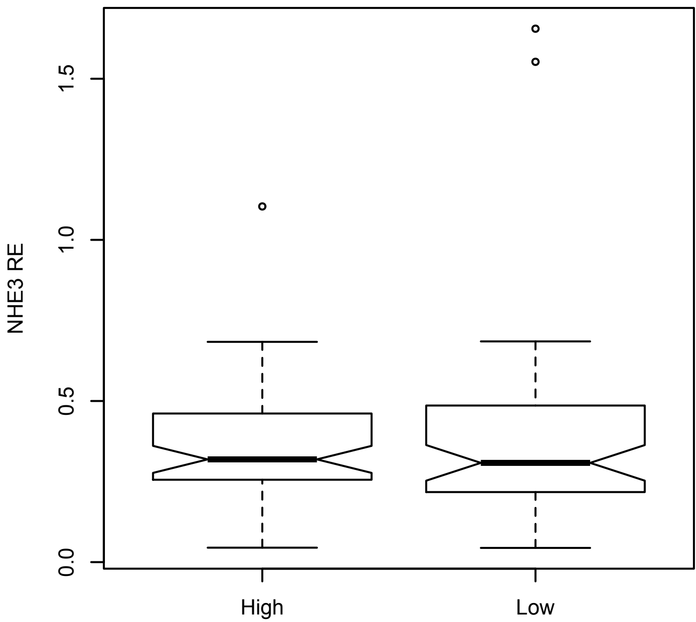

Ruminal Total SCFA (mmol/l)
50
100
150
High
Low

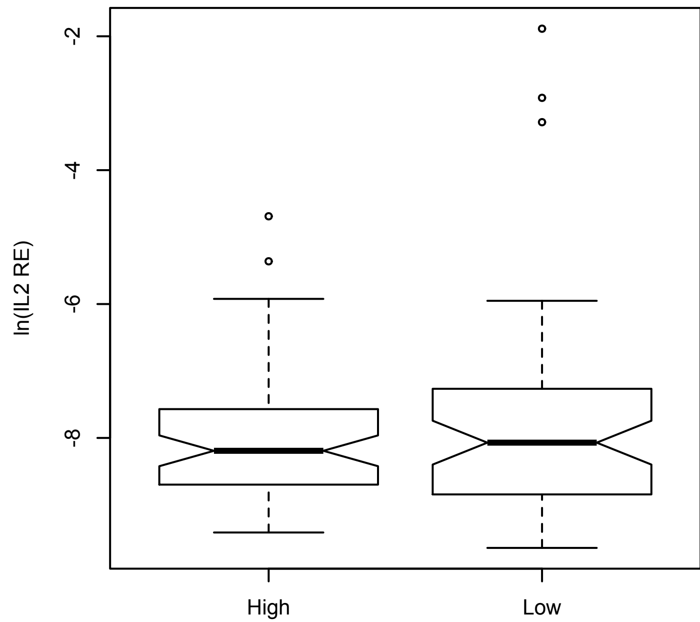

Ruminal Acetate:Propionate
1
2
3
4
5
High
Low

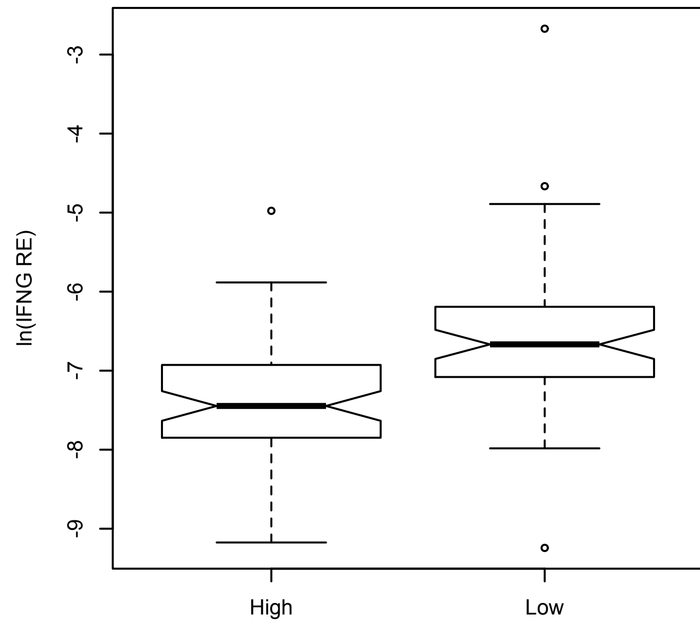

TLR4 RE
0.00
0.05
0.10
0.15
0.20
0.25
High
Low

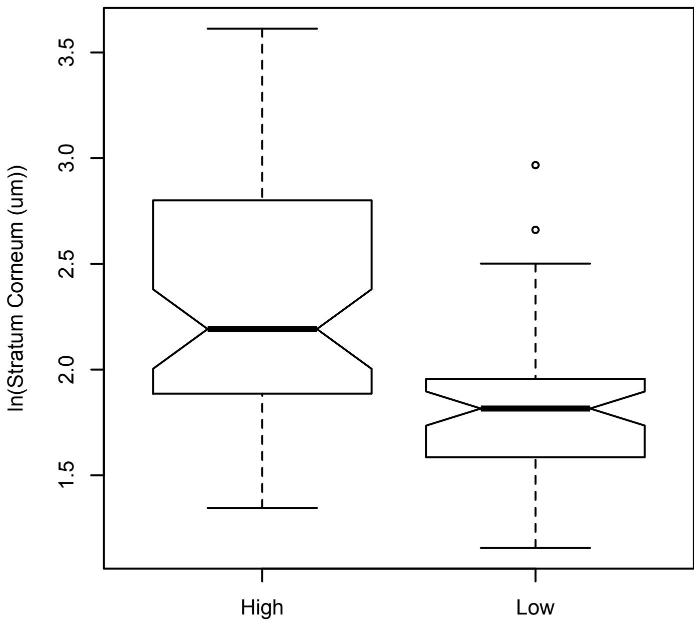

ln(IL1B RE)
-8
-7
-6
-5
-4
-3
High
Low

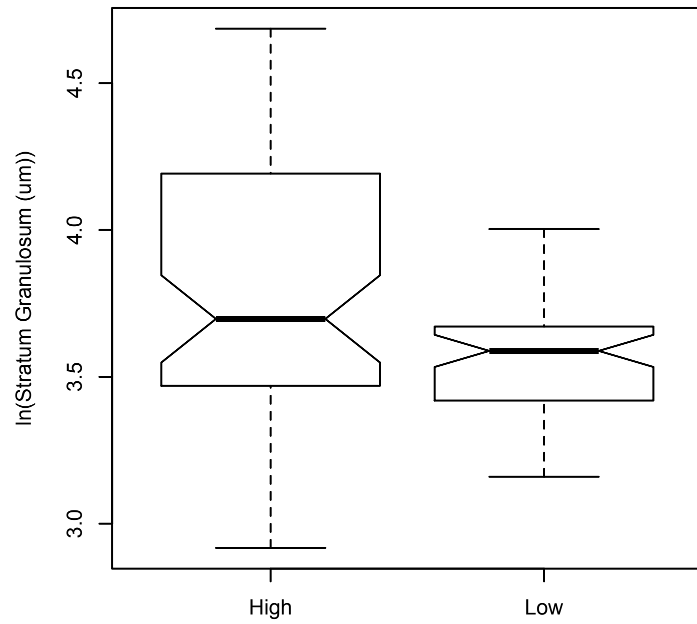

ln(CCL11 RE)
-5
-4
-3
-2
-1
0
High
Low

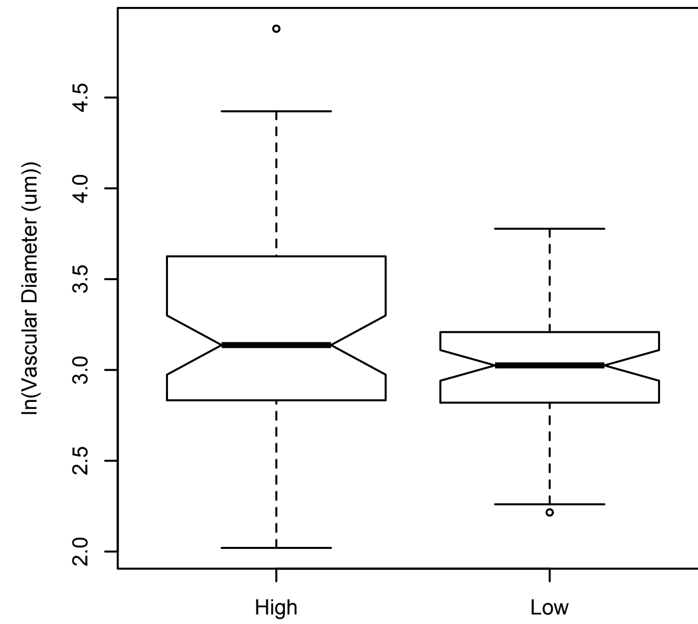

NHE3 RE
0.0
0.5
1.0
1.5
High
Low

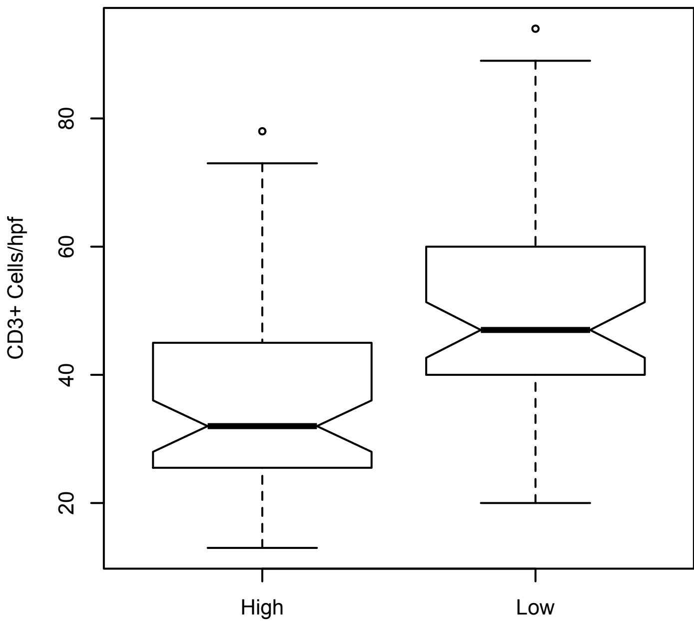

ln(IL2 RE)
-8
-6
-4
-2
High
Low

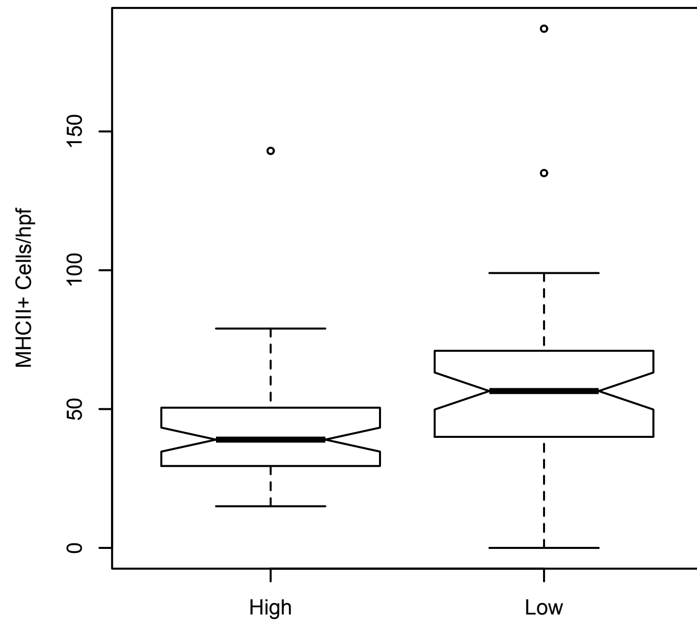

ln(IFNG RE)
-9
-8
-7
-6
-5
-4
-3
High
Low

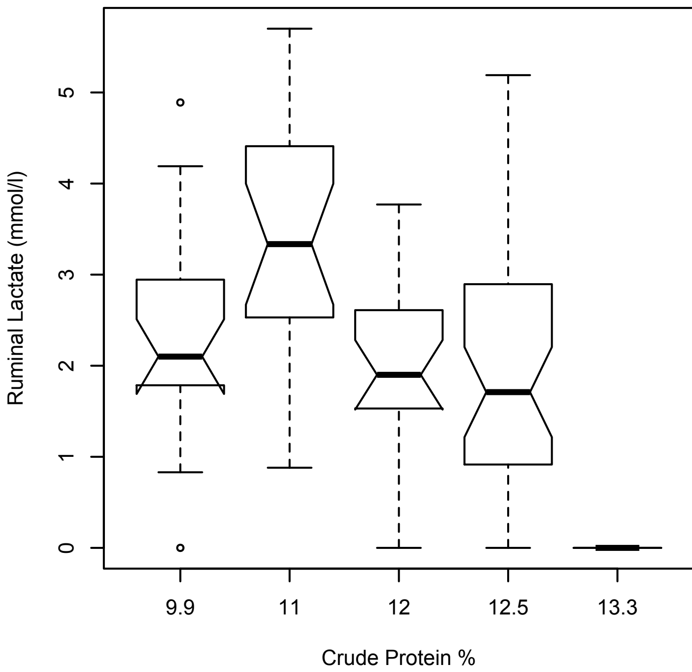
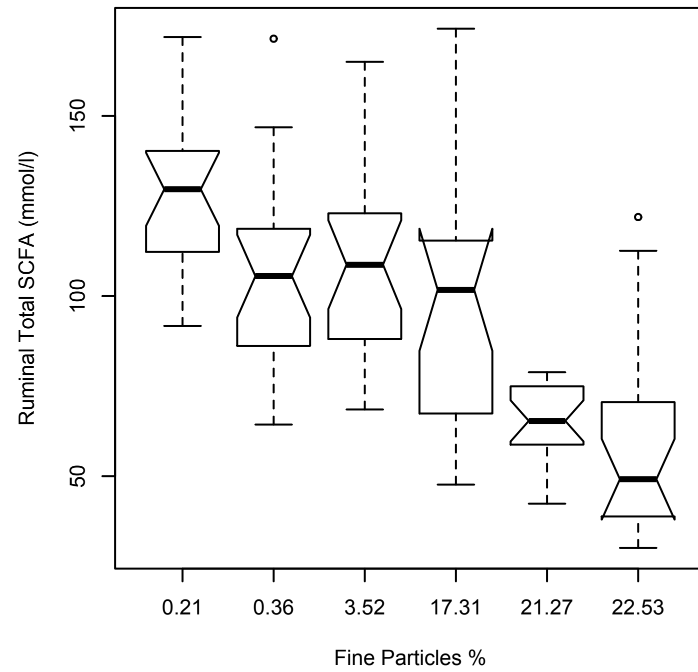
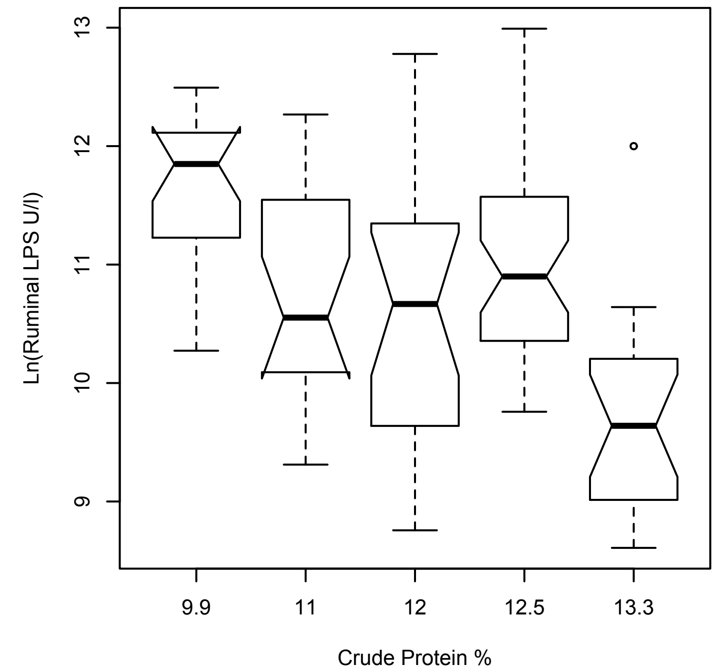
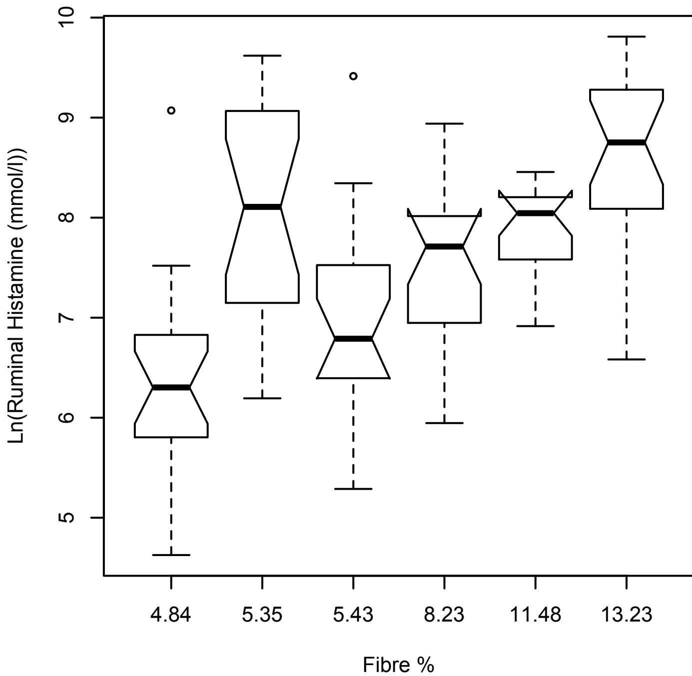
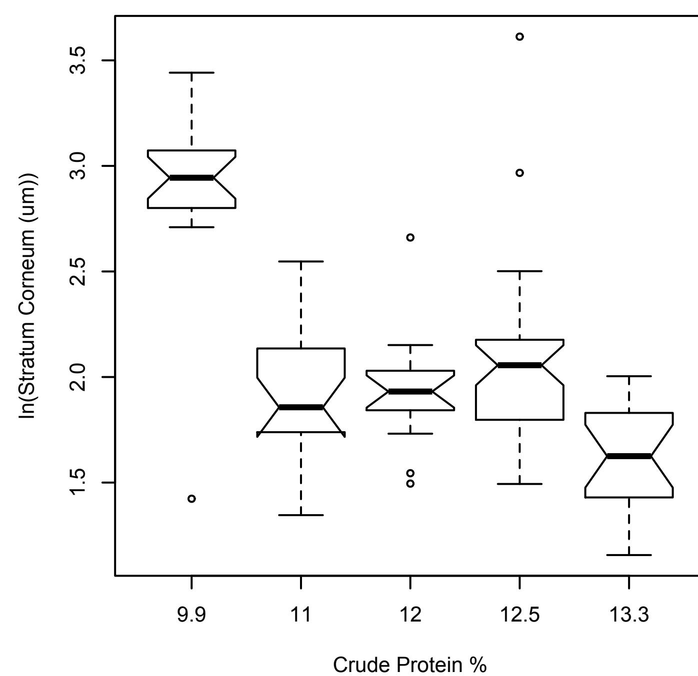
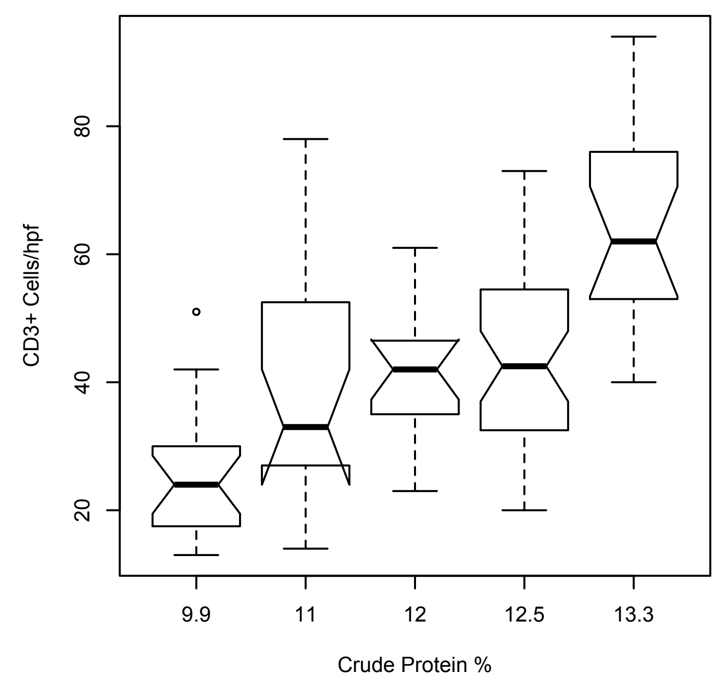

ln(Stratum Corneum (um))
1.5
2.0
2.5
3.0
3.5
High
Low


ln(Stratum Granulosum (um))
3.0
3.5
4.0
4.5
High
Low


ln(Vascular Diameter (um))
2.0
2.5
3.0
3.5
4.0
4.5
High
Low


CD3+ Cells/hpf
20
40
60
80
High
Low


MHCII+ Cells/hpf
0
50
100
150
High
Low


Ruminal Lactate (mmol/l)
0
1
2
3
4
5
9.9
11
12
12.5
13.3
Crude Protein %

Ruminal Total SCFA (mmol/l)
50
100
150
0.21
0.36
3.52
17.31
21.27
22.53
Fine Particles %

Ln(Ruminal LPS U/l)
9
10
11
12
13
9.9
11
12
12.5
13.3
Crude Protein %

Ln(Ruminal Histamine (mmol/l))
5
6
7
8
9
10
4.84
5.35
5.43
8.23
11.48
13.23
Fibre %

ln(Stratum Corneum (um))
1.5
2.0
2.5
3.0
3.5
9.9
11
12
12.5
13.3
Crude Protein %

CD3+ Cells/hpf
20
40
60
80
9.9
11
12
12.5
13.3
Crude Protein %